UNIVERSITY OF LEICESTER

NICOTINE CHRONIC TOLERANCE DEVELOPMENT AND WITHDARAWAL IN PLANARIA

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Nicotine chronic tolerance development and withdrawal in the planaria

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Abstract

In rodents, chronic nicotine exposure reduces sensitivity to its unconditioned effects, which then results in addictive-like behaviours (i.e., tolerance development). The studies reported here address the chronic effect of nicotine on an invertebrate model using two planaria species: *Schmidtea mediterranea* and *Dugesia sp.* In our experiments, animals were repeatedly exposed to nicotine in a specific context and then they received three consecutive tests: in the presence of water in the nicotine-associated context, to assess the development conditioned compensatory responses – CCRs -(Test1); a test with nicotine carried out in the nicotine-associated context (Test 2); and a test with nicotine in and a distinctive novel context (Test 3) to assess the role of context on the expression of chronic tolerance. Both *Dugesia sp.* and *Schmidtea mediterranea* showed evidence for the acute effect of nicotine on motility in a dose dependent manner, but only *Schmidtea mediterranea* showed evidence of the development of reliable chronic tolerance. *Schmidtea mediterranea* received repeated nicotine exposure for 5 hours in total during either 5 or 10 days, and the results showed evidence of tolerance development in the Tests 2 and 3, but not CCRs in the Test 1, after 10 days of exposure. On the contrary, animals exposed to the drug for 5 days (one hour/day) displayed CCRs in Test 1, but show no evidence of tolerance to the drug in Tests 2 and 3. We also found that the acute and chronic effects of nicotine are controlled by nicotinic receptor activation because the

acute effect of nicotine was partially attenuated, and the chronic tolerance was blocked by the co-administration of mecamylamine. Although chronic tolerance was observed in both nicotine-associated and novel contexts, chronic tolerance was stronger in the nicotine-associated than in the novel context. Overall, these results are consistent with the principles of the habituation model of tolerance (Baker & Tiffany, 1985). Also, these results are consistent with other findings in planaria and rodents suggesting that *Schmidtea mediterranea* is a useful preclinical model for the study of tolerance development following chronic exposure to drugs of abuse.

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Fatih

Dedication

For all frontline heath workers ……

Declaration

I hereby declare that this thesis has been composed by myself and that the research reported herein has been conducted by myself. Experiment 14, 15, 16, and 17 in Chapter 8 have been published in:

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List of Abbreviations

VTA	Ventral Tegmental area
NAc	Nucleus Accumbens
Ach	Acetylcholine
NIDA	The National Institute on Drug Abuse
CS	Conditioned Stimulus
US	Unconditioned Stimulus
IDI	Inter dose Interval
CR	Conditioned Response
CCR	Conditioned Compensatory Response
CNS	Central Nervus System
DA	Dopamine
5-HT	Serotonin
Ach	Acetylcholine
GABA	Gamma-aminobutyric Acid
HPLC	High-Performance Liquid Chromatography
SCH	Screw-like Hyperkinesia
WLP	Walnut Position
CPP	Conditioned Place Preference
DOPAC	3,4-Dihydroxyphenylacetic acid
HVA	Homovanillic Acid
5-HIAA	5-Hydroxyindoleacetic Acid

ANOVA	Analysis of Variances
η_p^2	Partial eta squared
FR	Fixed Ratio

1 Chapter 1: Drug Addiction and Motivational Theories

Addiction is a chronic and relapsing disorder characterised by compulsive drug-seeking and drug-taking behaviours and loss of control over consumption, despite adverse consequences (Berridge & Robinson, 2016). Dopamine transmission from the midbrain (VTA) to Ventral Striatum (NAc) increases following drug taking, gambling, sex, pornography and excessive eating – hence it is thought to be the motivational engine of these behaviours (Ikemoto & Bonci, 2014; Lewis, 2010). In addition, changes in the dopamine reward system associated with extensive drug use are thought to result in compulsive addictive behaviours (Robinson & Berridge, 2000).

Many human and animal studies (including vertebrates and invertebrates) have been conducted in order to identify the underlying neurological, biological, pathological and psychological reasons for the development, maintenance and relapse of drug addiction. These studies have largely found that repeated drug use cause dramatical changes in synaptic connections of neurons and brain plasticity. Therefore, drug addiction is defined as a brain disease by The National Institute on Drug Abuse (NIDA). Drug addiction negatively affects millions of people's lives, and cost billions of pounds to the economy of countries every single year.

Drug addiction is a complex phenomenon and can be explained from different perspectives such as social, medical, psychological. In this chapter, we will examine drug addiction theories from the motivational perspective and present four main theory of drug addiction. It is crucial to note that tolerance development is an essential component (explicitly or implicitly) in all these theories.

1.1 Opponent process theory of motivation

Positive (hedonic, appetitive) and negative (aversive) reinforcement mechanisms play an important role in the development, continuation and relapse of drug addiction. The opponent-process theory of motivation (Solomon & Corbit, 1974) conceptualized these two main reinforcement mechanisms as State A and State B. The opponent-process theory poses that initial drug administration produces: 1)- a hedonistic state (state A), which is related to the activation of excitatory properties of an administrated drug (or any other agent that disrupts homeostasis), and 2)- a negative emotional state (State B), which is opposite to State-A and automatically developed by CNS (negative feedback loop) in order to maintain homeostasis. The B-process is also present during stimulation, but manifested mostly after sudden termination of stimulus presentation. The hedonic A-state is fast and strong, whereas the opposite B-state is slow and weak after the first few administrations of stimuli. However, with repeated experience, organisms learn the relationships between environmental events and drug administration, and through learning the aversive B-state becomes faster and stronger, and reduced the magnitude of hedonic A-state (see Figure 1). In other words, through learning the B-state can be triggered earlier and achieve higher intensity, decreasing the size of the A-state.

Solomon and Corbit (1973) demonstrated that these opposite mechanisms (hence the name of the theory: Opponent processes) are similar across aversive and appetitive stimuli such as shock, love, social attachment and drug of abuse. For example, initial opioid administration produces a high level of pleasure (euphoria); however, unexpected termination of opioids injection after few stimulations elicits craving and withdrawal distress. Also, opioid injection after many stimulations results in less euphoria, relief and return to normal feeling; however, unexpected discontinuation after many stimulations produces intense craving and severe withdrawal signs for a long period of time. This

theory is critical to understand the contribution of reinforcing motivational factors such as positive and negative reinforcement on the development of addiction, and also (for the present discussion) the development of tolerance (hedonic habituation) and withdrawal syndromes.

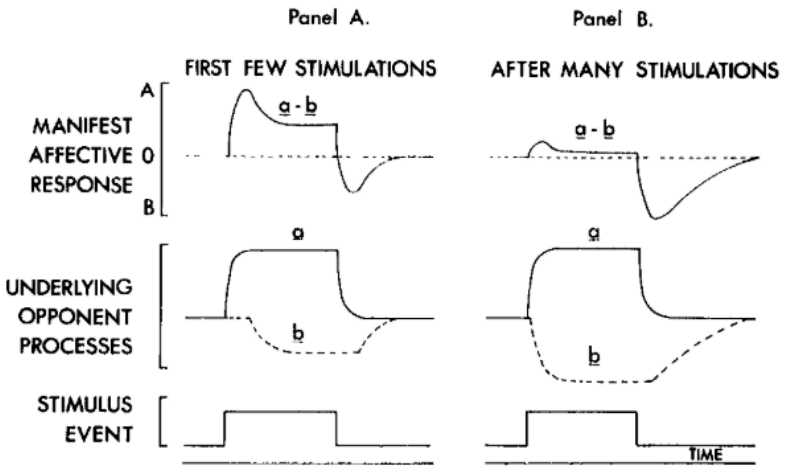


Figure 1. 1. A schematic illustration of the opponent-process theory (Solomon & Corbit, 1974).

1.2 Successive adaptation model

Wikler (1973) attempted to explain the development of addiction with successive adaptation model. Although it differs from opponent process, this model of successive adaptation is also related to associative learning and memory. Repeated drug (US) presentation in the presence of environmental stimuli allows for the formation of an association between the conditioned stimuli (CS) and the effects of the drug (US). In addition, CS presentation alone elicits an expression of conditioned response (CRs). The CRs might be in the similar or opposite direction to the initial drug-induced responses (URs). Eikelboom and Stewart (1982) proposed further that the direction of CRs is related to drug action on the afferent vs. efferent arms of the neural reflex circuit. If the CS triggers the afferent (inward) arm of the circuit, iso-directional and reflexive CRs would be produced that would be similar to initial drug effect. However, if CS triggers the

efferent arm (outward) of the circuit, compensatory opposite directional CRs would be observed. It is important to note that opposite directional CRs would result in the development of tolerance and physical dependence (withdrawal); similar directional CRs would result in sensitisation of the drug's effects. In other words, the direction of CRs is similar to the effect of the drug, as if the CS (stimulus) has acquired the properties of the US (drug). The summation of CRs and URs in the presence of drug and drug-related stimuli produces a stronger observed effect of the drug, which is called sensitisation. However, if the drug or associated cue activates the efferent arm of the central reflex (feedback) system, CRs mimics compensatory responses. These are responsible for the development of tolerance (with repeated drug experience), and withdrawal symptoms (in the absence of drug administration), an effect that has been observed across diverse physiological systems such as blood glucose level, blood pressure, body temperature, analgesia (pain sensitivity). It is important to note that the intensity of CRs changes with continuous CS-US pairings, can result in a stronger expression of tolerance, withdrawal and sensitisation to initial drug effect (Eikelboom & Stewart, 1982).

1.3 Dysregulation of reward system and Allostatic view of addiction

The allostatic view of addiction (Koob & Le Moal, 2001) defines addiction as the process of dysregulation of brain reward systems and adaptation to a dysregulated reward state. Allostatic state is different from the homeostatic state because homeostatic responses are automatically produced by CNS to maintain the internal equilibrium at the range of normal levels. However, allostasis responses are produced by CNS in adaptation to the dynamically dysregulated state of brain activity (produced by drugs). Therefore, allostatic state is defined as "a state of chronic deviation of regular system from its normal (homeostatic) operating level" (Koob & Le Moal, 2001).

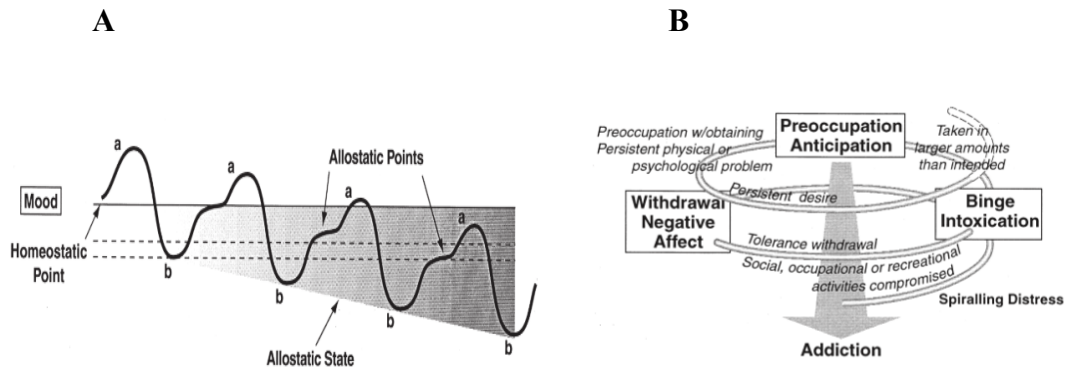


Figure 1. 2. A schematic illustration of the development of allostatic state (A) and three stages of addiction cycle (B) (Koob & Le Moal, 2001).

The allostatic view of addiction describes addiction as a three-stage spiralling cycle: Binge-intoxication, withdrawal negative effect, and pre-occupation-anticipation. In the first stage of the cycle, the binge-intoxication, drug is used due to its subjective pleasure. Therefore, drug-taking behaviour is expected to result in the same level of satisfaction as the initial experience. However, the allostatic state is based on a counter adaptive response mechanism, which is manifested as a withdrawal negative effect after the unexpected termination of drug-intake, which represents the second stage of the cycle (see Figure 1.2), called negative withdraw stage. This stage is critical in the theory, as it presumably motivates addicts to use drugs, in order to cease the withdrawal distress. However, it is important to note that every time the drug is used (failure of discontinuation) to relieve the pain of the withdrawal distress, it makes withdrawal stronger and makes the drug-induced subjective pleasure weaker. In other words, addicts gradually lose the sensitivity to the pleasure of drug-taking behaviour but continue drug consumption to eliminate withdrawal distress (negative reinforcement). This stage clarifies the development of physical dependence and relapse to some substances such as nicotine, morphine and alcohol. The final stage is called as pre-occupational/ anticipation where addiction is manifested as compulsive drug-seeking, and drug-taking behaviours and addicts lose the control of limiting drug use. This model is based predominantly on

negative reinforcement (second stage) where addicts take drugs to alleviate the withdrawal syndrome.

1.4 Incentive-sensitisation theory of addiction

The motivation for drug-seeking and taking behaviours in addicts is different from that of non-addicts. Because the drug-seeking and drug-taking behaviours become compulsive in addicts, these behaviours take over other daily activities, and addicts are not capable of stopping drug use. Unlike negative reinforcement view of addiction (see below), the incentive sensitisation theory (Robinson & Berridge, 1993) claimed that the negative reinforcement and positive reinforcement models are neither sufficient nor necessary to depict the full picture of addiction.

Subjective pleasure of drug (liking) and incentive-sensitisation to drug (wanting) are different from each other (Robinson & Berridge, 1993). Liking', is related to subjective pleasure and activation of dopamine reward mechanism following drug administration, which is similar to hedonic A-state of Opponent-process theory (Solomon & Corbit, 1974). Incentive-salience or 'wanting' is related to sensitisation of dopamine reward system with continuous CS-US pairing. Furthermore, sensitisation of incentive salience can produce compulsive drug seeking and drug-taking behaviours, which has been commonly used drug literature as the main characteristic of drug addiction.

The incentive-sensitisation theory focused on the psychological mechanisms of addiction, and defined drug addiction as the progressive evolution/transformation of drug-liking behaviours into drug-wanting behaviours as a consequence of incremental sensitisation of dopamine system. Associative learning plays an important role in incentive-sensitisation. Before any drug experience, neither drug nor drug-paired CSs have any corresponding value. Initial drug experience results in pleasure (euphoria),

conceptualized as 'liking' behaviours in the theory. However, across continuous experience with drug and drug-related CSs, the subjective pleasure of drug experience decreases due to the development of tolerance. However, the incentive value (wanting) of drug and drug-associated CS increases with continuous drug experience. In other words, the initial pleasure associated with drug-intake evolves into compulsive drug seeking and taking behaviours. This process called as incentive sensitisation (see figure below, Figure 1.3).

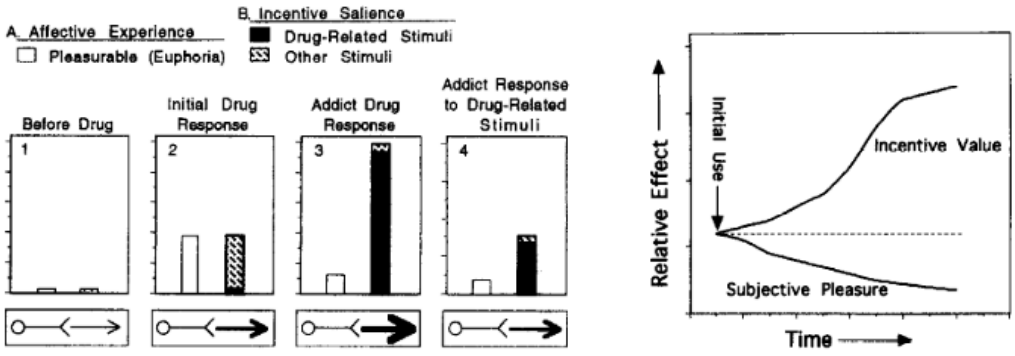


Figure 1. 3. A schematic illustration for the dynamic relationship between the subjective pleasurable effect of drug (liking) and incentive value of drug (wanting) (Robinson & Berridge, 1993).

1.5 Summary

In summary, despite some differences in all these approaches, they all account for the observation of the development of tolerance and that they all assume (perhaps in different ways) that tolerance is a contributing factor to drug addiction. Drug addiction is a complex phenomenon and drug of abuse negatively affects the lives of people and their loved ones. It also costs a lot to the medical and social systems of the countries. Understanding the relationship between appetitive and aversive motivational properties of drugs is fundamental to developing robust protocols to control drug addictive behaviours, treat addicts and prevent relapse. In the following chapter, we will elaborate on different types and theories of tolerance development.

2 Chapter 2: Theoretical Background of Drug Tolerance

Drug tolerance (tolerance henceforth) is the reduced behavioural responsiveness to unconditioned drug effects that results from repeated exposure. Kalant (1998), and Stewart and Badiani (1993) defined tolerance as an adaptation to a given dose of a drug, that may result in increased levels of consumption because in a tolerant animal higher doses are needed to achieve similar behavioural effects. Tolerance also shows a high degree of correlation with withdrawal symptoms – physical dependence on the effects of a certain drug (Siegel, 2008).

Tolerance is one of the key characteristics of addiction (Siegel, 1998; Baker & Tiffany, 1985). A common example of tolerance as the core characteristic of addiction in our daily lives is caffeine content in coffee. Consuming caffeine for the first time keeps people awake; however, at a certain point, they become tolerant to caffeine after repeated (i.e., chronic) consumption. Then individuals start consuming more caffeine such as drinking two cups of coffee instead of one to achieve the same effect. However, with the recurrent consumption, consumers are highly likely to become more tolerant. This iterative process leads to addiction to caffeine and any break to this process may cause headaches and discomfort (i.e., withdrawal).

A similar result regarding the association between tolerance and withdrawal was observed in nicotine addicts by Hughes & Hatsukami (1986). This study assessed tolerance by measuring the increase in the plasma cotinine—a biomarker for exposure to tobacco—level in 50 smokers, and withdrawal by measuring the level of carbon monoxide in their breath sample after four days of abstinence. The results demonstrated that tolerant people having higher plasma cotinine level experienced more withdrawal symptoms in the absence of smoking tobacco. This result suggested tolerance and

withdrawal symptoms are the manifestation of physical dependence and related in nicotine addicts.

Learning, on the other hand, can contribute to the development and expression of tolerance. A well-known example can be derived from the study of McCusker & Brown (1990) that measured cognitive and motor functions within two groups of people that usually had alcohol in a pub or in an office setting. When both groups drunk alcohol in a bar, the group which usually drank in a bar showed less impairment in cognitive and motor functions than the 'office' group. Another study (Remington et al., 1997) tested the role of context specificity on tolerance to alcohol. Alcohol consumption created less cognitive impairment in alcohol-associated beverages (i.e., beer) than when it was served with a novel mixture such as peppermint-flavoured beverages. These two studies on alcohol consumption behaviour of different groups provided concrete evidence that environmental conditions and flavours of the beverages can act as 'contexts', thus tolerance can become stronger with certain alcohol-associated contexts.

Rozin, Reff and Mark (1984) illustrated similar effects of caffeine in human participants. Twenty-four regular coffee drinkers were conditioned with (caffeinated) coffee four times a week, then they were tested in four different conditions (with caffeine or without caffeine; and in black coffee or apple juice): coffee with caffeine (CC), coffee no caffeine (CN), apple juice with caffeine (AC) and apple juice with no caffeine (AN). Caffeine served as the unconditioned stimulus and coffee was the conditioned stimulus in this experiment. The number of drops of saliva was measured 10 min before drinking (as baseline level) and 40 min after drinking the beverages. The changes in the number of drop of saliva before and after drinking beverages served as the dependent variable (DV). The results showed that people produced less saliva when caffeine was served in black coffee (familiar context) compared to apple juice (unfamiliar context). Context-

specific tolerance to caffeine was less effective with a familiar cue (i.e., coffee). In addition, participants that were tested with decaffeinated coffee showed inhibition of salivation. Their salivation release was lower than the baseline (coffee with caffeine) – which is evidence for a compensatory response, indicative of conditioned tolerance. This suggests that tolerance and withdrawal symptoms are associated and are controlled by homeostatic compensatory mechanisms.

2.1 Physiological Theories of Tolerance

A number of addiction studies intensively examined physiological mechanism of tolerance. There are four main physiological theories that explain the reasons for the reduced function of an administrated drug following chronic exposure:

1) alteration in the organism's drug metabolism, and the drug is metabolised faster (Mule & Woods, 1962);

2) alteration of the drug's action on CNS; decrease in the number of receptor sites that the drug can stimulate; decrease in the receptor sensitivity to drug (Schmidt & Livingston, 1933);

3) formation of silent receptors with initial drug administration that reduce the drug's effect with later administration (Collier, 1965);

4) immunity-like processes (Cochin & Kortnetsky, 1968).

Besides the aforementioned physiological tolerance theories, many drug addiction studies intensively addressed behavioural mechanisms of tolerance. This chapter is intended to provide a general introduction to behavioural theories of tolerance with an emphasis on the reasons for the reduced function of an administrated drug following chronic exposure and withdrawal responses following the discontinuation of the drug exposure.

2.2 Behavioural Theories of Tolerance

Behavioural Tolerance Theories are crucial in understanding the underlying mechanisms of tolerance. Behavioural tolerance theories can be divided into two main streams: tolerance with and tolerance without homeostatic compensatory responses.

Tolerance with homeostatic compensatory response theories suggest that tolerance is ruled by a conditioned compensatory response, which can be observed during the phase when the drug is excreted from the body (Siegel, 2008; Solomon, 1980). Therefore, compensatory responses were defined as a sign of distress from the drug's withdrawal. Withdrawal symptoms do not appear until an organism becomes tolerant (Wikler, 1973). Tolerance and withdrawal symptoms are thought to be controlled by similar homeostatic mechanisms (MacRae, Scoles & Siegel, 1987; Siegel et al., 2000). The severity and duration of withdrawal symptoms are linked to the magnitude of tolerance. For example, the opponent-process theory (Solomon, 1980) proposes that drug presentation produces a hedonistic effect that also disturbs the homeostatic mechanism, and the body produces a compensatory response to keep maintaining the homeostatic balance that can be observed following the termination of drug presentation. In other words, drug presentation elicits a large hedonistic effect during the first few stimulations, but this effect gets smaller after many stimulations, resulting in tolerance development. Also, the opponent process of homeostatic response that follows initial drug effect is a weak and slow response initially, but becomes faster and stronger with repeated drug presentation. Therefore, homeostatic response is offered as the main reason for the development of tolerance and drug withdrawal responses.

Another example of behavioural tolerance with homeostatic compensatory response is the Pavlovian conditioning tolerance theory proposed by Siegel (1975). This theory is strictly associative, and proposes a homeostatic mechanism that leads to the

development of tolerance modulated by drug-associated environmental cues. Several studies based on this theory also found that some manipulation in the experimental protocols retards the development of tolerance such as latent inhibition, CS presentation alone before CS-US pairing, or partial reinforcement effect (PRE), in which the CS is partially paired with the drug. The theory also offered that tolerance can be extinguished by non-drug CS presentations. However, this theory was silent about tolerance acquired independent of drug contingent cues.

Contrary to these two theories that suggest tolerance is an outcome of homeostatic compensatory responses, *tolerance as habituation theory* (Baker & Tiffany, 1985) proposed tolerance is the central feature of addiction, but the development of tolerance does not necessarily need homeostatic compensatory responses. Studies based on the habituation theory found a significant reduction in the magnitude of the drug effect with repeated presentations, but no evidence of the existence of homeostatic responses in the absence of drug administration (Tiffany et al., 1983).

As it can be seen, different studies based on the aforementioned behavioural theories of tolerance found contradictory results for the development of tolerance and withdrawal responses. Therefore, in this chapter, we will focus on the differences between the behavioural tolerance theories.

2.2.1 Opponent Process Theory

Opponent process theory (Solomon, 1980) explains how the reinforcing properties of rewards (natural rewards and drugs of abuse) change with repeated exposure (i.e., tolerance), and how drug craving and withdrawal symptoms develop with repetitive drug use. Solomon (1980) stated “the user not only becomes drug-tolerant but also becomes more intolerant of drug termination or absence.” This theory describes the development

of addictive behaviours with three motives: 1) - hedonistic contrast after initial stimulation, 2) - hedonic habituation with many stimulations and 3) - withdrawal syndrome following the termination of reinforcement.

The development of tolerance according to the opponent-process theory is based upon mutual interaction of two opposite motivational properties of reinforcement: 1)- hedonistic excitement termed as A process and 2)- withdrawal distress named as B process. The A and B processes both are elicited by the presentation of the drug. Process A is fast and very intense, then it gradually declines and come to a steady level. On the contrary, the B process is slow and weak with first stimulation but intensifies with repeated presentations. These two processes compete with each other, and the net result of the competition is a decreased magnitude of the excitatory properties of the reinforcer, which leads to the development of tolerance (Solomon, 1980). The other obvious difference between A and B is that B process can be learned, and hence becomes controlled by environmental stimuli. It is important to note that the B process is the main reason for the expression of withdrawal symptoms in the absence of reinforcement. Strengthening of the B process with many stimulations makes the organism more vulnerable to the hedonic effect of the stimulation of the reinforcer. If the A process is stronger than the B process, State A emerges; but if the B process is stronger than the A process, State B emerges. Severity and duration of the B process is accepted as the measurement for the degree of physiological dependence that is strengthened by exercise/rehearsal and weakened by disuse. Inter dose interval (IDI) is a crucial variable for the strength of the opponent process and the development of tolerance. Shorter IDI produces a stronger B process and that leads to stronger tolerance. However, a longer IDI prevents the growth of the B process that weakens the formation of tolerance (Solomon,

1980).

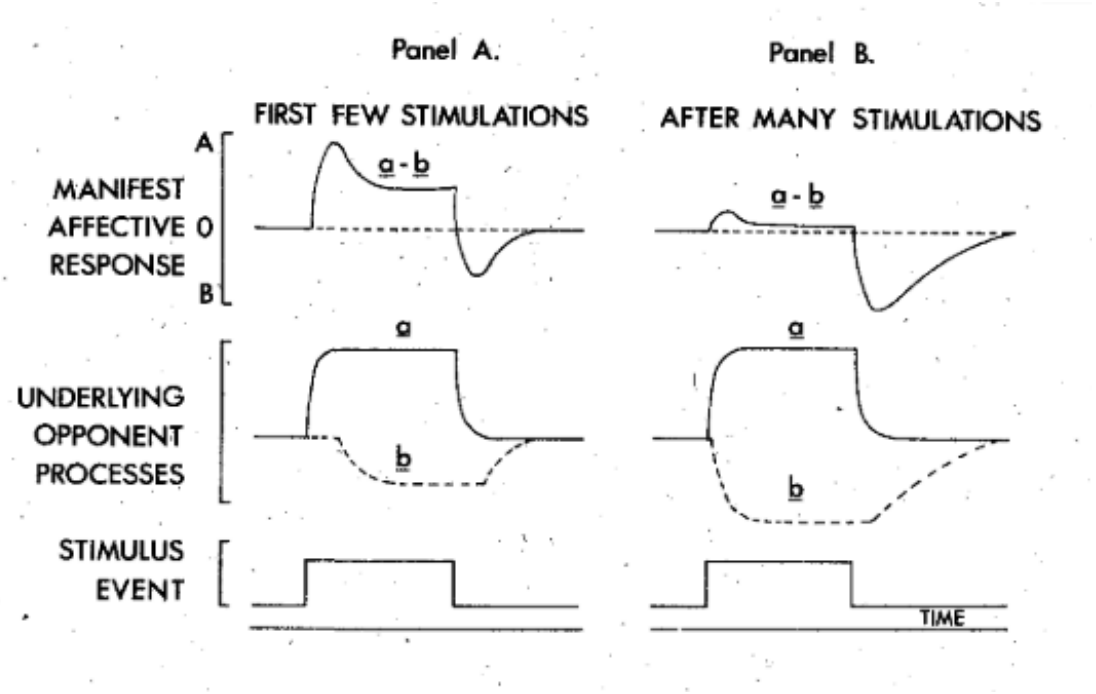


Figure 2. 1. A schematic demonstration of primary A process and opponent B process after few and many stimulations (Solomon, 1980).

Solomon (1980) reported an example of the change in the cardiac response of a dog presented with an electric shock. Shock presentation produced a sudden increase in heart beats early on, but then this response was gradually reduced and came to steady level State A. Unexpected termination of the shock produced a sudden deceleration in the response initially, but it gradually returned to baseline level (B process). Repeated shock stimulation led to a habituation effect; less excitement with shock presentation, but more intense withdrawal following the termination. In other words, continuous shock presentation gradually reduced its excitatory properties but, on the other hand, progressively increased the magnitude of withdrawal. This is a good analogy to understand how continuous drug taking changes the reinforcing effect of a drug and develops yearning for drug and withdrawal symptoms.

In summary, the opponent-process theory explains the development of drug tolerance as the competition between the hedonistic effect (A process) and opponent hedonic effect (B process) produced by drug stimulation. If the rewarding effect of the drug is stronger than the opponent hedonic effect, the organism is in State A, positive reinforcing state of pleasure. However, if the opponent hedonic effect is dominant, the organism is in State B, a negatively reinforcing state of aversive craving that leads to tolerance in the presence of the drug, and withdrawal symptoms in its absence.

However, this theory has a potential limitation. The opponent response in this theory is called withdrawal symptoms. Since these responses were observed immediately after stimulus termination, they might be a post/after-effect of stimulation or rebound response. Withdrawal responses can be seen a long time after drug termination.

2.2.2 Pavlovian Conditioning of Tolerance

According to the Pavlovian conditioning theory of tolerance, tolerance is an outcome of learning and anticipation of drug outcomes (Siegel, 1975; 2001; 2008; Siegel & Larson, 1996). Before conditioning, drug presentation creates an imbalance/disturbance in the homeostatic mechanism of the organism, and the organism produces a compensatory response to the drug-induced disturbance. Following repeated drug administration in a distinctive environment (which acts as a CS), drug-paired cues control this compensatory response, resulting in the development of a Conditioned Compensatory Response (CCR). CCR is an opposite response to the unconditioned drug effect that is triggered by CS presentation. Therefore, drug administration in the presence of the CS (distinctive environment) causes a reduction in the primary effect of the drug referred to as tolerance. On the other hand, exposure to the CS in the absence of drugs only activates the anticipatory opposite response. This response is accepted as a sign of

withdrawal and craving that can cause relapse long after the last drug administration. The CCRs have been investigated with different drugs with various response mechanisms.

To test this theory, Siegel (1975) investigated the development of conditioned tolerance to the analgesic effect of morphine (reducing pain sensitivity). This is a classic example of associative development of morphine tolerance. Rats were treated with morphine at 48 hours intervals in one of three different contexts: home cage, cold plate (room temperature), and hot plate (54.2° C) during three conditioning sessions. The three groups were then tested on the hot plate (at 54.2° C) and measured paw-licking latencies—paw licking is a sign of the experience of pain in the rat. The result suggested that animals displayed long paw-licking latencies after the first morphine treatment compared to saline injection, providing evidence of the analgesic effect of morphine. The latency, however, declined with repeated morphine injections. On the test day, animals that received repeated morphine treatments on the hot-plate showed shorter paw-licking latencies, suggesting the evidence of the development of tolerance to the analgesic effect of morphine. Additionally, animals that received morphine on the cold plate displayed tolerance to the analgesic effect of morphine as the hot-plate group did. However, animals that received morphine in their cage showed a long paw-licking latency on the test day as if they experienced morphine for the first time. These results suggest that drug cue association is important for the development of tolerance because testing in the alternative context eliminated the expression of tolerance. Moreover, when the cage group received three additional morphine injection on the hot plate, they became tolerant to the analgesic effect of morphine (shorter paw-licking latency) like the other groups. In summary, these results were evidence of situation-specific tolerance. It was assumed that the reduction in the systematic effect of morphine was due to CRs controlled by drug-associated cues, and it can be observed with placebo administration. When animals in these three groups (Hot

plate, Cage, and Saline) were tested with a saline injection, only the animals repeatedly treated with morphine in hotplate (Hot plate group) showed evidence of hyperalgesia (shorter paw-licking latencies when exposed to the hot plate), and no difference between the Cage and Saline groups. These results suggested that CCRs (hypersensitivity to pain) were controlled by drug-associated cues, but not by the systematic effect of the drug itself.

To support this theory further, Le at al. (1979) investigated the expression of conditioned tolerance to the hypothermic effects of alcohol under different environmental conditions. Initial alcohol exposure reduced rat's body temperature and successive alcohol treatments in a distinctive environment led to the tolerance of alcohol-induced hypothermic response. Partial attenuation of tolerance was observed when animals were tested in their home-cages after chronic treatment, and tolerance was reinstated when they were tested in the drug-paired distinctive environment again. This study showed that tolerance was context-specific, and it was mediated by hyperthermic CCRs. Moreover, in a similar study, Mansfield and Cunningham (1980) found that conditioned tolerance to the hypothermic effect of ethanol was associated to the hyperthermic compensatory response. This study also found attenuation of tolerance with non-reinforced extinction trials. These two studies offered evidence that supports the conditioned tolerance to alcohol as Siegel (1975) observed to morphine.

CCRs were also observed in different response mechanisms to the unconditioned effect of various drugs such as excessive salivary response to anticholinergic drugs (atropine) effect (Finch, 1938), reduction in heart rate to epinephrine effect (Subkov & Zilov, 1937), hyperglycaemia to insulin injection (Siegel, 1972), hyperalgesia (hypersensitivity to pain) to the analgesic effect of morphine (Siegel, 1975), and hyperthermia to hypothermic ethanol effect (Lê et al., 1987) (See Table2.1)

Studies	Drug	Response	Unconditioned Effect	Compensatory Response	Conclusions
Siegel, 1975	Morphine	Paw-licking latency (hot plate)	Analgesia ↑ (Reduced pain sensitivity)	Hyperalgesia ↓ (Increased Pain sensitivity)	• Context-specific tolerance • Hyperalgesia (CCRs) in the absence of morphine in the drug-paired context • Elimination of CCRs and tolerance following repeated placebo injection in the drug paired context • CCR and tolerance stored in long term memory because they were not affected delay after last morphine drug-paired
(Sherman, Strub and Lewis, 1984)	Morphine	Paw-licking latency or jump first	Analgesia ↑	Hyperalgesia ↓	Stress enhanced morphine analgesia
(Grisel et al., 1994)	Morphine	Tail-flick Latency	Analgesia ↑	Hyperalgesia ↓	
(Siegel, 1999)	Morphine	Tail-flick response latency	Analgesia ↑	Hyperalgesia ↓	Glucose facilitated development of tolerance to analgesic effect of morphine
(Lê et al., 1987)	Ethanol	Body temperature	Hypothermia ↓	Hyperthermia ↑	
(Siegel & Larson, 1996)	Ethanol	Body temperature	Hypothermia ↓	Not tested	Tolerance was attenuated by novel stimuli
(Siegel & Sdao-Jarvie, 1986)	Ethanol	Body temperature	Hypothermia ↓	Not tested	Novel context presentation eliminated tolerance
(Larson & Siegel, 1998)	Ethanol	Ataxia on tilting plane	Motor impairment ↑ (ataxia)	Hypertaxia ↓	• CCRs and tolerance were attenuated by novel stimuli • Long interval between drug-paired and testing inhibited tolerance
Subkov &Zilov (1937)	Epinephrine	Hearth beat rate	Tachycardia ↑	Bradycardia ↓	
Finch (1938)	Atropine	Salivary Response	Reduced Salivation ↓	Excessive Salivation ↑	
Siegel (1972)	Insulin	Blood Glucose Level	Hypoglycaemia ↓	Hyperglycaemia ↑	
(Eikelboom & Stewart, 1979)	Morphine	Body temperature	Hyperthermia↑	Hyperthermia ↑	CCRs

					Hypothermic response was observed in the pre-injection area before drug injection.
(Tiffany et al., 1983)	Morphine	Flinch/ Jump to Shock	Analgesia ↑	No compensatory response ↔	• Tolerance to analgesic morphine effect • Without hyperalgesic compensatory response

Table 2. 1. Demonstration of unconditioned effects and conditioned responses to a variety of drugs and response systems.

2.2.2.1 Extinction and Reinstatement of Tolerance

Tolerance and conditioned compensatory responses can be extinguished with repeated placebo administration in the presence of drug-paired cues. Siegel (1975) tested the effect of extinction sessions on the expression of CCRs. In this experiments, rats were given repeated morphine injections, followed by exposure to the conditioning context in the experimental group (a control group remained in their home cages during the context extinction sessions). The animals were finally tested with placebo in the conditioning context. Animals that received two weeks of rest following the last morphine drug-paired session showed hyperalgesia as a compensatory response (hypersensitivity to pain) when tested in the conditioning context. However, repeated placebo administration in the presence of drug-paired cues (extinction sessions) in the experimental group gradually extinguished the CCRs, in that the paw-lick response latencies returned to the baseline level. This result suggests that tolerance to morphine is stored into long-term memory because animals were still tolerant to morphine after a delay of time, but it can be extinguished with extinction. Additionally, a morphine injection after the extinction sessions reinstated tolerance to morphine. The fact that the conditioned tolerance response to morphine extinguishes and can be reinstated by simply exposing the animals to an additional morphine injection strongly suggests that tolerance development is ruled the same processes that are known to be standard Pavlovian conditioning. However, this

study and other Pavlovian conditioning tolerance studies were mostly silent about the non-contingent central effect of the drug alone (Siegel, 2008).

2.2.3 Tolerance as Habituation without Conditioned Compensatory Responses

The aforementioned studies suggested that tolerance to drug effects resulted from homeostatic regulatory responses. However, they overlooked the development of tolerance that may be independent of drug-environment contingencies. Baker and Tiffany (1985) suggested another theory which captures tolerance as habituation. This theory suggests that tolerance can be seen with both associative and non-associative routes, as an alternative to Siegel's (1975) Pavlovian Conditioning Tolerance Theory. Habituation theory minimizes the contribution of homeostatic regulatory responses on tolerance responses and proposes that opponent responses are not essential for tolerance development.

Tolerance as a habituation theory is derived from Wagner's priming theory of habituation (Wagner, 1976). According to this theory, tolerance is an outcome of the prime of the stimulus-related memory which attenuate the magnitude of responding to unconditioned stimulus. In other words, tolerance – the attenuation of responding to drug – is observed if the properties of stimulus is retrieved from memory. According to Habituation theory (Wagner, 1981), the magnitude of URs is modulated by how stimuli are surprising or expected (primed). For example, if the presentation of a stimulus is surprising for the organism, the processing of its features in the short-term memory (STM) is effective and leads to strong unconditioned responses (URs). On the other hand, if the stimulus is expected/predicted/primed, the organism is familiar with it, and the stimulus becomes less surprising for the organism. In that case its features are not processed in the STM, and as a result it produces diminished, weakened URs.

Exposure to an eliciting stimulus can result in two forms of priming leading to habituation: self-generated priming and associatively generated priming. These two forms of priming result in decreased neural processing of the features of the stimulus, reduces the vulnerability to the disruptive effect of drugs and leads to tolerance (Wagner, 1981). Self-generated priming explains short-term habitation without associative learning. This concept asserts that tolerance is an outcome of previous experience of drug exposure history. Inter-trial interval (ITI) plays an important role in the development of self-generated priming of habituation. If stimulus presentation (drug exposure) is with shorter ITIs, stronger habituation is observed. However, if the presentation of stimuli is widely spaced in time, habituation is retarded and reduced. This is because of the retention in registration and retrieval of the properties of stimulus properties to STM. The second form of habituation is associatively generated priming. Drug treatment with reliable drug-cues signals (or contextual cues) result in the establishment of associations between the drug-cues and the effects of the drug (in Siegel's theory, for example, the compensatory response of the organism to the drug insult). These cues therefore can reduce the unconditioned drug effects, and drug presentation in the presence of the drug-cues elicits smaller URs compared to drug without reliable cues.

In order to investigate this habituation theory further, Tiffany et al. (1983) tested conditioned tolerance to the analgesic effect of morphine in rats measuring the jumping response (as an index of sensitivity to electric shock). Rats received an electric shock after saline or morphine injection in a distinctive context or home cage during drug-paired sessions. Then, they were all tested with morphine in the distinctive context to test context-specific tolerance and after administration of vehicle to measure CCRs. Tolerance results replicated previous findings of Siegel's (1975) study regarding

conditioned context-specific tolerance to the analgesic effect of morphine. However, they did not observe any CCRs, a finding that is at odds with Siegel's observations.

Additionally, tolerance data showed that the morphine-treated group remained less sensitive to the shock than the saline-treated group on the test day. The researchers presented an explanation, suggesting that the conditioned environment itself may produce stress that reduces sensitivity to the shock response. In other words, stress caused by the conditioned environment may mask the expression of CRs. To test this assumption, the authors of the study made two experimental manipulations: 1) long exposure (i.e., extinction) to the distinctive environment before test sessions, and 2) creating a less stressful conditioning context (dark environment). Test data revealed that stressful conditions in the conditioned environment reduced response sensitivity because animals in these groups remained more sensitive to shock than the control group. Tolerance was tested with a morphine injection and CCRs were tested with saline injections. They observed less morphine sensitivity in the group trained and tested in the same context, relative to the other two groups, but no evidence of CCRs was found. Overall, these results suggest there was evidence of context-mediated conditioned tolerance, but tolerance was not mediated by CCRs as tolerance studies of Pavlovian conditioning (Siegel, 1972; 1975; Macrae et al., 1987)

Furthermore, Eikelboom and Stewart (1979) tested tolerance and compensatory responses to morphine by measuring the changes in their rectal temperature under three different environmental conditions: home cage, pre-injection context, and injection context. Morphine tolerant rats showed two different conditioned responses in the absence of morphine in two different environmental conditions. First response was conditioned anticipatory hypothermic response in the pre-injection area before drug injection. This anticipatory response was a counter directional response to initial hyper

thermic morphine action that adjust the equilibrium of body temperature and leads to tolerance. Second response was iso-directional hyper thermic response to drug injected context in the absence of morphine. This second response confirmed the evidence of stimulus substitution theory as Pavlov observed in 1927. However, it is at odds with the findings of Siegel (1975), which showed CCRs in the drug contingent context in the absence of morphine.

2.3 Summary

In this chapter, we reviewed the main tolerance theories and studies that attempted to explain the underlying behavioural mechanism of tolerance. Tolerance is characterised by a decrease in the physiological effects of a drug, so that a) larger doses are needed in order to achieve similar effects (Kalant, 1998); or b) the initial dose produces less effects with repeated administration. In particular, three different kinds of tolerance have been identified, on the basis of the number of exposures to the drug. Acute tolerance happens within the administration of a single dose of the drug: the physiological effects of the drug at a given concentration are smaller when looking at the descending portion of the drug's blood concentration —relative to the same concentration in the ascending portion of the curve (e.g., Perkins et al., 1991). Rapid tolerance is observed as less effect of the drug during a second administration of the drug, usually given between 8 to 24 hours after the first; in contrast, chronic tolerance is that observed after multiple—usually 3 or more—administrations of the drug (e.g., Stolerman et al., 1973). It is this chronic tolerance which is the focus of the present study. Two main experimental criteria were used in the present study for assessing chronic tolerance: 1) chronic exposure phase (a gradual reduction in the effect of drug with repeated drug exposure) and 2) tolerance during test phase (less

effect of nicotine on experimental group having history of chronic drug exposure than control group tested nicotine for the first time).

The aforementioned tolerance studies provide different sources of evidence for tolerance development: repeated drug exposure reduces the initial effect of the drug and decreases unconditioned responses (URs) to the drug. However, the direction of the conditioned responses (CRs) following the termination of drug presentation were different across these studies. For example, some studies found opposite directional conditioned (compensatory) responses (Solomon, 1980; Siegel, 1975); however, other studies found iso-directional conditioned responses (Eikelboom & Stewart, 1979) after the drug termination. Furthermore, Tiffany et al. (1983) did not observe CRs after drug termination; therefore, it is suggested that CCRs is not an essential component of drug tolerance.

Tolerance as a habituation theory is derived from Wagner's priming theory of habituation (Wagner, 1976). According to Wagner's model, habituation (and hence tolerance) occurs due to the action of either of two mechanisms: associative priming and self-generated priming (see Wagner, 1976; and Prados et al., 2020, for a detailed explanation). Associatively generated priming enables environmental cues associated with drug effects to attenuate, in the long-term, the unconditioned effects of drugs, resembling the well-known diminution of unconditioned effects observed in basic learning procedures (Kimmel, 1966). Self-generated priming allows a representation of the drug effects to be primed in short-term memory by a previous drug exposure and reduces the unconditioned effects of drugs. However, it is important to highlight that these researchers used measured different responses to assess the development of tolerance and CCRs to the analgesic effect of morphine across these studies, such as paw-licking latency regarding pain-sensitivity on the hot-plate (Siegel, 1975), frequency of the jumping

response to an electric shock (Tiffany et al., 1983), and rectal temperature changes around the morphine injection (Eikelboom & Stewart, 1979). Cunningham (1993) highlighted that the diverse response systems might be ruled by various mechanisms. Therefore, one possible reason for these differences might be the different measurement systems researchers used across these studies. Overall, these contradictory results in the tolerance literature suggest that tolerance is a complex mechanism, and only a single theory of drug tolerance cannot explain the development of tolerance and compensatory responses as a whole.

3 Chapter 3: Nicotine Tolerance and Withdrawal in Vertebrates

Drug tolerance is manifested as a reduced sensitivity (and hence unconditioned response) to a drug with repeated administration. It is also defined as an adjustment/ adaptation to the effectiveness of drug and drug-induced disturbances (Stewart & Badiani, 1993). In the previous chapter, we reviewed a range of behavioural theories of tolerance development and also studies investigating this phenomenon (see table 2.1). Of note, some studies (Siegel, 1975; Siegel, 1999; Sherman, Strub & Lewis, 1984) found that tolerance is highly related to withdrawal symptoms (Hughes & Hatsukami, 1986). Drug tolerance is an outcome of the development of the opposite reaction to the unconditioned effect of a drug, which attenuates the observed behavioural response to the drug. This compensatory adaptive response (or conditioned compensatory response; CCR) also manifests as withdrawal and craving response following unexpected discontinuation of drug administration when cues previously associated with the drug effects are presented.

However, other theories have suggested that drug tolerance is an outcome of the priming of the drug-related memory, and that compensatory adaptive responses are not an essential component drug tolerance (Baker & Tiffany, 1985). The priming of the drug-related memory determines how drug is surprising (failure of tolerance) or expected/primed (observation of tolerance), which might happen with or without reliable drug contingent cues. These theories are mainly based on morphine and ethanol studies (see table 2.1). However, little is known about the development of chronic tolerance to nicotine and its relationship with withdrawal.

Nicotine is the main component of tobacco that makes smoking addictive. Continuous nicotine exposure causes adaptive changes that lead to tolerance. As addiction to other drugs of abuse, nicotine addiction is a chronically relapsing disorder (DiFranza & Wellman, 2005). Multiple studies have observed that tolerant organisms are affected

less from nicotine exposure than non-tolerant organisms. Also, cessation of smoking causes severe withdrawal symptoms in tolerant organisms such as anxiety, stress and irritability (Hughes et al. 1991). Relief from withdrawal distress is believed to be one of the main drivers for the resumption of smoking (i.e., relapse) and tobacco addiction. In this chapter we will review nicotine tolerance studies using vertebrates. We focus on 1) acute and chronic effects of nicotine with various behavioural and physiological responses; 2) the relationship between tolerance and withdrawal responses; and 3) prolonged (i.e., chronic) nicotine tolerance.

3.1 Acute and Chronic effect of nicotine

In one of the earliest studies, Domino & Lutz (1973) assessed acute and chronic effects of nicotine (0.025 mg/kg) on operant behaviour of bar pressing in rats for water reinforcement. Initially, animals were trained to press a bar 15 times in order to gain access to water for 4 seconds. Animals received a nicotine injection twice per day for a period of 15 days. The results showed that the initial nicotine administration significantly impaired the initial development of the operant response; however, repeated nicotine injections for 15 days led to a gradual increase in the magnitude of the operant responses. The initial nicotine injection (day 1) suppressed the reaction for 35 min. Then the delay of bar pressing response was reduced over the days of nicotine injection and came to zero on the final day of final the treatment (day 15). These results revealed a gradual reduction in the pharmacological action of nicotine over repeated treatment, suggesting the development of tolerance to the initial effect of nicotine.

Stolerman et al. (1973) examined acute and chronic tolerance (1 mg/kg) to the depressant action of nicotine by measuring locomotor activity in rats performing in a Y-shaped runway apparatus. The number of entries to the arm of the apparatus and the

amount of rearing behaviour were measured as dependent variables (DVs) to assess changes in the sensitivity to nicotine. Acute nicotine treatment in non-tolerant rats showed a significant reduction in the number of these two behavioural responses in a dose-dependent manner (which is similar to the depressant effect of nicotine): larger doses produced lower motor activity.

Clarke and Kumar (1983) tested the acute effect of nicotine in rats. Rats were injected with different nicotine doses (0.1, 0.2, 0.4 mg/kg and saline) and the changes in their locomotor activity in photocell cages was measured for 80 min (4 x 20 minutes bins) immediately after the injections. The results of the acute challenge showed that locomotor activity of animals treated with saline considerably and gradually declined by the time of the test. However, nicotine treated animals showed a sudden decrease within the first 20 min (suggesting ataxia and depressant action) which was greater than that observed in the saline group. Then they showed higher locomotor activity than saline group until the end of the test (suggesting a stimulant action) in a dose-dependent manner. In other words, acute nicotine challenge elicited bi-phasic locomotor response in a dose-dependent manner. Additionally, co-injection of mecamylamine (0, 0.5 and 1.0 mg/kg) with nicotine (0, 0.4 mg/kg) eliminated the depressant behavioural action of nicotine (observed in first 20 min) in a dose-dependent manner; however, mecamylamine alone did not elicit significant behavioural change. In other words, mecamylamine co-injection antagonised behavioural depressant action elicited by acute nicotine challenge.

Pauly et al. (1992) assessed the development of tolerance to nicotine (2.0 mg/kg) by using a classic experimental-control group design with male mice. This study measured two behavioural responses; Y-maze crosses, and rearing, and three physiological responses: body temperature, heart rate and plasma corticosterone (CCS). Animals in the experimental group were injected with 2.0 mg/kg nicotine, and those in

the control group were injected saline three times per day for a period of 12 days. On the following day, behavioural and physiological sensitivity to nicotine was tested. The results showed that animals which received chronic nicotine pre-treatment were less sensitive to the acute nicotine challenge for Y-maze crosses, Y-maze rears, heart rate, and body temperatures. Also, repetition of nicotine challenge following two weeks of nicotine cessation showed that animals that previously received chronic nicotine pre-treatment were still tolerant to nicotine relative to the saline group. The plasma CCS level was also tested during 0, 4, 8, and 12 days of chronic nicotine injection, and 15 days following nicotine cessation. The results showed that the plasma level of the experimental group was not different than the saline group on days 0, 4 and 8 during the chronic injection regimen. However, the plasma CSS level was significantly increased for the experimental group on the final day of chronic injection in relation with the development of nicotine tolerance. This tolerance-associated CCS increase was still significant 15 days following nicotine cessation. Overall, chronic nicotine injection resulted in the development of tolerance to the behavioural and physiological effect of nicotine. Also, tolerance to nicotine sustained even after two weeks of nicotine cessation.

Irvine et al. (2001) tested the development of tolerance to the anxiolytic and anxiogenic effects of nicotine (0.1 mg/kg). Elevated plus-maze test of anxiety was used to assess: 1) the percentage number of open arm entries; and 2) the percentage of time spent on the open arms. Forty-eight animals were randomly divided into three groups: vehicle, acute nicotine and chronic nicotine (pre-treated with nicotine for seven days). Half of the animals in each group were tested 5 minutes after the nicotine injection to measure anxiolytic effect of nicotine; and the other half was tested 30 min after the nicotine injection to measure the anxiogenic effect of nicotine. The behavioural test after 30 min of the nicotine injection showed that there was a significant reduction in both

responses after the first nicotine exposure (in the acute group), suggesting the anxiolytic effect of nicotine. However, the nicotine-induced anxiogenic response did not change in the chronic nicotine group, suggesting the evidence of tolerance to only the anxiogenic effect of nicotine after chronic pre-treatment.

Additionally, the behavioural test after 5 min of the nicotine (0.1 mg/kg) injection (Irvine et al., 2001) revealed that the chronic nicotine group performed higher (both entry and time responses) than the acute and the vehicle groups, suggesting the anxiolytic effect of nicotine. However, there was no difference between the behaviour of the acute nicotine and the control groups. Since the anxiolytic effect was detected for the first time after 7 days of pre-treatment, researchers applied a longer nicotine pre-treatment (two weeks) with another group of animals to test the development of tolerance to the anxiolytic effect of nicotine. The results showed that the behavioural response of the group which received two weeks of nicotine pre-treatment was not different from the reaction of the vehicle and acute groups. This result was interpreted as tolerance to the anxiolytic effect of nicotine by the authors. This interpretation of tolerance was open to discussion because previous drug tolerance studies showed that tolerance is developed to the initial effect of the drug (Siegel, 1975; Siegel & Larson, 1998). However, tolerance to the anxiolytic effect of nicotine in this study was not to the initial effect of the drug (day1), it was to the effect observed after a week (day7). Therefore, tolerance to the anxiolytic effect of nicotine in this study is open to discussion.

The acute effect of nicotine (1mg/kg) was investigated with an attention task (five-choice serial reaction time task) on rats (Stolerman et al., 2000). They used a nose-poke task for food reinforcement after the presentation of a light. Correct responses, omission errors and reaction times were used as DVs. The results showed that a nicotine injection produced a dose-dependent increase in correct responses and decrease omission errors

and delay of reaction time. These results suggested that nicotine improved cognitive and psychomotor performance as a result of increased attention. However, this study did not examine the chronic effect of nicotine on attention.

3.2 Tolerance and Withdrawal Response

Acute and chronic tolerance to nicotine was measured by activity in rats (Stolerman et al., 1973). Three groups of rats were used as following: 1) single nicotine exposure group that received nicotine (1mg/kg) injection 5 hours before the nicotine test; 2) repeated nicotine group that received nicotine (1mg/kg) injection three times per day for a period of 3 days; and 3) saline group that received repeated saline injection three times per day for a period of 3 days. Then, these three groups were given a 3 min behavioural test in a Y-shaped runway apparatus 5 min after the saline or nicotine injection. The number of rears and entries into the arms were counted. The behavioural entry results showed that animals in the acute and chronic nicotine groups did more entries than the saline group, suggesting a depressant action of nicotine. Although the chronic nicotine group seemed less sensitive (more entries) to the effect of nicotine than acute nicotine group, this difference was not statistically significant. Additionally, re-test with nicotine after seven days of abstinence showed no significant difference between all groups. Furthermore, the results of the rearing responses showed evidence of both acute and chronic tolerance to nicotine. In other words, animals in the chronic and acute groups performed greater rear responses than the saline-treated animals; also, the difference between acute and chronic groups was significant. However, the second tolerance test following seven days of rest (nicotine cessation) did not reveal evidence of acute tolerance but there was evidence of chronic tolerance. Furthermore, the saline tests following acute and chronic nicotine treatment did not produce compensatory (i.e., withdrawal)

responses. Overall, this study showed the development of chronic tolerance to the unconditioned nicotine effect, which was independent from the withdrawal responses, a finding which is consistent with habituation theory of tolerance (Baker & Tiffany, 1985)

Stolerman et al. (1973) also examined prolonged chronic tolerance after cessation of nicotine (1mg/kg) treatment. Rats were treated with saline or nicotine (1 mg/kg) 3 times per day for a period of 8 days. Then, they were tested with nicotine and water after 5, 20 and 80 days following the cessation of nicotine exposure. The number of entries into the arms of the Y maze was counted. The nicotine test result showed the evidence of prolonged tolerance after 5, 20 and 80 days of abstinence, while the nicotine tolerance after 80 days of cessation was weaker. The saline tests results showed no evidence of withdrawal responses, which is again consistent with the habituation theory of tolerance (Baker & Tiffany, 1985). Furthermore, the length of nicotine pre-treatment positively associated with the development of nicotine tolerance. Because tolerance was eliminated after seven days of cessation following three days of nicotine pre-treatment (see Stolerman et al., 1973, Experiment 2); it was still significant after 5, 20, and 80 days of cessation following eight days of nicotine pre-treatment (experiment 4; Stolerman et al., 1973). Overall, tolerance to nicotine persists long after nicotine cessation but without much evidence for withdrawal responses.

Additionally, Irvine et al. (2001) tested the abstinence-induced withdrawal responses by saline injection to tolerant rats that previously received six days of nicotine pre-treatment. Animals spent less time in the open arms and performed less open-arm entries; this outcome was interpreted as an anxiogenic withdrawal response to the absence of nicotine. However, a very small dose of nicotine (5 ng) injection after withdrawal, which did not induce any behavioural response in non-tolerant rats, reversed the withdrawal response in tolerant rats. These results suggest that that adaptive changes to

nicotine result in hyposensitivity to nicotine when it is present, and hypersensitivity when it is absent.

Finally, Clarke & Kumar (1983) tested for withdrawal following chronic administration of nicotine. Two groups of animals were trained and tested in four phases. Initially, they received four weeks of daily nicotine (0.4 mg/kg) or saline injections, and both groups were tested with nicotine once in every week (Phase 1). Then, they were tested with five different nicotine concentrations (0, 0.1, 0.2, 0.4 and 0.8 mg/kg) every three consecutive days (Phase 2). After that, the animals were tested following a saline injection after 1, 4, 8, 15 days of nicotine cessation (Phase 3), and finally were tested with nicotine on the 23rd day following of nicotine cessation (Phase 4). The result of phase 1 showed evidence of tolerance to the depressant effect of nicotine a week after daily nicotine injections. Phase 2 (nicotine tests) results revealed that the depressant locomotor action of nicotine subsided into stimulative locomotor action for the nicotine group. The results of the saline tests in Phase 3 showed no difference between saline and nicotine treated groups. However, it is important to note that the animals in the saline group were not naïve to nicotine because they had already been exposed to nicotine during the test trials, which might be the reason for the failure to observe evidence of the withdrawal symptoms. When re-tested with nicotine after three weeks of nicotine cessation in Phase 4, animals in the nicotine group were still tolerant to the depressant action of nicotine. This result revealed tolerance to nicotine even after nicotine cessation. Furthermore, tolerance to depressant action of nicotine was prevented by mecamylamine co-administration. Overall, this study showed the evidence of tolerance to depressant behavioural action of nicotine during and after daily nicotine injection, which was eliminated by mecamylamine co-administration.

3.3 Summary

In summary, the aforementioned nicotine studies suggested that acute nicotine treatment elicits depressant activity in several behavioural and physiological response systems. However, repeated nicotine treatment leads to a reduction in the depressant effect of nicotine. Blocking nAChRs, for example with mecamylamine, plays an essential role in the attenuation of both the acute and the chronic effect of nicotine. Chronic tolerance can persist long after cessation of nicotine. Finally, withdrawal symptoms seem not to be an essential component of chronic nicotine tolerance, as Stolerman et al. (1973) and Irvine et al. (2011) showed no withdrawal responses in the presence of tolerance to nicotine's action, which is consistent with the habituation theory of tolerance (Baker & Tiffany, 1985)

4 Chapter 4: Invertebrates as a Model for Addiction Studies

In drug addiction studies, vertebrates (e.g., rats and mice) are generally used as an alternative model for humans (Badiani, Caprioli & De Pirro, 2019 ; Buttarelli et al., 2008). There is, however, a growing interest in the use of invertebrate animals in learning and addition studies. Numerous studies have suggested that a diversity of invertebrate species learn in standard conditioning tasks including arthropods like *Drosophila* (Devineni & Heberlein, 2009) and the honeybee *Apis mellifera* (Abramson et al., 2004), molluscs like the crayfish (Nathaniel et al., 2010), the *Aplysia* (Chen et al., 2014), and plathyhelminthes like the planaria (Prados et al., 2013). In addition, some invertebrate species used in research of learning, memory and addiction do share some characteristics with vertebrate species: they have centralized nervous systems (CNS), similar neurotransmitter systems, and similar learning and memory basic abilities (Sarnat & Netsky, 1985). Therefore, invertebrates can be considered an alternative model to understand the behavioural and pharmacological mechanisms of addiction.

4.1 Scientific Potential of Planaria for Studying Addiction

Planaria have been suggested as a valuable model for behavioural and biochemical studies to investigate drug abuse (Pagán et al., 2009; Palladini et al., 1996; Passarelli et al., 1999; Umeda et al., 2005). Despite its small size compared with vertebrates, they are members of the platyhelminth phylum, the most distant phylum from vertebrates that presents a centralised nervous system (Sandmann et al., 2011). The planarian's nervous system has been described as the ancestor of the vertebrates' brain (Sarnat & Netsky, 1985).

Sarnat & Netsky (1985) have also highlighted the notable similarities between the brains of humans and planarians, stating that "several neurotransmitter substances identified in the human brain also occur in the planaria nervous system" such as dopamine

(DA), serotonin (5-HT), acetylcholine (Ach), and gamma-aminobutyric acid GABA (see also Buttarelli et al., 2008; Rawls et al., 2008, 2011). Also, planarians brains show the presence of dendritic spines which are a putative site of memory storage (Turel et al, 2020). Planaria show neurobiological vertebrate-like features that make them an interesting model for animal's cognition and drug addiction research.

The existence and role of neurotransmitters can be assessed by using standard techniques like High Performance Liquid Chromatography (HPLC). Using this technique, studies have shown in planaria that there is a close relationship between the changes in neurotransmitter content and behavioural responses. For example, Itoh & Igarashi (2000) investigated circadian rhythms in planarians. Animals were kept under a 12-12 hours light-dark cycle. Serotonin levels were measured at 4-hour intervals using HPLC. Results showed that serotonin levels were higher during the light cycle than in the middle of the dark cycle, which is the same as in other vertebrates. This revealed a measurable role of the serotonergic system in the circadian rhythms in the planaria. In addition, the presence of different neurotransmitter systems in planarians has been revealed by their behavioural sensitivity to different drugs and selective receptor ligands. In the following section, I will describe some of these studies.

4.1.1 Acute Exposure Pharmacological Studies with Planaria

4.1.1.1 Dopaminergic and Cholinergic Systems

The striking similarity between the neurotransmitters systems (i.e., dopaminergic, cholinergic) of mammalians and planarians make the later a good model for the screening of compounds relevant to drug abuse research. Several studies have characterised distinctive stereotypical behaviours in planaria (Buttarelli et al., 2000; Farrell et al., 2008). For example, stimulation of dopaminergic and cholinergic receptor systems results

in a variety of distinctive behavioural responses such as C-like positions, screw-like hyperkinesias, and bridge like positions.

A pharmacological study (Palladini et al., 1996) investigated the behavioural responsiveness to manipulations of the dopaminergic system in planaria. This study characterised distinct behavioural patterns produced by stimulation of D1 and D2 receptors. Selective stimulation of D1 dopamine receptors elicited screw-like hyperkinesia (SCH), whereas selective stimulation of the D2 dopamine receptor elicited C-like hyperkinesia. Dopaminergic antagonists partially attenuated these hyperkinesia behaviours. Exposure to the dopamine reuptake inhibitor, nomifensine, which increases dopaminergic transmission, elicited both C-like and SCH (screw-like) behaviours. Pre-treatment with a D2 antagonist inhibited C-like hyperkinesia responses, but not SCH responses. On the other hand, pre-treatment with a D1 antagonist eliminated the SCH action elicited by nomifensine, but not the C-like responses. These results suggest good behavioural sensitivity to the effects of selective D1 and D2 antagonists.

The role of the dopaminergic system in the control of stereotyping behaviours has also been addressed in studies assessing the behavioural effects of acute exposure to mephedrone, which increases dopamine release (0-1000mM; (Ramoz et al., 2012)). C-like stereotypical behaviours and motility were measured during a 10 min exposure to the drug. The results showed that acute mephedrone administration significantly reduced locomotor activity and increased C-like stereotypical behaviours compared to control animals. These stereotypical behaviours were dopamine-sensitive because treatment with selective D1 antagonist SCH23390 (0.3 mM) in combination with mephedrone significantly attenuated mephedrone-induced stereotypical behaviours.

Nishimura et al. (2010) investigated the role of cholinergic neurons on the control of locomotion in planarians. Planarians treated with the cholinergic agonist

physostigmine (10 mM), showed increased muscle contraction behaviour. However, pre-treatment with Ach receptor antagonists, tubocurarine (100 mM) and atropine (10 mM) two hours before the treatment with physostigmine weakened the muscle contraction responses, extending the latency for the contraction response.

Buttarelli et al. (2000) also addressed the role of the cholinergic system in the behavioural control of the planaria. Animals were treated with nicotine, a cholinergic nicotinic receptor agonist, and they measured the response latency and intensity to the effects of the drugs, as well as its post-effects. Nicotine-induced walnut position (WLP) responses were registered. The nicotine effect was first observed 15 minutes after the exposure to a lower dose of the drug (20 and 50 mg/ml) and that effect remained for 10 minutes after the animals were withdrawn from the drug. However, the nicotine effect was faster, in that the behavioural response appeared 10 minutes after exposure, and lasted for longer, 15 minutes after withdrawal from the drug at higher doses (100 and 500 mg/ml). These results suggest that the behavioural effects of nicotine on motor behaviours are dose dependent. Additionally, exposure to higher nicotine concentrations increased the duration of the response of WLP, the duration of post effects, and shortened the latency of the nicotine effect. Of note, post effects of the drug after drug removal was interpreted as "accumulation of neuroactive substances inside flatworm."

Rawls et al. (2011) tested the effect of different nicotine concentrations measuring the number of C-like hyperkinesias and locomotor activity (grid-line crosses) in five-min intervals. Acute exposure to nicotine increased hyperkinesia behaviours of animals, but reduced their locomotor activity in a concentration dependent manner compared to a control group that received water treatment. This study shows that increased hyperkinetic behaviour is associated to a reduction in the locomotor activity of the planaria. In addition, Pagán and colleagues (2015) investigated the effect of the cholinergic nicotinic antagonist

curare (1 mM) on nicotine-induced seizure-like movements. Nicotine treatment with different doses (0 - 2 mM) elicited concentration-dependent seizure-like movements; however, curare attenuated these movements, suggesting that the effect of nicotine depended on activation of cholinergic receptors. In summary, these studies with cholinergic drugs show that the functions cholinergic nervous system is conserved in planaria and it is similarly sensitive to cholinergic antagonists as it observed in rodents (Clarke & Kumar, 1983).

Buttarelli et al. (2000) also investigated the interaction between cholinergic and dopaminergic systems in the planaria. Animals were treated with dopaminergic agonist such as nomifensine (5-50 mg/ml) and apomorphine (2.5-10 mg/ml), and these elicited screw-like behaviours; however, pre-treatment with acetylcholinesterase inhibitor physostigmine (20 mg/ml) prevented the behavioural effect of nomifensine and reduced the intensity of behavioural changes induced by apomorphine. Moreover, physostigmine elicited bridge-like behavioural responses; however, pre-treatment with the dopamine agonist apomorphine reduced the intensity of the observed behaviours. Finally, the cholinergic antagonist atropine (1 mg/ml) produced screw-like behaviours; however, pre-treatment with SCH23388 (1 mg/ml), a classic D1 dopamine antagonist, reduced the intensity of atropine-induced hyperkinesias. These results show that planaria have both dopaminergic and cholinergic neurotransmitters systems, and these two interact so that stimulation of one system can reduce the transmission of the other.

4.1.1.2 Serotonin and Opioid Systems

Farrell et al. (2008) investigated the locomotor effect of serotonergic agonists (8-OH-DPAT and mCPP) and antagonists (WAY-100635) in planarians. Exposure to serotonergic agonists produced seizure-like behaviours and decreased locomotor activity in a dose-dependent manner. Pre-treatment with the serotonergic antagonist attenuated

these abnormal responses and reversed the effect of agonists (hypoactivity). This study concluded that 5-HT receptors are present in the planaria and are involved in the control of the planaria's locomotor activity.

Buttarelli et al. (2002) assessed the interaction between cannabinoid and opioid systems in the planaria. Spontaneous motor activity and stereotypical responses were measured for 20 min in plain water and also in the presence of a cannabinoid agonist, WIN55212.2 using different concentrations (20 – 250 mg/ml). Drug treatment increased motor activity and induced stereotypical responses. Pre-treatment with a cannabinoid antagonist drug, SR141716A (50 mg in 3 ml water), attenuated induced motor and stereotypical responses but did not cause any behavioural changes alone. However, pre-treatment with the opioid antagonist naloxone (30 or 100 mg) weakened induced motor responses in a dose-dependent manner. These results showed the existence of a functional interaction between the opioid and the cannabinoid receptor systems in the planaria.

As mentioned above, the existence of a serotonin system in the planaria has been confirmed by using HPLC (Itoh, 2005; Umeda et al., 2005). In conclusion, the aforementioned planaria studies with acute exposure to different drugs targeting different neurotransmitter systems demonstrated that mammalian-like neurotransmitter systems are well conserved in planaria and they function in a similar way as they do in vertebrates. Therefore, the planaria can be considered a useful model for the screening of compounds relevant to drug-induced physiological challenges such as those observed with drugs of abuse, and to study the pharmacology and behavioural control of various neurochemical systems (Pagán et al., 2009; Farrell et al., 2008).

4.1.2 Acute Withdrawal Responses with Planaria

Withdrawal from drugs of abuse like nicotine after acute and chronic exposure is associated with the display of some atypical behaviours both in mammals and planarians; we typically refer to this behavioural pattern as drug-induced abstinence behaviour. The abstinence-induced behaviours have been frequently been used to assess physical dependence to drugs of abuse such as morphine (Grisel et al., 1994; Siegel, 1975), ethanol (Larson & Siegel, 1998; Lê et al., 1987), caffeine (Rozin et al., 1984) and nicotine (Pomerleau et al., 1983)

Raffa & Desai (2005) tested abstinence-induced behaviours in planaria with cocaine. Animals were tested with water or cocaine after overnight (18-24 h) cocaine exposure. Changes in locomotor activity were measured three times after withdrawal: at 0-5, 30-35 and 60-65 min. Animals tested with water after overnight exposure showed atypical behaviours such as: "'HeadBop' ('nodding' movement of head while moving forward), 'Squirming' (uncoordinated, 'jerky' movements), 'Clinging' (scrunching, typically intertwining with another planaria), 'HeadSwing' (axial rotation of head about long axis 'helicopter' motion—while tail is anchored), 'TailTwist' (tip of body twisted, usually accompanied by decrease in locomotor activity) and 'Corkscrew' (spiral motion around long axis)" (Raffa & Desai, 2005, pp 201). These abnormal behaviours were related to the omission of cocaine, because control animals tested with cocaine following cocaine or water pre-treatment, and animals tested with water following water pre-treatment, did not display them. It is important to note that these withdrawal behaviours were intense within the first five min of withdrawal and were then attenuated over the rest of withdrawal period (up to 65 minutes).

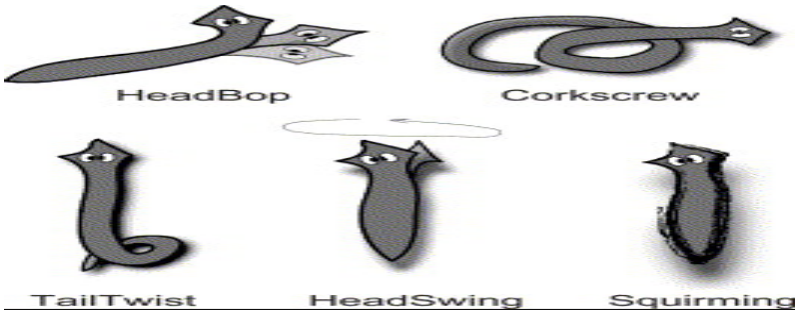


Figure 4. 1. A systematic illustration of atypical behaviours of planaria in water following cocaine exposure (Raffa & Desai, 2005).

Rawls et al. (2011) assessed nicotine-induced withdrawal behaviours. They measured changes in the locomotor activity of planaria for 5 min. Four groups of animals were pre-treated and tested in four different conditions: water-water, water-nicotine, nicotine-nicotine and nicotine-water (withdrawal group). Although the locomotor activity was not affected in the control groups, discontinuation of nicotine exposure following 60 minutes of nicotine pre-treatment (0.03 mM) reduced locomotor activity. The reduced locomotion was interpreted as a sign of withdrawal distress.

Additionally, Raffa et al. (2013) examined how opioid and nicotinic receptor types were involved in withdrawal behaviour related to nicotine's absence. Withdrawal behaviours were measured for 5 min in water following 60 min pre-treatment with nicotine or nicotine co-incubation with acetylcholinergic and opioid receptors antagonists. Discontinuation of nicotine (nicotine-water) significantly reduced the locomotor activity of planarians compared to three control groups (water-water, nicotine-nicotine, and water-nicotine). However, co-incubation with nAchRs antagonist mecamylamine (50 mM), opioid receptor antagonist naloxone (10 mM), selective MOR antagonist CTAP (10 mM), and selective DOR antagonist naltrindole (10 mM) attenuated nicotine-induced withdraw response. These results suggest that the withdrawal behaviour are related to nicotinic receptor activation (for it was blocked with mecamylamine) and

further suggest the involvement of opioid receptor, as opioid antagonists also attenuated the negative motivational state due to withdrawal from nicotine.

Ramoz et al., (2012) assessed withdrawal behaviours with mephedrone (1 mM) in planarians by using the same experimental design as Raffa et al. (2013). Withdrawal behaviours were assessed by measuring motility of animals when animals were tested with mephedrone or vehicle for 5 min after 60 min of pre-treatment with mephedrone or vehicle. Discontinuation of mephedrone (mephedrone-water) elicited reduction in locomotor activity compared to three control groups (water-water, mephedrone-mephedrone, and water-mephedrone). Additionally, similar results regarding withdrawal behaviours were observed with 10 mM mephedrone.

In conclusion, the studies reviewed in this section suggest that withdrawal behaviours can be observed after a single (i.e., acute) drug exposure. This is somewhat at odds with the observation in addicts and mammalian models of drug abuse, which tend observed withdrawal after chronic exposure. That is, rodent studies suggest that withdrawal behaviours are the outcome of physical dependence and that it happens after adaptation to a given drug following chronic exposure (e.g., Siegel, 1975; 2008). Thus, multiple drug administrations might be needed for the development of physical dependence. It is also important to note that the withdrawal responses in these studies were in the same direction with the unconditioned effect of the drugs used. Therefore, the changes in behavioural responses after discontinuation of a single exposure might be conceptualised as a post-effect of drug exposure because: 1) animals were tested immediately after a single drug exposure; and 2) the responses were not compensatory to unconditioned drug effect as previous drug tolerance and withdrawal theories have shown (Siegel, 1975; Solomon, 1980)

4.2 Pavlovian Conditioning and Planaria

Pavlovian conditioning is a learning preparation in which an animal is typically exposed to a conditional stimulus (CS) closely followed by the presentation of a biologically relevant event, the unconditional stimulus (US). This learning procedure enables subjects to predict the occurrence of the outcome (the US) with the presentation of CS. Planarians have been suggested to be a good model for learning and memory studies. An early experiment by Thompson and McConnell (1955) showed an example of classical conditioning in planaria. Researchers used one Experimental group, trained with a 3-sec light (the CS) and the presentation of a 1-sec shock (the US) in the last sec of the light stimulus. Two control groups were used in this experiment: the Light Control group received 3 sec of light (and no US); and the Response Control group received neither the light nor the shock. All animals received 3 blocks of 50 light-shock trials; the inter trial interval (ITI) was 20 sec, and each block was separated by a 5 min interval. Longitudinal contraction responses during the first two seconds of the light were measured as the conditioned responses (CRs). Thompson and McConnell (1955) found that the frequency of the CR significantly increased during training for the Experimental Group compared to the Light Control and Response Control groups. This result suggests that CRs can be established in planarians like in vertebrates.

Baxter & Kimmel (1963) investigated the conditioning and extinction of CRs in planarians using the procedure developed by Thompson and McConnell (1955). Animals were conditioned with light (CS) and shock (US) with a paired or unpaired design for 5 days of 50 CS-US trials. The paired groups received CS-US delivery in systematic paired order, but unpaired groups received CS or US first. Frequency of the longitudinal contraction response within the first two sec of the light was counted as CRs. The results showed that there was a gradual increase in the number of CRs for the paired group

compared to the unpaired group. When the light was presented alone without the shock during the extinction trials, the CRs of the paired group dropped to the level of the unpaired group. These results show evidence of acquisition and extinction of CRs in the planaria.

Kimmel & Garrigan (1973) further examined extinction learning in planaria. This study investigated the effect of the number of conditioning trials (150 vs 250) and CS-US intervals (2 or 4 sec) on the development and extinction of CRs (freezing response to shock) to a standard 60-w frosted bulb (CS) paired and unpaired design. Animals in the paired conditioning groups received daily 50 CS and 50 US pairings in a systematic order; however, animals in unpaired control groups received the same numbers of stimuli each day in unsystematic order. Conditioning results revealed that animals showed higher levels of CR in the paired than in the unpaired group, suggesting the development of CRs to the CS. The magnitude of the CR was also stronger with more conditioning trials (250) than with less trials (150). Extinction data results showed that the animals that received less conditioning trials (150) were more resistant (slower to extinguish) to extinction than animals that received more conditioning trial (250). These results suggest that more conditioning results in stronger expectation and its withdrawal leads frustration in animals. Therefore, the magnitude of CR for animals that received more conditioning trial (250) was even lower than the unpaired group. Moreover, conditioning with a shorter interval (2 sec) was more resistant to extinction than longer intervals (4 seconds) less paired group. Overall, this study shows how procedural differences (the number of conditioning trials, the length of the interval between events) play an important role in extinction learning in planarians as they do in vertebrate models like rats. Therefore, the planaria can be considered a good model for complex conditioning because the CRs of

planaria were also affected by procedural differences like observed in other vertebrate models.

More recently, Prados et al. (2013) examined basic Pavlovian conditioning as well as cue competition phenomena in planaria. In this study, an electrical shock (4.5 v) that elicited the unconditioned longitudinal contraction response was used as the US; they also used a light and vibration as CSs, using a procedure inspired by the original report by Thompson and McConnell (1955) but with significant parametric differences. The CS was presented for 10 sec and followed by shock for 0.5 sec during 20 conditioning trials, with a 5 min inter trial interval (ITI). There were two experimental groups: paired group and unpaired group. While the onset of light was regularly paired with the shock during conditioning in the paired group, the shock was presented randomly during conditioning in the unpaired group. The results showed the development of the contraction CRs in the paired group. Although there was an initial increase of the contraction response in the presence of the light in the control, unpaired groups, this early response habituated. This shows that the development of the CR in the group paired is a genuine example of Pavlovian conditioning suggesting that the animals learn to anticipate the shock-US in the presence of the light-CS contiguity. The remaining experiments assessed cue competition phenomena (blocking and overshadowing). Conditioning with a compound of two stimuli weakens the learning of one of the two stimuli, relative to a group that received training with the same stimulus but in the absence of a second stimulus, and this is called overshadowing (Pavlov, 1927). Animals showed weaker CRs to single elements (light or vibration alone) after compound conditioning with the shock-US than when the element had been paired with the shock alone. This result suggests that the elements overshadowed each other. Moreover, if one of the elements of the compound was pre-conditioned before compound conditioning, it blocked the learning of the added element,

an instance of blocking effect (see Kamin, 1969, for the original demonstration of blocking in rats). These results suggest a common learning mechanism across different species.

Planaria are known to be an excellent model for memory and brain regeneration. Their entire body, including the brain, can regenerate after head amputation. They can learn associations and store information for at least two weeks which is enough for brain regeneration. Shomrat & Levin (2013) tested the memory retention of planarians after head regeneration. Animals were trained to find food (a small drop of liver) in a rough-texture environment and then decapitated. The animals were tested after their head was entirely regenerated. The results showed that animals that had previously received training reached the food earlier than non-trained animals. This study suggests that memories can survive brain regeneration in planaria, and they are a good model to understand the dynamics of brain regeneration and long-term memory.

These aforementioned learning studies with planaria suggest they are capable of learning simple and complex procedures in pavlovian conditioning, similar to what has been observed in vertebrates such as the development and extinction of CRs, overshadowing and blocking phenomena. Learning principles are similar across the species of animals, and this ability is well conserved in the simple organism planaria. Therefore, planaria is an important model to investigate the evolutionary history of learning and comparative psychology.

4.2.1 Conditioned Place Preference (CPP) with Acute Exposure

Conditioned place preference (CPP) is a paradigm that evaluates the rewarding effect of drugs building on the concept of classical conditioning, by pairing drug effects with distinctive environmental cues (Tzschentke, 2007). Continuous drug presentation in a distinctive environment establishes cue-drug associations. The appetitive value of the

drug is evaluated by assessing the time subjects spend in the drug-paired contextual environment in a subsequent test in the absence of the drug (van deer Koy, 1987). Some researchers have carried out research in this area. Childs and de Wit (2013) tested the rewarding effect of amphetamine in humans using a CPP procedure. In this study, there were two different groups (paired and unpaired), and amphetamine and placebo were administrated in two different rooms. While people in the paired group continuously received the drug in one room and placebo in another room, their administration was randomised for the unpaired group (so that they would not be able to develop a preference for one of the contexts). By the end of conditioning phase, participants were asked which room they would like to stay next time. Most people in the paired group reported they would like to stay in methamphetamine-paired room. However, there were no differences between the rooms for participants in the unpaired group. These results suggest that CPP is a valid procedure for humans to test the rewarding properties of amphetamine.

Planaria studies have also assessed the rewarding effect of drugs of abuse after single drug exposure in a distinctive environment. In these CPP experiments, researchers used biased CPP protocol in which the animals develop a preference for an area that was initially aversive (brightly illuminated) because the planarians are photophobic (Raffa et al., 2013). Given the choice, the animals avoid the light and stay in the dark side of a petri dish. Then, the aversive context was paired with salient drug, and in the final test the animals showed a preference for the brightly illuminated area (a reversed preference due to conditioned place preference). For example, cotinine is known to contribute the reinforcing effect of nicotine, and the rewarding properties of cotinine were assessed using a CPP protocol after single exposure in planarians (Phelps et al., 2019). Phelps et al. (2019) used two contexts, one brightly illuminated, and one kept in semi-darkness. Animals were then tested in a task in which they could choose between the light and dark

contexts. Test results showed that animals spend greater time in the cotinine-paired light area which was initially aversive compared to the dark area.

In a similar study, Ramoz et al. (2012) investigated the rewarding properties of mephedrone in planaria after a single exposure to the target context. Mephedrone presentation in the non-preferred light area for 30 min increased the time spend in the non-preferred area in a dose-dependent manner. This suggests that mephedrone has rewarding properties for the planarians that were increased with the dose. However, this CPP response might be a post-effect of the drug because animals were tested immediately after the single exposure. Therefore, there is a pressing need to look at CPP studies in planarians that involved chronic exposure, and where testing is conducted after a retention interval from training; this is the focus of the following section.

4.2.2 CPP after Chronic Drug Exposure

Hutchinson et al. (2015) tested the rewarding agents cocaine (1 mM and 10 mM) and mephedrone (1 mM and 10 mM) using a CPP paradigm in planarians. Initially, animals were placed on a two-sided petri dish with rough (sandpaper) and smooth (shiny white card) surface sides. The time spent by the animals in each side was recorded to identify their least and most preferred surfaces (rough or smooth). The conditioning phase lasted 10 days. On five of these days, the animals were exposed to either a cocaine or a mephedrone solution in their least preferred surface for 15 min; on the remaining days they were exposed to water in their preferred context. The drug and water days were alternated throughout the conditioning phase. The animals were then tested again in two-sided dishes to assess whether there was a change in preference, (a CPP response). The CPP response was tested three times, 2, 6, and 13 days after conditioning. The results showed that animals conditioned with cocaine spend more time on the cocaine-paired surface than in the water-paired surface on tests 1 and 3, suggesting the CPP response

mediated by cocaine was retained over a period of two weeks. However, the CPP using mephedrone as the rewarding agent was not significant. These results suggest that cocaine was an effective rewarding agent for CPP, and the memory was retained long-term (over two weeks) making the planarians comparable to vertebrate species.

Rawls et al. (2011) investigated CPP using nicotine (0.1 mM) as the rewarding agent in planarians. Initially, the animals were placed into petri-dishes in which half of it was dark, and the other half was illuminated (the light side). Since planarians are photophobic, most of them spend more time in the dark environment. Then, they were treated with nicotine in their less preferred surface (the light environment), and water in their most preferred side (the dark environment) during conditioning. Following this, they were tested again in the half-dark and half-light petri-dishes. The results revealed that, after conditioning, the animals spent more time in the light environment in which nicotine had been presented. Repetitive nicotine presentations in the less preferred side altered planarians place preferences. This shows that the planaria is good model to examine the rewarding properties of nicotine. Using the same CPP preparation just described, Raffa et al. (2013) investigated the rewarding effects of amphetamine, and the results were very similar to those reported Rawls et al. (2011).

Mohammed Jawad et al., (2018) investigated the development, extinction and reinstatement of CPP responses using as the rewarding agent a 10% sucrose solution. They found that repeated sucrose administration in the non-preferred area resulted in animals spending more time during a subsequent test on the non-preferred sucrose-paired area - thus revealing the development of a conditioned place preference with sucrose as a rewarding agent. However, this preference was reduced to the pre-conditioning levels with repeated extinction trials (with the two-sided dishes) in the absence of sucrose. Sucrose exposure in an alternative environment following the extinction reinstated the

sucrose related CPP response, another finding that replicates observations in rodents and humans (Rescorla & Heth, 1975). Additionally, Mohammed Jawad et al (2018) assessed the development of tolerance to sucrose by measuring the locomotor activity planaria. Animals received sucrose in a distinctive context (paired context) and water in another context (control context) in alternating days of conditioning. They observed that the hypo locomotive effect of sucrose gradually diminished with repeated exposure, suggesting the development of tolerance to hypo-locomotor effect of sucrose. Following conditioning, animals were tested with sucrose and water in the both sucrose-paired and alternative contexts. They were more tolerant to the effect of sucrose in the paired context, suggesting context-mediated tolerance. In addition, they observed hyperactive compensatory responses in the presence of the sucrose-paired context but not in the alternative context when they were tested with water, suggesting the development of conditioned compensatory responses (CCRs). Finally, this study showed that the development of sucrose mediated CPP is dependent on the dopamine reward system, because pre-treatment with the D1 dopamine receptor antagonist, SCH23390, blocked the development of CPP; on the other hand, the conditioned compensatory responses remain unaffected by the treatment with the dopamine antagonist. In summary, this study suggested that the rewarding properties of sucrose are controlled by the dopamine system, as it has been observed in vertebrate species; and thus, that planaria is a good model for pharmacological manipulations of the reward system. Overall, CPP studies with planarians have shown that the planaria is a good model to investigate rewarding effect of various drugs.

4.3 Chronic Exposure: Tolerance, Withdrawal and Sensitisation

Drug tolerance, sensitisation and withdrawal responses have been heavily studied with humans, vertebrates (e.g., rats and mice) and invertebrates in order to understand physiological, neurobiological, and behavioural mechanisms of drug addiction. As previously defined, tolerance is the diminished effect of a drug observed with repeated exposure. It is also defined as adaptation to a given dose of drug that can result in increased levels of consumption to achieve the same effects. Tolerance is highly correlated with withdrawal that appears after discontinuation of drug presentation (Hughes & Hatsukami, 1986). Sensitisation is the reverse of tolerance, and is defined as an augmented behavioural response to psychoactive drugs such as cocaine. These three phenomena are highly related to other psychological symptoms of addiction such as lack of control, excessive time invested in pursuing drugs, and persistence of drug taking despite adverse consequences (Søvik & Barron, 2013). These three phenomena have been tested with planarians to further our understanding of the basic mechanisms of addiction to various drugs.

Rawls et al. (2010) investigated behavioural sensitisation and cross sensitisation with cocaine (0.01 - 3 mM), glutamate (0.1 – 10 mM) and caffeine (0.1, 1 and 3 mM) at different concentrations. C-like hyperkinesia responses were measured during 1 and 5 min of drug exposure. Acute exposure to glutamate and cocaine produced concentration-dependent hyperactivity. Repeated exposure to those drugs enhanced hyperactivity, suggesting the evidence of behavioural sensitisation (twice on day 1 and final exposure was given on day 4). This behavioural sensitisation to cocaine and glutamate in planaria was linked to the duration of drug abstinence following initial exposure. The longer the abstinence was, the stronger the sensitisation was. Animals which received six days of abstinence after initial exposure to cocaine or glutamate before a second exposure showed

greater sensitisation than animals which only had two days of abstinence. This study also showed evidence of cross sensitisation. Cross-sensitisation is defined as enhanced behavioural response to one drug following exposure to another drug, and tends to lead to the inference that the two drugs share some mechanisms of action. Cocaine treatment after glutamate pre-treated produced behavioural sensitisation and vice-versa. This study highlighted that not all psychoactive chemicals cause behavioural sensitisation. For example, initial caffeine exposure produced concentration-related hyperactivity, but repeated exposure did not produce behavioural sensitisation unlike what was observed with cocaine and glutamate. Overall, this is the first study to observe that a drug of abuse produces behavioural sensitisation and cross sensitisation in planaria. Behavioural sensitisation to psychoactive drugs is conserved in the simple organism in planaria. However, this research can be questioned whether 1- or 5-min exposure is enough to assess drug action on CNS.

Hutchinson et al. (2015) investigated chronic effect of mephedrone and cocaine (1mM and 10 mM) on locomotor activity of planaria. Animals were conditioned with those drugs or water for five episodes and tested with water three times (2, 6, and 13 days) after conditioning. The conditioning data showed that initial exposure to the drugs at 1 mM concentration significantly reduced their locomotor activity and animals appeared to be tolerant to hypo-locomotor effect of cocaine and mephedrone with repeated exposure. Additionally, chronic withdrawal data showed that animals pre-conditioned with mephedrone at 10 mM concentration elicited hypo-locomotor withdrawal response compared to animals pre-conditioned with cocaine at 10 mM concentration and water. These results suggested tolerance to mephedrone and cocaine at lower concentration and withdrawal to mephedrone at higher concentrations. It is important to note that the tolerance results contradicted to other cocaine studies with planaria that showed that

repeated cocaine exposure cause locomotor sensitisation (Rawls et al., 2010). Additionally, chronic withdrawal response to mephedrone was at odds with Siegel's (1975) Pavlovian conditioning model of tolerance, because withdrawal response was at the similar direction to unconditioned effect drug.

Rawls et al. (2011) investigated sensitisation and tolerance responses to nicotine at different concentrations. C-like hyperkinesia responses were measured during 5 minutes of exposure to different nicotine concentrations or water within three different test sessions (twice on day 1 and final exposure was on day 4). This study revealed bi-phasic effects of nicotine regarding different concentrations. Repeated exposure to lower nicotine concentrations (0.1 and 0.3 mM) increased the stereotypical behaviours compare to initial exposure, suggesting behavioural sensitisation; however, repeated exposure to higher nicotine concentrations (1 and 3 mM) resulted in tolerance development, that is a reduction in the hyperkinesia behaviour. This research can be questioned whether 5 min exposure is enough to assess drug action on CNS.

Nicotinic Acetylcholine Receptors (nAChRs) both acts on the motor system that elicits contraction/seizure like responses and neural system that leads to substance of abuse and addiction. nAChRs are classified into two main groups: muscle-type and neural type (Corringer et al., 2000; Miller & Gotti, 2009). A recent study (Nishimura et al., 2010) investigated the role of cholinergic neurons on the function of motor behaviours of planaria. Administration of physostigmine (an AChE inhibitor, 3 μM - 10 mM) elevated the amount of Ach and induced a sudden muscle contraction response in concentration dependent manner. The latency of the contraction responses was measured as a dependent variable. Pre-treatment with tubocurarine (a muscle nAChR antagonist, 100 μM) (100 mM) and atropine (a non-selective muscarinic ACh receptor (mAChR) antagonist, 10 μM] (10 mM) two hours before the treatment with physostigmine weakened the muscle

contraction responses, extending the latency for the contraction response. However, pre-treatment with mecamylamine (a non-competitive antagonist of the nicotinic acetylcholine receptors) did not have any effect on the contraction responses. Therefore, these results suggested that contraction responses of planaria were modulated by muscle type nAChRs and muscranic acetylcholinergic receptor types but not by neural type.

Similarly, Clarke & Kumar (1983) tested the motor function of nicotine in rats. Rats were injected with different nicotine doses (0.1, 0.2, 0.4 mg/kg and saline) and the changes in their locomotor activity. Acute nicotine exposure reduced motility in concentration dependent manner; however, tolerance to the initial effects of nicotine was observed over the course of repeated exposure to nicotine. They also observed that pre-treatment with mecamylamine (a non-competitive antagonist of the nicotinic acetylcholine receptors) blocked the initial (acute) effect of nicotine. In another study (McCallum et al., 1999) observed that mecamylamine blocked the acute action of nicotine, and the development of tolerance. These two studies were conducted on rats, and suggested that both the acute effects of nicotine and the development of tolerance following chronic exposure depend on activation of nicotinic receptors.

Moreover, Sal and colleagues (2021) (see chapter 8) assessed whether mecamylamine, a nAChRs antagonist, attenuated the decreased motility caused by acute nicotine exposure and the development of tolerance caused by chronic nicotine exposure. Mecamylamine co-administration (0.05 mM) attenuated the effect of nicotine during the chronic exposure days. Additionally, tolerance development was significant across both tolerance tests with nicotine, and mecamylamine during chronic exposure successfully blocked the development of tolerance. These results confirm that nicotine-induced tolerance development depends on nicotine receptor activation, because mecamylamine blocked the development of tolerance, and also attenuated the acute effects of nicotine.

4.4 Summary

As outlined above, there is growing interest in the use of planaria to study pharmacological and behavioural effects of addiction. Despite its small size compared to vertebrates, they have well conserved mammalian-like CNS and functional neurotransmitter systems that show similar interactions as seen in vertebrates. Planaria shows basic and complex learning and memory phenomena, behaviours characteristics of drug abuse such as withdrawal, tolerance and sensitisation. Although planaria is a promising model for pre-clinical studies, the neurophysiology of invertebrates sometimes differs from the mammalian version. For example, cholinergic transmission is excitatory in vertebrates organisms, but in many invertebrates, acetylcholine increase chloride conductance which is inhibitory.

The purpose of the experiments that follow in this thesis was to assess 1) the development of tolerance to nicotine during repeated nicotine exposure in a specific context; 2) the expression of CCRs to nicotine-associated CS in the absence of nicotine; 3) the expression of nicotine tolerance in the presence of nicotine-associated cues; and 4) the role of a novel context on the expression of nicotine tolerance. The effect of different regimen of nicotine exposure on the development of tolerance was also investigated. The changes in neurotransmitter levels after acute and chronic nicotine exposure (such as dopamine and serotonin) were also assessed with High-Performance Liquid Chromatography (HPLC). Furthermore, the role of nicotinic Acetylcholine Receptors (nAChRs) on the acute and chronic effect (tolerance) of nicotine was examined with mecamylamine co-administration. Moreover, contribution of anticipatory responses with post-treatment with nicotine and nicotine after-effect with pre-treatment with nicotine were also examined. The important research questions are addressed across 4 experimental chapters (Thesis Chapter 6-9), as follows:

Chapter 6: The experiments outlined in Chapter 6 address tolerance and withdrawal behaviours with *Dugesia sp.* over intermittent or massive nicotine regimens. This includes the changes in locomotor activity (covered distance in cm) during and after exposure to nicotine. In addition, Chapter 6 assessed the role of different concentration on the development of tolerance in in nicotine-paired (where nicotine repeatedly presented) and novel context. Furthermore, these studies examined the association between neurotransmitter systems and acute vs. chronic effect of nicotine.

Chapter 7: The experiments in Chapter 7 asses the chronic effect of nicotine with two main regimens of nicotine exposure: 5-days and 10-days. The main objective of these experiments was to determine whether chronic exposure to nicotine (5-days and 10-days) result in 1) less effect of nicotine (i.e., tolerance to unconditioned effect of nicotine), 2) learned tolerance (i.e., context dependency and CCRs) using *Schmidtea mediterranea*.

Chapter 8: The experiments reported in Chapter 8 assess the conditioning (Seiegel, 1975; Solomon, 1980) and habituation (Baker & Tiffany, 1985) theories of tolerance development by monitoring the locomotor activities of *Schmidtea mediterranea* during nicotine exposure. We also assessed Conditioned Compensatory Responses (CCRs) following chronic exposure to nicotine to test the contribution of CCRs to chronic nicotine tolerance. We assessed whether whether mecamylamine, a nAChRs antagonist, blocks (or attenuates) the decreased motility caused by acute nicotine exposure and the development of tolerance caused by chronic nicotine exposure.

Chapter 9: The purpose of the meta-analysis reported in Chapter 9 was to obtain more accurate prediction regarding the effect of context on the development of tolerance and withdrawal to nicotine following chronic exposure.

5 Chapter 5: General Methods

The general methods described in this chapter are valid for all experiments that reported in this thesis. Any changes therein are outlined in the relevant experimental chapters. The specific drugs (nicotine and mecamylamine) and concentrations used are described in the relevant experimental chapters that follow.

5.1 Animals

5.1.1 Species

Two different species of planarians were used in this Thesis: brown planaria, *Dugesia sp.*, purchased from Blades Biological Ltd (Cowden, Edenbridge, Kent, UK), and *Schmidtea mediterranea* bred in a colony at the University of Leicester.

5.1.2 Animal husbandry

Dugesia sp. were kept in plastic containers filled with tap water treated with 1 ml/l AquaSafe© (Tetra, Germany). They were fed raw chicken for 3 hours every three or four days, and their water was changed immediately after feeding. One week before starting the experiments, the animals were placed into ice-cube trays where they were kept individually and food deprived until the onset of the experiment. They were housed in an incubator with a constant temperature of 20° C. A dimly lit green light inside the incubator provided a 9/15 light/dark cycle (lights on at 9 AM).

Schmidtea mediterranea were bred in a colony at the University of Leicester and kept in the *Montjuic Water,* a solution of 5 mmol/l NaCl, 1.0 mmol/l $CaCl_2$, 1.0 mmol/l $MgSO_4$, 1.0 mmol/l $MgCl_2$, 1.0 mmol/l KCl and N/A mmol/l $NaHCO_3$, that has been shown to be the ideal medium for the animals to healthily grow and develop (see, for example, Brubacher et al., 2014). The colony was kept in an incubator at 20° C and a 9/15 light/dark cycle (lights on at 9 AM). The animals were fed raw ox liver for 3 hours twice per week and the water was changed immediately after every feeding. One week before

the start of the experiment, the animals were food deprived and housed individually in small plastic containers (in an ice cube tray) located in an incubator. All the procedures in this research were performed in accordance with the Policy on Research Involving the Use of Animals (University of Leicester, UK).

5.1.3 Ethical approval

Although the planaria are not a protected species, and therefore the procedures used in this thesis are not regulated, we informally consulted with members of the Animal Ethics Committee of the University of Leicester, who approved the research project.

5.2 Materials

During the experimental sessions, the planarians were placed in Petri dishes containing 10 ml of treated water to monitor their behaviour. The features of the dishes could be used as the experimental context in the experimental designs. We used plastic Petri dishes in Experiments 1-7 (Chapter 6) carried out with *Dugesia sp.*; and glass Petri dishes in Experiment 8 (also with *Dugesia sp.*, Chapter 6), and all the experiments with *Schmidtea mediterranea* reported in Chapter 7 and 8. The specific characteristics of the dishes are described below.

The plastic petri dishes used in the experiments reported in Chapter 6 were 9 cm in diameter. The surface of the dishes was polished by hand with sandpaper resulting in a smooth semi-transparent surface. These dishes served as the distinctive context that we referred to as *smooth* in our experiments (typically used during the chronic exposure to the drug nicotine). We also used plastic dishes covered with white sand glued to the dish using transparent silicone; these dishes served as the *rough* context in our experiments, used in the tests in which the content dependency of tolerance was assessed. As said above, the dishes were filled with 10 ml of tap water treated with Aquasafe or a nicotine

solution (nicotine hydrogen tartrate salt, Sigma-Aldrich, UK, dissolved in autoclaved distilled water).

The watch glass soda lime dishes used in the experiments reported in Chapter 7 were 10 cm in diameter. Some of the dishes used were grooved by hand with a dental drill; these dishes served as the soft exposure context used during the chronic exposure to nicotine. Other dishes were covered with white sand glued with transparent silicone; these dishes were used as the distinctive rough context in the tests that assessed the contextual dependency of tolerance. The dishes were filled with 20 ml of either treated water or a nicotine solution (nicotine hydrogen tartrate salt, Sigma-Aldrich, UK, dissolved in autoclaved distilled water).

The experiments reported in Chapter 6 (with *Dugesia sp.*) were run inside a small chamber (an iron frame box 60 x 60 x 80 cm with a wooden floor and ceiling) surrounded by black curtains to prevent any light coming from outside of the experimental area. The experimental chamber was illuminated by a Philips CorePro B22 LED GLS Bulb 5.5 W (40W) bulb mounted on the top centre of the wooden ceiling. During the experimental sessions, the animals' activity was tracked by using a Video-Track System (ViewPoint, Lyon, France).

The experiments reported in Chapter 7 and 8, using Watch glass soda lime dishes, were run in groups of up to sixteen by using four wooden boxes (26 x 26 x 36 cm), which each could hold four dishes. These boxes were illuminated by dimmable LED panel lights (Model: 15-24 x 1W) placed at the bottom of the box; the light was set at 39 lux (see Figure 5.1). The dishes were placed directly on top of the LED panel. A camera on the top center of the wooden box could simultaneously record the activity of the four animals using *SharpCap* capture software; these videos were subsequently analyzed using a

video-track system (*ViewPoint*, Lyon, France) allowing us to register the activity of the four animals in each box during the experimental sessions (see Prados et al., 2020).

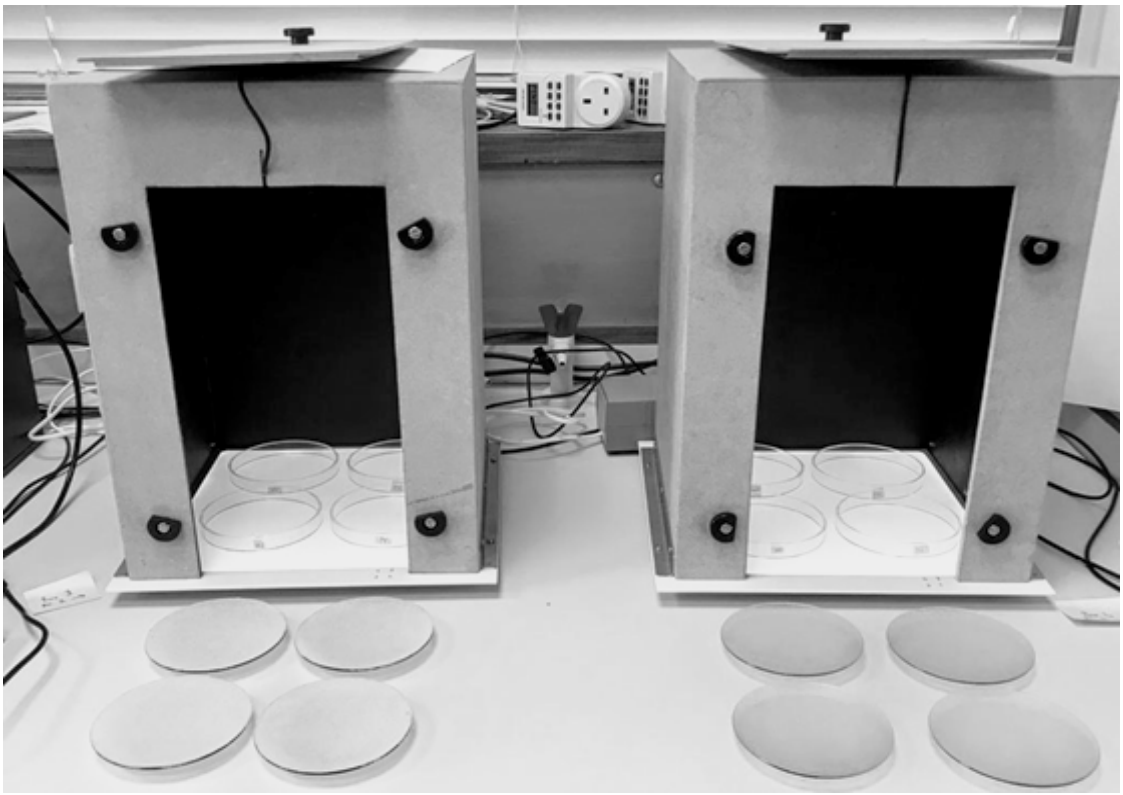


Figure 5. 1. The demonstration of wooden boxes and glass petri-dishes.

5.2.1 High-performance liquid chromatography (HPLC)

Chromatographic separation was achieved using a 2.00 x 150 mm C18, 5µm column (LUNA(2): Phenomenex, UK) perfused with a 75 mM phosphate running buffer (75 mM NaH_2PO_4) , 1 mM ethylenediaminetetra acetic acid (EDTA); 0.6 mM octane sulfonic acid, 10% methanol, pH 7.4) at a flow rate of 300 µl/min delivered by a high pressure pump. Detection of electroactive compounds was achieved with an Antec electrochemical detector, incorporating a VT-03 low volume flow cell (Antec, Netherlands), with the working electrode set at 800 mV relative to a Ag/AgCl reference electrode. Samples supernatant from tissue homogenates were injected manually through

a Rheodyne high pressure valve, incorporating a 20 µl sample loop. Supernatant samples were analysed in duplicate and levels of dopamine, DOPAC, HVA were measured.

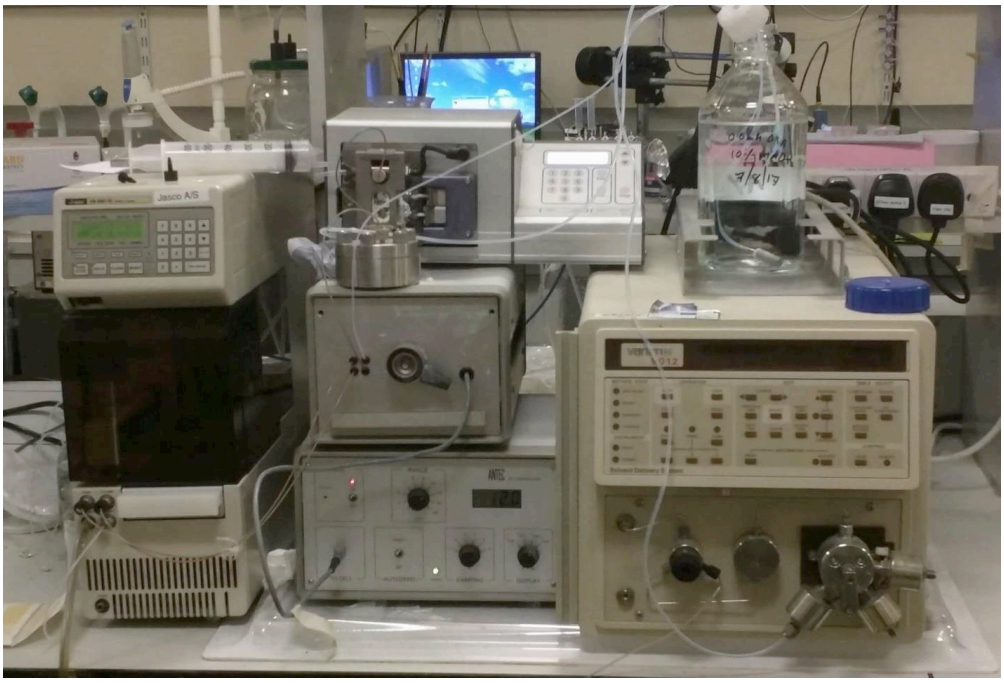


Figure 5. 2. The of HPLC system. Comprising; Autosampler, injection valve, detector, buffer reservoir, high pressure pump.

5.3 Experimental procedures

5.3.1 Behavioural Protocol

In the behavioural experiments reported in this Thesis, the animals were allowed to freely move when exposed to treated water or a nicotine solution. In every experimental session, the planarians activity, locomotion, was automatically recorded using the video track devices described above. The animals were manipulated using a soft artistic brush (to move them from the home container to the experimental dishes, for example). Animals taken from the colony were randomly assigned to different experimental groups and placed into ice-cube trays for three days in order to allow them to adapt to their new home environment. During this adaptation phase, the animals were daily handled with the brush every day to mitigate the disruptive effect of brush manipulations on their behaviours

during the experiment. Animals were food-deprived for seven days before starting the experiment; we adopted the procedure used in other experiments carried out in our laboratory in which sucrose was used as the rewarding agent.

The experiments reported in this Thesis all followed the same general procedure with three phases: 1) habituation to the drug-paired context; 2) chronic exposure to nicotine; and 3) test (see Figure 5.3, for a summary of the experimental designs).

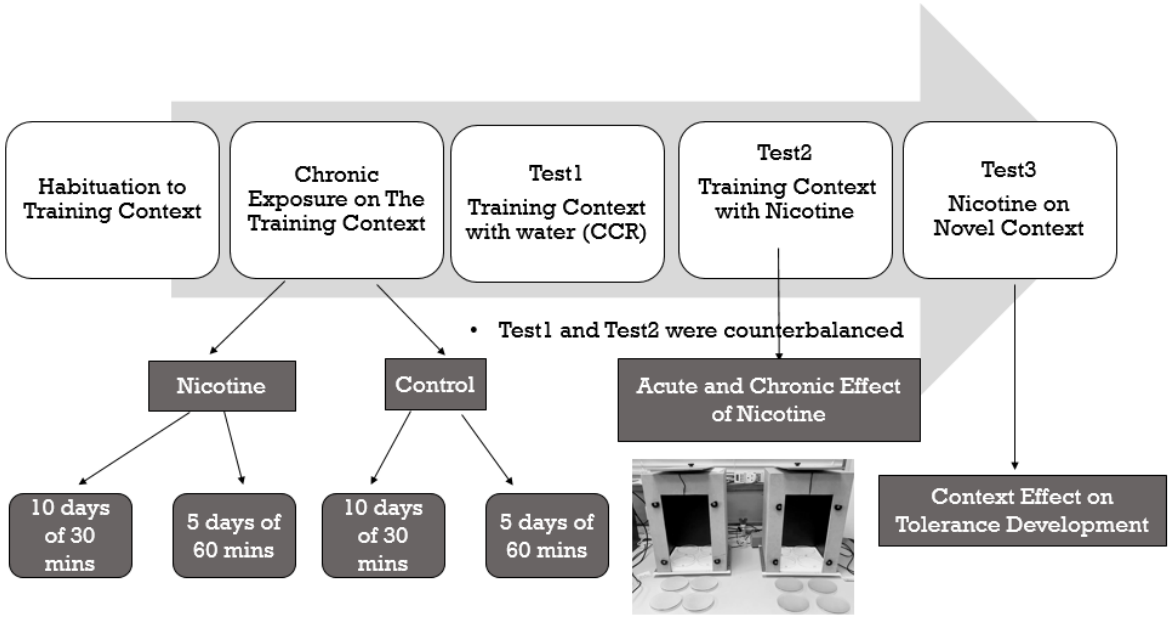


Figure 5. 3. Summary of Experimental design.

5.3.1.1 Habituation

During the habituation session of the experiments, animals were placed on the polished or grooved dishes for 30 minutes in the experimental context for them to habituate to the experimental setting on the day before the start of the chronic exposure to nicotine. Their locomotor activity was recorded during the habituation session to establish their base-line locomotor activity. If there is a difference between the locomotor activities of groups, planarians were exchanged between groups to match the levels of activity across groups before starting the chronic exposure procedure.

5.3.1.2 Chronic Exposure

A day after habituation, the chronic exposure started at a rate of one trial per day over a certain number of days, depending on the experiment. A chronic exposure trial started by placing the animal in one of the polished or grooved dishes containing either a nicotine solution (the experimental condition, Group Nicotine) or treated water (the control condition, Group Water). The animals were allowed to freely move during the length of each trial (which varied across experiments). We mainly used two different chronic exposure protocols, the 10x and the 5x protocols. In the 10x, the animals received ten 30 min daily exposure sessions; in the 5x protocol, the animals received five 1 hour daily exposure sessions. The animals' locomotor activity was recorded during each session and we compared the activity of the experimental and control groups in bins of ten minutes.

5.3.1.3 Test

Following chronic exposure, all the animals were given three test trials over three consecutive days. During Test 1, all the planarians were placed on the nicotine-paired context with water for 30 minutes; exposure to the context in the absence of any drugs aimed to reveal any conditioned responses elicited by the contextual cues—that is, the conditioned compensatory responses or CCR which, according to conditioning theory (Siegel, 1975), underlie the development of tolerance. During Test 2, all the planarians were exposed to nicotine to compare its acute (in the Group Water, exposed for the first time to nicotine) and chronic (in the Group Nicotine, exposed to nicotine during the chronic exposure phase) effects. As noted above, the locomotor activity of the animals was recorded and analysed in 10 min bins. The order in which the animals received the Test 1 and Test 2 was counterbalanced across animals.

Test 3 was conducted in a novel environment to test whether the development of tolerance was context dependent. All the animals were exposed to nicotine on a novel distinctive dish with a rough surface (white sand was glued to the dishes using transparent silicone). If tolerance to the nicotine effects is controlled by the contextual cues, we should expect an attenuation of tolerance in the novel context (Siegel, 1975).

5.3.2 Experimental Design

We mainly used two different chronic exposure protocols: 10x, ten days of exposure with 30 min of exposure; and 5x, five days of exposure with 1h exposure. The differences in the chronic exposure and test sessions across the experiment as follows.

Across the experiment, we made two important manipulations on dependent variables (DVs). The first manipulation was in drug concentration. We used different nicotine concentrations across the experiments. For example, in Experiment 1, Chapter 6, we monitored the locomotor activity of the animals exposed to three different concentrations (0.01, 0.025 and 0.1 mM) to identify optimal concetration that would allow us to observe the development of tolerance at doses which are not toxic (that do not harm the planarians). This study showed that 0.025mM and 0.01 mM nicotine concentrations were better concentrations to use in subsequent experiments because some planaria was died in the high nicotine concentration of 0.1 mM (see Experiment 1). Then, we used different concentrations across the experiments (see Table 5.1)

Experiment Number	Chapter Number	Treatment	Tests (mins)	Concentrations (mM)	Extra
1	6	5 x 10 min	-	0.01, 0.025 and 0.1 nicotine	
2	6	-	10	0.025 nicotine	
3	6	10 x 30 min	30	0.025 nicotine	HPLC

4	6	10 x 30 min	30	0.01 nicotine	
5	6	2 x 150 min	30	0.01 nicotine	
6	6	4 x 150 min	30	0.01 and 0.025 nicotine	HPLC
7	6	16 hours over night	30	0.025 and 0.1 nicotine	
8	6	10 x 30 min	30	0.025 nicotine	*Dugesia sp.* *vs.* *Schmidtea mediterranea*
9	7	10 x 30 min 5 x 60 min	60	0.025 nicotine	
10	7	Replication 10 x 30 min 5 x 60 min	60	0.025 nic	
11	7	mecamylamine manipulation with 5 x 60 min	60	0.025 nicotine 0.5 mecamylamine	
12	7	10 x 30 min		0.05 nicotine	
13	7	30 min pre-exposure to context with 10 x 30 min	30	0.05 nicotine	Anticipatory Response
14	8	10 x 30 min	30	0.025 nicotine	
15	8	mecamylamine manipulation with 10 x 30 min	60	0.025 nicotine 0.5 mecamylamine	
16	8	30 min post-exposure to context with 10 x 30 min	30	0.05 nicotine	Abstinence-induced Responses
17	8	30 min post-exposure to context with 10 x 30 min	30	0.1 nicotine	Abstinence-induced responses

Table 5. 1**.** Summary of the experimental conditions (exposure protocol and nicotine concentration) used in each experiment.

5.3.3 HPLC

In two experiments (Chapter 6; Experiments 3 and 7), HPLC analysis was conducted 24 hours following the last test (Test 3). HPLC with electrochemical detection was used to measure oxidable compounds such as dopamine and its metabolites (DOPAC and HVA), and serotonin and its metabolites (5-hydroxyindoleacetic acid: 5-HIAA) applying electrochemical detectors in the tissue.

5.3.3.1 Homogenisation Procedure

Each individual planaria was dried, weighted and then homogenised in 0.1 M perchloric acid for approximately 2 mins to ensure that the tissue was well dispersed. The homogenate solution was then transferred into 1.5 ml Eppendorf and centrifuged using a bench centrifuge for 45 min at 4°C. The supernatant was then carefully aspirated off using a Pasteur pipette ready for HPLC analysis, taking care not to disturb the pellet.

5.4 Data Analysis

5.4.1 Behavioural Analysis of Locomotor Activity

The locomotor activity was measured during all sessions (chronic exposure and test) and organized in 10-min bins for data analyses. In the majority of experiments reported in this thesis, the data from the chronic exposure phase (10 days, 30 min of exposure per day) was analysed by using a 2 (Group: Nicotine *vs*. Water) x 10 (Days: 1–10) x 3 (Bins: 1-3) mixed ANOVA. ANOVAs during chronic exposure were followed up with within-subjects linear contrasts in each group to ascertain if there was a change in motility across days of chronic exposure, which would be indicative of tolerance development. The data from the Test 1 trial were analysed by using a 2 (Group: Nicotine *vs*. Water) x 6 (Bins: 1-6) mixed ANOVAs to assess the development of CCRs. The data from Tests 2 and 3 were analysed together using a 2 (Group: Nicotine *vs*. Water) x 2 (Tests: 2–3) x 6 (Bins: 1-6) ANOVA to assess the development of tolerance to nicotine and its contextual dependency. The reported effect size for ANOVAs is partial eta squared (η_p^2). When violations of sphericity were observed, the Huynh-Feldt adjustment was used. All the analyses were conducted using IBM SPSS Statistics for Windows, Version 26.0.

5.4.2 Meta-analysis

Meta-analysis is a statistical technique for combining the findings from multiple independent studies. It is often used to assess the effectiveness of manipulations, by combining data from two or more experiments. Meta-analysis of numerous experiments provides a precise estimate of the effect of manipulations, giving due weight to the size of the different experiments included (Smith & Glass, 1977). R. A. Fisher (1944) stated "When a number of quite independent tests of significance have been made, it sometimes happens that although few or none can be claimed individually as significant, yet the aggregate gives an impression that the probabilities are on the whole lower than would often have been obtained by chance" (p. 99).

In particular, we were interested in conducting a Meta-analysis with the data from the *Schmidtea mediterranea* experiments (Chapters 7 and 8). In these experiments, we often observed evidence for the development of tolerance and that tolerance tended not to be context dependent. However, individual studies with the *Schmidtea mediterranea* in Chapter 7 and 8 showed the development of tolerance was successful sometimes in Test 2 (nicotine test on the drug-paired context), and sometimes in Test 3 (nicotine test on the novel context). Obviously, there was the question of whether tolerance was context dependent, because we observed small variations across the results of the different experiments. Therefore, a meta-analysis would help us to address that question, allowing us to compare all the Test 2 and Test 3 across experiments. The results of the meta-analysis will be reported in Chapter 8.

5.4.3 HPLC Data Analysis

Peak areas for compounds of interest were determined using a PC-based integrator (Chrom Perfect) and concentrations were calculated for each with reference to peak areas in a standard solution (50 nM), made freshly each day. Mean concentrations were

calculated for each tissue sample, by taking an average of the two duplicates, and then this value was corrected for the weight of tissue. The result of HPLC analysis will be reported in Chapter 6, Experiments 3 and 7.

6 Chapter 6: Assessment of the chronic effects of Nicotine on tolerance development in Planaria (*Dugesia sp.*)

6.1 Introduction

This chapter presents series of experiments with Brown Planaria (*Dugesia sp.*) concerned with the development of chronic tolerance to nicotine. The main objective of these experiments was to determine whether chronic exposure to nicotine results in less effect of nicotine (i.e., tolerance to the unconditioned effects of nicotine), and also on the assessment of learned tolerance (i.e., context dependency and CCRs) using commercially available *Dugesia sp*. Based on previous findings in rodents and humans, there were four main hypotheses in these experiments:

First, we expected a reduction in the unconditioned hypoactive response to nicotine over the course of repeated administrations. That would reveal adaptation to the effects of nicotine.

Second, we expected to observe CCRs with planaria after chronic nicotine treatment, when animals were tested in the presence of distinct stimuli previously paired with the effects of nicotine, but in the absence of the drug. Pavlovian conditioning contributes to homeostatic regulation in many biological systems and serves to maintain the stability of organisms (Siegel, 1975). It is assumed that the unconditional effects of drugs cause a physiological disturbance, and imbalance that threatens the existence of the animal. Through learning, animals develop an anticipatory homeostatic response in the presence of stimuli previously paired with the effects of the drug. This anticipatory response tends to be opposite in nature to the unconditioned effects of the drug, and hence it attenuates the initial effect of drug and helps the organism achieve the balance/homeostasis (Siegel, 2008). Therefore, we expected to observe CCRs after

chronic nicotine treatment when planaria were tested in the absence of the drug but in the presence of stimuli paired with the drug effects.

Third, we expected to observe less hypo-activity in the nicotine pre-treated group compared to the vehicle pre-treated group when they were tested with nicotine in the drug-paired context. This was to compare the chronic (long-term) and acute (short-term) effects of nicotine. The fourth hypothesis was that, as tolerance development is context dependent (Siegel, 1975, Siegel, 2008), we expected not to observe tolerance when the animals were tested in a novel context.

Nicotine is a toxic substance (Norazlina et al., 2010) and exposure to high concentrations of nicotine may harm planaria. Therefore, we aimed to identify a nicotine concentration that does not harm animals and reliably produces a change in behaviour in Experiment 1. We assessed the effects of different concentrations of nicotine (water, 0.01 mM, 0.025 mM, and 0.1 mM) on the locomotor activity. Thirty-two brown planaria (*Dugesia sp.*) were used in the present study. The planaria were exposed to different concentrations of nicotine for 10 min every alternative day (days 1, 3, 5, 7, 9). There were eight animals in each group, but two worms died because of the toxic effects of the higher dose of nicotine (0.1 mM). Because of a technical problem, the camera system did not record the locomotor activity on Day 1. The planaria were held in the same way described in the general methods chapter. The plastic Petri dishes (9 cm in diameter) were used in this study.

6.2 Experiment 1: Dose Dependent Nicotine Effect in Planaria

6.2.1 Results and discussion

Figure 6.1 displays the mean distance covered by planaria in different concentrations. Nicotine administration reduced locomotor activity of planaria in a

concentration dependent manner compared to the control group which was exposed to water. This result was confirmed with a 4 (Concentration: water, 0.01, 0.025, 0.1 mM) x 4 (Days: 3, 5, 7 and 9) mixed ANOVA that revealed a main effect of Concentration, $F(3, 26) = 3.82$, $p = 0.02$, and Days, $F(3, 78) = 3.16$, $p = .03$, but no interaction between these factors, $F(9, 78) = 1.25$, p = .28. Additionally, *t*-tests analysis were used to find out the unconditioned response to the exposure to nicotine (at different concentrations) — comparisons were made relative to control animals exposed to treated water. The results showed that 0.1 mM nicotine ($M = 32$ cm, $SE = 6$ cm); $t(12) = 3.39$, $p = 0.05$, and 0.025 mM nicotine, ($M = 49.5$ cm, $SE = 5.2$ cm); $t(12) = 2.38$, $p = .035$, caused a significant decrease in motility counts compared to the planaria treated with water group ($M = 57.5$ cm, $SE = 5.2$ cm). A marginal difference was observed in the planaria treated with 0.01 mM nicotine ($M = 41.5$ cm, $SE = 5.2$ cm) compared with the water group, $t(14) = 2.04$, $p = 0.06$.

In summary, the behavioural results indicated that nicotine reduces the locomotor activity of planaria in a concentration dependent manner (Rawls et al., 2011).The highest (0.1 mM) and medium (0.025 mM) nicotine concentrations significantly (and lower nicotine concentration [0.01 mM] marginally) reduced the activity compared to the water-treated group. Since some planaria died in the high nicotine concentration (0.1 mM), we inferred that 0.025mM and 0.01 mM nicotine concentrations were better concentrations to use in subsequent experiments.

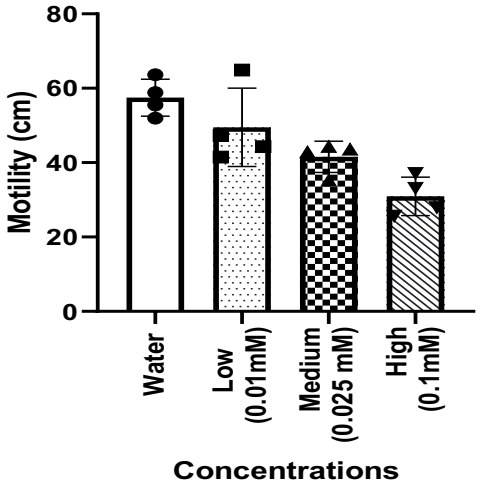


Figure 6. 1. This figure shows the mean locomotor activity of planaria (*Dugesia sp.*) to different nicotine concentrations. n = 6-8 planaria/group.

6.3 Experiment 2: Texture Effect on the locomotor Activity

In the Experiment 1, we identified nicotine concentrations, 0.025 mM and 0.01 mM, that produced an effect in locomotor activity but were not harmful for the planaria. The purpose of this experiment was to test the effect of different textures (Rough and Smooth) on the pharmacological action of 0.025 mM of nicotine. We assessed whether the changes in locomotor activity of the planaria was caused by either pharmacological action of nicotine rather than the textures (smooth and rough) where nicotine was given. Planaria were tested during a single exposure to nicotine or water either on the smooth or rough textures for 10 min, and the data were recorded in 10 min bins. We allocated 64 animals to four groups: Nicotine on Smooth Surface, Nicotine on Rough Surface, Water on Smooth Surface, and Water on Rough surface. The data for four animals in the Nicotine Groups, and three animals in the Water Groups were discarded from the analysis because the camera created noise during data collection. Therefore, only data from 57 animals were analysed.

6.3.1 Results and discussion

As expected, based on pilot data, planaria that received nicotine showed lower locomotor activity (M = 34.2 cm, SE = 4.8 cm) than the planaria that experienced water (M = 52.4 cm, SE = 4.7 cm). This impression was confirmed with a 2 (Group: Nicotine *vs.* Water) x 2 (Texture: Rough *vs.* Smooth) mixed ANOVA that revealed a main effect of Group, $F(1, 53) = 7.41$, $p < .05$, $\eta_p^2 = .12$; neither the effect of Textures, $F(1, 53) = 0.66$, $p = .42$, $\eta_p^2 = .012$, nor the Group x Textures interaction, $F(1, 53) = 0.75$, $p = .39$, $\eta_p^2 = .014$, was significant. The significant Group effect and absence of a Group by Texture interaction effect imply that nicotine reduced their locomotor activity, and it was independent of the texture.

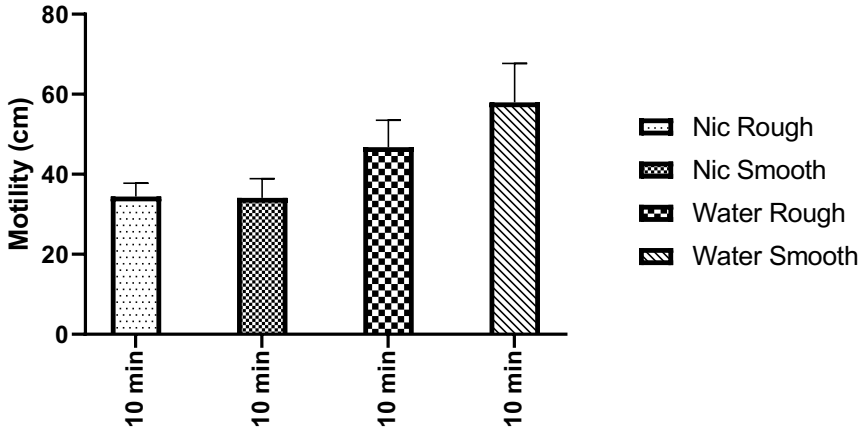


Figure 6. 2**.** This figure shows the mean locomotor activity of planaria (*Dugesia sp.*) for 10 minutes in nicotine and water groups on different surfaces. n=13-16 planaria per group.

6.4 Experiment 3: Tolerance Development with Intermittent Nicotine Exposure (0.025 mM) and assessment of neurochemical changes with HPLC

Experiment 1 established that the unconditioned response to nicotine was reduced locomotor activity—comparison made to control animals exposed to treated water. The purpose of Experiment 3 was to assess the development of tolerance to the hypo

locomotive effect of nicotine using a chronic procedure that better mimics the chronic exposure regimens used in other animals such as rodents, and indeed chronic consumption in humans. We used a relatively low concentration of nicotine (0.025 mM) which Experiment 1 had determined it produced reliable hypo locomotion in planaria. Thirty-four *Dugesia sp.* were used in the present study.

We also investigated neurochemical changes in neurotransmitters such as dopamine and its metabolites (DOPAC and HVA), and serotonin and its metabolite (5-hydroxyindoleacetic acid: 5-HIAA) 24 hours following the last test (Test 3) using High-Performance Liquid Chromatography (HPLC). Twenty-four hours after the last test session (Test 3), each planaria was individually homogenized by sonication in 0.1 M per-chloric acid containing 0.1 M EDTA and ethylhomocholine. There were three groups of animals in this HPLC analysis, 16 animas in each group, including 1) Chronic Group: treated with nicotine during the conditioning and test days, 2) Acute Group: exposed to nicotine only during the test sessions, and 3)- Control Group: selected from the colony. The HPLC procedure is explained in detail in the General Method chapter (see 5.3.3).

6.4.1 Behavioural Results and Discussion

6.4.1.1 Chronic Exposure

The data of the chronic exposure phase of the experiment is displayed in Figure 6.3 A. As expected, based on our previous findings, planaria that received nicotine displayed less locomotor activity (M = 26.9 cm, SE = 5.1 cm) than the planaria that experienced water (*M* = 63.1 cm, *SE* = 5.2 cm) on the first day. However, there was no evidence of the development of tolerance because there was an increase in locomotor activity for both Group Nicotine (*M* = 45 cm, *SE* = 3.3 cm) and Group Water (*M* = 84.2 cm, *SE* = 3.3 cm) on the final day. These impressions were confirmed with a 2 (Group: Nicotine vs Water) x 10 (Days: 1 – 10) x 3 (Bins: 1-3) mixed ANOVA that revealed a

main effect of Group, $F(1, 32) = 75.40$, $p < .001$, $\eta_p^2 = .70$, Days , $F(7.9, 254.5) = 3.69$, $p < .001$, $\eta_p^2 = .10$, and Bins, $F(1.4, 44.6) = 103.28$, $p < .001$, $\eta_p^2 = .76$, and a significant interaction of Group x Bins, $F(1.4, 44.6) = 13.58$, $p < .001$, $\eta_p^2 = .30$. However, there was no significant interaction of Group x Days, $F(7.9, 254.5) = 0.73$, $p = .67$, $\eta_p^2 = .02$, or Days x Bins, $F(11.7, 374) = 1.33$, $p = .20$, $\eta_p^2 = .04$. The remaining three-way interaction Group x Days x Bins was also non-significant, $F(11.7, 374) = 1.35$, $p = .19$, $\eta_p^2 = .04$. Within-subjects linear contrasts revealed an effect of Day in Group Nicotine, $F(1, 16) = 6.02$, $p < .03$, $\eta_p^2 = .27$, but not in Group Water, $F(1, 16) = 2.11$, $p = .17$, $\eta_p^2 = .12$, suggesting an increase in locomotor activity in Group Nicotine but not Water. These results suggest that the chronic exposure procedure used in the present experiment is effective in developing long-term tolerance to the effects of nicotine in the planaria.

6.4.1.2 Test 1, Conditioned Compensatory Responses

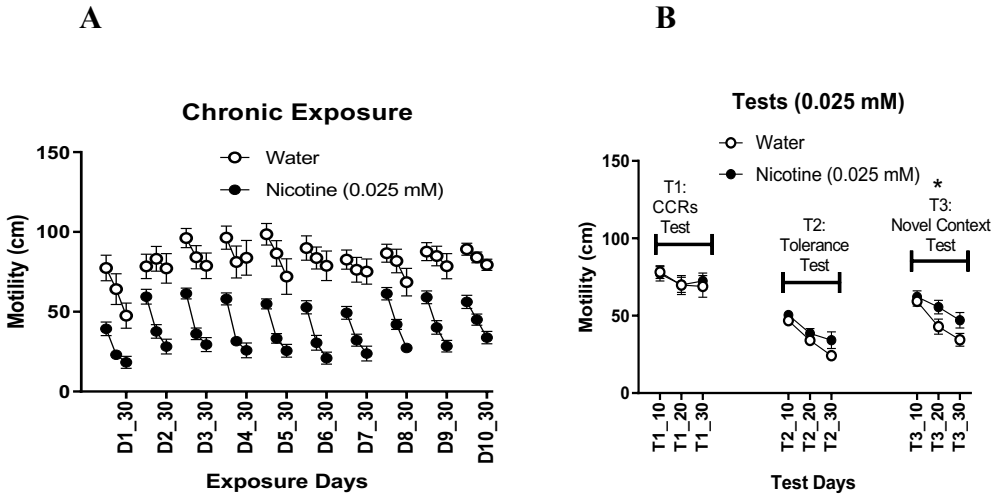
Test 1 was conducted in the drug-paired context with water to assess the development of CCRs. As can be observed in Figure 6.3 B (Left panel, T1), planaria in the Group Nicotine, exposed to nicotine ($M = 73.1$ cm, $SE = 4.9$ cm), behaved in a similar way to animals in the Group Water ($M = 72.3$ cm, $SE = 4.9$ cm). This impression was confirmed by a 2 (Group: Nicotine vs Water) x 3 (Bins: 1-3) mixed ANOVA, which revealed no effect of Group, $F(1, 32) = 0.14$, $p = .91$, $\eta_p^2 = .001$, a significant effect of Bins, $F(2, 64) = 4.55$, $p = .14$, $\eta_p^2 = .12$, but no interaction between these factors, $F(2, 64) = 0.36$, $p = .70$, $\eta_p^2 = .01$.

6.4.1.3 Test 2 and Test 3

These tests were conducted to assess the development of tolerance to the unconditioned (hypo locomotion) effects of nicotine. Group Nicotine received the drug for the 11th time whilst animals in the Group Water received it for the 1st time in the Drug-paired Context (Test 2). Additionally, animals in both groups were tested with nicotine

again, but in a Novel Context (Test 3). Figure 6.3 B (central and right panels, T2 and T3) shows that planaria in Nicotine Group displayed more locomotor activity (M = 48 cm, SE = 2.3 cm) than planaria in Control Group (M = 40.3 cm, SE = 2.3 cm) during tolerance tests, suggesting the expression of tolerance to nicotine. This impression was confirmed by a 2 (Group: Nicotine vs Water) x 2 (Tests: Test 2 and Test 3) x 3 (Bins: 1-3) mixed ANOVA, which revealed significant effects of Group, $F(1, 32) = 5.84$, $p = .03$, $\eta_p^2 = .14$, Tests, $F(1, 32) = 44.14$, $p < .001$, $\eta_p^2 = .58$, and Bins, $F(1.6, 52.3) = 35.8$, $p<.001$, $\eta_p^2 = .53$; the interactions were all non-significant: Tests x Bins, $F(2, 64) = 0.04$, $p = .96$, $\eta_p^2 = .001$, Group x Tests, $F(1, 32) = 0.81$, $p = .37$, $\eta_p^2 = .02$, Group x Bins, $F(1.6, 52.3) = 1.53$, $p = .23$, $\eta_p^2 = .04$, Group x Tests x Bins, $F(2, 64) = 0.44$, $p = .63$, $\eta_p^2 = .014$.

It is important to see that we were not able to replicate the same results with the same experimental procedure with the same concentration (see Experiment 8).



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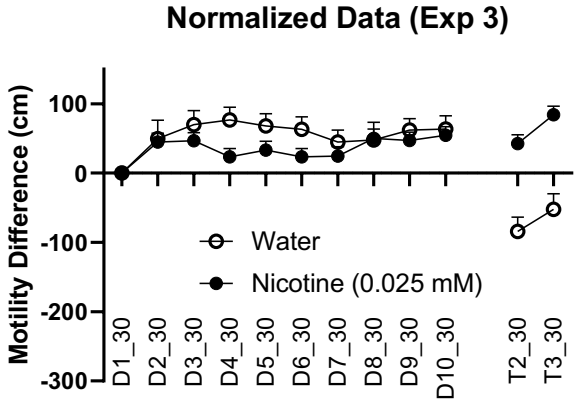


Figure 6. 3. Experiment 3. Mean distance covered by planaria in the drug-paired context throughout 10 days of 30 min in the presence of nicotine or water (A), 30 min of CCRs test in the presence of only water (T1), 30 min of tolerance test in the presence of nicotine (T2), and 30 min of novel context test in the presence of nicotine with an alternative context (T3) (B). Results represented in 10 minutes bins. Data normalized to the first training session and represented in 30 min bins (C). Bars represent standard errors. n=17 planaria in each group.

6.4.2 HPLC Results and Discussion

We examined the effect of acute and chronic nicotine exposure on the changes in Dopamine and its metabolites, DOPAC and HVA, and the serotonin metabolite 5-HIAA that were measured in the dialysates by HPLC. The HPLC data is displayed in Figure 6.4 and the data for each neurotransmitter and metabolites were analysed using one-way ANOVA. There was no main effect of Groups on DOPAC, $F(2,45) = 2.36$, $p = .105$, a significant effect on DA, $F(2,45) = 9.39$, $p < .001$, no significant effect on 5-HIAA, $F(2,44) = 2.12$, $p = .13$, no significant effect on HVA, $F(2,45) = 1.33$, $p = .27$, and a marginal effect on 5-HT, $F(2,45) = 2.98$, $p = .061$. These results indicated that there was a significant difference between Chronic, Acute and Control groups on DA and 5-HT, but not on the remaining metabolites. Post-hoc analysis on Groups for DA neurotransmitter level revealed a significant difference between Group Control and Group Acute ($p < 0.001$); Group Control and Group Chronic ($p = .002$), but there was no difference between Group Acute and Group Chronic ($p = .37$). Additional post-hoc analysis on Groups for 5-HT neurotransmitter level revealed a significant difference between Group Control and

Group Acute (p = .031), and a marginal difference between Group Control and Group Chronic (p = .053). However, there was no significant difference between the acute and chronic groups (p = .81). Overall, these results suggested that nicotine exposure increased the concentrations of DA and 5-HT neurotransmitters; however, HPLC results failed to show a difference between acute and chronic groups, unlike the behavioural findings.

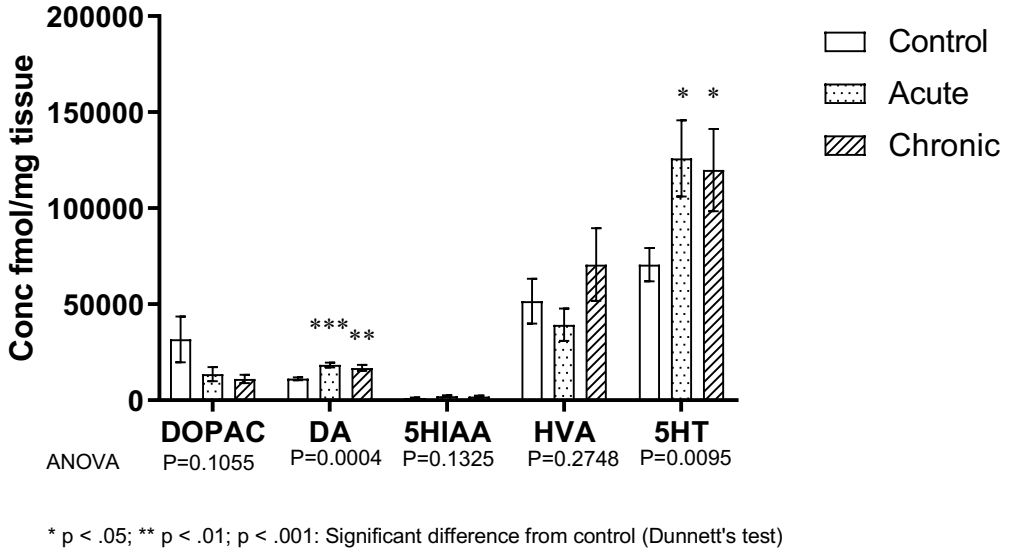


Figure 6. 4**.** The average DOPAC, DA, 5-HIAA, HVA, and 5-HT concentrations fmol/mg tissue of Chronic, Acute, and Control Groups. n=16 planaria in each group.

Neurotransmitters	DOPAC	DA	5-HIAA	HVA	5-HT
Control vs acute	.087	.0001	.046	.530	.053
Control vs Chronic	.052	.002	.297	.330	.031
Acute vs Chronic	.805	.369	.314	.113	.811

Table 6. 1**.** This Table shows the results of the post-hoc analysis between groups for each neurotransmitter.

6.5 Experiment 4: Tolerance Development with Intermittent Nicotine Exposure (0.01 mM)

Experiment 3 suggested that the locomotor activity of the animals tends to increase with repeated exposure to nicotine (an instance of tolerance development). However, we did not observe chronic tolerance development at the neurochemical level in the HPLC analyses, unlike the behavioural findings. Other nicotine studies with rats found similar results using HPLC technique (Damsma et al., 1989). Researchers observed an increase in cellular DA level and its metabolites of animals treated with acute and chronic nicotine, but there was no difference between these groups. Furthermore, other studies in rodents using nicotine (Stolerman et al., 1974) and morphine (Dafter & Odber, 1989) have also observed absence of tolerance development with high doses. Therefore, it is possible that 0.025 mM of nicotine concentration was too high for the planaria. The goal of Experiment 4 was to replicate the previous experiment with a lower nicotine concentration (0.01 mM), which marginally reduced the locomotor activity (see Experiment 1), and assess the reliability of the previous findings in the behavioural level. Sixteen animals were accidentally tested earlier, therefore the data for chronic exposure was for 9 days (from day1 to day9) for all the animals. We allocated a total 24 planaria to two groups, Nicotine and Water. One animal in Group Water and one in Group Nicotine died over the course of the experiment, resulting in $n = 11$ for Group Nicotine, and $n = 11$ in Group Water. The planaria were held in the same way described in the general methods chapter. The plastic Petri dishes (9 mm diameter) were used in this study. The experimental design and hypothesises were the same as in Experiments 3.

6.5.1 Results and discussion

6.5.1.1 Chronic Exposure

As expected, based on our previous findings, planaria that received Nicotine (M = 45.7 cm, SE = 7.6 cm) showed less locomotor activity than the planaria that experienced Water (M = 59.7 cm, SE = 7.6 cm) on the first day of chronic exposure. Although some variability was observed across days of chronic exposure, there did not seem to be any development of tolerance because there was an increase in the locomotor activity in both groups across days. The data of the chronic exposure phase of the experiment is presented in Figure 6.5 A. A 2 (Group: Nicotine vs Water) x 9 (Days: 1 – 9) x 3 (Bins: 1-3) mixed ANOVA revealed main effects of Group, $F(1, 20) = 6.07$, $p = .02$, $\eta_p^2 = .23$, and Bins, $F(2,40) = 116.37$, $p < .001$, $\eta_p^2 = .85$, as well as a significant interaction of Group x Bins, $F(2,40) = 31.86$, $p < .001$, $\eta_p^2 = .61$. There was no effect of Days, $F(8,160) = 2.01$, $p = .40$, $\eta_p^2 = .09$, and the Days x Bins interaction was also n on-significant, $F(12.2, 536.5) = 1.31$, $p = .21$, $\eta_p^2 = .03$. The remaining three-way interaction Group x Days x Bins was significant, $F(12.2, 536.5) = 2.16$, $p = .01$, $\eta_p^2 = .05$. Within-subjects linear contrasts revealed no effect of Day in Group Nicotine, $F(1, 10) = 0.15$, $p = .71$, $\eta_p^2 = .015$, or in Group Water, $F(1, 10) = 0.61$, $p = .45$, $\eta_p^2 = .057$, suggesting no significant increase in locomotor activity in either Group Nicotine or Water. These results suggests that the chronic exposure procedure used in the present experiment is not effective in producing long-term tolerance to the effects of nicotine in the planaria.

6.5.1.2 Test1

Test 1 was conducted on the drug-paired context with water to assess the development of CCRs. As can be observed in Figure 6.5 B (left panel, T1), planaria chronically trained with nicotine (M = 65.1 cm, SE = 6.9 cm) behaved in a similar way to control animals (M = 73 cm, SE = 9.1 cm). A 2 (Group: Nicotine vs Water) x 10 (Days:

1 – 10) x 3 (Bins: 1-3) mixed ANOVA revealed no effect of Group, F (1, 15) =0.50, p=0.4, η_p^2=.03, a significant effect of Bins, F (2, 30) = 4.89, p = .015, η_p^2 = .15, but no interaction between these factors, F (2, 30) = 0.363, p = .7, η_p^2=.011.

6.5.1.3 Test 2 and Test 3

These tests were conducted to assess the development of tolerance to the unconditioned (hypo locomotion) effects of nicotine, as well as to assess the context dependence of the tolerance developed to nicotine during the chronic exposure. Group Nicotine received the drug for the 11th time whilst animals in the Group Water received it for the first time in the nicotine-paired context in Test 2. The animals in both groups were tested in the presence of nicotine again, but in a novel distinctive context, during Test 3. Figure 6.5 B (central and right panels, T2 and T3) shows that planaria previously exposed to nicotine (M = 50.8 cm, SE = 4.3 cm) behaved in a similar way to animals in the Group Water (M = 57.8 cm, SE = 4.2 cm) both during the Test 2 (in the nicotine-paired context) and Test 3 (in the new context), suggesting no evidence of tolerance in either context. These impressions were confirmed by a 2 (Group: Nicotine *vs*. Water) x 2 (Tests: Test 2 and Test 3) x 3 (Bins: 1-3) mixed ANOVA, which revealed no main effect of Group F(1, 19) = 1.32, p = .26, η_p^2 = .06, and Tests, F(1, 19) = 0.59, p = .45, η_p^2 = .03, but a main effect of Bins, F(2, 38) = 26.3, p < .001, η_p^2 = .58. The remaining interactions were all non-significant: Group x Tests interaction, F(1, 19) = 0.013, p = .91, η_p^2 = .001, Group x Bins interaction, F(2, 38) = 0.61, p = .54, η_p^2 = .03, Tests x Bins interaction, F(2, 38) = 0.32, p = .72, η_p^2 = .02, and three-way interaction Group x Tests x Bins, F(2, 38) = 0.48, p = .62, η_p^2 = .02.

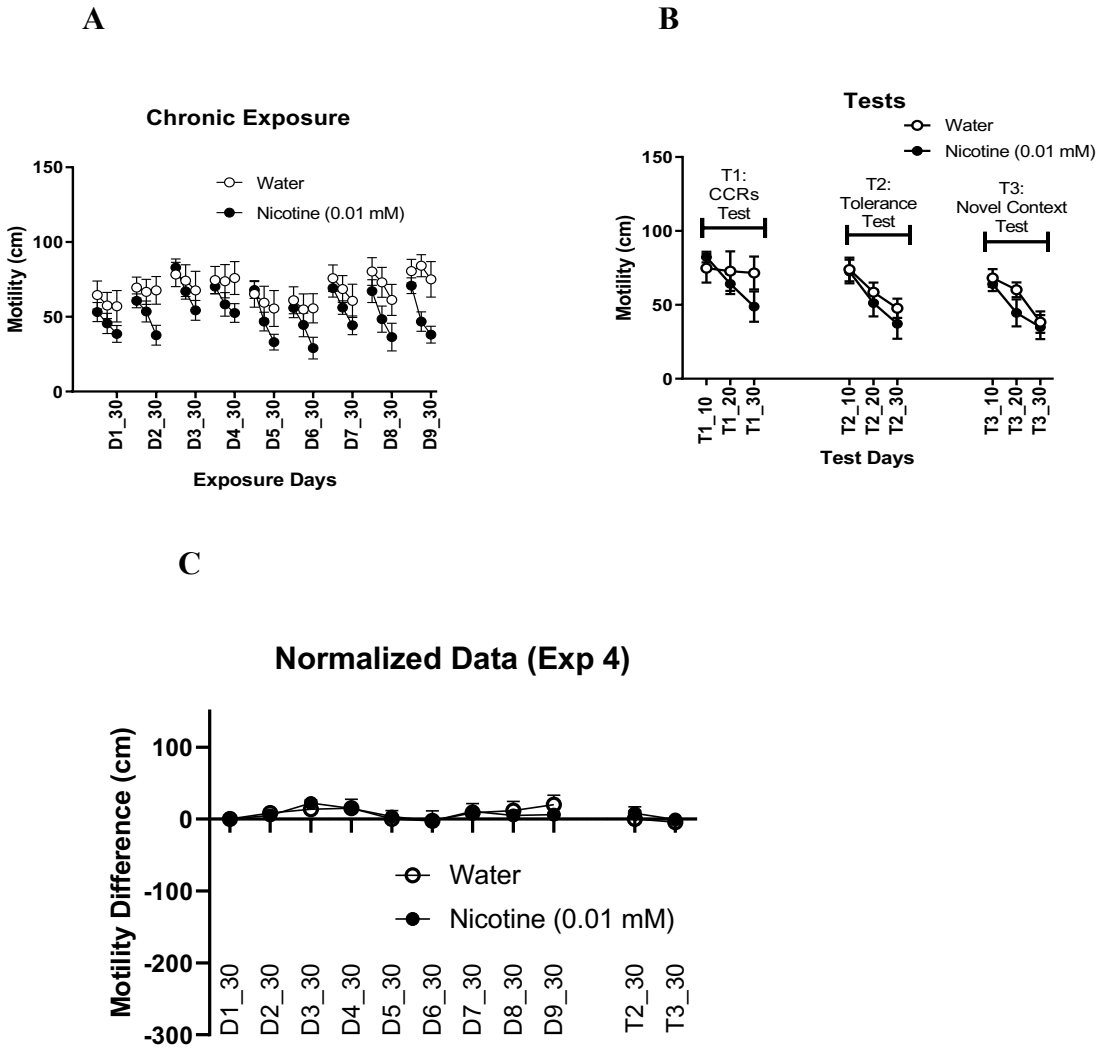


Figure 6. 5. Experiment 4. Mean distance covered by planaria in the drug-paired context throughout 10 days of 30 min in the presence of nicotine or water (A), 30 min of CCRs test in the presence of only water (T1), 30 min of tolerance test in the presence of nicotine (T2), and 30 min of novel context test in the presence of nicotine with an alternative context (T3) (B). Results represented in 10 minutes bins. Data normalized to the first training session and represented in 30 min bins (C). Bars represent standard errors. n=11 planaria in each group.

6.6 Experiment 5: Tolerance Development with Massive Nicotine Exposure (0.01 mM)

In Experiment 4, we were not able to replicate the tolerance development following intermittent nicotine administration. Intermittent here means that animals were exposed to nicotine for 30 minutes in a daily session and then returned to the home context for 23.5 hours before the next exposure session. A way of observing a successful tolerance with nicotine could be with the use of a long, massive exposure. Feng et al. (2006) tested the effect of chronic nicotine on the tolerance development in *C elegans*. They observed

that animals exposed to nicotine for the first time (16 min) displayed greater locomotor activity than naïve animals. However, when animals were tested with nicotine (16 min) after overnight (16 h) nicotine exposure, they performed similar behavioural activity to naïve animals on nicotine-free plates and showed the evidence of tolerance. The goal of Experiment 5 was to investigate chronic effect of nicotine with massive exposure. We allocated 64 planaria to two groups, nicotine and Water. Since the camera system created noise during data collection, the data of 3 animals from Group Nicotine and 5 animals from Group Water were discarded from the analysis, resulting in n = 31 for Group Nicotine and n =29 in Group Water. The planaria were held in the same way described in the general methods chapter. The plastic Petri dishes (9 mm diameter) were used in this study. The planaria always received water or nicotine on the smooth (drug-paired) surface 2.5 hours (150 min) during two days with 0.01 mM nicotine solution (total 5 hours). All subsequent tests were performed as in previous experiments.

6.6.1 Results and Discussion

6.6.1.1 Chronic Exposure

As expected (based on pilot data), exposure to nicotine reduced the locomotor activity of the animals: the planaria exposed to nicotine showed on average a 50% reduction in locomotor activity (M = 37.4 cm, SE = 3.4 cm) relative to planaria exposed to treated water (M = 74.6 cm, SE = 3.5) on the first day of chronic exposure. There did not seem to be any development of tolerance because there was no increase in the locomotor activity of Group Nicotine on the second day of chronic exposure. The data of the chronic exposure phase of the experiment is displayed in Figure 6.6 A. A 2 (Group: Nicotine vs Water) x 2 (Days: 1 – 2) x 3 (Bins: 1-15) - 15 bins means 150 min of nicotine exposure- mixed ANOVA that revealed a main effect of Group, $F(1, 58) = 71.21$, $p < .001$, η_p^2=.55, and Bins, $F(6.5, 377.8) = 60.61$, $p < .001$, $\eta_p^2 = .51$, as well as significant

Group x Bins interaction , $F(6.5, 377.8) = 7.35$, $p < .001$, $\eta_p^2 = .11$. However, there was no significant effect of Days, $F(1, 58) = .001$, $p = .97$, $\eta_p^2 = .00$, and the remaining interactions were not significant: Group x Days, $F(1, 58) = 0.64$, $p = 0.43$, $\eta_p^2 = .011$, Days x Bins, $F(6.6, 383) = 0.80$, $p = .37$, $\eta_p^2 = .014$, Group x Days and Bins, $F(6.6, 383) = 0.98$, $p = .44$, $\eta_p^2 = .017$. The result suggested that the locomotor activity of animals was different between groups, and it changed over the bins. However, there was no increase in the locomotor activity of Group Nicotine over the course of nicotine exposure, suggesting that the development of tolerance was not successful. These results confirm that the chronic exposure procedure used in the present experiment was not effective in developing long-term tolerance to the effects of nicotine in the planaria.

6.6.1.2 Test 1

Test 1 was conducted in the drug-paired context with water to assess the development of CCRs. As can be observed in Figure 6.6 B (left panel, T1), planaria previously exposed to nicotine ($M = 83.7$ cm, $SE = 3.6$ cm) showed marginally lower locomotor activity than animals in the Group Water ($M = 93$ cm, $SE = 3.7$ cm). This impression was confirmed by a 2 (Group: Nicotine *vs*. Water) x 3 (Bins: 1-3) mixed ANOVA, which revealed an effect of Bins, $F(2, 116) = 4.58$, $p = 0.01$, $\eta_p^2 = .07$, a marginal effect of Group, $F(1, 58) = 3.18$, $p = 0.08$, $\eta_p^2 = .05$, but no Group x Bins interaction, $F(2, 116) = 2.10$, $p = 0.12$, $\eta_p^2 = .035$. The marginal effect of Group went in the opposite direction to what we expected.

6.6.1.3 Test 2 and Test 3

Figure 6.6 B (central and right panels, T2 and T3) displays tolerance test results in the drug-paired (Test 2) and the novel contexts (Test 3). Animals in Group Nicotine ($M = 69.6$ cm, $SE = 1.8$ cm) behaved in a similar way to the animals in the Group Water ($M = 72.5$ cm, $SE = 1.8$ cm), in both contexts. This impression was confirmed by a 2

(Group: Nicotine *vs*. Water) x 2 (Tests: Test 2 and Test 3) x 3 (Bins: 1-3) mixed ANOVA, which revealed no effect of Group, $F(1, 58) = 1.32$, $p = .25$, $\eta_p^2 = .02$, a main effect of Tests, $F(1, 58) = 13.03$, $p = .001$, $\eta_p^2 = .18$, Bins, $F(1.7, 98.4) = 104.4$, $p < .001$, $\eta_p^2 = .64$, as well as a significant Tests x Bins interaction, $F(1.3, 75.8) = 7.97$, $p = .003$, $\eta_p^2 = .12$. The remaining interactions were all non-significant: Group x Tests, $F(1, 58) = 0.49$, $p = .49$, $\eta_p^2 = .008$, Group x Bins, $F(1.6, 98.4) = 0.25$, $p = .75$, $\eta_p^2 = .004$, and Group x Tests x Bins, $F(1.3, 75.8) = 0.34$, $p = .62$, $\eta_p^2 = .006$.

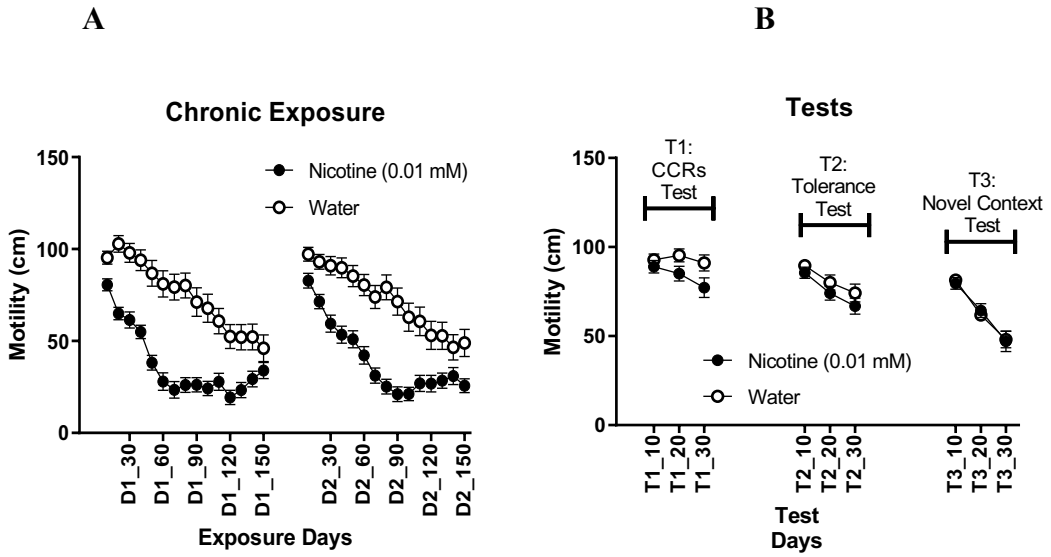


Figure 6. 6. Experiment 5. Mean distance covered by planaria in the drug-paired context throughout 2 days of 2.5 hour in the presence of nicotine or water (A), 30 min of CCRs test in the presence of only water (T1), 30 min of tolerance test in the presence of nicotine (T2), and 30 min of novel context test in the presence of nicotine with an alternative context (T3) (B). Results represented in 10 minutes bins. Bars represent standard errors. n=29-31 planaria in each group.

6.7 Experiment 6: Tolerance Development with Massive Nicotine Exposure and two doses (0.01 mM and 0.025 mM), followed by HPLC neurochemical analysis.

In the all previous experiments, planaria were treated with nicotine for a total of 5 hours before they were tested; however, we did not observe tolerance development using both intermittent (30 min x 10 days) and massive (2.5 hours x 2 days) nicotine regimens. As Feng et al. (2006) reported, animals were adapted to the hyper locomotor effect of nicotine after overnight (16 h) nicotine exposure. This suggests that tolerance

development could be better observed with a prolonged nicotine treatment. Therefore, we decided to increase the total length of nicotine treatment from 5 to 10 hours.

In this experiment, the planaria were treated with either water (W), a low nicotine concentration (L; 0.01 mM) or a high nicotine concentration (H; 0.025 mM) for 2.5 hours during four days of chronic exposure (a total of 10 hours). Following the chronic exposure sessions, all the animals were tested in three different nicotine concentrations (0; 0.01; and 0.025) over three days. Tests were counterbalanced (e.g., W-L-H, L-H-W or H-W-L). Animals were always tested in the drug-paired context.

We also investigated underlying neurochemical changes neurotransmitter systems after chronic nicotine exposure with HPLC, using the same experimental procedure described for Experiment 3. There were three groups of animals in this HPLC analysis, including Chronic High Group (treated with nicotine concentration of 0.025 mM during the conditioning days), Chronic Low Group (treated with nicotine concentration of 0.01 mM during the conditioning days) and Acute Group (exposed to nicotine only during the test sessions). No animal was taken from the colony. There were 16 animas in each group.

6.7.1 Results and discussion

6.7.1.1 Chronic Exposure

The data of the chronic exposure phase of the experiment in Figure 6.7 A showed that over the course of the chronic exposure, the animals treated with High Nicotine (*M* = 31.2 cm, *SE* = 3.7 cm) and Low Nicotine (*M* = 33.6 cm, *SE* = 3.6 cm) showed lower levels of locomotor activity than the animals exposed to water (*M* = 47.8 cm, *SE* = 3.7 cm). This result replicates the results of previous experiments. Additionally, there seems to be a decrease in the effects of nicotine across days, suggesting tolerance development. These impressions were confirmed with a 3 (Group: Nicotine L *vs*. Nicotine H *vs.* Water) x 4 (Days: 1 – 4) x 15 (Bins: 1-15) mixed ANOVA that revealed main effects of Group,

$F(1, 40) = 12.16$, $p = .001$, $\eta_p^2 = .23$, Days, $F(3, 120) = 2.79$, $p = .04$, $\eta_p^2 = .065$, Bins, $F(6.4, 257.9) = 46.48$, $p < .001$, $\eta_p^2 = .54$, an a significant Days x Bins interaction, $F(16.2, 1028.2) = 3.12$, $p < .001$, $\eta_p^2 = .07$. The remaining interactions were all non-significant: Group x Days, $F(3, 120) = 0.73$, $p = .54$, $\eta_p^2 = .02$, Group x Bins, $F(6.4, 257.9) = 1.21$, $p = .30$, $\eta_p^2 = .03$, and Group x Days x Bins, $F(16.2, 1028.2) = 1.47$, $p = .10$, $\eta_p^2 = .03$. Within-subjects linear contrasts using three different nicotine concentrations revealed a marginal effect of Day in Group High Nicotine, $F(1, 13) = 3.72$, $p = .076$, $\eta_p^2 = .22$, but not in groups Low Nicotine, $F(1, 13) = 1.46$, $p = .25$, $\eta_p^2 = .10$, and Water, $F(1, 13) = 1.44$, $p = .25$, $\eta_p^2 = .10$. These results suggest that there is a slight reduction in the effect of higher concentration with repeated exposure.

6.7.1.2 Test 1, Conditioned Compensatory Responses (CCRs)

The Test 1 was conducted in the drug-paired context with treated water to assess the development of CCRs. As can be observed in Figure 6.7 B (left panel, T1), planaria in the Group Water (M = 67.9 cm, SE = 6.3 cm) behaved in a similar way to animals in the Group High Nicotine (M = 66.2 cm, SE = 6.3 cm, p = .27) and Group Low Nicotine (M = 57.9 cm, SE = 6.3 cm, p = .27). This impression was confirmed by a 3 (Group: High Nicotine, Low Nicotine *vs*. Water) x 3 (Bins: 1-3) mixed ANOVA, which revealed no effect of Group, F (2, 39) =0.71, p = .50, η_p^2 =.035, a main effect of Bins, F (1.7, 68) =12.65, $p < .001$, η_p^2=.24, and no interaction between these factors, F (1.7, 68) =0.36, p=0.80, η_p^2=.02. Animals chronically treated with the low and high concentrations did not produce CCRs in the absence of nicotine.

6.7.1.3 Test 2, Tolerance Test with Low Nicotine Concentration

Test 2 was conducted to assess the development of tolerance to the unconditioned effects of nicotine in the drug-paired context with the lower nicotine concentration, 0.01 mM. As can be observed in Figure 6.7 B (central panel, T2), planaria in the Group Water

(*M* = 56.3 cm, *SE* = 6.2 cm) behaved in a similar way to animals in the Group High Nicotine (*M* = 62.3 cm, *SE* = 6.2 cm, *p* = .49) and Group Low Nicotine (*M* = 47.6 cm, *SE* = 6.2 cm, *p* = .32). This impression was confirmed by a 3 (Group: High Nicotine, Low Nicotine *vs*. Water) x 3 (Bins: 1-3) mixed ANOVA, which revealed no effect of Group, F (2, 39) =1.45, p=0.25, η_p^2=.07, a main effect of Bins, F (1.8, 72.3) =27.4, p<0.001, ηp^2=.41, and no interaction between these factors, F (3.7, 72.3) =1.66, p=0.17, η_p^2=.08). Testing animals with the low nicotine concentration (0.01 mM) did not elicit any differences in the activity of the groups, suggesting no expression of tolerance.

6.7.1.4 Test 3, Tolerance Test with High Nicotine Concentration

Test 3 was conducted to assess the development of tolerance to the unconditioned effects of nicotine in the drug-paired context with high nicotine concentration (0.025 mM). As can be observed in Figure 6.7 B (right panel, T3), planaria in the Group Water (*M* = 48.8 cm, *SE* = 6.2 cm) behaved in a similar way to animals in the Group High Nicotine (*M* = 56.3 cm, *SE* = 6.2 cm, *p* = .40) and Group Low Nicotine (*M* = 45.3 cm, *SE* = 6.2 cm, *p* = .70). This impression was confirmed by a 3 (Group: High Nicotine, Low Nicotine *vs*. Water) x 3 (Bins: 1-3) mixed ANOVA, which revealed no effect of Group, F (2, 39) =0.81, p=0.45, η_p^2=.04, a main effect of Bins, F (2, 78) =27.4, p<0.001, ηp^2=.41, and no interaction between these factors, F (4.78, 72.3) =1.44, p=0.23, η_p^2=.07. The high concentration test did not reveal the development of tolerance for animals treated with nicotine earlier.

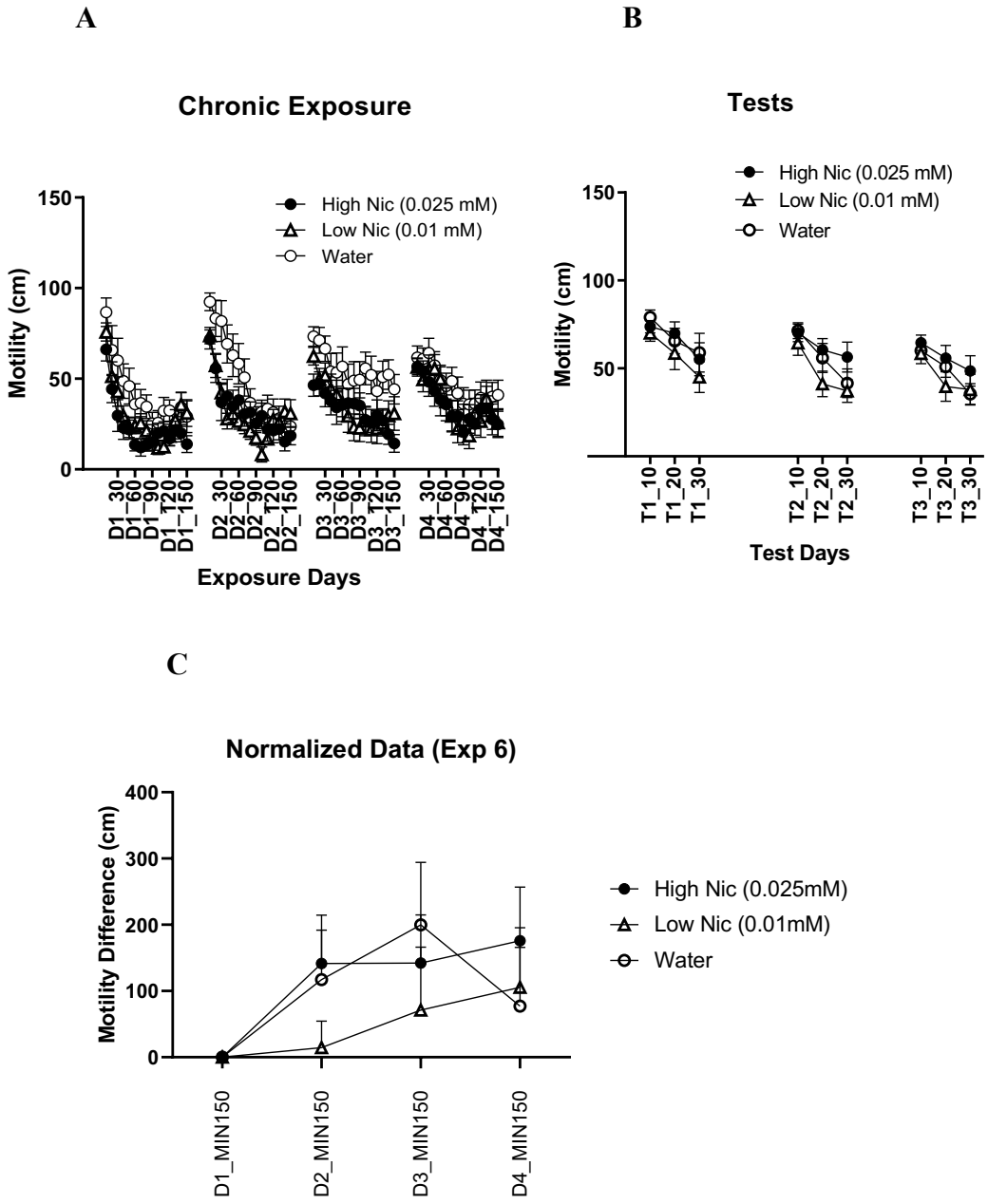


Figure 6. 7. Experiment 6. Mean distance covered by planaria in the drug-paired context throughout 4 days of 2.5 hour in the presence of high (0.025 mM) and low nicotine (0.01 mM) or water (A), 30 min of CCRs test in the presence of only water (T1), 30 min of tolerance test in the presence of low nicotine (T2), and 30 min of novel context test in the presence of high nicotine (T3) (B). Results represented in 10 minutes bins. Data normalized to the first training session and represented in 30 min bins (C). Bars represent standard errors. n = 14-15 each group.

6.7.2 HPLC Results

We examined the effects of nicotine concentrations (high chronic, low chronic and acute group) on the changes in neurotransmitter levels (DOPAC, DA, 5-HIAA, HVA, 5-HT). The results are displayed in Figure 6.8. Data for each neurotransmitter were analysed using one-way ANOVA. There was no main effect of nicotine concentration on

DOPAC, F (2,26) = 0.19, p = .83, DA, F (2,29) = 0.79, p = .46, 5-HIAA, F (2,28) = 0.40, p = .67, HVA, F (2,13) = 0.09, p = .92, and 5-HT, F (2,26) = 0.21, p =.81. These results indicated that there was no significant differences between high chronic, low chronic, and acute groups over the different neurotransmitters. We used to observe the difference in the neurochemical changes among groups in Experiment 3; however, we did not here. The reason is not having a colony control group where animals were directly taken from the colony and never exposed to nicotine during the tests in Experiment 6 contrary to Experiment 3. In Experiment 6, all three groups, including water group presented in the figure, were exposed to nicotine during Tests 2 and 3, and there was not a control colony group.

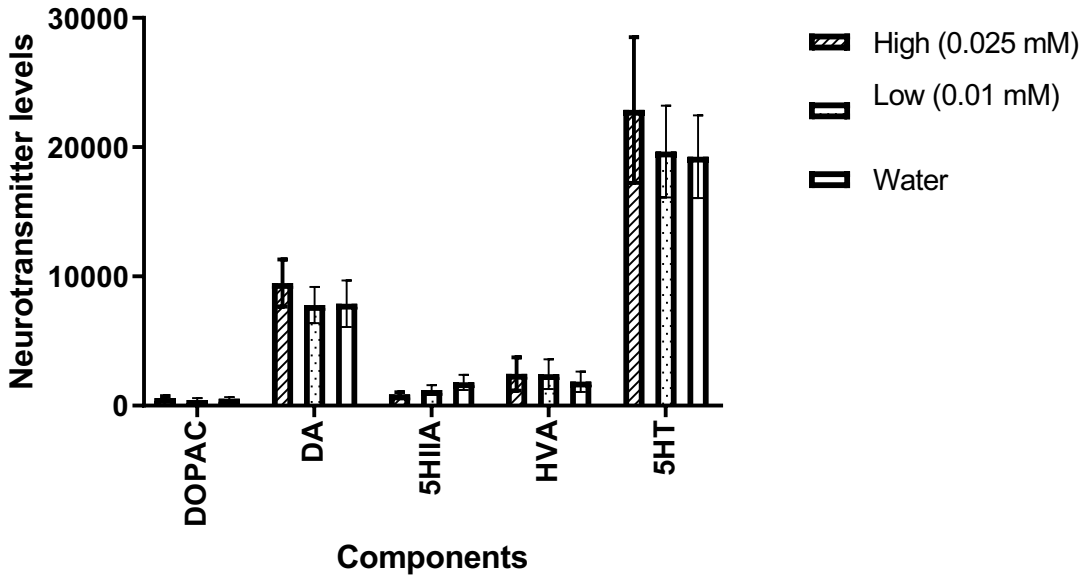


Figure 6. 8**.** The average neurotransmitter levels for DOPAC, DA, 5-HIAA, HVA, and 5-HT in high chronic nicotine, low chronic nicotine and acute nicotine = 5-12 the planaria/group.

6.8 Experiment 7: Tolerance Development with Long-term Nicotine Exposure (0.025 mM and 0.1 mM)

In the previous experiments, we did not successfully observe any changes in behavioural responses of planaria during five and ten hours of chronic exposure regarding tolerance development and CCRs. Feng et al. (2006) found that 16-hour (i.e., overnight) nicotine exposure resulted in the development of tolerance in *C elegans*. In Experiment 7 we assessed the effect of chronic nicotine with 16-hour long-term overnight exposure using two different nicotine concentrations: 0.025 mM and 0.1 mM.

Because of equipment limitations, we could not record the 16-hour exposure session. Therefore, we conducted a 30 min test immediately after the 16-hour overnight exposure (initial test). This was followed by three tests with water, low and high dose (counterbalanced) that replicate the procedure described for Experiment 6 (one test every 24 hours).

6.8.1 Results and discussion

6.8.1.1 Immediate Test

Animals were treated with different nicotine concentrations overnight, during a 16-hour period. Immediately after the overnight exposure, they were tested in the same concentrations that they were exposed to overnight. The purpose of the initial test was to assess if the 16-hour exposure resulted in differences in locomotor activity (i.e., chronic tolerance). The results of the initial test are displayed in Figure 6.9 (left panel). Nicotine reduced animals' locomotor activity in a concentration dependent manner. The locomotor activity of the animals was as follows: Group Water: M = 117.3 cm, SE = 8.6 cm, Group Low Nicotine: M = 79.6 cm, SE = 8.6 cm, and Group High Nicotine, M = 48.1 cm, SE = 8.9. A 3 (Group: High Nicotine vs. Low Nicotine vs. Water) x 3 (Bins: 1-3) mixed ANOVA revealed a significant main effect of Group, $F(2, 44) = 15.45$, $p < .001$, η_p^2 =

.41, Bins, $F(1.6, 78.3) = 24.6$, $p < .001$, $\eta_p^2 = .36$, but no interaction between these factors, $F(3.5, 78.3) = 0.89$, $p = .46$, $\eta_p^2 = .04$.

6.8.1.2 Test 1

Test 1 was conducted on the drug-paired context with water to assess the development of CCRs. As can be observed in Figure 6.9 (central panel, T1), planaria in the Group Water (M = 96.2 cm, SE = 8.5 cm) behaved in a similar way to animals in the Group High Nicotine (M = 96.7 cm, SE = 9.2 cm, p = .96) and Group Low Nicotine (M = 92.9 cm, SE = 8.8 cm, p = .79). This impression was confirmed by a 3 (Group: High Nicotine, Low Nicotine *vs*. Water) x 3 (Bins: 1-3) mixed ANOVA, which revealed no main effect of Group, F (2, 39) =0.54, p= .95, η_p^2=.003, Bins, F (1.6, 62) =2.83, p= .08, ηp^2=.07, and no interaction between these factors, F (3.2, 62) =0.55, p = .66, η_p^2=.03. The Water test did not produce CCRs for animals treated chronically with Low and the High concentrations of nicotine.

6.8.1.3 Test 2 Tolerance with Lower Nicotine Concentration

Test 2 was conducted to assess the development of tolerance to the unconditioned effects of nicotine in the drug-paired context with low nicotine concertation (0.01 mM). As can be observed in Figure 6.9 (central panel, T2), planaria in the Group Water (M = 66.5 cm, SE = 7.8 cm) behaved in a similar way to animals in the Group High Nicotine (M = 66.2 cm, SE = 8.1 cm, p = .98) and Group Low Nicotine (M = 76.7 cm, SE = 8.1 cm, p = .37). This impression was confirmed by a 3 (Group: High Nicotine, Low Nicotine *vs*. Water) x 3 (Bins: 1-3) mixed ANOVA, which revealed no effect of Group, F (2, 40) =0.54, p=.59, η_p^2=.03, a main effect of Bins, F (1.8, 70) = 16.56, $p < .001$, η_p^2=.29, but no interaction between these factors, F (3.5, 70) = 0.70, $p = .58$, $\eta_p^2 = .03$. Testing animals with the low nicotine concentration (0.01 mM) did not elicit any differences in the activity of the groups.

6.8.1.4 Test 3, Tolerance Test with High Nicotine Concentration

Test 3 was conducted to assess the development of tolerance to the unconditioned effects of nicotine in the drug-paired context with high nicotine concentration (0.025 mM). As can be observed in Figure 6.9 (right panel), planaria in the Group Water (M = 43.6 cm, SE = 5.2 cm) behaved in a similar way to animals in the Group High Nicotine (M = 50.4 cm, SE = 5.4 cm, p = .38) and Group Low Nicotine (M = 43.1 cm, SE = 4.9 cm, p = .94). This impression was confirmed by a 3 (Group: High Nicotine, Low Nicotine *vs*. Water) x 3 (Bins: 1-3) mixed ANOVA, which revealed no effect of Group, F (2, 40) =0.59, p=0.56, η_p^2=.03, a main effect of Bins, F (1.9, 74.3) = 36.50, p < .001, ηp^2=.48, and no interaction between these factors, F (3.7, 74.3) = 0.87, p = .48, η_p^2=.04. High concentration test did not reveal the development of tolerance for animals in Low and High Nicotine concentrations.

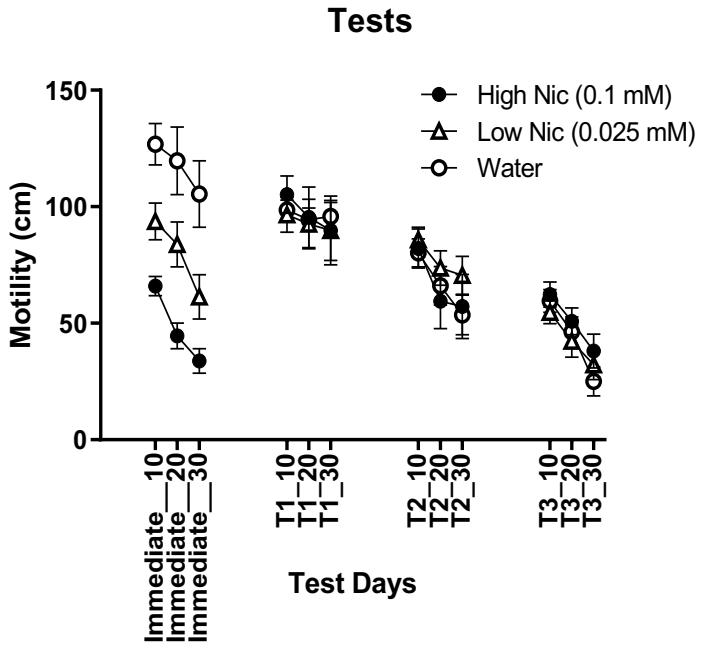


Figure 6. 9. Experiment 7. Mean distance covered by planaria in the drug-paired context immediately after overnight exposure in the presence of high (0.025 mM) and low nicotine (0.01 mM) or water (A), 30 min of CCRs test in the presence of only water (T1), 30 min of tolerance test in the presence of low nicotine (T2), and 30 min of novel context test in the presence of high nicotine (T3) (B). Results represented in 10 minutes bins. Bars represent standard errors. n = 14-15 each group.

6.9 Experiment 8: Comparison of Tolerance development between two strains of planaria: Dugesia sp. and Schmidtea mediterranea

Previous tolerance experiments reported in this chapter did not show a robust and consistent evidence of tolerance development with *Dugesia sp.* at both the behavioural and the neurochemical levels. Drug tolerance studies with different strains of animals showed that strain may play an essential role for the observation of tolerance development. Grieve and Littleton (1979) compared the function of tolerance development to ethanol with different strains of mice (C57BL, TO Swiss and DBA2). This study showed that the development of tolerance was very rapid with C57BL mice, but slower with TO Swiss mice; also, little evidence of tolerance was observed with DBA2 mice. Therefore, we need to consider the possibility that the failure to observe reliable nicotine tolerance development in planaria might be due the species of planaria we used (brown planaria, *Dugesia sp.*). The purpose of Experiment 8 was to compare the function of nicotine tolerance with different strains of planaria: *Dugesia sp.* and *Schmidtea mediterranea*. We used a total 40 *Dugesia sp.* and 32 *Schmidtea mediterranea*.

Animals were tested in 10 cm in diameter watch glass soda lime dishes; the surface of the dishes had been grooved by hand with a dental drill. These dishes served as the drug-paired context used during the chronic exposure to nicotine, and the Tests 1 and 2; similar dishes covered with a rough sandy surface were used as the alternative context in Test 3. The dishes could be filled with 20 ml of treated water or a nicotine solution (nicotine hydrogen tartrate salt, Sigma-Aldrich, UK, dissolved in autoclaved distilled water). We used a relatively low concentration of nicotine (0.025 mM) which pilot experiments had indicated produces reliable hypo-locomotion in planarians. We used 10 days of chronic exposure and then test 1 (CCRs), Test 2 and Test 3 with the different contexts as we described in the behavioural protocol section (see 5.3.1)

6.9.1 Results and discussion for Dugesia sp.

6.9.1.1 Chronic Exposure

On the first day of Chronic Exposure, the animals treated with nicotine showed on average more than 50% reduction in locomotor activity (M = 50.2 cm. SE = 6.1 cm) relative to planaria exposed to treated water (M = 123.7 cm, SE = 6.2 cm). Although some variability was observed across days, there did not seem to be a decrease in the effects of nicotine across days. The data of the chronic exposure phase of the experiment is displayed in the Figure 6.10 A. These impressions were confirmed with a 2 (Group: Nicotine *vs*. Water) x 10 (Days: 1 – 10) x 3 (Bins: 1-3) mixed ANOVA that revealed main effects of Group, $F(1, 38) = 121.24$, $p < .001$, $\eta_p^2 = .76$, Bins, $F(1.8, 68.8) = 122.8$, $p < .001$, $\eta_p^2 = .76$, as well as significant interactions Group x Bins, $F(1.8, 68.8) = 55.6$ $p < .001$, $\eta_p^2 = .59$, and Days x Bins, $F(10.5, 400.2) = 2.19$, $p = .02$, $\eta_p^2 = .05$. The remaining main effects and interactions were all non-significant: Days, $F(7.6, 290) = 1.84$, $p = .07$, $\eta_p^2 = .05$, Group x Days interaction, $F(7.6, 290) = 1.82$, $p = .08$, $\eta_p^2 = .05$, Group x Days x Bins interaction, $F(10.5, 400.2) = 1.49$, $p = .13$, $\eta_p^2 = .04$. Within-subjects linear contrasts revealed no effect of Day in Group Nicotine, $F(1, 19) = 2.11$, $p = .14$, $\eta_p^2 = .11$, or in Group Water, $F(1, 19) = 0.026$, $p = .87$, $\eta_p^2 = .001$, suggesting no significant increase in locomotor activity in both Group Nicotine and Water. These results suggest that, unlike what was observed in the previous experiment, the chronic exposure procedure used in the present experiment was not effective in developing long-term tolerance to the effects of nicotine in the planaria.

6.9.1.2 Test 1, Conditioned Compensatory Responses (CCRs)

The Test 1 was conducted in the drug-paired context with treated water to assess the development of CCRs. As can be observed in Figure 6.10 B (left panel, T1), planaria in the Group Nicotine (locomotor activity M = 111.3 cm, SE = 6.5 cm), behaved in a

similar way to animals in the Group Water (M = 117.2 cm, SE = 6.5 cm). This impression was confirmed by a 2 (Group: Nicotine *vs*. Water) x 3 (Bins: 1-3) mixed ANOVA, which revealed no main effects of Group, $F(1, 38) = 0.41$, $p = .52$, $\eta_p^2 = .01$, and Bins, $F(2, 76) = 0.50$, $p = .61$, $\eta_p^2 = .01$, and no interaction between these factors, $F(2, 76) = 0.64$, $p = .53$, $\eta_p^2 = .02$.

6.9.1.3 Test 2 and Test 3

These tests were conducted to assess the development of tolerance to the unconditioned (hypo locomotion) effects of nicotine, as well as to assess the context dependence of the tolerance developed to nicotine during the chronic exposure. Group Nicotine received the drug for the 11th time whilst animals in the Group Water received it for the first time in the drug-paired context in Test 2. The animals in both groups were tested in the presence of nicotine again, but in a novel distinctive context, during Test 3. As can be observed in Figure 6.10 B (central and right panels, T2 and T3), planaria previously exposed to nicotine (M = 57.8 cm, SE = 4.7 cm) behaved in a similar way to animals in the Group Water (M = 50.6 cm, SE = 4.7 cm) both during the Test 2 (in the drug-paired context) and Test 3 (in the new context), suggesting no evidence of tolerance in either context. These impressions were confirmed by a 2 (Group: Nicotine *vs*. Water) x 2 (Tests: Test 2 and Test 3) x 3 (Bins: 1-3) mixed ANOVA, which revealed a main effect of Bins, $F(1.6, 62.9) = 116.5$, $p < .001$, $\eta_p^2 = .75$ and significant Tests x Bins interaction, $F(1.6, 62.3) = 4.97$, $p = .01$, $\eta_p^2 = .12$. However, there was no main effects of Group, $F(1, 38) = 1.11$, $p = .30$, $\eta_p^2 = .03$, Tests, $F(1, 38) = 1.04$, $p = .31$, $\eta_p^2 = .03$. The remaining interactions were all non-significant: Group x Tests interaction, $F(1, 38) = 1.16$, $p = .29$, $\eta_p^2 = .03$, Group x Bins interaction, $F(1.6, 62.9) = 0.18$, $p = .79$, $\eta_p^2 = .005$, and Group x Tests x Bins interaction, $F(1.6, 62.3) = 0.95$, $p = .37$, $\eta_p^2 = .02$.

A **B**

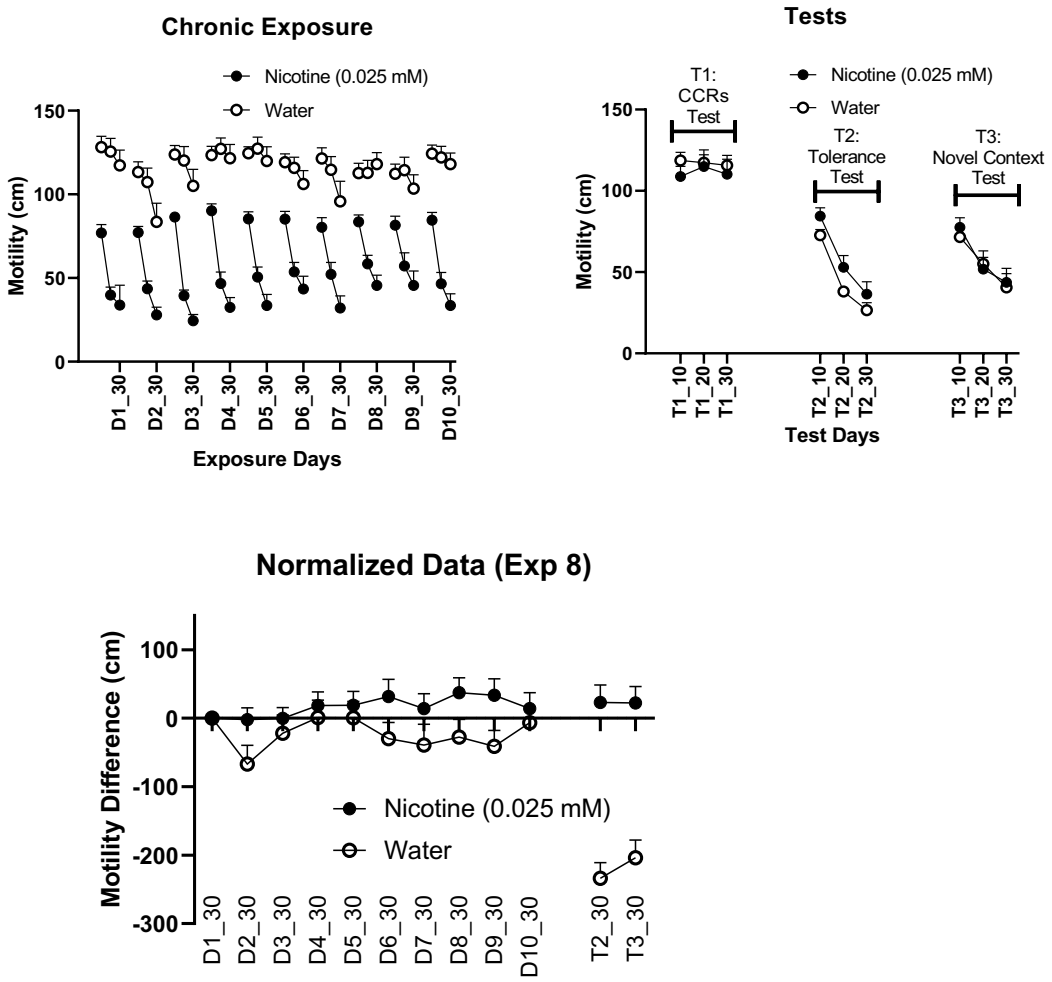


Figure 6. 10. Experiment 8. Mean distance covered by planaria in the drug-paired context throughout 10 days of 30 min in the presence of nicotine or water (A), 30 min of CCRs test in the presence of only water (T1), 30 min of tolerance test in the presence of nicotine (T2), and 30 min of novel context test in the presence of nicotine with an alternative context (T3) (B). Results represented in 10 minutes bins. Bars represent standard errors. n=20 planaria in each group.

6.9.2 Results and discussion for Schmidtea mediterranea

We observed the development of tolerance without compensatory responses in *Schmidtea mediterranea*. A series of tolerance experiment with *Schmidtea mediterranea* has already been published (Sal, Prados & Urcelay, in press, *Pharmacology, Biochemistry and Behaviour*). The experiments with *Schmidtea mediterranea* are reported in Chapter 8. The experiment that replicates the procedure of Experiment 8 (with *Dugesia sp.*) but using *Schmidtea mediterranea* is reported as Experiment 13 (see Chapter 8).

6.10 Summary

Chronic nicotine exposure reduces sensitivity to the effects of nicotine, which then results in behavioural changes (i.e., tolerance development). In planaria, changes in locomotor activity following acute nicotine administration have been reported (Rawls, 2011), but it is unknown whether chronic exposure leads to tolerance development. Because planaria can be used as a first-stage preclinical model, in this chapter we assessed the acute and chronic effects of nicotine on the locomotor activity of planaria (*Dugesia sp.*). In different experiments, we used different concentrations and lengths of exposure. We tested for tolerance development as assessed by a reduced sensitivity of nicotine's effects, and the presence of conditioned compensatory responses. We observed that acute nicotine administration produced hypoactivity in a consistent concentration-dependent manner. However, we observed similar hypoactive responses to nicotine following acute and chronic administrations.

Previous planaria studies showed the existence of different neurotransmitter systems such as dopaminergic. (Palladini et al., 1996; Ramoz et al., 2012) cholinergic system (Nishimura et al., 2010; Buttarelli et al., 2000) and the interaction between cholinergic and dopaminergic system (Buttarelli et al., 2000). These studies identified these neurotransmitter systems by screening distinctive hyperkinesia responses with the stimulation of compounds relevant to drug of abuse. However, limited number of planaria studies investigated changes in neurotransmitter systems using High-Performance Liquid Chromatography (HPLC). In this chapter, we assessed the changes in neurotransmitter levels (such as dopamine and serotonin) after acute and chronic nicotine exposure using HPLC. The results showed a significant difference between control and nicotine treated animals for the level of DA and serotonin concentration; however, the absolute concentration of DA was lower than serotonin. The propensity of dopamine to oxidase

might be the potential reason because animals homogenized with perchloric acid which minimizes the oxidation of dopamine. We also observed no difference between acute and chronic effect of nicotine at the neurochemical level in the HPLC analyses, unlike the behavioural findings. Other nicotine studies with rats found similar results using HPLC technique (Damsma et al., 1989). Researchers observed an increase in cellular DA level and its metabolites of animals treated with acute and chronic nicotine, but there was no difference between these groups.

Along with the absence of tolerance, we did not observe systematic conditioned responses with *Dugesia sp.* However, our recent experiment with the similar manipulation but in a different strain, *Schmidtea mediterranea*, showed tolerance development. In summary, with the parameters tested, we observed the development of tolerance to the effect of nicotine with *Schmidtea mediterranea* but not with *Dugesia sp.*

7 Chapter 7: Chronic effect of Nicotine in Planaria (*Schmidtea mediterranea*): Tolerance and Compensatory Responses

7.1 Introduction

In rodents, chronic exposure reduces sensitivity to the unconditioned effects of nicotine, which then results in addictive-like behaviours (i.e., tolerance development). Habituation model of tolerance (Baker & Tiffany, 1985) suggested that dose level and inter-dose interval (ITI) have an important impact on drug signalling that effect the development of drug tolerance. Also, several tolerance studies with nicotine (Feng et al., 2006) highlighted the importance of different regimens of exposure and on the development of tolerance (i.e., one-time overnight exposure versus intermittent exposure).

In this Chapter we will present four experiments regarding the chronic effect of nicotine on *Schmidtea mediterranea* with different experimental schedules: 10x and 5x. In both schedules, the animals were exposed to nicotine for 5 hours in total during either during 5 days (5x schedule) or 10 days (10x schedule). The main objective of these experiments was to determine whether chronic exposure to nicotine (with 5x and 10x) result in 1)- less effect of nicotine (i.e., tolerance to the unconditioned effect of nicotine), 2)- learned tolerance (i.e, context dependency and CCRs) using *Schmidtea mediterranea*. Based on previous findings in rodents and humans, there were four main hypotheses in these experiments:

The first hypothesis was that chronic nicotine exposure would produce sensitivity to the effect of nicotine, which then results in behavioural changes and tolerance development. That potentially would reveal adaptation to the effects of nicotine.

Second, we expected to observe CCRs with planaria after chronic nicotine treatment. Pavlovian conditioning contributes to homeostatic regulation in different

systems to maintain the stability of an organism (Siegel, 1975). The unconditional effect of drugs causes a physiological disturbance, and imbalance that threatens the existence of the animal. Through learning, animals develop an anticipatory homeostatic response to the presentation of drug-associated stimuli that attenuates the initial effect of drug and achieve the balance/ homeostasis (Siegel, 2008). Therefore, we expected to observe CCRs with planaria after chronic nicotine treatment.

Third, we expected to observe less hypo-activity in the nicotine pre-treated group compared to the vehicle pre-treated group when they were tested with nicotine in the drug-paired context. This was to compare the chronic and acute effects of nicotine.

Our fourth hypothesis was that, tolerance development is context dependent (Siegel, 1975, Siegel, 2008). We expected to observe no (or less) tolerance development in a novel environment.

7.2 Experiment 9: Tolerance and Compensatory Reponses with Different Regimens of Exposure (10x and 5x) - 60 min Tests

In the experiment 14 (presented in chapter 8), we observed tolerance development in planaria during and following chronic nicotine treatment. Siegel (1975) suggested that Conditioned Compensatory Responses (CCRs) are the underlying reason for tolerance development. Feng et al. (2006) found that different schedules of nicotine administration cause different behavioural responses to nicotine. For example, on the one hand, repeated intermittent chronic nicotine exposure led to behavioural sensitisation; on the other hand, uninterrupted massive chronic nicotine exposure resulted in the development of tolerance. In other words, having different histories of chronic nicotine administration results in differential expression of behavioural responses to nicotine. Thus, the goal of this experiment was to examine the role of different regimens of chronic nicotine

administration on the development of tolerance and CCRs. We allocated a total 64 animals to four groups, 10x Nicotine, 10x Water, 5x Nicotine and 5x Water. One animal in Group 10x Water and one in Group 5x Nicotine died over the course of the experiment, resulting in n = 16 for Group 10x Nicotine, n = 15 in Group 10x Water, n = 15 in Group 5x Nicotine, and n = 16 in Group 5x Water. We used a relatively low concentration of nicotine (0.025 mM) which pilot experiments had indicated produces reliable hypo-locomotion in planarians.

Animals in the 10x Nicotine group experienced 30 min of nicotine exposure over the 10 consecutive drug-paired days; animals in the 5x Nicotine group were treated with nicotine for one hour during five consecutive days. The control groups were treated in the same way but exposed to treated water instead of nicotine. Then the animals in the 5x and 10x schedule received the same three test sessions of 60 min: 1) – CCRs test in the drug-paired context; 2) – tolerance test in the drug-paired context; and 3)- tolerance test in the novel context. It is important to note that planaria pre-treated with nicotine showed a trend for development of CCRs relative to the Control Group when drug free water was presented in the drug-paired context in the previous experiment 14 (See chapter 8). Since in that experiment the test session was 30 mins, it may be possible that the absence of compensatory responses was due to insufficient time during the test session to observe the expression of CCRs. Therefore, we decided to increase the duration of the test from 30 min to 1h in this experiment.

7.2.1 10x schedule results

7.2.1.1 Chronic Exposure

The data of chronic exposure phase of the experiment is presented in Figure 7.1 A. As expected, based on previous data, nicotine reduced locomotor activity of planaria. On the first day of chronic exposure, Planaria that received nicotine (M = 63.3 cm, SE =

4.4 cm) showed significantly lower locomotor activity than the planaria that experienced water (M = 114.9 cm, SE = 5 cm). However, the activity of nicotine treated animals remained the same during the chronic exposure. These impressions were confirmed with a 2 (Group: Nicotine vs Water) x 10 (Days: 1 – 10) x 3 (Bins: 1-3) mixed ANOVA that revealed main effects of Group, $F(1, 29) = 139.3$, $p < .001$, $\eta_p^2 = .83$, and Bins, $F(1.8, 51.9) = 35.24$, $p < .001$, $\eta_p^2 = .55$, but no effect of Days, $F(6.9, 202.2) = 1.93$, $p = .07$, $\eta_p^2 = .06$. There were significant interactions of Group x Days, $F(6.9, 202.2) = 4.26$, $p < .001$, $\eta_p^2 = .13$, and Group x Bins, $F(1.8, 51.9) = 65.95$, $p < .001$, $\eta_p^2 = .69$, as well as a significant three-way significant Group x Bins x Days, $F(14, 407.3) = 2.7$, $p = .001$, $\eta_p^2 = .08$. The Bins x Days interaction was non-significant, $F(14, 407.3) = 1.16$, $p = .29$, $\eta_p^2 = .04$. Within-subjects linear contrasts revealed no effect of Day in Group Nicotine, $F(1, 15) = 0.31$, $p = .86$, $\eta_p^2 = .002$, suggesting no change in locomotor activity in Group 10x Nicotine, but there was a marginal effect of Day in Group Water, $F(1, 14) = 3.53$, $p = .08$, $\eta_p^2 = .20$, but this was due to random variation across days. These results suggest that the chronic exposure procedure with 10x regimen that used in the present experiment is not effective in developing long-term tolerance to the effects of nicotine in the planaria.

7.2.1.2 Test1

Test 1 was conducted in the drug-paired context with water to assess the development of CCRs. As can be observed in Figure 7.1 B (left panel), planaria previously exposed to nicotine (M = 123.2 cm, SE = 8.1 cm) behaved in a similar way than control animals (M = 102.8 cm, SE = 7.8 cm). This impression was confirmed by a 2 (Group: Nicotine vs Water) x 3 (Bins: 1-6) mixed ANOVA, which showed no significant effect of Group, $F(1, 29) = 0.14$, $p = .71$, $\eta_p^2 = .005$, or Bins, $F(2, 58.1) = 1.75$, $p = .12$, $\eta_p^2 = .06$, but revealed a significant interaction between these factors, $F(2, 58.1) = 7.15$, $p = .002$, $\eta_p^2 = .19$. Further analysis of this interaction with an independent sample

t-test to compare both groups in each Bin during the CCRs test revealed no evidence of CCRs because there was no significant difference between Group Nicotine and Group Water for the first Bin, $t(29) = .95$, p = .35 and the last Bin, $t(29) = 1.74$, p = .093, revealing that water test after chronic nicotine treatment did not cause the development of the CCRs. Although the Nicotine Group tends to show CCRs in the last Bin, it was not statistically significant, and that was not replicated in the further experiments.

7.2.1.3 Test 2 and Test 3

These tests were conducted to assess the development of tolerance to the unconditioned (hypo locomotion) effects of nicotine. Group Nicotine received the drug for the 11th time whilst animals in the Group Water received it for the first time in Drug-paired Context (Test 2). Additionally, animals in both groups were tested with nicotine again, but in the Novel Context (Test 3). Figure 7.1 B (T2 and T3, mid and right panels) shows that planaria in Nicotine Group (M = 49.1 cm, SE = 3.5 cm) displayed more locomotor activity than planaria in Control Group (M = 37.6 cm, SE = 3.7 cm) during the tolerance tests, suggesting the expression of tolerance to nicotine. This impression was confirmed by a 2 (Group: Nicotine vs Water) x 2 (Tests: Test 2 and Test 3) x 6 (Bins: 1-6) mixed ANOVA, which revealed main effects of Group, F (1, 29) = 4.98, p = .03, η_p^2 = .15, Tests, F (1, 29) = 6.08, p = .02, η_p^2 = .17, and Bins, F(4.2, 120.8) = 52.08, p < .001, η_p^2=.64, as well as a significant interaction of Tests x Bins, F(4.6, 134) = 5.04, p < .001, η_p^2 = .15. The remaining interactions were not significant: Group x Tests, F(1, 29) = 0.89, p = .35, η_p^2 = .03, Group x Bins, F(4.2, 120.8) = 1.85, p = .75, η_p^2 = .01, Group x Tests x Bins, F(4.6, 134) = 0.24, p = .98, η_p^2 = .001.

The main effect of Test confirms that activity was lower during Test 3, but the lack of a Group x Test interaction suggests that chronic nicotine exposure with 10x schedule was successful for the development of tolerance. However, contrary to what

could be expected from Siegel's theory (e.g., Siegel, 1975), the tolerance to the effects of nicotine was not context dependent. Just to summarise, the development of tolerance was observed in both the trained (Test 2) and novel contexts (Test 3).

7.2.2 5x Schedule Results

7.2.2.1 Chronic Exposure

As expected, nicotine administration reduced locomotor activity of planaria. Planaria that received nicotine showed significantly lower locomotor activity (M = 52.6 cm, SE = 5.2 cm) than the planaria that experienced water (M = 93.4 cm, SE = 5.1 cm) on the first day. However, the activity of nicotine treated animals remained the same for the first day (M = 52.6 cm, SE = 5.2 cm) and the last days (M = 47.7 cm, SE = 4.6 cm) of the chronic exposure. Therefore, there was no development of tolerance during the chronic nicotine exposure with 5x schedule. The data of chronic exposure phase of the experiment is presented in Figure 7.1 C. These impressions were confirmed with a 2 (Group: Nicotine vs Water) x 5 (Days: 1 – 5) x 6 (Bins: 1-6) mixed ANOVA that revealed main effects of Drug, $F(1, 29) = 117.01$, $p < .001$, $\eta_p^2 = .80$, and Bins , $F(3.9, 113.5) = 39.7$, $p < .001$, $\eta_p^2 = .58$, but no significant effect of Days, $F(4, 116) = 1.26$, $p = .29$, $\eta_p^2 = .04$, The analysis also showed significant interactions of Drug x Days, $F(4, 116) = 3.59$, $p = .008$, $\eta_p^2 = .11$, Drug x Bins, $F(3.9, 113.5) = 37.39$, $p < .001$, $\eta_p^2 = .56$, and Bins x Days, $F(13.3, 384.7) = 1.94$, $p = .02$, $\eta_p^2 = .06$. The remaining three-way Group x Bins x Days interaction was non-significant, $F(13.3, 384.7) = 1.61$, $p = .08$, $\eta_p^2 = .05$. Within-subjects linear contrasts revealed no effect of Day in Group Nicotine, $F(1, 14) = 1.38$, $p = .26$, $\eta_p^2 = .09$, and a significant effect of Group Water, $F(1, 15) = 12.63$, $p = .003$, $\eta_p^2 = .46$, suggesting an increase in locomotor activity in Group Water but not Nicotine. These results suggest that the chronic exposure procedure with 5x schedule used in the present

experiment is not effective in developing long-term tolerance to the effects of nicotine in the planaria.

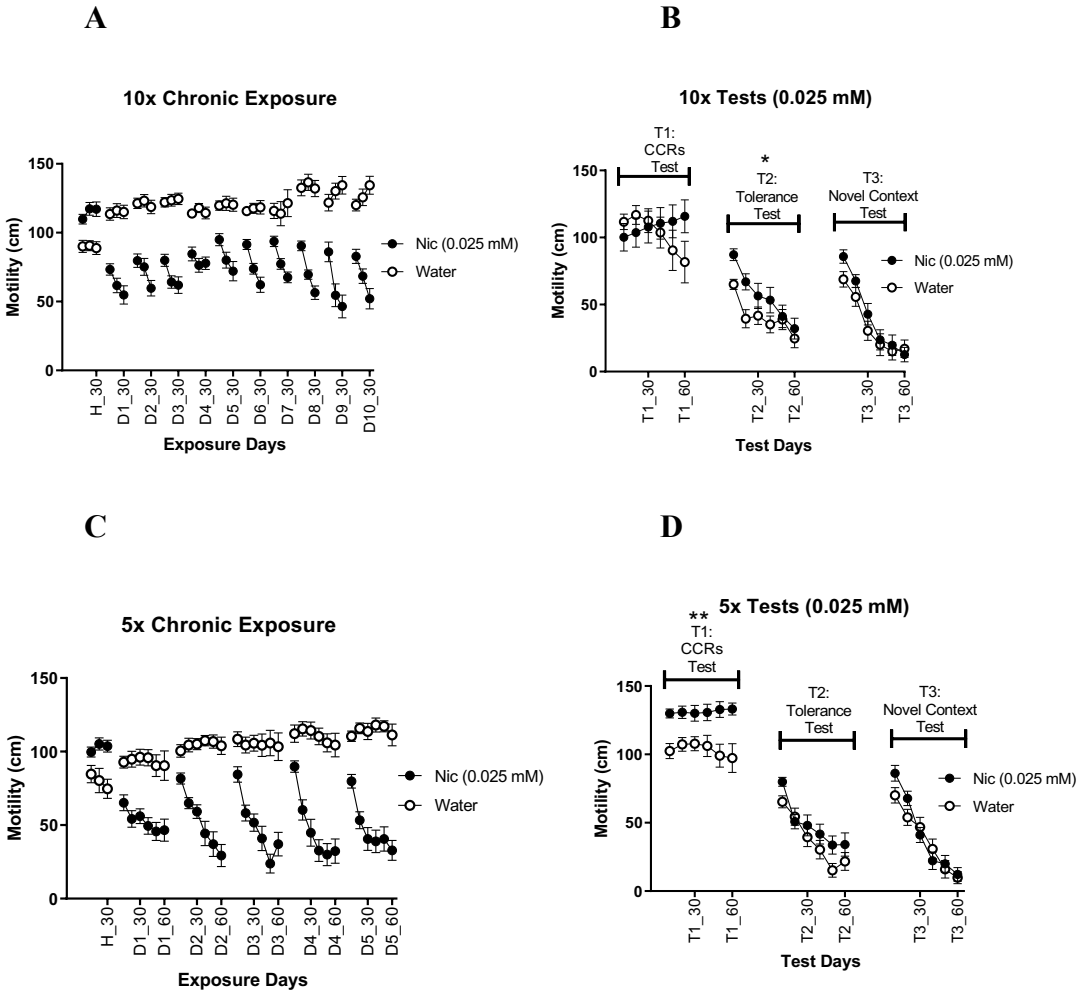
7.2.2.2 Test 1

Test 1 was conducted in the Drug-paired context with water to assess the development of CCRs. As can be observed in Figure 7.1 D (left panel), planaria previously exposed to nicotine displayed more activity (M = 131 cm, SE = 5.4 cm) than planaria in Control Group (M = 103.4 cm, SE = 5.3 cm), suggesting the expression of Conditioned Compensatory Response (CCR) to hypo-locomotive effects of nicotine, revealed in the presence of water in the drug-paired context following chronic nicotine exposure. This impression was confirmed by a 2 (Group: Nicotine vs Water) x 6 (Bins: 1-6) mixed ANOVA, which revealed a main effect of Group, $F(1, 29) = 13.40$, $p = .001$, $\eta_p^2 = .32$, but no effect of Bins, $F(2.9, 83) = 0.40$, $p = 0.85$, $\eta_p^2 = .01$, and no interaction between these factors, $F(2.9, 83) = 1.34$, $p = .34$, $\eta_p^2 = .19$.

7.2.2.3 Test 2 and Test 3

These tests were conducted to assess the development of tolerance to the unconditioned (hypo locomotion) effects of nicotine. Group Nicotine received the drug for the 6th time whilst animals in the Group Water received it for the first time in Drug-paired Context (Test 2). Additionally, animals in both groups were tested with nicotine again, but in a Novel Context (Test 3). As can be observed in Figure 7.1 D (mid and right panels), planaria previously exposed to nicotine (M = 44.8 cm, SE = 3.1 cm) behaved in a similar way than control animals (M = 37.8 cm, SE = 2.9 cm) in both contexts, suggesting that, with this regimen, there was no tolerance development to the effects of nicotine in planaria. This impression was confirmed by a 2 (Group: Nicotine vs Water) x 2 (Tests: Test 2 and Test 3) x 6 (Bins: 1-6) mixed ANOVA, which revealed no effect of Group, $F(1, 29) = 2.63$, $p = 0.12$, $\eta_p^2 = .08$, or Tests, $F(1, 29) = 0.57$, $p = .46$, $\eta_p^2 = .02$.

There was a main effect of Bins, $F(3.9, 114) = 70.6$, $p < .001$, $\eta_p^2 = .71$, as well as a significant Tests x Bins interaction, $F(4, 116.9) = 4.24$, $p = .03$, $\eta_p^2 = .13$. The remaining interactions were all non-significant: Group x Tests, $F(1, 29) = 0.63$, $p = .43$, $\eta_p^2 = .02$, Group x Bins, $F(3.9, 114) = 1.14$, $p = .34$, $\eta_p^2 = .04$, Group x Tests x Bins, $F(4, 116.9) = 2.23$, $p = .07$, $\eta_p^2 = .07$.



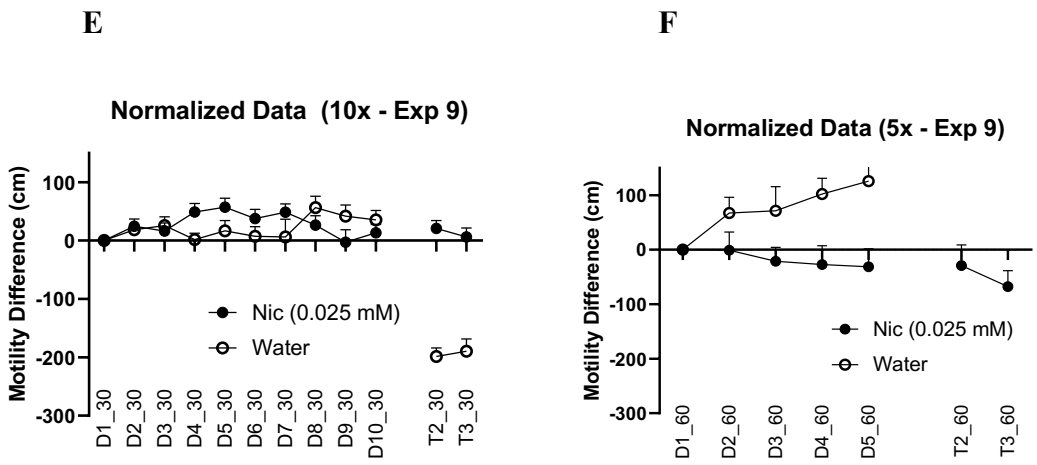


Figure 7. 1. Experiment 9. Mean distance covered by planaria in the drug-paired context throughout 10 days of 30 mins (A) or 5 days of 60 min (C) in the presence of nicotine or water, 1h of CCRs test in the presence of only water (B-T1 and D- T1), 1h of tolerance test in the presence of nicotine (B-T2 and D-T2), and 1h of novel context test in the presence of nicotine with an alternative context (B-T3 and D-T3). Results represented in 10 minutes bins. Data normalized to the first training session and represented in 30 min bins for 10x (E) and for 5x (F). Bars represent standard errors. N=15-16 planaria in each group.

Overall, these results from the 10x and 5x schedule of chronic nicotine administration suggest that different regimens of nicotine exposure induced different behavioural changes in planaria. The 10x schedule showed the evidence of non-context dependent tolerance development to the unconditioned effects of nicotine the 5x schedule revealed evidence of the development of CCRs.

7.3 Experiment 10: Replication of 10x and 5x Schedules of Tolerance with 60 min Tests

In the previous experiment (Experiment 9), we observed tolerance development but not CCRs with 10x schedule. We also found CCRs but not tolerance development with 5x schedule. However, due to random variation, in the previous experiment animals in water and nicotine groups showed differences in locomotor activity during the habituation session—before tolerance training. Therefore, the purpose of this experiment was to replicate the previous experiment but while controlling for locomotor activity during habituation. We achieved this by matching both groups in terms of their locomotor activity during the habituation session. We allocated a total 64 animals to four groups,

10x Nicotine, 10x Water, 5x Nicotine and 5x Water. One animal in Group 10x Water and 5x Water died over the course of the experiment, resulting in n = 16 for Group 10x Nicotine, n = 15 in Group 10x Water, n = 16 in Group 5x Nicotine, and n = 15 in Group 5x Water. The flatworms were held in the same way described for previous experiments. The same petri-dishes and experimental procedure employed in the previous experiments were used in the present experiment.

7.3.1 10x Schedule Results

7.3.1.1 Chronic Exposure

As expected, planaria exposed to nicotine (M = 60 cm, SE = 4.8 cm) showed less locomotor activity than the planaria that experienced water (M = 122.5 cm, SE = 5 cm) on the first day of the chronic exposure. Although some variability was observed across days, there did not seem to be any development of tolerance because there was an increase in the locomotor activity of both groups across days. The data of chronic exposure phase of the experiment is presented in Figure 7.2 A. This impression was confirmed by a 2 (Group: Nicotine vs Water) x 10 (Days: 1 – 10) x 3 (Bins: 1-3) mixed ANOVA that revealed main effects of Drug, $F(1, 29) = 96.4$, $p < .001$, $\eta_p^2 = .77$, Days, $F(9, 261) = 3.38$, $p = .001$, $\eta_p^2 = .10$, Bins, $F(1.4, 39.8) = 90.6$, $p < .001$, $\eta_p^2 = .76$, as well as significant interactions Drug x Bins, $F(1.4, 39.8) = 86.3$, $p < .001$, $\eta_p^2 = .75$, and Days x Bins, $F(14.7, 425.9) = 1.31$, $p = .02$, $\eta_p^2 = .06$. The remaining interactions were non-significant: Drug x Days, $F(9, 261) = 1.002$, $p = .44$, $\eta_p^2 = .03$, Drug x Days x Bins, $F(14.7, 425.9) = 0.77$, $p = 0.71$, $\eta_p^2 = .03$. Within-subjects linear contrasts revealed an effect of Day in Group Nicotine, $F(1, 15) = 14.37$, $p = .002$, $\eta_p^2 = .49$, and in Group Water, $F(1, 14) = 7.81$, $p = .014$, $\eta_p^2 = .36$, suggesting an increase in locomotor activity in both groups . These results seem to suggest that the chronic exposure procedure with 10x schedule used in the present experiment might not be effective in developing long-term tolerance to the effects of

nicotine in the planaria. However, any development of tolerance might be masked by the increase in the activity of the control animals.

7.3.1.2 Test 1

Test 1 was conducted on the drug-paired context with water to assess the development of CCRs. As can be observed in Figure 7.2 B (left panel), planaria previously exposed to nicotine (127 cm ± 5.6) behaved in a similar way as control animals (126 cm ± 5.8). This impression was confirmed by a 2 (Group: Nicotine vs Water) x 6 (Bins: 1-6) mixed ANOVA, which revealed a main effect of Bins, $F(5, 145) = 6.36$, $p < .001$, $\eta_p^2 = .18$, but no main effect of Group $F(1, 29) = 0.02$, $p = .89$, $\eta_p^2 = .001$, and no interaction between these factors, $F(5, 145) = 0.81$, $p = .54$, $\eta_p^2 = .03$.

7.3.1.3 Test 2 and Test 3

These tests were conducted to assess the development of tolerance to the unconditioned (hypo locomotion) effects of nicotine in Drug-paired Context (Test 2) in Novel Context (Test 3). Figure 7.2 B (mid and right panels, T2 and T3) shows that planaria in Nicotine Group (65.8 ± 3.8) displayed more locomotor activity than the animals in the Control Group (56.5 cm ± 3.9). This impression was confirmed by a 2 (Group: Nicotine vs Water) x 2 (Tests: Test 2 and Test 3) x 6 (Bins: 1-6) mixed ANOVA, which revealed main effects of Bins, $F(2.9, 84.1) = 64.8$, $p < .001$, $\eta_p^2 = .69$, and Tests, $F(1, 29) = 12.08$, $p = .02$, $\eta_p^2 = .29$, as well as significant interactions of Group x Tests, $F(1, 29) = 7.72$, $p = .01$, $\eta_p^2 = .21$, Tests x Bins, $F(3.4, 99.8) = 7.29$, $p < .001$, $\eta_p^2 = .20$. The main factor Group, however, was non-significant, $F(1, 29) = 2.87$, $p = .10$, $\eta_p^2 = .90$. The remaining interactions were also non-significant: Group x Bins, $F(2.9, 84.1) = 0.46$, $p = .71$, $\eta_p^2 = .02$, Group x Tests x Bins, $F(3.4, 99.8) = 2.33$, $p = .70$, $\eta_p^2 = .075$. The significant Group by Test interaction seems to suggest that tolerance to nicotine was

mediated by the context in which the animals were tested. In other words, nicotine tolerance was significant in one context, but not in the other context.

Further analyses of the Group x Test interaction suggested that there was a context dependent tolerance to the primary effect of nicotine. The development of tolerance was observed in the Drug-paired Context (Test 2) but not in the Novel Context (Test 3). The analysis of Test 2 data with a 2 (Group: Nicotine vs Water) x 6 (Bins: 1-6) mixed ANOVA that revealed a main effect of Group, $F(1, 29) = 9.78$, $p = .004$, $\eta_p^2 = .25$, and Bins, $F(3.5, 101.9) = 18.24$, $p < .001$, $\eta_p^2 = .39$, but no interaction between these factors, $F(3.5, 101.9) = 1.04$, $p = .38$, $\eta_p^2 = .03$. Additionally, the analysis of Test 3 data with a 2 (Group: Nicotine vs Water) x 6 (Bins: 1-6) mixed ANOVA that showed no effect of Group, $F(1, 29) = 0.18$, $p = .67$, $\eta_p^2 = .006$; there was a significant effect of Bins, $F(2.7, 80.6) = 53.6$, $p < .001$, $\eta_p^2 = .65$, but the Group x Bins interaction was non-significant, $F(2.7, 80.6) = 1.61$, $p = .20$, $\eta_p^2 = .05$.

7.3.2 5x Schedule Results

7.3.2.1 Chronic Exposure

The data of chronic exposure phase of the experiment is presented in Figure 7.2 C. As in previous experiments, nicotine reduced locomotor activity of planaria. Planaria that received nicotine showed significantly lower locomotor activity (M = 56 cm, SE = 6 cm) than the planaria that experienced water (M = 110.6 cm, SE = 6.2 cm) on the first day. However, the activity of nicotine treated animals did not increase from the first day (M = 56 cm, SE = 6 cm) to the last day (M = 48.5 cm, SE = 8.1 cm) of the chronic exposure. Therefore, there was no apparent development of tolerance during the chronic nicotine exposure phase. These impressions were confirmed with a 2 (Group: Nicotine vs Water) x 5 (Days: 1 – 5) x 6 (Bins: 1-6) mixed ANOVA that revealed a main effect of Group, $F(1, 29) = 70.2$, $p < .001$, $\eta_p^2 = .71$, Bins , $F(2.7, 78.9) = 51.2$, $p < .001$, $\eta_p^2 = .64$,

as well as significant interaction of Bins x Days, $F(11.1, 322) = 1.09$, $p = .31$, $\eta_p^2 = .04$. There was no main effect of Days, $F(4, 116) = 0.96$, $p = .43$, $\eta_p^2 = .03$, and the remaining interactions were all not significant: Group x Days, $F(4, 116) = 1.65$, $p = .17$, $\eta_p^2 = .03$, Group x Bins, $F(2.7, 78.9) = 11.34$, $p < .001$, $\eta_p^2 = .28$, Group x Bins x Days $F(11.1, 322) = 0.86$, $p = .58$, $\eta_p^2 = .03$. Within-subjects linear contrasts revealed no effect of Day in Group Nicotine, $F(1, 15) = 0.63$, $p = .44$, $\eta_p^2 = .04$, nor in Group Water, $F(1, 14) = 0.014$, $p = .71$, $\eta_p^2 = .01$, suggesting no increase in locomotor activity in Group Nicotine and Group Water. These results again seem to suggest that the chronic exposure procedure used in the present experiment is not effective in developing long-term tolerance to the effects of nicotine in the planaria.

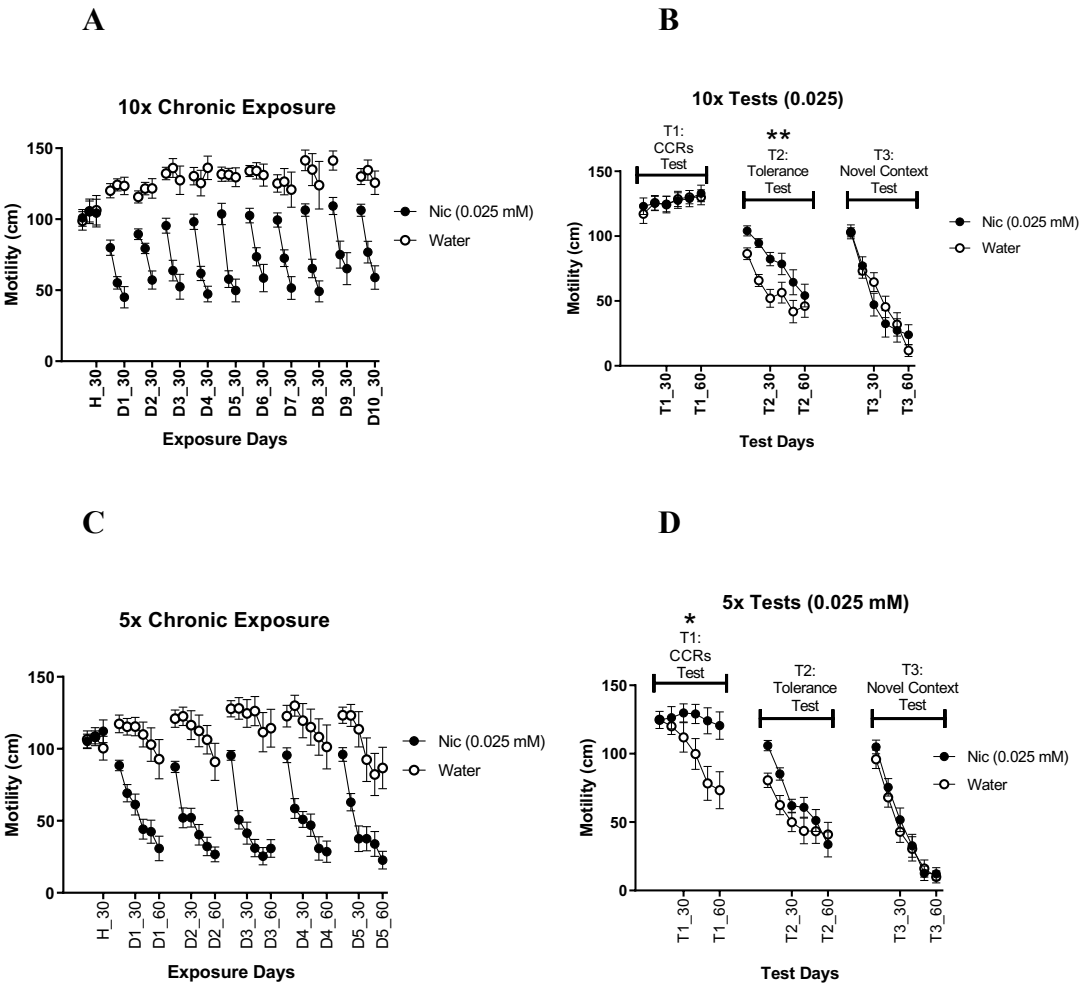
7.3.2.2 Test 1

Test 1 was conducted in the drug-paired context with water to assess the development of CCRs. As can be observed in Figure 7.2 D (left panel), planaria previously exposed to nicotine displayed more activity (125.8 ± 7.6) than planaria in Control Group (101 cm ± 7.9), suggesting the expression of Conditioned Compensatory Response (CCR) to hypo-locomotive nicotine effect in the presence of water in drug-paired context following chronic nicotine exposure. This impression was confirmed by a 2 (Group: Nicotine vs Water) x 6 (Bins: 1-6) mixed ANOVA, which revealed a significant effect of Group, $F(1, 29) = 5.04$, $p = .03$, $\eta_p^2 = .15$, Bins, $F(2.7, 79.7) = 8.7$, $p < .001$, $\eta_p^2 = .23$, as well as significant Group x Bins interaction, $F(2.7, 79.7) = 6.12$, $p = .001$, $\eta_p^2 = .17$.

7.3.2.3 Test 2 and Test 3

These tests were conducted to assess the development of tolerance to the unconditioned (hypo locomotion) effects of nicotine in Drug-paired Context (Test 2) and Novel Context (Test 3). As can be observed in Figure 7.2 D (mid and right panels),

planaria previously exposed to nicotine (57.3 cm ± 3.6) behaved in a similar way than control animals (50.6 cm ± 3.8), suggesting there was no tolerance to nicotine in both contexts (trained and new) in planaria. This impression was confirmed by a 2 (Group: Nicotine vs Water) x 2 (Tests: Test 2 and Test 3) x 6 (Bins: 1-6) mixed ANOVA, which revealed no effect of Group $F(1, 29) = 1.61$, $p = .21$, $\eta_p^2 = .05$. The analysis showed, however significant effects of Tests, $F(1, 29) = 11.26$, $p = .002$, $\eta_p^2 = .28$, and Bins, $F(4, 115.6) = 91.6$, $p < .001$, $\eta_p^2 = .76$, and a significant interaction of Tests x Bins, $F(3.7, 108.8) = 7.44$, $p < .001$, $\eta_p^2 = .20$. The remaining interactions were all non-significant: Group x Tests, $F(1, 29) = 0.56$, $p = .46$, $\eta_p^2 = .02$; Group x Bins interactions, $F(3.9, 115.6) = 1.16$, $p = .19$, $\eta_p^2 = .05$.; and Group x Tests x Bins, $F(3.7, 108.8) = 0.83$, $p = .50$, $\eta_p^2 = .03$.



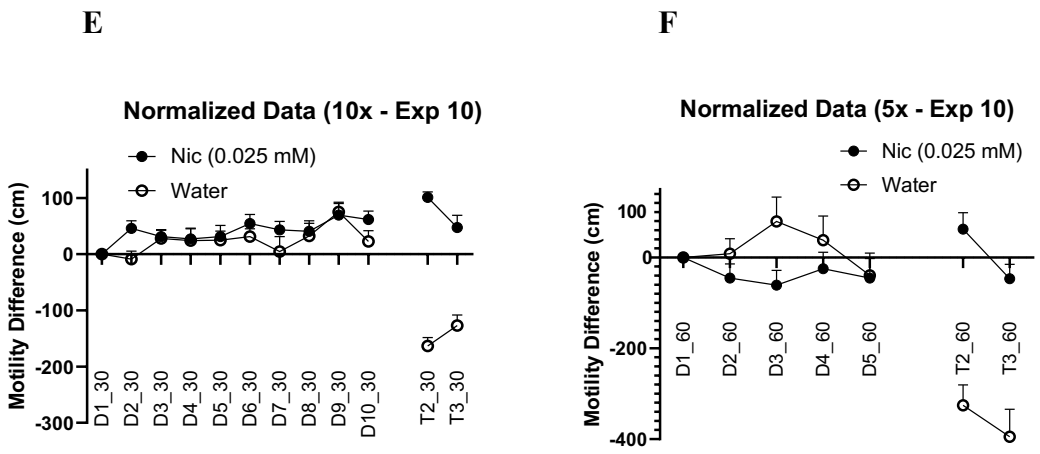


Figure 7. 2. Experiment 10. Mean distance covered by planaria in the drug-paired context throughout 10 days of 30 mins (A) or 5 days of 60 min (C) in the presence of nicotine or water, 1h of CCRs test in the presence of only water (B-T1 and D- T1), 1h of tolerance test in the presence of nicotine (B-T2 and D-T2), and 1h of novel context test in the presence of nicotine with an alternative context (B-T3 and D-T3). Results represented in 10 minutes bins. Data normalized to the first training session and represented in 30 min bins for 10x (E) and for 5x (F). Bars represent standard errors. Bars represent standard errors. N=15-16 planaria in each group.

Overall, Experiment 10 revealed a similar pattern of results as the previous Experiment 9, but controlling for baseline differences in locomotion (during the habituation session). Different schedules of nicotine exposure revealed different behavioural responses in planaria. Chronic exposure with 10x schedule showed the evidence of context dependent tolerance to the unconditioned effects of nicotine, and with 5x schedule produced evidence of CCRs

7.4 Experiment 11: Mecamylamine Experiment with 5x Schedule

In the previous experiments using 10x schedule (see Experiment 15 in Chapter 8), we observed that mecamylamine administration during chronic exposure training partially attenuated the primary reinforcing effect of nicotine and blocked the development of tolerance to nicotine's effects. Previous experiments with 5x schedule (Experiments 9 and 10) showed the evidence of CCRs but not tolerance to the unconditioned effects of nicotine. The purpose of this experiment was to assess whether mecamylamine, a nAChRs antagonist, modulates the nicotine-induced CCRs using 5x schedule. We hypothesized that the behavioural changes induced by chronic nicotine exposure with 5x schedule would be blocked by mecamylamine. Therefore, we used 2

(Drug 1: nicotine vs water) x 2 (Drug 2: mecamylamine vs water) experimental design for this experiment. We allocated a total 112 animals to four groups, Nicotine, Nicotine + Mecamylamine, Mecamylamine and Water. Two animals in Group Nicotine + Mecamylamine and one animal in Group Mecamylamine died over the course of experiment, resulting in n = 26 for Group Nicotine + Mecamylamine, n = 27 for Group Mecamylamine, and n =28 for Group Water and Group Nicotine. We used a relatively low concentration of nicotine (0.025 mM), and mecamylamine (0.05 mM). The flatworms were housed and maintained in the same way as described for previous experiments. The 5x schedule was used as described in previous experiments.

7.4.1 5x Results

7.4.1.1 Chronic Exposure

The data of chronic exposure phase of the experiment are presented in Figure 7.3 A and B. As previously observed, exposure to nicotine reduced the locomotor activity of the animals: the planarians exposed to nicotine showed less locomotor activity (M = 52.3 cm, SE = 3.6 cm) than the planarians that experienced nicotine plus mecamylamine (M =69.6 cm, SE = 3.4 cm). Additionally, planarians exposed to water (M = 107.9 cm ± 3.6) behaved a similar way than planarians exposed to only mecamylamine (97.9 cm ± 3.7), suggesting mecamylamine did not cause any changes alone. This impression was confirmed by a 2 (Drug 1 [Nicotine vs Water]) x 2 (Drug 2 [Mecamylamine vs Water]) x 5 (Days: 1-5) x 3 (Bins: 1-3) ANOVA that revealed a significant effect of Drug 1, $F(1, 105) = 130.47$, $p < .001$, $\eta_p^2 = .55$, as well as significant interaction of Drug 1 x Bins, $F(2.6, 267.6) = 33.9$, $p < .001$, $\eta_p^2 = .24$, Drug 1 x Days x Bins, $F(11.5, 1205) = 1.84$, $p = .04$, $\eta_p^2 = .02$, Drug 1 x Drug 2, $F(1, 105) = 13.75$, $p < .001$, $\eta_p^2 = .12$, Drug 1 x Drug 2 x Bins, $F(2.6, 267.6) = 7.03$, $p < .001$, $\eta_p^2 = .06$. However, there was no main effect of Drug 2, $F(1, 105) = 1.01$, $p = .32$, $\eta_p^2 = .01$. The remaining interactions were not significant:

Drug 1 x Days, $F(3.8, 397.3) = 2.21$, $p = .07$, $\eta_p^2 = .021$, Drug 2 x Bins, $F(2.6, 267.6) = 1.88$, $p = .17$, $\eta_p^2 = .02$, Drug 2 x Days, $F(3.8, 397.3) = 1.78$, $p = .13$, $\eta_p^2 = .02$, Drug 2 x Days x Bins, $F(11.5, 1205) = 1.52$, $p = .11$, $\eta_p^2 = .014$, Drug 1 x Drug 2 x Days, $F(3.8, 397.3) = 0.16$, $p = .95$, $\eta_p^2 = .002$, Drug 1 x Drug 2 x Days x Bins, $F(11.5, 1205) = 1.51$, $p = .12$, $\eta_p^2 = .014$.

The significant Drug 1 x Drug 2 interaction suggested that the effect of nicotine was attenuated by mecamylamine. Further analysis of this interaction confirmed that mecamylamine attenuated the effect of nicotine, because Group Nicotine displayed less motility than Group Nicotine +Mecamylamine, $F(1, 52) = 13.48$, $p = .001$, $\eta_p^2 = .21$. Group Mecamylamine did not differ from Group Water, $F(1, 53) = 3.128$, $p = .08$, $\eta_p^2 = .056$, revealing that mecamylamine did not cause significant changes in motility when given alone (see Figure 7.3, left panel).

7.4.1.2 Test 1

Test 1 was conducted on the drug-paired context with water to assess the development of CCRs and the effect of mecamylamine on the development of CCRs. As can be observed in Figure 7.3 C (left panel, T1), planarians previously exposed to nicotine (M = 105.5 cm, SE = 5.2 cm) behaved in a similar way to animals in the Group Water (M = 113.9 cm, SE = 5.2 cm). This impression was confirmed by a mixed ANOVA 2 (Drug 1 [Nicotine vs Water]) x 2 (Drug 2 [Mecamylamine vs Water]) x 6 (Bins: 1-6) that revealed no effect of Drug 1 (nicotine), $F(1, 99) = 0.43$, $p = .52$, $\eta_p^2 = .004$, or Drug 2 (mecamylamine), $F(1, 99) = 0.17$, $p = .68$, $\eta_p^2 = .002$; we observed, however, a significant interaction of Drug 1 x Drug 2, $F(1, 99) = 4.79$, $p = .031$, $\eta_p^2 = .046$. There was no effect of Bins, $F(3.1, 303.6) = 1.97$, $p = .12$, $\eta_p^2 = .02$, Bins x Drug 1 interaction , $F(1, 99) = 1.05$, $p = .37$, $\eta p2 = .01$, Bins x Drug 2 interaction, $F(1, 99) = 0.19$, $p = .66$, $\eta_p^2 = .002$. However the triple interaction Bins x Drug 1 x Drug 2 was significant, $F(1, 99) = 4.16$, p

= .006, η_p^2 = .04. The significant Drug 1 x Drug 2 interaction suggested that effect of nicotine was attenuated by mecamylamine. Further analysis of this interaction confirmed that mecamylamine did not excerpt an effect on animals previously exposed to nicotine, $F(1, 50) = 1.83$, $p = .18$, $\eta_p^2 = .03$, nor on those previously exposed to Water, $F(1, 49) = 2.96$, $p = .09$, $\eta_p^2 = .057$. In another word, mecamylamine did not have a differential effect on the animals exposed to nicotine and water (Group Nicotine + Mecamylamine and Group Water). These results suggest that both Nicotine and Water Groups covered similar amounts during the test, and the history of nicotine (Drug 1) or mecamylamine (Drug 2) exposure did not exert any significant effects on the CCR test. In another word, there was no differences between the groups exposed to Nicotine and Water (with no Mecamylamine) that would not replicate the results of the previous experiment.

7.4.1.3 Test 2 and Test 3

These tests were conducted to assess the effect of nicotine induced-tolerance development. Based on previous experiments with 5x schedule, we expected that animals previously exposed to nicotine would cover the same distance as control group that was not exposed to nicotine before. Figure 7.3 C (central and right panels, T2 and T3; see also Figure 7.3 D for a summary of Tests 2 and 3) shows that planarians in Group Nicotine displayed higher levels of locomotor activity (M = 55.4 cm, SE = 3.2 cm) than the animals in the Group Water (M = 44.8 cm, SE = 3.2 cm) in both contexts, suggesting a context independent development of tolerance to the effects of nicotine. However, planarians previously exposed to nicotine plus mecamylamine (M = 51.7 cm, SE = 3.5 cm) behaved in a similar way to animals previously exposed to mecamylamine only (M = 46.7 cm, SE = 3.3 cm), suggesting no effect on mecamylamine on tolerance, suggesting an attenuation of the tolerance development in the animals exposed to Nicotine + Mecamylamine.

These impressions were confirmed by a 2 (Drug 1 [Nicotine vs water) x 2 (Drug 2 [mecamylamine vs water]) x 2 (Tests: Test 2 and Test 3) x 6 (Bins: 1-6) mixed ANOVA, which revealed an effect of Drug 1, $F(1, 103) = 5.40$, $p = .22$, $\eta_p^2 = .05$, but no effect of Drug 2, $F(1, 103) = 0.075$, $p = .78$, $\eta_p^2 = .001$, and no interaction between Drug 1 and Drug 2 $F(1, 103) = 0.71$, $p = 0.40$, $\eta_p^2 = .007$. There was also a significant effect of Test, $F(1, 103) = 4.24$, $p = .04$, $\eta_p^2 = .04$, Bins, $F(4, 407.6) = 286.2$, $p < .001$, $\eta_p^2 = .73$, as well as significant interactions of Drug 1 x Bins, $F(4, 407.6) = 2.41$, $p = .49$, $\eta_p^2 = .02$, Drug 2 x Bins, $F(4, 407.6) = 4.25$, $p = .002$, $\eta_p^2 = .04$, Test x Bins, $F(3.7, 378.6) = 5.38$, $p < .001$, $\eta_p^2 = .05$. The remaining interactions were not significant: Test x Drug 1, $F(1, 103) = 0.02$, $p = .89$, $\eta_p^2 = .001$, Test x Drug 2, $F(1, 103) = 1.42$, $p = .24$, $\eta_p^2 = .014$, Test x Drug 1 x Drug 2, $F(1, 103) = 0.029$, $p = .86$, $\eta_p^2 = .001$, Bins x Drug 1 x Drug 2, $F(4, 407.6) = 1.06$, $p = .37$, $\eta_p^2 = .01$, Test x Bins X Drug 1, $F(4, 407.6) = 0.07$, $p = .98$, $\eta_p^2 = .001$, Test x Bins X Drug 2, $F(4, 407.6) = 0.25$, $p = .89$, $\eta_p^2 = .002$, Test x Bins x Drug 1 x Drug 2, $F(4, 407.6) = 0.97$, $p = .42$, $\eta_p^2 = .009$. These results suggest that there is an evidence of context-independent tolerance which was not modulated by mecamylamine. Further analysis on tests days confirmed that there was evidence of the development of tolerance on Test 2 (with a marginal effect), $F(1, 103) = 3.57$, $p = .06$, $\eta_p^2 = .034$ and Test 3, $F(1, 105) = 4.48$, $p = .04$, $\eta_p^2 = .041$. Also the nicotine-induced tolerance was not modulated by the co-administration of mecamylamine on Test 2 (no interaction between Nicotine and Mecamylamine), $F(1, 103) = 0.33$, $p = .57$, $\eta_p^2 = .003$ and on Test 3, $F(1, 105) = 0.62$, $p = .43$, $\eta_p^2 = .006$.

Overall, mecamylamine co-administration attenuated the effect of nicotine during the chronic exposure days. Contrary to previous findings of 5x schedule, water test on the nicotine-paired environment did not elicited CCRs. Moreover, in contrast to previous findings of 5x schedule, nicotine tests elicited context independent tolerance as we had

observed with 10x schedule in the Previous Experiments (6, 7 and 8). Furthermore, Nicotine-induced tolerance development depends on nicotine receptor activation, because mecamylamine blocked the development of tolerance as we observed in the previous mecamylamine experiment (Experiment 14) with the 10x schedule.

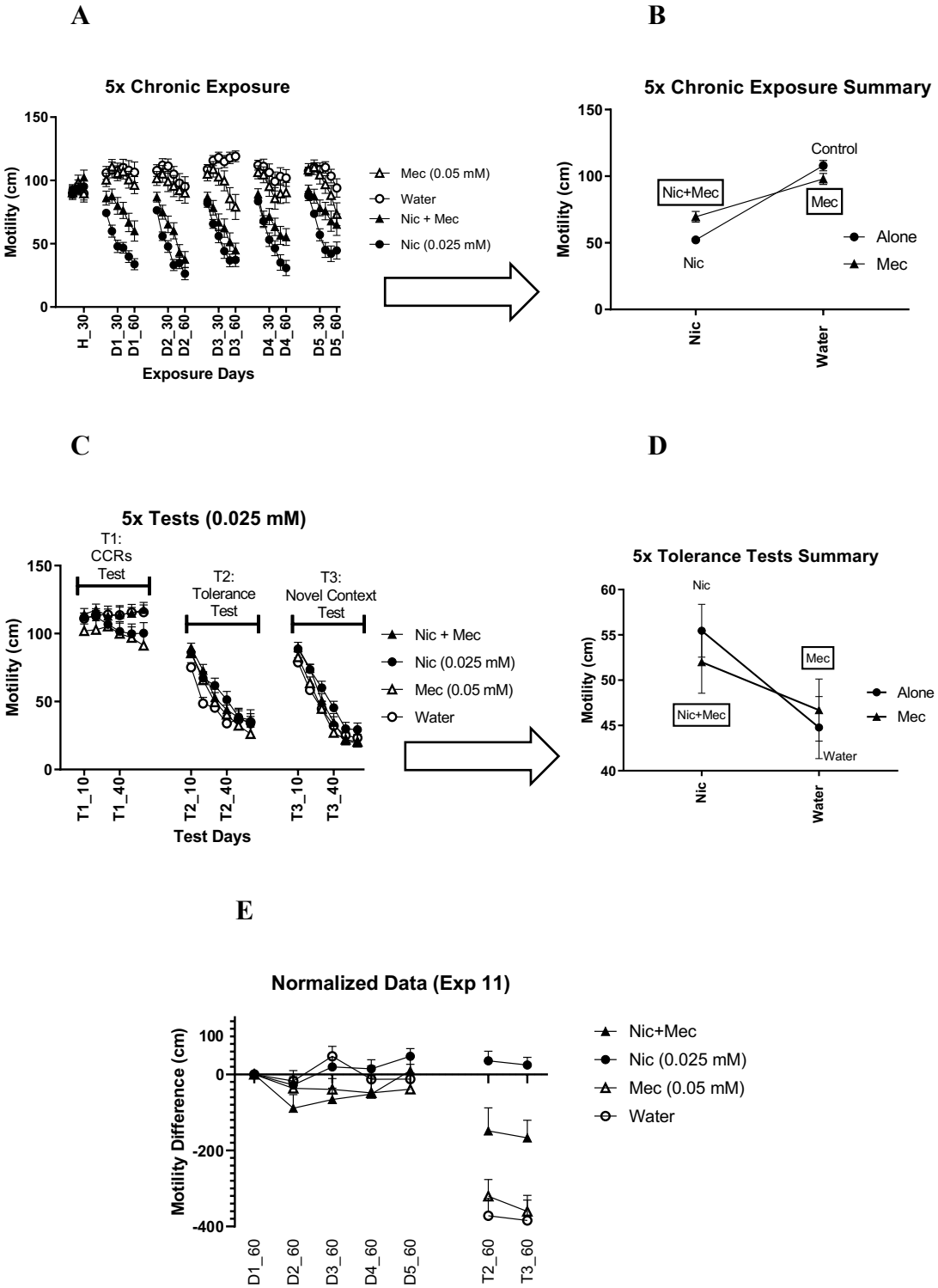


Figure 7. 3. Experiment 11. Mean distance covered by planaria in the drug-paired context throughout 10 days of 30 mins in the presence of nicotine or water (A), 60 min of CCRs test in the presence of water (T1), 60 min of tolerance test in the presence of nicotine (T2), and 60 min of novel context test in the presence of nicotine with an alternative context (T3). The summary data of 10 days of chronic nicotine exposure (C), and the summary data of nicotine in novel context (D). Results represented in 10 minutes bins. Data normalized to the first training session and represented in 30 min bins (E). Bars represent standard errors.Bars represent standart errors. N=28 planaria in each group.

7.5 Experiment 12: 10x Schedule with higher dose (0.05 mM)

In the previous experiments (Experiments 9 and 10), we observed CCRs to the primary effect of nicotine with 5x schedule. However, we failed to replicate this effect in the experiment in which we assessed the effect of mecamylamine (Experiment 11): the animals exposed to Nicotine and Water according to the 5x schedule did not differ in the Test 1 when tested with vehicle in the drug-associated context (unlike in Experiments 9 and 10). Animals within the 5x schedule were exposed to nicotine twice as long daily as animals in the 10x schedule (Experiments 9 and 10). Our findings showed that nicotine exposure with the 10x schedule reduced locomotor activity of animals from 80 cm to 50 cm within 30 min; which was similar to the reduction in the first 30 min of exposure for the nicotine group trained with 5x schedule. However, the continuation of nicotine exposure, 31- 60 min, reduced locomotor activity more and the locomotor activity came down to 20 cm per 10 min. It is possible that, since the Nicotine group with 5x schedule was exposed to nicotine twice as long daily as nicotine group with 10x schedule, this could be of relevance. Solomon (1980) posited that the primary A-process is modulated by the intensity of the unconditioned stimulus (US) that stimulate drug-antagonistic B-process. Therefore, we assumed that increasing the intensity/strength of the A process (depressant stimulation) with a higher nicotine concentration might result in a stronger B process which would reveal the development of CCRs. For this reason, we doubled the concentration of nicotine (0.05 mM) for the present experiment using the 10x schedule (Experiment 12). We assumed that doubling the drug concentration could lead to the development of CCRs with 10x schedule as we observed in 5x schedule. We allocated a

total of 48 animals to two groups, Nicotine and Water. One animal in the Nicotine Group died over the course of the experiment, resulting n =23 in Group nicotine and, n = 24 in Group Water. The flatworms were housed and maintained in the same way as described for previous experiments. The 10x schedule was used as described in previous experiments.

7.5.1 Results

7.5.1.1 Chronic Exposure

The data of chronic exposure phase of the experiment is presented in Figure 7.4 A. Planaria that received nicotine (M = 31.8 cm, SE = 3.2 cm) showed less locomotor activity than the planaria that experienced water (M = 92.5 cm, SE = 3.1 cm) on the first day of the chronic exposure. Although some variability was observed across days, there did not seem to be any development of tolerance because there was an increase in the locomotor activity of the water group across days. This impression was confirmed by a 2 (Group: Nicotine vs Water) x 10 (Days: 1 – 10) x 3 (Bins: 1-3) mixed ANOVA that revealed main effects of Drug, $F(1, 45) = 501.4$, $p < 0.001$, $\eta_p^2 = .91$, and Days, $F(7.7, 375.9) = 3.08$, $p = .003$, $\eta_p^2 = .06$, Bins, $F(2, 90) = 167.7$, $p < .0001$, $\eta_p^2 = .79$, as well as significant interactions of Drug x Days, $F(7.7, 345.9) = 3.87$, $p < .001$, $\eta_p^2 = .08$, Drug x Bins, $F(2, 90) = 167.7$, $p < .001$, $\eta_p^2 = .79$, Days x Bins, $F(16.3, 734.3) = 1.71$, $p = .04$, $\eta_p^2 = .04$, and a triple interaction of Drug x Days x Bins, $F(16.3, 734.3) = 1.79$, $p = .03$, $\eta_p^2 = .04$. Within-subjects linear contrasts revealed an effect of Day in Group Water, $F(1, 23) = 15.94$, $p = .001$, $\eta_p^2 = .41$, but not in Group Nicotine, $F(1, 22) = .17$, $p = .69$, $\eta_p^2 = .007$, suggesting an increase in locomotor activity in Group Water but not Nicotine. These results suggest that the chronic exposure procedure with 10x schedule used in the present experiment is not effective in showing the development of long-term tolerance to the effects of nicotine in the planaria.

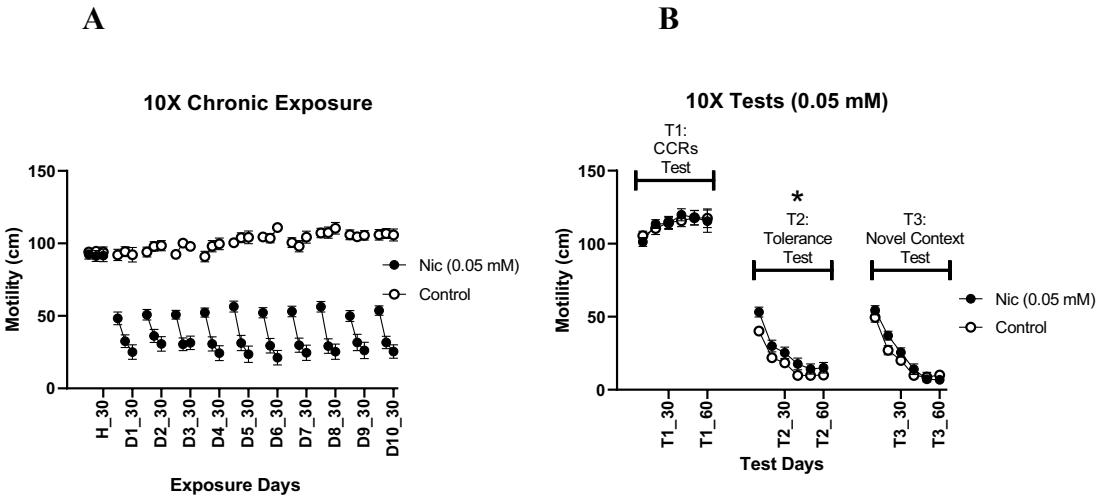
7.5.1.2 Test1

Test 1 was conducted on the drug-paired context with water to assess the development of CCRs. As can be observed in Figure 7.4 B (left panel, T1), planaria previously exposed to nicotine (113.6 cm ± 3.9) behaved in a similar way as control animals (113.5 cm ± 3.8). This impression was confirmed by a 2 (Group: Nicotine vs Water) x 6 (Bins: 1-6) mixed ANOVA, which revealed a main effect of Bins, $F(2.1, 93.7) = 7.07$, $p = .001$, $\eta_p^2 = .14$, but no main effect of Group $F(1, 45) = 0.01$, $p = .99$, $\eta_p^2 = .00$, and no interaction between these factors, $F(2.1, 93.7) = 0.57$, $p = .57$, $\eta_p^2 = .013$.

7.5.1.3 Test2 and Test3

These tests were conducted to assess the development of tolerance to the unconditioned (hypo locomotion) effects of nicotine in Drug-paired Context (Test 2) in Novel Context (Test 3). Figure 7.4 B (mid and right panels, T2 and T3) shows that planaria in the Nicotine Group (25 ± 2.1 cm) displayed marginally more locomotor activity than planaria in Control Group (19.6 cm ± 2.1 cm). This impression was confirmed by a 2 (Group: Nicotine vs Water) x 2 (Tests: Test 2 and Test 3) x 6 (Bins: 1-6) mixed ANOVA, which revealed marginal effect of Group, $F(1, 45) = 3.30$, $p = .076$, $\eta_p^2 = .07$, a significant effect of Bins, $F(4.3, 192.5) = 140.8$, $p < .001$, $\eta_p^2 = .76$, and a significant interaction of Tests x Bins, $F(3.9, 177) = 4.94$, $p < .001$, $\eta_p^2 = .10$. However there was no effect of Tests, $F(1, 45) = .88$, $p = .77$, $\eta_p^2 = .002$, and the remaining interactions were non-significant: Group x Tests, $F(1, 45) = 2.32$, $p = .13$, $\eta_p^2 = .05$, Group x Bins, $F(4.3, 192.5) = 1.87$, $p = .10$, $\eta_p^2 = .04$, and Group x Tests x Bins, $F(3.9, 177) = .95$, $p = .43$, $\eta_p^2 = .02$. The significant Group x Tests interaction suggests that tolerance to nicotine was mediated by the context. In other words, nicotine tolerance was significant in one context, but not in the other context.

Further analyses of the Group x Tests interaction suggested that there was a context dependent tolerance to primary effect of nicotine. The analysis of Test 2 data with a 2 (Group: Nicotine vs Water) x 6 (Bins: 1-6) mixed ANOVA that revealed a main effect of Group, $F(1, 45) = 4.78$, $p = .034$, $\eta_p^2 = .10$, Bins, $F(4, 179.3) = 64.32$, $p < .001$, $\eta_p^2 = .59$, but no interaction between these factors, $F(4, 179.3) = .84$, $p = .49$, $\eta_p^2 = .02$. Additionally, the analysis of Test 3 data with a 2 (Group: Nicotine vs Water) x 6 (Bins: 1-6) mixed ANOVA that showed no effect of Group, $F(1, 45) = 1.13$, $p = .29$, $\eta_p^2 = .02$, an effect of Bins, $F(4.3, 193.7) = 113.4$, $p < .001$, $\eta_p^2 = .72$, and a marginal interaction between Group and Bins, $F(4.3, 193.7) = 2.22$, $p = .06$, $\eta_p^2 = .05$. Evidence for tolerance was observed therefore in the Drug-paired Context (Test 2) but not in the Novel Context (Test 3).



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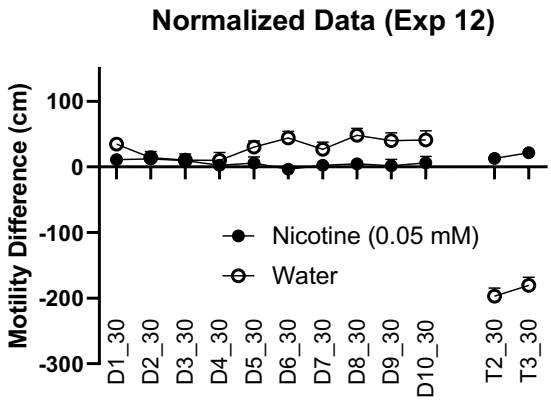


Figure 7. 4. Experiment 12. Mean distance covered by planaria in the drug-paired context throughout 10 days of 30 mins in the presence of nicotine or water (A), 60 min of CCRs test in the presence of only water (T1), 60 min of tolerance test in the presence of nicotine (T2), and 60 min of novel context test in the presence of nicotine with an alternative context (T3) (B). Results represented in 10 minutes bins. Data normalized to the first training session and represented in 30 min bins (C). Bars represent standard errors. Bars represent standard errors. N=23-24 planaria in each group.

7.6 Experiment 13: Development of Anticipatory Responses during Chronic Nicotine Exposure (0.05 mM)

In previous experiments (Experiments 9 and 10), using the 10x schedule, we observed the development of tolerance to hypo locomotive effects of nicotine; however, we did not find evidence of CCRs. Therefore, our results suggest that tolerance was not controlled by conditioned compensatory responses (CCRs), which is at odds with Siegel's (1975) studies. In addition, Solomon's (1980) *Opponent Process Theory* suggests initial presentation of unconditioned stimuli elicits both a hedonistic drug effect, the A-process, and a compensatory response called the B-process. These two processes counteract each other, and the net result is the reduction in the magnitude of the hedonistic status induced by the US presentation with repeated exposure, resulting in tolerance development. Solomon (1980) described three features of the opponent B-process: 1) - takes longer time to onset, 2) - strengthen gradually with repeated exposure and 3) –is slow to decay therefore persists after discontinuation of the US presentation. Additionally, Solomon

posited that environmental stimuli could be associated with either A- or B-process with repeated exposure. If drug contingent cue activates the A-process, iso-directional response which is followed by drug-antagonistic response is observed; however, if the B-process is activated, conditioned compensatory responses (CCRs) are observed. Therefore, we assumed that the failure to observe CCRs may result from the use of a simultaneous procedure, in which CS and US are presented always at the same time. Simultaneous conditioning does not always get reflected in performance, so it may be possible that by using an arrangement where presentations of the CS predict the later drug, that may better allow for the observation of tolerance.

Therefore, we hypothesized that animals might be able to activate CCRs to the effect of nicotine if they were exposed to the contextual cues alone for 30 min before the nicotine administration. The rationale was to allow the context to signal the nicotine administration, allowing them to develop a CCRs to the hypo locomotive effects of nicotine. For example, morphine (Grisel et al., 1994; Sherman, Strub & Lewis, 1984; Siegel, 1999) and ethanol (Larson & Siegel, 1998; Siegel & Larson, 1996; Siegel & Sdao-Jarvie, 1986) studies with Siegel's Pavlovian Tolerance Model found the development of tolerance when cue and drug repeatedly presented and observed the expression of compensatory responses when the drug-paired cue was presented alone (cue- no drug) before the joint presentations of the cue and drug events. Thus, the goal of this experiment was to assess if establishing a predictive relationship between the surface and the effects of nicotine would facilitate the expression of anticipatory CCRs. We allocated a total 48 animals to two groups, Nicotine and Water. There were 24 animals in each Group Nicotine, and Group Water. We used the same concentration of nicotine (0.05 mM) as we used for the previous experiment (Experiment 12). Animals were pre-exposed to the environmental stimuli by placing them into 20 ml of water on the smooth surface for 30

min. Then, 10 ml of water was taken off using a syringe (without disturbing the animals) and replaced by 10 ml of nicotine solution (to get a 0.05 mM concentration) or water. After that their motility was recorded for 30 more minutes.

7.6.1 Anticipatory Responses

7.6.1.1 Anticipatory Responses during Chronic Exposure

We hypothesized that establishing a predictive relationship between the context and nicotine administration would better allow for the expression of anticipatory homeostatic responses. The data presented in Figure 7.5 A shows that conditioned activity increases anticipatory to nicotine administration but not water administration in the presence of drug-paired context. These impressions were confirmed with a 2 (Group: Nicotine vs Water) x 10 (Days: 1 – 10) x 3 (Bins: 1-3) mixed ANOVA, that revealed a main effect of Group, $F(1, 46) = 6.12$, $p = .02$, η_p^2=.12, Days, $F(7.6, 349.4) = 4.80$, $p < .001$, $\eta_p^2 = .09$, no effect of Bins, $F(1.7, 78.8) = 1.36$, $p = .26$, $\eta_p^2 = .03$, as well as a significant interaction of Group x Bins, $F(1.7, 78.8) = 3.94$, $p = .03$, $\eta_p^2 = .08$. The remaining interactions were non-significant: Group x Days, $F(7.6, 349.4) = 0.51$, $p = .83$, $\eta_p^2 = .001$, Days x Bins, $F(14.1, 651) = 0.99$, $p = .47$, $\eta_p^2 = .02$, and Group x Days x Bins, $F(14.1, 651) = 0.58$, $p = .88$, $\eta_p^2 = .01$. Within-subjects linear contrasts revealed an effect of Day in Group Nicotine, $F(1, 23) = 10.33$, $p = .004$, $\eta_p^2 = .31$, but not in Group Water, $F(1, 23) = 2.67$, $p = .12$, $\eta_p^2 = .10$, suggesting an increased anticipatory response to nicotine but not water during the days of chronic exposure.

7.6.1.2 Anticipatory Responses before Test 1

Test 1 was conducted with water on the drug-paired context to assess the development compensatory responses before the administration of nicotine. As it can be observed in Figure 7.5 B (left panel), planaria previously trained with nicotine did not display a higher level of anticipatory response (higher locomotor activity) than planaria

previously trained with water. This impression was confirmed by a 2 (Group: Nicotine vs Water) x 3 (Bins: 1-3) mixed ANOVA, which revealed no effect of Group, $F(1, 46) = 0.02$, $p = .89$, $\eta_p^2 = .001$, a significant effect of Bins, $F(1.8, 84.5) = 7.17$, $p = .001$, $\eta_p^2 = .13$ and but no interaction effect of Group x Bins, $F(1.8, 84.5) = 1.13$, $p = .32$, $\eta_p^2 = .02$.

7.6.1.3 Anticipatory Responses before Test 2 and Test 3

These tests were conducted to assess anticipatory responses before tolerance tests. As it can be observed in Figure 7.5 B (mid and right panels), there is no evidence of the development of anticipatory responses before tolerance tests. This impression was confirmed by a 2 (Group: Nicotine vs Water) x 2 (Tests: Test 2 and Test 3) x 3 (Bins: 1-3) mixed ANOVA that revealed no effect of Group, $F(1, 46) = 1.27$, $p = .27$, $\eta_p^2 = .03$, Tests, $F(1, 46) = 3.03$, $p = .09$, $\eta_p^2 = .06$, a significant effect of Bins, $F(2, 92) = 3.77$, $p = .03$, $\eta_p^2 = .08$, significant interaction effect of Tests x Bins, $F(2, 92) = 3.68$, $p = .03$, $\eta_p^2 = .07$, but the remaining interactions were not significant: Group x Tests $F(1, 46) = 0.24$, $p = .62$, $\eta_p^2 = .005$, Group x Bins, $F(2, 92) = 1.78$, $p = .18$, $\eta_p^2 = .04$, Group x Tests x Bins, $F(2, 92) = 0.54$, $p = .58$, $\eta_p^2 = .01$

7.6.2 Nicotine Exposure

7.6.2.1 Chronic Exposure

The data of chronic exposure phase of the experiment is presented in Figure7.5 C. Over the course of the chronic exposure, planaria treated with nicotine showed lower levels of motility than the animals in the control group, exposed to water, replicating the results of previous experiments. We also observed a certain increase in the levels of activity in the group Nicotine. These impressions were confirmed with a 2 (Group: Nicotine vs Water) x 10 (Days: 1 – 10) x 3 (Bins: 1-3) mixed ANOVA that revealed a main effect of Group, $F(1, 46) = 479{,}7$ $p < .001$, $\eta_p^2 = .91$, Days, $F(6.6, 371.1) = 6.90$, $p < .001$, $\eta_p^2 = .13$, Bins, $F(1.6, 74.7) = 185.5$, $p < .001$, $\eta_p^2 = .80$, significant interaction of

Group x Bins, $F(1.6, 74.7) = 149.9$, $p < .001$, $\eta_p^2 = .76$, Days x Bins, $F(15.3, 707.62) = 3.56$, $p < .001$, $\eta_p^2 = .07$, and Group x Days x Bins, $F(15.3, 707.62) = 2.48$, $p = .001$, $\eta_p^2 = .05$. However, Group x Days interaction was not significant, $F(8.1, 371.1) = 0.85$, $p = .56$, $\eta_p^2 = .02$. Therefore, the development of tolerance to nicotine over the days of the repeated nicotine exposure was not significant during the chronic exposure phase. Within-subjects linear contrasts revealed an effect of Day in Group Nicotine, $F(1, 23) = 24.65$, $p < .001$, $\eta_p^2 = .52$, but not in Group Water, $F(1, 23) = 3.29$, $p = .083$, $\eta_p^2 = .12$, suggesting an increase in locomotor activity in Group Nicotine but not Water. These results suggest that the chronic exposure procedure used in the present experiment is effective in revealing the development of long-term tolerance to the effects of nicotine in the planaria.

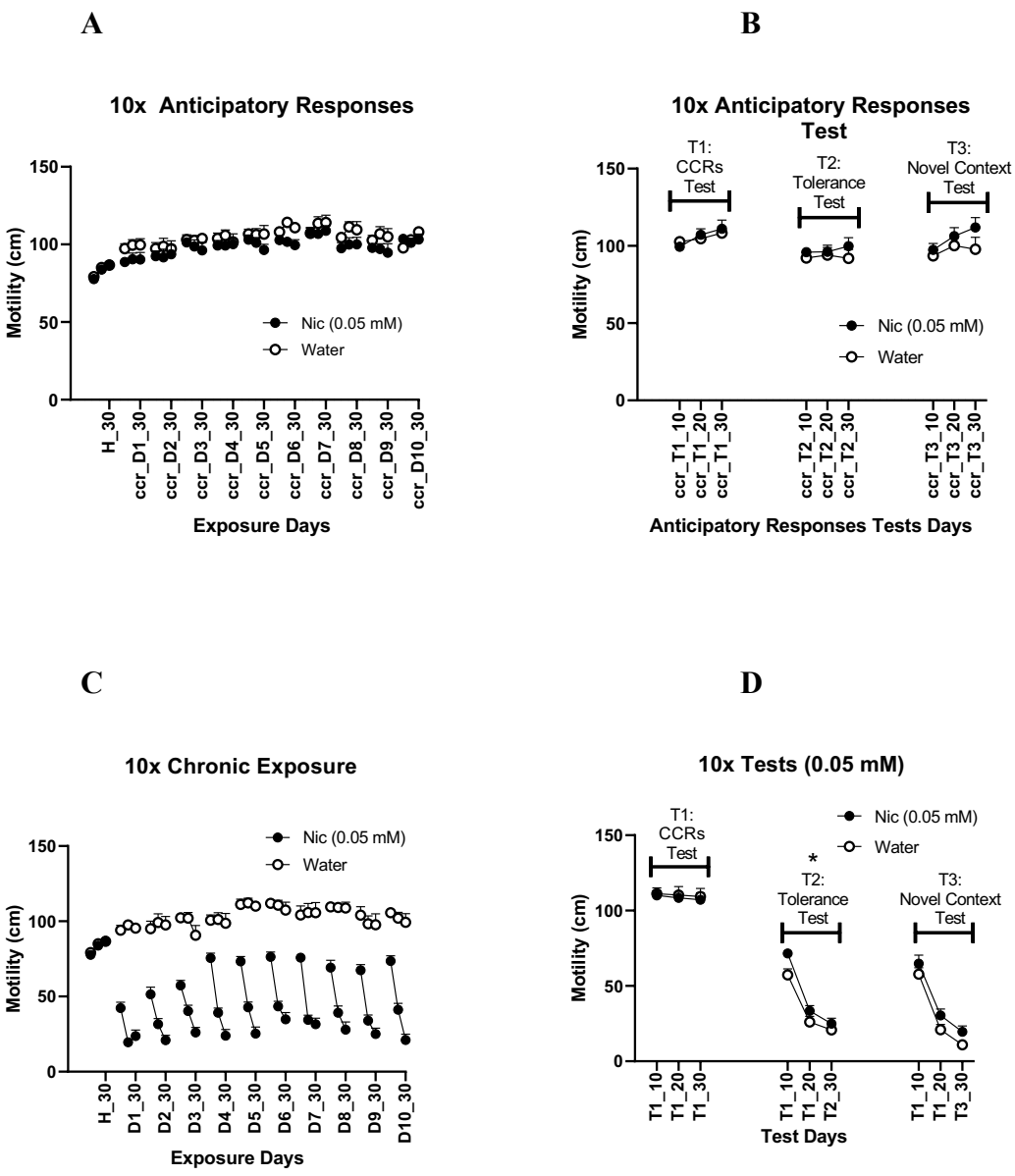
7.6.2.2 Test 1

Test 1 was conducted on the drug-paired context with water to assess the development of CCRs. As can be observed in Figure 7.5 D (left panel, T1), planaria previously exposed to nicotine behaved in a similar way than control animals. This impression was confirmed by a 2 (Group: Nicotine vs Water) x 3 (Bins: 1-3) mixed ANOVA, which revealed no main effect Group, $F(1, 46) = 0.084$, $p = .77$, $\eta_p^2 = .002$, Bins, $F(1.7, 80.3) = 0.65$, $p = .50$, $\eta_p^2 = .014$, no interaction between these factors, $F(1.7, 80.3) = 0.41$ $p = .84$, $\eta_p^2 = .001$.

7.6.2.3 Test 2 and Test 3

These tests were conducted to assess the development of tolerance to the unconditioned (hypo locomotion) effects of nicotine. Group Nicotine received the drug for the 11th time whilst animals in the Water Group received it for the first time in Drug-paired Context (Test 2). Additionally, animals on both groups were tested with nicotine again, but it was in Novel Context (Test 3). Figure 7.5 D (mid and right panels, T2 and T3) shows that planaria in the Nicotine Group displayed higher locomotor activity than

planaria in Control Group with both contexts, suggesting the development of no context dependent tolerance to nicotine. This impression was confirmed by a 2 (Group: Nicotine vs Water) x 2 (Tests: Test 2 and Test 3) x 3 (Bins: 1-3) mixed ANOVA, which revealed an effect of Group $F(1,46) = 6.24$, $p = .02$, $\eta_p^2 = .12$, Bins, $F(2, 92) = 268.75$, $p < .001$, $\eta_p^2 = .85$, no effect of Tests, $F(1, 46) = 2.97$, $p = .09$, $\eta_p^2 = .06$, and no interaction of Group x Tests, $F(1,46) = 0.003$, $p = .96$, $\eta_p^2 = .001$, Group x Bins, $F(2, 92) = 0.59$, $p = .55$, $\eta_p^2 = .01$,Tests x Bins, $F(1.6, 75.6) = 0.79$, $p = .46$, $\eta_p^2 = .02$, or Group x Tests x Bins, $F(1.6, 75.6) = 1.35$, $p = .26$, $\eta_p^2 = .03$.



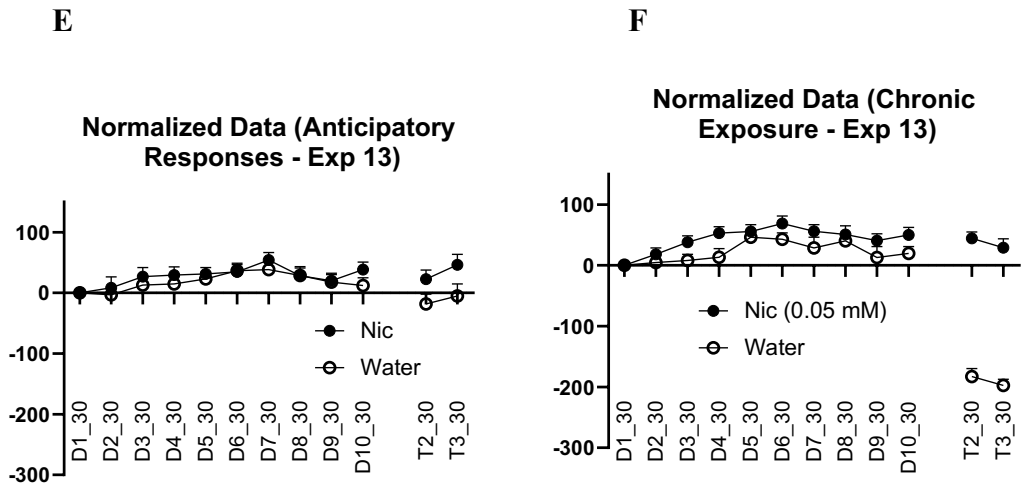


Figure 7. 5. Experiment 13**.** Mean distance covered by planaria on the drug-paired context in the presence water before nicotine or water treatment throughout 10 days of 30 mins (A), 30 min of compensatory response test before CCRs test (T1), 30 min of compensatory response test before tolerance test on the drug-paired context (T2), 30 min of compensatory response test before tolerance test in the novel context (T3) (B). **Tolerance.** Mean distance covered by planaria on the drug-paired context throughout 10 days of 30 mins in the presence of nicotine or water (C), 30 min of CCRs test in the presence of only water (T1), 30 min of tolerance test in the presence of nicotine (T2), and 30 min of novel context test in the presence of nicotine with an alternative context (T3) (D). Data normalized to the first training session and represented in 30 min bins during pre-exposure to context alone (E) and chronic exposure to the context and nicotine (F). Bars represent standard errors.

7.7 Summary

Chapter 7 assessed the chronic effect of nicotine in planaria with different regimens of exposure; one with 10x, ten days of exposure with 30 min of exposure; another protocol was 5x, five days of exposure with 1h exposure. The results of this chapter illustrated that *Schmidtea mediterranea* is a good model to investigate the chronic effect of nicotine, and different schedules of nicotine exposure revealed different behavioural responses in planaria. The result of Tests for the first two experiments (Experiment 9 and 10) demonstrated that animals treated with nicotine with 5x schedule had developed a hyperactivity CCRs to nicotine associated surface but not developed tolerance—in the sense that they did not appear to be more resistant to the effects of nicotine in the tests 2 and 3 where they were exposed to nicotine in a familiar and novel context). On the other hand, animals trained with 10x schedule have developed tolerance to the effect of nicotine but did not show evidence for the development of CCRs. These

data suggest that different schedules of chronic nicotine treatment/ exposure produced different behavioural response associated with tolerance.

Animals were randomly allocated; therefore, the baseline locomotor activity of planaria was not taken into consideration for the earlier experiments. However, we observed different regimens of nicotine exposure induced various behavioural changes in planaria in Experiment 9. In another word, we found the development of test tolerance but not CCRs after 10-days of exposure with 30 min of exposure, and the display of CCRs but not test tolerance following 5-days of exposure with 1h exposure. In order to test whether baseline activity of planaria was an important factor for the display of test tolerance to hypo-locomotor effect of nicotine, we equated the baseline motility of animals for the next experiment (Experiment 10) and replicated the same experimental protocol and the manipulations. The results were similar to what we found for the previous experiment (Experiment 9). Thus, it can be concluded that equated baseline motility is not an important element for the display of test tolerance.

We further investigated the underlying reason why 10-days regimen revealed tolerance but not CCR after, 5-days regiment elicited CCR but not tolerance. Nicotine is a toxic substance, and exposure to higher nicotine toxicity might be the reason. Because animals experienced 5-days schedule of nicotine treatment received 1h exposure per day (higher daily nicotine toxicity); on the other hand, animals experienced 10-days schedule exposed to nicotine 30 min per day. Solomon' s opponent process theory suggested that initial presentation of unconditioned stimuli elicits both a hedonistic drug effect, The A-process and compensatory B-process. These two processes counteract with each other, and the intensity/ strength of A -proposes might result in a stronger B-process which would reveal the development of CCRs. Previous nicotine studies with rats (Clarke and Kuma, 1983) and planaria (Rawls et al., 2011) showed that increasing the concentration

(toxicity of nicotine) cause more impairment in animals motor behaviours, as we found. Therefore, increasing the toxic effect of nicotine (A-process) with a higher concentration might results in a stronger B-process which would reveal the development of CCRs. For this reason, we doubled the concentration of nicotine (0.05 mM) using the 10x schedule for Experiment 12 in order to get a stronger CCRs as we observed in 5x schedule. We observed the display of test tolerance without CCRs.

Additionally, Solomon posited that environmental stimuli could be associated with either A- or B-process with repeated exposure. If drug contingent cue activates the A-process, iso-directional response which is followed by drug-antagonistic response is observed; however, if the B-process is activated, conditioned compensatory responses (CCRs) are observed. Therefore, the failure to observe CCRs may result from the use of a simultaneous procedure, in which CS and US are presented always at the same time. Simultaneous conditioning does not always get reflected in performance, so it may be possible that by using an arrangement where presentations of the CS predict the later drug, that may better allow for the observation of CCR, if animals were exposed to the contextual cues alone for 30 min before the nicotine administration. For example, morphine (Grisel et al., 1994; Sherman, Strub & Lewis, 1984; Siegel, 1999) and ethanol (Larson & Siegel, 1998; Siegel & Larson, 1996; Siegel & Sdao-Jarvie, 1986) studies with Siegel's Pavlovian Tolerance Model found the development of tolerance when cue and drug repeatedly presented and observed the expression of compensatory responses when the drug-paired cue was presented alone (cue- no drug) before the joint presentations of the cue and drug events. Therefore, the rationale was to establish a predictive relationship between the surface and the effects of nicotine and allowing them to develop a CCRs to the hypo locomotive effects of nicotine. However, the results showed the development of

test tolerance without the expression of CCR following chronic nicotine exposure, similar to the previous findings (Experiment 9, 10 and 12).

We also investigated the role of nicotinic acetylcholinergic receptors on the development of CCRs in Experiment 11. We used only the 5x schedule by adding two groups of animals co-exposed to mecamylamine alongside with water and nicotine. However, this study produced null results regarding the development of CCRs. In Experiment 12, we doubled the concentration of nicotine (0.05 mM) because increasing the depressant effect of nicotine might produce a stronger opponent response (Solomon, 1980). Also, In Experiment 13, animals received 30 min of pre-exposure to the context before nicotine presentation in the same context so that the context would signal drug delivery that leads to the development of preparatory/ anticipatory responses to nicotine. There were null results regarding the development of context-dependent anticipatory responses; however, we observed the development of tolerance but no evidence of CCRs as we found for previous studies with *Schmidtea mediterranea* using 10x schedule.

Chapter 8 is an adapted reproduction of a paper recently accepted for publication:

Sal, F., Prados, J., and Urcelay, G. P. (2020). Nicotine chronic tolerance development and withdrawal in the planaria (*Schmidtea mediterranea*). *Pharmacology, Biochemistry and Behaviour, in press*. https://doi.org/10.1016/j.pbb.2020.173075

8 Chapter 8: Nicotine tolerance and Withdrawal

8.1 Introduction

Nicotine addiction is a major preventable cause of death in humans and is characterized by multiple unsuccessful attempts to quit smoking cigarettes. As with addiction to other drugs of abuse, nicotine addiction seems to be driven by a combination of a) the rewarding effects of nicotine; b) tolerance development; and c) the presence of withdrawal symptoms following chronic exposure to the drug. Wikler (1973) was among the first to identify withdrawal and negative reinforcement as mechanisms driving the development of addiction (see also Solomon & Corbit, 1973). Also, it has been shown that nicotine tolerance correlates with the severity of nicotine addiction (Fagerström, 1978). Tolerance is characterised by a decrease in the physiological effects of a drug, so that a) larger doses are needed in order to achieve similar effects (Kalant, 1998); or b) the initial dose produces less effects with repeated administration. In particular, three different kinds of tolerance have been identified, on the basis of the number of exposures to the drug. Acute tolerance happens within the administration of a single dose of the drug: the physiological effects of the drug at a given concentration are smaller when looking at the descending portion of the drug's blood concentration —relative to the same concentration in the ascending portion of the curve (e.g., Perkins et al., 1991). Rapid tolerance is observed as less effect of the drug during a second administration of the drug, usually given between 8 to 24 hours after the first; in contrast, chronic tolerance is that

observed after multiple—usually 3 or more—administrations of the drug (e.g., Stolerman et al., 1973). It is this chronic tolerance which is the focus of the present study.

Classic theories of addiction assume tolerance and withdrawal to develop in parallel; consequently, the magnitude of the withdrawal response would be related to the degree of tolerance development. This is consistent with the idea that both are manifestations of physiological dependence (Kalant et al., 1971), and that learning mechanisms (triggered by experience with the drug) are involved in the manifestation of tolerance and withdrawal (Solomon & Corbit, 1973). In humans, this observation has been confirmed in nicotine addicts. For example, Pomerleau et al. (1983), monitored the changes in heart rate per plasma nicotine increments following smoking, and found evidence of higher levels of tolerance in heavy smokers than in light smokers. In addition, heavy smokers showed more abstinence signs following an overnight deprivation period. The relationship between tolerance and withdrawal was established at the individual level in a study by Hughes & Hatsukami (1986) in which tolerance to the effects of nicotine was found to correlate with signs of withdrawal discomfort (subjectively assessed by the smokers themselves as well as by independent observers).

The relationship between tolerance and withdrawal is well captured by psychophysiological theories of drug tolerance. A central tenet of these theories is that drugs such as nicotine produce homeostatic challenges and that environmental or contextual cues (hereafter called conditioned stimuli, or CS) become associated with the homeostatic challenge (Siegel, 1983; 2008; Solomon & Corbit, 1973). That is, drug presentation disturbs the homeostasis of the organism, and the organism produces a compensatory response to counteract the homeostatic imbalance produced by the disruptive effect of the drug. Following chronic drug administration in the presence of distinctive CSs, the compensatory responses that restore homeostatic balance come under

the control of CSs through conditioning and result in a conditioned response typically referred to as Conditioned Compensatory Responses (CCRs). With sufficient experience, in the presence of the CS (contextual cues where the drug has been administered) the animals express CCRs which counteract and weaken the effects of the drug; in other words, they develop tolerance to the effects of the drug (Siegel, 1975).

The conditioning model of drug tolerance anticipates that chronic tolerance is under the control of CSs, and hence after an organism has had extensive experience with a drug, the observation of tolerance would be stronger in the presence of drug-predicting CSs (the contextual cues where the drug effects were experienced) than in their absence. Similarly, following tolerance development, presentation of drug-paired CSs in the absence of the drug should reveal CCRs. A number of studies have found that cue-induced compensatory responses (CCRs) are opposite to drug-induced unconditioned responses, and these are observed following discontinuation of the drug (e.g., Larson & Siegel, 1998; Rozin et al., 1984; Siegel, 1975). Therefore, CCRs have been interpreted as withdrawal symptoms that arise as a consequence of the omission of the expected effects of the drug; in this context, the CCR per se would result in a homeostatic imbalance. In summary, there is abundant evidence observed in humans and nonhuman animals for the presence of CCRs following chronic exposure to drugs and for the claim that the development of tolerance and withdrawal follows similar principles as other basic learning processes (Siegel et al., 2000; Siegel & Allan, 1998; see Siegel, 2001, for a comprehensive review).

In contrast, a number of studies on nicotine addiction have reported an absence of correlation between tolerance development and withdrawal responses. Stolerman et al. (1973), investigated the development of tolerance to nicotine by measuring the motility of rats. With repeated nicotine exposure animals become tolerant to the depressant action

of nicotine; however, rats did not show an abstinence syndrome when the nicotine was omitted. Similarly, Domino & Lutz (1973) tested tolerance to nicotine measuring rates of bar pressing on a fixed ratio (FR) schedule for water reinforcement. Animals injected with nicotine suppressed bar pressing behaviours; however, with repeated nicotine treatments over a two-week period the bar pressing response rate steadily increased revealing the development of tolerance to the drug. However, treatment with a saline solution after repeated nicotine administrations (that is, testing the animals in the presence of the contextual cues associated with the nicotine treatment) did not produce conditioned compensatory responses (increased bar pressing behaviour). These results are consistent with the habituation theory of tolerance put forward by Baker and Tiffany (1985), according to which tolerance simply reflects a process of habituation; from this perspective, homeostatic CCRs (Siegel, 1975; Solomon, 1980) are not necessary for the development of tolerance.

In this present chapter, we will present four study to assess the conditioning and habituation theories of tolerance development by monitoring the locomotor responses of planarians during chronic nicotine exposure. The planarians nervous system presents structural and physiological similarities to the nervous system of vertebrates: centralized and bilateral with similar neural networks, transmitters, and neuromodulators (Buttarelli et al., 2008; Rawls et al., 2011; Sandmann et al., 2011). They are suitable for the observation of conditioned place preference (CPP, Hutchinson et al., 2015; Mohammed Jawad et al., 2018.; Turel et al., 2020), a canonical test for the rewarding effect of drugs of abuse and natural reinforcers (Tzschentke, 2007). In the study of basic learning processes, planarians show blocking and overshadowing (Prados et al., 2013), two phenomena suggesting the operation of selective processes as seen in rodents and humans. Planarians express cholinergic receptors and are sensitive to cholinergic agonists

and antagonists including nicotine (Buttarelli et al., 2000). A previous report has suggested the observation of tolerance in planarians after three exposures to nicotine (Rawls et al., 2011). Tolerance in smokers, however, is likely to reflect the operation of adaptations that occur after repeated, chronic experience with nicotine. To model the development of tolerance and nicotine dependence, in the Experiment 14 reported below, we monitored the hypo-locomotor effects of nicotine in planarians following a regimen of nicotine treatment that better resembles the process of interest in humans. We measured the motility using an automated equipment that neutralizes observer bias. We also assessed the development of CCRs following chronic exposure to nicotine, and whether tolerance to the effects of nicotine diminishes in the absence of drug-paired CSs. Experiment 15 tested whether the development of tolerance to nicotine depends on nicotinic receptor activation. Experiments 16 and 17, assessed withdrawal responses following chronic nicotine exposure with higher doses.

8.2 Experiment 14. Nicotine-induced Tolerance Development with Low concentration (0.025 mM)

Preliminary experiments carried out in our laboratory had established that the unconditioned response to the exposure to nicotine (at different concentrations) was reduced motility—comparison made to control animals exposed to treated water; these preliminary studies also suggested that the motility of the animals tend to increase with repeated exposure to nicotine (an instance of tolerance development). The goal of Experiment 14 was to assess the development of tolerance to the hypo-locomotive effects of nicotine using a chronic exposure procedure that mimics the chronic exposure regimens used in other animals such as rodents—and indeed chronic self-administration in humans. We allocated a total 32 animals to two groups, Nicotine and Water. One

animal in group Water died over the course of the experiment, resulting in n = 16 for Group Nicotine, and n = 15 in Group Water. We used a relatively low concentration of nicotine (0.025 mM) which pilot experiments had indicated produces reliable hypo-locomotion in planarians.

8.2.1 Results

8.2.1.1 Chronic Exposure

As expected (based on pilot data), exposure to nicotine reduced the motility of the animals: the planarians exposed to nicotine showed on average a 50% reduction in motility (M = 62.5 cm, SE = 3.2) relative to planarians exposed to treated water (M = 124.6 cm, SE = 3.3 cm) on the first day of exposure. The activity of nicotine treated animals gradually increased during the chronic exposure phase and their motility on the last exposure day was % 35 higher from the first day (M = 84.6 cm, SE = 4.1 cm). The data of the chronic exposure phase of the experiment is displayed in the Figure 8.1 A. A visual inspection of the data suggests an increase level of motility in the Group Nicotine whereas the animals in the Group Water tend to maintain a consistent level of activity. These impressions were confirmed with a 2 (Group: Nicotine *vs*. Water) x 10 (Days: 1 – 10) x 3 (Bins: 1-3) mixed ANOVA that revealed a main effect of Group, $F(1, 29) = 106.98$, $p < .001$, $\eta_p^2 = .79$, Days, $F(9, 261) = 3.18$, $p = .001$, $\eta_p^2 = .10$, Bins, $F(2, 58) = 40.31$, $p < .001$, $\eta_p^2 = .58$, as well as significant interactions Group x Days, $F(9, 261) = 3.68$, $p < .001$, $\eta_p^2 = .11$, Group x Bins, $F(2, 58) = 61.475$, $p < .001$, $\eta_p^2 = .68$, and Days x Bins, $F(18, 522) = 1.66$, $p = .042$, $\eta_p^2 = .05$. The remaining three-way interaction Group x Days x Bins was non-significant, $F(18, 522) = 0.68$, $p = .82$, $\eta_p^2 = .02$. Within-subjects linear contrasts revealed an effect of Day in Group Nicotine, $F(1, 15) = 33.01$, $p < .001$, $\eta_p^2 = .68$, but not in Group Water, $F(1, 14) = 0.003$, $p = .95$, $\eta_p^2 < .01$, suggesting an increase in motility in Group Nicotine but not Water. These results confirm that the

chronic exposure procedure used in the present experiment is effective in developing long-term tolerance to the effects of nicotine in the planaria.

8.2.1.2 Test 1, Conditioned Compensatory Responses (CCRs)

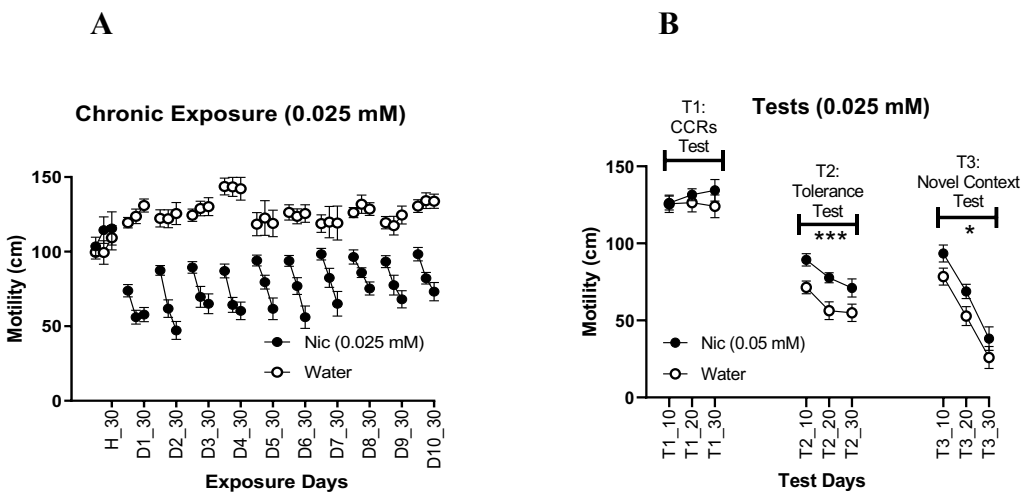
The Test 1 was conducted in the exposure context with treated water to assess the development of CCRs. As can be observed in Figure 8.1 B (left panel, T1), planarians in the Group Nicotine, exposed to nicotine (M = 130.8 cm, SE = 4.8), behaved in a similar way to animals in the Group Water (M = 125.4 cm, SE = 4.9). This impression was confirmed by a 2 (Group: Nicotine *vs*. Water) x 3 (Bins: 1-3) mixed ANOVA, which revealed no main effects of Group, $F(1, 29) = 0.61$, $p = .44$, $\eta_p^2 = .02$, and Bins, $F(2, 58) = 0.67$, $p = .51$, $\eta_p^2 = .02$, and no interaction between these factors, $F(2, 58) = 1.14$ $p = 0.33$, $\eta_p^2 = .04$.

8.2.1.3 Test 2 and Test 3

These tests were conducted to assess the development of tolerance to the unconditioned (hypo locomotion) effects of nicotine, as well as to assess the context dependence of the tolerance developed to nicotine during the chronic exposure. Group Nicotine received the drug for the eleventh time whilst animals in the Group Water received it for the first time in the exposure context in Test 2. The animals in both groups were tested in the presence of nicotine again, but in a novel distinctive context, during Test 3. Figure 8.1 B (central and right panels, T2 and T3) shows that planarians in Group Nicotine displayed more motility (M = 73.1 cm, SE = 3.2) than planarians in Group Water (M = 56.5 cm, SE = 3.3) both during the Test 2 (in the exposure context) and Test 3 (in the new context), suggesting the expression of tolerance to nicotine independent of context. These impressions were confirmed by a 2 (Group: Nicotine *vs*. Water) x 2 (Tests: Test 2 and Test 3) x 3 (Bins: 1-3) mixed ANOVA, which revealed significant main effects of Group $F(1, 29) = 12.48$, $p = .001$, $\eta_p^2 = .30$, Tests, $F(1, 29) = 5.87$, $p = .02$, $\eta_p^2 = .17$,

and Bins, $F(2, 58) = 73.25$, $p < .001$, $\eta_p^2 = .72$, as well as a significant interaction between Tests x Bins, $F(2, 58) = 21.92$, $p < .001$, $\eta_p^2 = .43$. The remaining interactions were non-significant: Group x Tests, $F(1, 29) = 0.21$, $p = .65$, $\eta_p^2 = .007$, Group x Bins, $F(2, 58) = .29$, $p = 0.75$, $\eta_p^2 = .01$, and the three-way Group x Tests x Bins interaction, $F(2, 58) = 0.24$, $p = .98$, $\eta_p^2 = .001$.

The main effect of Test confirms that activity was lower during Test 3, but the lack of a Group x Test interaction suggests that tolerance to nicotine effects was not dependent on context, as similar tolerance development was observed in the trained (Test 2) and novel contexts (Test 3).



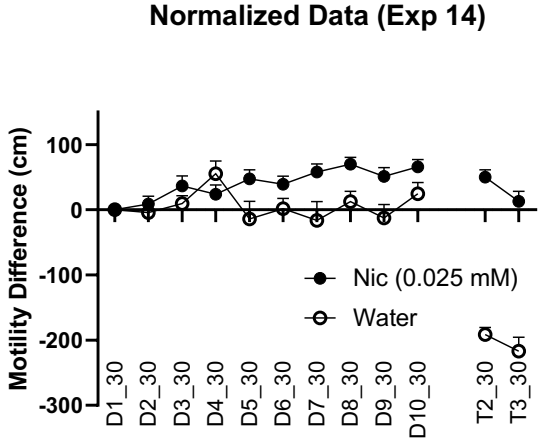


Figure 8. 1. Experiment 14. Mean distance covered by planarians in the exposure context throughout 10 sessions of 30 min in the presence of nicotine or water (A), 30 min of CCRs test in the presence of only water, $p = .44$ (T1), 30 min of tolerance test in the presence of nicotine $p = .001$ (T2), and 30 min of novel context test in the presence of nicotine with an alternative context, $p = .058$ (T3) (B). Results represented in 10 minutes bins. Data normalized to the first training session and represented in 30 min bins (C). Bars represent standard errors. n=15-16 planarians in each group.

8.3 Experiment 15: Nicotine-induced Tolerance Development with Low concentration (0.025 mM) and assessment of the effect of Mecamylamine

In Experiment 14, we observed that initial nicotine exposure decreased motility, and chronic exposure to nicotine resulted in the development of tolerance. Clarke and Kumar (1983) observed similar results with rats. Acute nicotine exposure reduced motility; however, tolerance to the initial effects of nicotine was observed over the course of repeated exposure to nicotine. They also observed that pre-treatment with mecamylamine (a non-competitive antagonist of the nicotinic acetylcholine receptors) blocked the initial (acute) effect of nicotine. In another study (McCallum et al., 1999) observed that mecamylamine blocked the acute action of nicotine, and the development of tolerance. These two studies were conducted on rats, and suggest that both the acute effects of nicotine and the development of tolerance following chronic exposure depend on activation of nicotinic receptors. Therefore, the purpose of this experiment was to assess in planaria whether mecamylamine, a nAChRs antagonist, blocks (or attenuates) the decreased motility caused by acute nicotine exposure and the development of tolerance caused by chronic nicotine exposure. We used a 2 (Drug 1: nicotine vs water) x 2 (Drug 2: mecamylamine vs water) factorial design for this experiment. We allocated a total 112 animals to four groups, Nic, Nic+Mec, Water and Mec. One animal in Group Nic and two animals in Group Nic+Mec died over the course of the experiment, resulting in $n = 27$ for Group Nic, $n = 26$ in Group Nic+Mec, $n = 28$ for Groups Water and Mec. We used the same concentration of nicotine as in Experiment 14, and 0.05 mM mecamylamine (as used in Raffa et al., 2013). Test sessions were 60 minutes long, instead

of the 30 mins used in Experiment 14. The flatworms were held in the same way as described in Experiment 14.

8.3.1 Results

8.3.1.1 Chronic Exposure

Nicotine administration reduced motility of planaria, and mecamylamine administration partially blocked the effects of nicotine during the chronic exposure. The data of chronic exposure phase of the experiment is displayed in Figure 8.2 A. Planaria that experienced nicotine showed significantly less motility (M = 66.3 cm, SE = 3) than the planaria that experienced nicotine plus mecamylamine (M = 81.6 cm, SE = 3). However, planaria exposed to water (M = 113.7 cm, SE = 3) behaved a similar way than planaria exposed to mecamylamine alone (M = 117 cm, SE = 3), suggesting mecamylamine did not have any effect when administered alone. This impression was confirmed by a 2 (Drug 1 [Nicotine vs Water]) x 2 (Drug 2 [Mecamylamine vs Water]) x 10 (Days: 1-10) x 3 (Bins: 1-3) ANOVA that revealed a significant effect of Drug 1, $F(1, 108) = 186.5$, $p < .001$, $\eta_p^2 = .63$, as well as significant of Drug 1 x Bins interaction, $F(1.6, 178.4) = 119.10$, $p < .001$, $\eta_p^2 = .52$, but no main interaction effect of Drug 1 x Days, $F(8.7, 941.7) = 1.14$, $p = .33$, $\eta_p^2 = .01$, Drug 1 x Days x Bins, $F(15, 1617.3) = 0.877$, $p = .59$, $\eta_p^2 = .008$. There is also a main effect of Drug 2 $F(1, 108) = .31$, $p = .003$, $\eta_p^2 = .08$, Drug 2 x Bins interaction, $F(1.6, 178.4) = 8.35$, $p = .001$, $\eta_p^2 = .07$, but the remaining interactions were non-significant: Drug 2 x Days, $F(8.7, 941.7) = 1.58$, $p = .12$, $\eta_p^2 = .01$, Drug 2 x Days x Bins, $F(15, 1617.3) = 1.31$, $p = .19$, $\eta_p^2 = .012$. Furthermore, we also found a marginal interaction between Drug 1 and Drug 2, $F(1, 108) = 3.91$, $p = .050$, $\eta_p^2 = .03$, but the remaining interactions were not significant: Drug 1 x Drug 2 x Bins, $F(1.6, 178.4) = 0.50$, $p = .57$, $\eta_p^2 = .005$, Drug 1 x Drug 2 x Days, $F(8.7, 941.7) = 055$, $p = .84$, $\eta_p^2 = .005$, Drug 1 x Drug 2 x Days x Bins, $F(15, 1617.3) = 0.90$, $p = .56$, η_p^2

= .008. The (marginally) significant Drug 1 x Drug 2 interaction suggested the effect of nicotine was attenuated by co-treatment with mecamylamine. Further analysis of this interaction confirmed that mecamylamine attenuated the effect of nicotine, because Group Nic displayed less motility than Group Nic+Mec, $F(1, 54) = 15.01$, $p < .001$, $\eta_p^2 = .22$. Group Mec did not differ from Group Water, $F(1, 54) = 0.49$, $p = .48$, $\eta_p^2 = .01$, revealing that mecamylamine did not cause any changes in motility when given alone (see Figure 8.2 A, left panel).

8.3.1.2 Test 1, Conditioned Compensatory Responses (CCRs)

The Test 1 was conducted in the exposure context with treated water to assess the development of CCRs and the effect of mecamylamine on the CCRs. As it can be observed in Figure 8.2 B (left panel, T1), both nicotine ($M = 111.4$ cm, $SE = 5.6$ cm) and water ($M = 114.4$ cm, $SE = 5.8$ cm) groups covered similar amounts during the test, and the history of nicotine (Drug 1) or mecamylamine (Drug 2) exposure did not have any significant effects on the CCR test. This impression was confirmed by a 2 (Drug 1 [Nicotine vs Water]) x 2 (Drug 2 [Mecamylamine vs Water]) x 6 (Bins: 1-6), which revealed no main effect of Drug 1 (nicotine), $F(1, 105) = 1.42$, $p = .23$, $\eta_p^2 = .01$, no main interaction effect of Drug 1 x Bins, $F(3.1, 318.9) = 0.77$, $p = .51$, $\eta_p^2 = .01$, Drug 1 x Drug 2, $F(1, 102) = 0.36$, $p = .55$, $\eta_p^2 = .003$.

8.3.1.3 Test 2 and Test 3

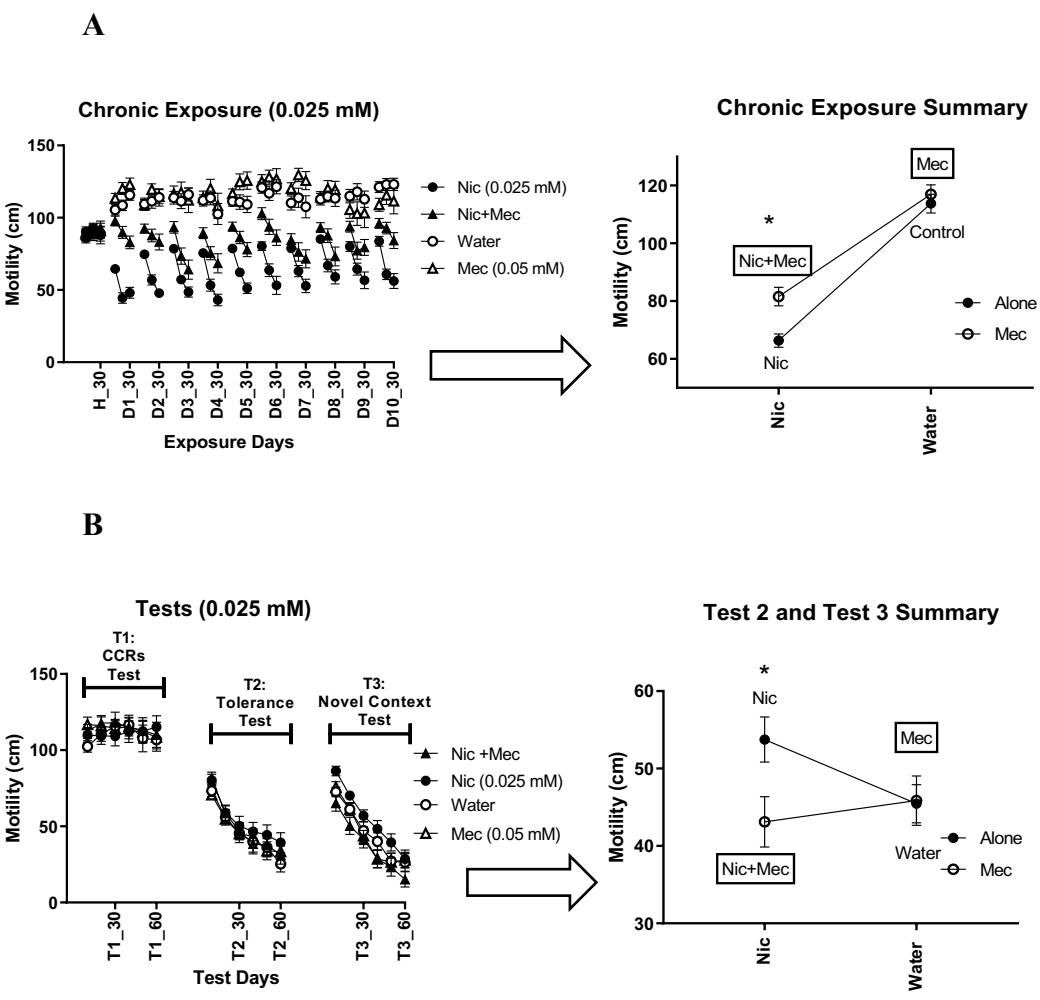
These tests were conducted to assess the effect of mecamylamine on the development of tolerance to chronic nicotine exposure. We expected that animals previously exposed to nicotine would cover more distance than the control group that was not exposed to nicotine before (i.e., to replicate the findings of Experiment 14), and that the administration of mecamylamine would block this effect. Figure 8.2 B (mid and left panels, T2 and T3; see also the summary graph) shows that planarians in Group Nic

displayed more motility (M = 55.5 cm, SE = 3) than planarians in Group Water (M = 45.5 cm, SE = 2.9) both during the Test 2 (in the exposure context) and Test 3 (in the new context), suggesting the expression of tolerance to nicotine. However, Group Nic+Mec (M = 42.9 cm, SE = 3.1) showed similar levels of motility as Group Water (see above), suggesting that mecamylamine attenuated the development of tolerance to the effects of nicotine, but did not cause any changes alone (Group Mec [M = 45.9 cm, SE = 2.9] behaved a similar way as Group Water). A mixed ANOVA on Test 2 and Test 3 data (Drug 1 [Nicotine vs Water) x Drug 2 [Mecamylamine vs Water] x Tests [Test 2 vs Test 3] and Bin [6] as factors) revealed no effect of Drug 1, $F(1, 105) = 1.05$, $p = .31$, $\eta_p^2 = .01$, a marginal effect of Drug 2, $F(1, 105) = 3.55$, $p = .06$, $\eta_p^2 = .033$, and importantly a Drug 1 x Drug 2 interaction, $F(1, 105) = 4.06$, $p = .04$, $\eta_p^2 = .04$. We also observed a significant effect of Bins, $F(3.3, 344.9) = 146.05$, $p < .001$, $\eta_p^2 = .58$, and an interaction between Tests x Bins, $F(3.9, 405.9) = 3.16$, $p = .015$, $\eta_p^2 = .03$. None of the remaining effects or interactions were significant (largest F = 3). The significant Drug 1 x Drug 2 interaction suggested that nicotine induced tolerance development was sensitive to mecamylamine blockade.

We followed up that interaction with a 2 (Drug 1: Nicotine *vs*. Water) x 2 (Tests: Test 2 and Test 3) x 3 (Bins: 1-3) a mixed ANOVA, to assess whether we observed tolerance in the absence of mecamylamine. The analysis revealed significant effect of Drug 1, $F(1, 53) = 5.73$, $p = .02$, $\eta_p^2 = .09$, Bins, $F(3, 157.6) = 6.59$, $p = .02$, $\eta_p^2 = .098$, and Tests x Bins interaction $F(3.6, 189.7) = 3.42$, $p = .01$, $\eta_p^2 = .06$, but no effect of Tests, $F(1, 53) = 0.004$, $p = .95$, $\eta_p^2 = .001$. The remaining interactions were all non-significant (all Fs <1). These results suggested that tolerance to nicotine across the tests was significant. Moreover, a similar analysis with the groups that received mecamylamine revealed no effect of Drug 1, $F(1, 52) = 0.41$, $p = .52$, $\eta_p^2 = .008$, Tests, $F(1, 52) = 2.67$,

$p = .11$, $\eta_p^2 = .05$, but a significant effect of Bins, $F(3.7, 191.4) = 73.1$, $p < .001$, $\eta_p^2 = .58$. None of the remaining interactions was significant (largest $F = 2.46$). These results suggest that animals treated with chronic nicotine showed tolerance development, and that mecamylamine blocked that effect across the both tolerance tests.

Overall, mecamylamine attenuated the effect of nicotine during the chronic exposure days. Additionally, tolerance development was significant across both tolerance tests with nicotine, and mecamylamine during chronic exposure successfully blocked the development of tolerance. These results confirm that nicotine-induced tolerance development depends on nicotine receptor activation, because mecamylamine blocked the development of tolerance, and also attenuated the acute effects of nicotine.



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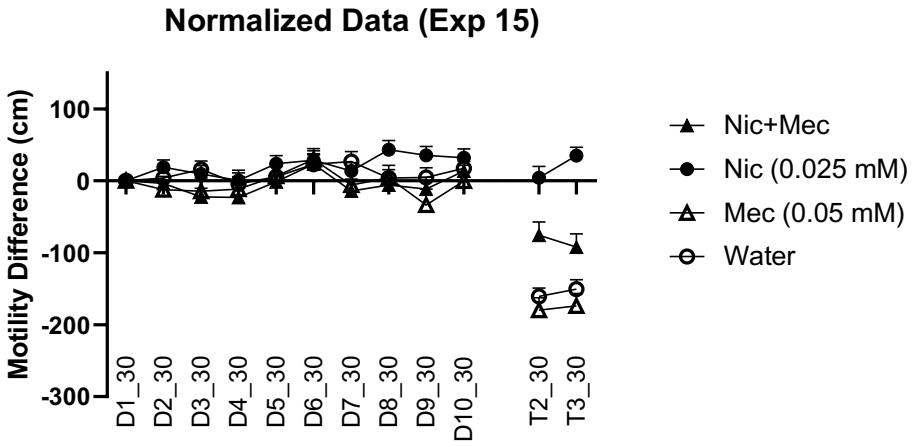


Figure 8. 2**.** Experiment 15. Mean distance covered by planarians in the exposure context throughout 10 days of 30 min in the presence of nicotine, water, mecamylamine or mecamylamine plus nicotine (A), 60 min of CCRs test in the presence of only water (T1), 60 min of tolerance test in the presence of nicotine (T2), and 60 min of novel context test in the presence of nicotine with an alternative context (T3) (B). Results represented in 10 minutes bins. Data normalized to the first training session and represented in 30 min bins (C). Bars represent standard errors. n=28 planarians in each group.

8.4 Experiment 16. Abstinence-induced Behaviour and Tolerance Development with a medium dose (0.05 mM).

The goal of Experiment 16 was to investigate the after-effects of nicotine using the chronic exposure procedure used in Experiments 14 and 15 (10 daily exposure sessions); this would complement and expand the analysis by Rawls et al. (2011) who used an acute exposure procedure (a single exposure to nicotine). The procedure of Experiment 16 replicates the one described for previous experiments; however, following each daily exposure session throughout the experiment, the animals were given an additional 30 min in the exposure context but in the absence of nicotine to monitor the after effects of nicotine; also, following all test sessions, the animals were given an additional 30 min session with treated water in the exposure context (Tests 1 and 2) and in the novel context (after the Test 3). We allocated a total 48 animals to two groups,

Nicotine and Water. Two animals in Group Nicotine died over the course of the experiment, resulting in $n = 22$ for Group Nicotine, and $n = 24$ in Group Water. We used a higher concentration of nicotine (0.05 mM) than the one used in the previous experiments because it would better approximate the dose used in previous planaria studies (Pagan et al., 2009; Rawls et al., 2011).

8.4.1 Results

8.4.1.1 Chronic exposure

The data of the chronic exposure phase of the experiment is displayed in Figure 8.3 A. As expected based on our previous findings, planarians exposed to nicotine showed less motility ($M = 25.4$ cm, $SE = 3.6$) than the planarians in the control condition, exposed to treated water ($M = 82.1$ cm, $SE = 3.5$) on the first day of the chronic exposure. Although some variability was observed across days, there did not seem to be a development of tolerance because there was an increase in the motility of the both groups across days. A 2 (Group: Nicotine *vs*. Water) x 10 (Days: 1 – 10) x 3 (Bins: 1-3) mixed ANOVA revealed main effects of Group, $F(1, 44) = 619.9$ $p < .001$, $\eta_p^2 = .93$, Days, $F(7.2, 321.1) = 4.89$, $p < .001$, $\eta_p^2 = .10$, and Bins, $F(1.9, 4.7) = 141.6$ $p < .001$, $\eta_p^2 = .76$, as well as significant interactions Group x Days, $F(7.2, 321.1) = 3.4$, $p = .001$, $\eta_p^2 = .07$, and Group x Bins, $F(1.9, 4.7) = 160.6$, $p < .001$, $\eta_p^2 = .78$, and a significant three-way interaction Group x Days x Bins, $F(12.2, 536.5) = 2.16$, $p = .01$, $\eta_p^2 = .05$. The remaining Days x Bins interaction was non-significant, $F(12.2, 536.5) = 1.31$, $p = .21$, $\eta_p^2 = .03$. Within-subjects linear contrasts revealed an effect of Day in Group Nicotine, $F(1, 21) = 22.33$, $p < .001$, $\eta_p^2 = .51$, but not in Group Water, $F(1, 23) = 2.27$, $p = .14$, $\eta_p^2 = .09$, suggesting an increase in motility in Group Nicotine but not Water. These results confirm the findings of Experiment 14, but with a higher dose, in that we observed development of tolerance to nicotine with a chronic exposure procedure in the planaria.

8.4.1.2 Test 1

Test 1 was conducted on the exposure context with water to assess the development of CCRs. As can be observed in Figure 8.3 B (left panel, T1), planarians previously exposed to nicotine (M = 88.9 cm, SE = 3.6 cm) behaved in a similar way to animals in the Group Water (M = 93.5 cm, SE = 3.5 cm). This impression was confirmed by a 2 (Group: Nicotine *vs*. Water) x 3 (Bins: 1-3) mixed ANOVA, which revealed a significant effect of Bins, $F(2, 88) = 4.1$, $p = .02$, $\eta_p^2 = .08$, but no effect of Group, $F(1, 44) = 0.86$, $p = .35$, $\eta_p^2 = .02$, and no interaction between these factors, $F(2, 88) = 2.57$, $p = .08$, $\eta_p^2 = .05$.

8.4.1.3 Test 2 and Test 3

These tests were conducted to assess the development of tolerance to the unconditioned effects of nicotine (the hypo-locomotion response). Group Nicotine received the drug for the eleventh time whilst animals in Group Water received it for the first time in the exposure context (Test 2). Additionally, animals in both groups were tested with nicotine again, but in a novel distinctive context in Test 3. Figure 8.3 B (central and right panels, T2 and T3) shows that planarians in Group Nicotine displayed higher levels of motility (M = 41.1 cm, SE = 2.2 cm) than the animals in the Group Water (M = 29.2 cm, SE = 2.1 cm) in both contexts, suggesting a context independent development of tolerance to the effects of nicotine. These impressions were confirmed by a 2 (Group: Nicotine *vs*. Water) x 2 (Tests: Test 2 and Test 3) x 3 (Bins: 1-3) mixed ANOVA, which revealed main effects of Group $F(1, 44) = 15.70$, $p < .001$, $\eta_p^2 = .26$, and Bins, $F(2, 58) = 187.05$, $p < .001$, $\eta_p^2 = .81$, but no effect of Tests, $F(1, 44) = 3.27$, $p = .08$, $\eta_p^2 = .07$. There was a significant Group x Bins interaction, $F(2, 88) = 4.23$, $p = 0.02$, $\eta_p^2 = .09$. The remaining interactions were all non-significant: Group x Tests, $F(1,44) = 1.19$, $p = .28$, $\eta_p^2 = .03$, Tests x Bins, $F(2, 88) = 1.24$, $p = .29$, $\eta_p^2 = .03$, and the three-way Group x

Tests x Bins interaction, $F(2, 88) = 0.22$, $p = .80$, $\eta_p^2 = .005$. These results suggest development of tolerance in the absence of context dependence.

8.4.1.4 Nicotine after-effect during chronic exposure

The data of after-effect sessions during the chronic exposure phase of the experiment is displayed in Figure 8.3 C. Animals pre-treated with nicotine showed lower motility than planarians pre-treated with water during the added 30 min exposure to treated water across the chronic exposure phase. However, the motility of animals pre-treated with nicotine gradually increased across the days, consistent with the notion of tolerance development (in that case of the after-effect of exposure to nicotine). These impressions were confirmed with a 2 (Group: Nicotine *vs*. Water) x 10 (Days: 1-10) x 3 (Bins: 1-3) mixed ANOVA that revealed main effects of Group, $F(1, 44) = 25.11$, $p < .001$, $\eta_p^2 = .36$, Days, $F(6.9, 304.4) = 7.23$, $p < .001$, $\eta_p^2 = 0.14$, and Bins, $F(1.8, 80.4) = 28.27$, $p < .001$, $\eta_p^2 = .39$, as well as a significant interaction of Group x Bins $F(1.8, 80.4) = 26.62$, $p < .001$, $\eta_p^2 = .38$. The remaining interaction were all non-significant: Group x Days, $F(6.9, 304.4) = 1.64$, $p = .12$, $\eta_p^2 = .04$, Days x Bins, $F(14.5, 638.4) = 1.34$, $p = .17$, $\eta_p^2 = .03$, and the three-way Group x Days x Bins interaction, $F(14.5, 638.4) = 1.15$, $p = .31$, $\eta_p^2 = .03$. Within-subjects linear contrasts revealed an effect of Day in Group Nicotine, $F(1, 21) = 15.37$, $p < .01$, $\eta_p^2 = .42$, but not in Group Water, $F(1, 23) = 1,33$, $p = .26$, $\eta_p^2 = .05$, suggesting an increase in motility in Group Nicotine but not Water. This pattern of results suggests an after-effect of the nicotine treatment on the day of chronic exposure that progressively weakens by the end of the chronic exposure phase—indicating the development of tolerance of the nicotine after-effect.

8.4.1.5 Nicotine after-effect following Test 1

The after-effect responses were assessed following Test 1 (CCR test in the absence of the drug) by monitoring the animals during an additional 30 min period. The

results of this additional 30 min period are displayed in Figure 8.3 D (left panel, T1); planarians in the Groups Nicotine and Water behaved in a very similar way. A 2 (Group: Nicotine v*s.* Water) x 3 (Bins: 1-3) mixed ANOVA, revealed no significant effects of Group, $F(1, 44) = 0.02$, $p = .88$, $\eta_p^2 = .001$, and Bins, $F(1.7, 73.2) = 0.83$, $p = .41$, $\eta_p^2 = .02$; the interaction between these factors was also non-significant, $F(1.7, 73.2) = 1.73$, $p = 0.19$, $\eta_p^2 = .04$

8.4.1.6 Nicotine after-effect following Test 2 and 3

These tests were conducted to investigate the after-effect of nicotine following acute (Group Water) and chronic nicotine exposure (Group Nicotine): animals in the Group Water were exposed for the first time to nicotine in the Test 2, and only for the second time during Test 3 in a new environment (the animals in Group Nicotine were exposed to the drug for the eleventh and twelfth time). The results of the additional 30 min exposure to treated water in the exposure context after Test 2, and in the novel context in Test 3 are displayed in Figure 8.3 D (central and right panels, T2 and T3). A visual inspection of the results suggests that both groups behaved in a very similar way. A 2 (Group: Nicotine *vs*. Water) x 2 (Tests: Test 2 and Test 3) x 3 (Bins: 1-3) mixed ANOVA, revealed a main effect of Tests, $F(1, 44) = 7.76$, $p = .01$, $\eta_p^2 = .15$, a significant Group x Bins interaction, $F(1.8, 81.9) = 5.88$, $p = .005$, $\eta_p^2 = .02$, and a significant Tests x Bins interaction, $F(2, 88) = 4.94$, $p = .01$, $\eta_p^2 = .10$. The remaining main factors and interactions were all non-significant: Group, $F(1, 44) = 0.53$, $p = .82$, $\eta_p^2 = .001$; Bins, $F(1.8, 81.9) = 22.10$, $p < .001$, $\eta_p^2 = .33$; Group x Tests interaction, $F(1, 44) = 0.54$, $p = .46$, $\eta_p^2 = .01$; and the three-way Group x Test x Bins interaction, $F(2, 88) = 0.43$, $p = .65$, $\eta_p^2 = .01$.

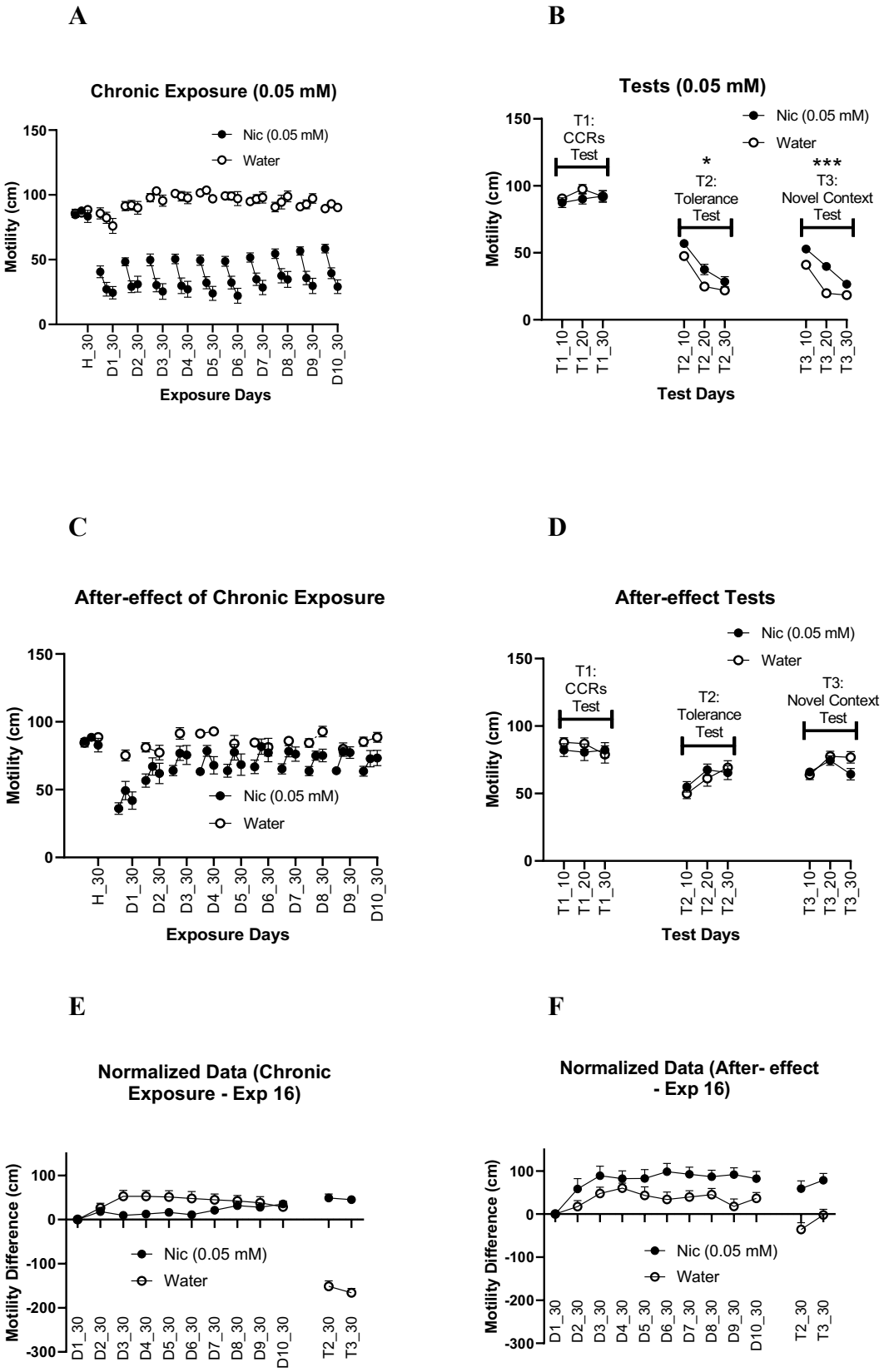


Figure 8. 3. Experiment 16. Mean distance covered by planarians on the exposure context throughout 10 days of 30 min in the presence of nicotine or water (A), 30 min of CCRs test in the presence of only water (T1), 30 min of tolerance test in the presence of nicotine (T2), and 30 min of novel context test in the presence of nicotine with an alternative context (T3) (B). **After-Effect.** Mean distance covered by planarians on the drug-paired context throughout 10 days of 30 min in the absence of nicotine after nicotine administration (C), 30 min abstinence test after the CCRs test (T1), 30 min of abstinence test after tolerance test (T2), and 30 min of abstinence test

after novel context test (T3) (D). Results represented in 10 minutes bins. Data normalized to the first training session and represented in 30 min bins for the chronic exposure (E) and the after-effect (F). Bars represent standard errors. n=22-24 planarians in each group.

8.5 Experiment 17. Abstinence-induced Behaviour and Tolerance Development with a high nicotine dose.

The goal of Experiment 17 was twofold: first, to replicate Experiment 16 with a higher nicotine concentration (0.1 mM); this was the nicotine concentration used by Pagan et al., (2009) in their study of withdrawal-like behaviour, and the lowest dose used by Rawls et al. (2011) used in their study of withdrawal-like behaviour and on the development of tolerance to nicotine. In addition, as we have observed, acute nicotine exposure of planaria causes a decrease in motility, and (in particular at high doses) but others have also seen an increase in C-shaped responses (Rawls et al, 2011), which may be similar to stereotypies such as rearing or head twitching in rats. Rawls and colleagues' (2011) data suggests that C-shaped responses and motility are inversely related (*i.e*, as C-shaped increase, the corresponding motility decreases). Therefore, a second goal was to quantify C-shaped responses to assess whether chronic exposure results in any changes in C-shaped behaviours. Following the results by Rawls and colleagues (2011), we did not expect a high rate of C-shaped behaviours because they did not observe that in their report with a similar dose (0.1 mM) as we used here. We allocated a total 48 animals to two groups, Nicotine and Water, resulting in n = 24 for Group Nicotine, and n = 24 in Group Water. Other than that, the experimental procedure replicates the one described in Experiment 16.

8.5.1 Results

8.5.1.1 Chronic exposure

Over the course of the chronic exposure, the animals treated with nicotine showed lower levels of motility than the animals in the control group, exposed to treated water, replicating the results of previous experiments but with a higher nicotine concentration.

Although some variability was observed across days, there did not seem to be a decrease in the effects of nicotine across days. The data of the chronic exposure phase of the experiment in Figure 8.4 A suggest that motility in Group Nicotine was actually higher on the first day of exposure than on the last day of the exposure phase: the animals covered 33.8 (± 2.7) cm on Day 1 21.03 (± 2.6) cm on Day 10. These results do not suggest the development of tolerance during the chronic exposure to a relatively high concentration of nicotine; quite the opposite, this pattern resembles the development of sensitization to the effects of the drug. These impressions were confirmed with a 2 (Group: Nicotine *vs*. Water) x 10 (Days: 1 – 10) x 3 (Bins: 1-3) mixed ANOVA that revealed a main effect of Group, $F(1, 46) = 858.9$, $p < .001$, $\eta_p^2 = .95$, and Bins, $F(2, 92) = 67.3$, $p < .001$, $\eta_p^2 = .59$, as well as significant Group x Days, $F(9, 414) = 2.58$ $p = .007$ $\eta_p^2 = .05$, and Group x Bins interactions, $F(2, 92) = 62.6$, $p < .001$, $\eta_p^2 = .58$. The remaining factor and interactions were all non-significant: Days, $F(9, 414) = 1.025$ $p = .42$, $\eta_p^2 = .02$; Days x Bins, $F(18, 828) = 0.69$, $p = .82$, $\eta_p^2 = .015$, and the three way Group x Days x Bins interaction, $F(18, 828) = 0.82$, $p = .67$, $\eta_p^2 = .02$. Within-subjects linear contrasts revealed an effect of Day in Group Nicotine, $F(1, 23) = 6.77$, $p = .01$, $\eta_p^2 = .22$, but not in Group Water, $F(1, 23) = 0.71$, $p = .40$, $\eta_p^2 = .03$. However, it should be noted that with this high dose, motility in Group Nicotine *decreased* rather than increased, revealing no tolerance development whilst the animals were under the effects of nicotine.

8.5.1.2 Test 1

Test 1 was conducted on the exposure context with water to assess the development of CCRs. As can be observed in Figure 8.4 B (left panel, T1), the two groups performed in similar ways. A 2 (Group: Nicotine *vs*. Water) x 3 (Bins: 1-3) mixed ANOVA, revealed a significant effect of Bins, $F(2, 92) = 0.39$, $p = .68$, $\eta_p^2 = .008$.

However, neither the main factor Group, $F(1, 46) = 0.68$, $p = .41$, $\eta_p^2 = .015$, nor the Group x Bins interaction, $F(2, 92) = 7.51$, $p = .001$, $\eta_p^2 = .14$, was significant.

8.5.1.3 Test 2 and Test 3

Figure 8.4 B (central and right panels, T2 and T3) displays the results of Tests 2 and 3. The Group Nicotine displayed higher levels of motility than the Group Water in both contexts, suggesting the development of a context independent tolerance to nicotine. This impression was confirmed by a 2 (Group: Nicotine *vs*. Water) x 2 (Tests: Test 2 and Test 3) x 3 (Bins: 1-3) mixed ANOVA, which revealed significant main effects of Group $F(1, 46) = 6.57$, $p = .01$, $\eta_p^2 = .12$, and Bins, $F(2, 92) = 116.3$, $p < .001$, $\eta_p^2 = .72$; the factor Tests, however, was not significant , $F(1, 46) = 0.14$, $p = .71$, $\eta_p^2 = .003$. The main effect of Group, together with the absence of effect of the Tests factor suggest the development of context independent chronic tolerance to nicotine. The analysis also revealed a significant Test x Bins interaction, $F(2, 92) = 5.31$, $p = 007$, $\eta_p^2 = .10$; the remaining interactions were all non-significant: Group x Tests, $F(1, 46) = 0.15$, $p = .70$, $\eta_p^2 = .003$; Group x Bins, $F(2, 92) = 2.31$, $p = .11$, $\eta_p^2 = .05$; and the three-way Group x Tests x Bins interaction, $F(1, 46) = 0.66$, $p = .52$, $\eta_p^2 = .014$.

8.5.1.4 Nicotine after-effect during chronic exposure

The data corresponding to the additional 30 min of exposure to treated water in the exposure context following each of the chronic exposure trials is displayed in Figure 8.4 C. Although we did not observe the development of tolerance (increased motility as the animal acquires experience with the drug) during the actual exposure trials, we observed the development of tolerance to the after-effects of the drug during the additional 30 min exposure to water. A 2 (Group: Nicotine *vs*. Water) x 10 (Days: 1 – 10) x 3 (Bins: 1-3) mixed ANOVA revealed main effects of Group, $F(1, 46) = 99.31$ $p < .001$, $\eta_p^2 = .68$, Days, $F(9, 414) = 10.22$, $p < .001$ $\eta_p^2 = .18$, and Bins, $F(1.5, 68.9) = 25.15$,

$p < .001$, $\eta_p^2 = .35$, as well as significant Group x Days interaction, $F(9, 414) = 3.67$, $p < .001$, $\eta_p^2 = .07$, and Group x Bins interaction, $F(1.5, 68.9) = 59.63$, $p < .001$, $\eta_p^2 = .56$. The remaining interactions were non-significant: Days x Bins, $F(11.8, 542.4) = 1.29$, $p = .19$, $\eta_p^2 = .03$, and the three-way Group x Days x Bins, $F(11.8, 542.4) = 1.17$, $p = .28$, $\eta_p^2 = .02$. Within-subjects linear contrasts revealed an effect of Day in Group Nicotine, $F(1, 23) = 79.51$, $p < .001$, $\eta_p^2 = .77$, but a marginally significant in Group Water, $F(1, 23) = 4.04$, $p = .056$, $\eta_p^2 = .15$. Thus, the assessment of the after effect of nicotine revealed tolerance development as was observed in previous experiments in this study.

8.5.1.5 Nicotine after-effect following Test 1

The after-effect responses were assessed following Test 1 (CCR test in the absence of the drug) by monitoring the animals during an additional 30 min period. The results of this additional 30 min period are displayed in Figure 8.4 D (left panel, T1); planarians in the Groups Nicotine and Water behaved in a very similar way. A 2 (Group: Nicotine *vs*. Water) x 3 (Bins: 1-3) mixed ANOVA, revealed no effects of Group, $F(1, 46) = 0.24$, $p = .62$, $\eta_p^2 = .005$, Bins, $F(1.7, 76.7) = 0.18$, $p = .83$, $\eta_p^2 = .004$, and no interaction between these factors, $F(1.7,76.7) = 0.13$, $p = .88$, $\eta_p^2 = .003$.

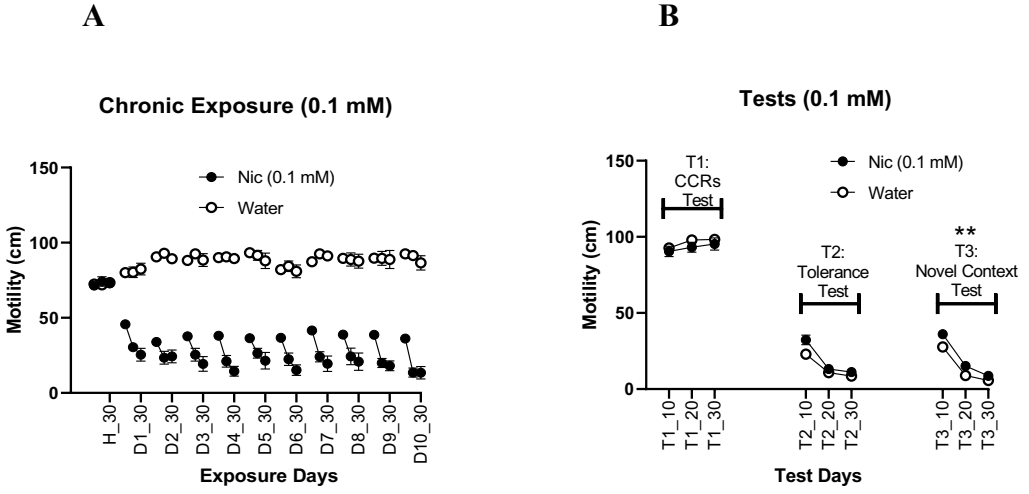
8.5.1.6 After-effect following Test 2 and Test 3

Figure 8.4 D (central and right panels, T2 and T3) shows that the Group Nicotine displays higher levels of motility than Group Water during the additional 30 min that followed the Test 2 and Test 3. A 2 (Group: Nicotine *vs*. Water) x 2 (Tests: Test 2 *vs.* Test 3) x 3 (Bins: 1-3) mixed ANOVA, revealed significant main effects of Group, $F(1, 46) = 9.19$, $p = .004$, $\eta_p^2 = .17$, Tests, $F(1, 46) = 8.51$, $p = .005$, $\eta_p^2 = .16$, and Bins, $F(1.7, 79.1) = 75.9$, $p < .001$, $\eta_p^2 = .62$. The interactions between these factors were all non-significant: Group x Tests, $F(1, 46) = 1.35$, $p = .25$, $\eta_p^2 = .03$; Group x Bins, $F(1.7, 79.1) = 2.43$, $p =$

.10, η_p^2 = .05; Test x Bins, $F(1.8, 82.8) = 0.61$, p = .81, η_p^2 = .001; and the three-way Group x Test x Bins, $F(1.8, 82.8) = 1.35$, p = .26, η_p^2 = .03.

8.5.1.7 C-shaped behaviours and the development of tolerance

During Test 2, when the Water Group experienced nicotine for the first time whilst the Nicotine Group experienced it for the 11th time, we counted C-shaped hyperkinesias every three minutes (starting at mins 0, 3, 6, 9, 12, 15, 18, 21, 24, 27) using 30-sec samples (total, 300 seconds). We wanted to assess if there were differences between groups that received acute or chronic nicotine. We used an independent samples t-test to compare the C-shaped hyperkinesias in Water and Nicotine Groups. The results revealed no differences between the groups, $t(46) = 0.65$, p = .52, suggesting that C-shaped behaviours were similar (Group Water, M = 1.04, SE = 0.24; Group Nicotine, M = 1.25, SE = 0.21). These results suggest that the higher motility observed in Group Nicotine is not due to a decrease in the number of C-shaped behaviours, if any these were descriptively higher in Group Nicotine relative to Group Water. Thus, the development of tolerance does not seem to be driven by a decrease in C-shaped behaviours.



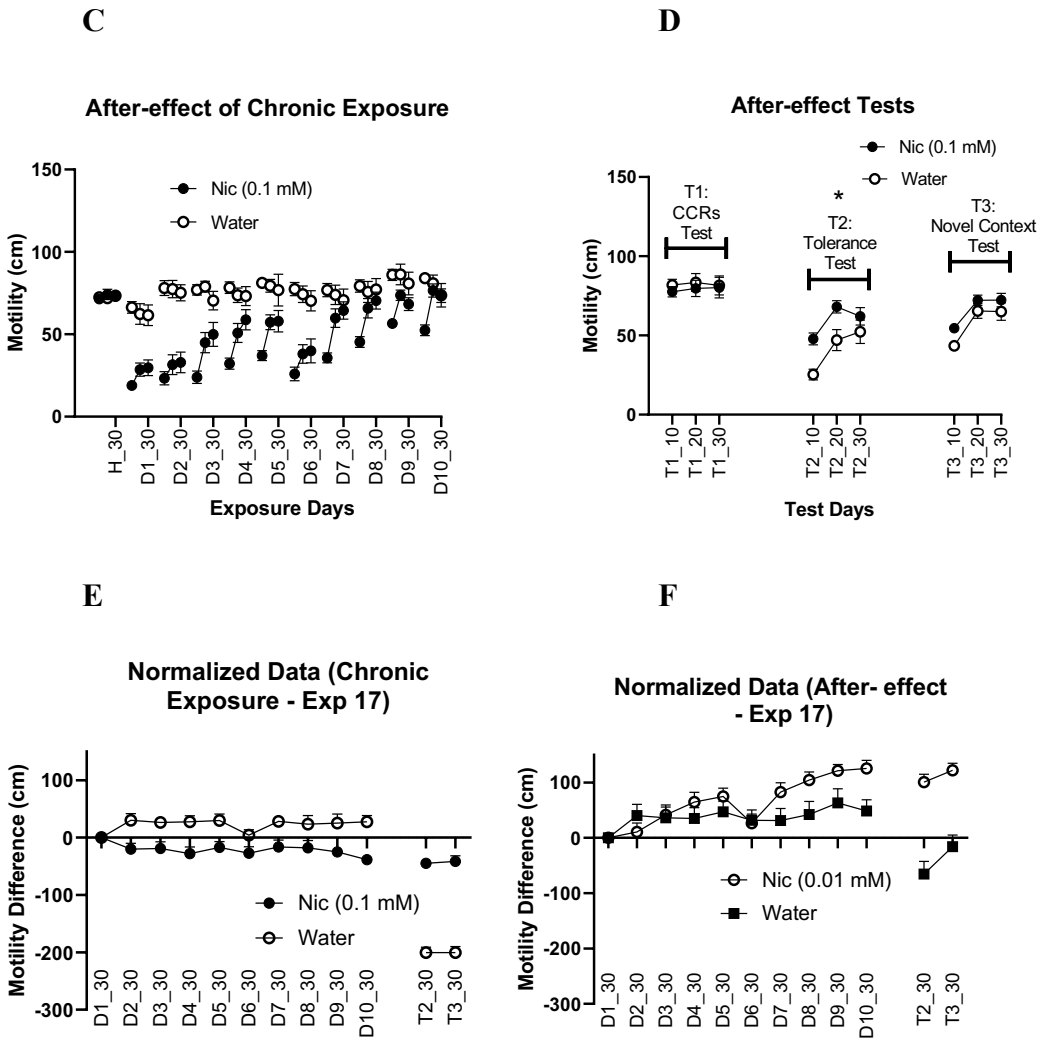


Figure 8. 4. Experiment 17. Mean distance covered by planarians on the exposure context throughout 10 days of 30 min in the presence of nicotine or water (A), 30 min of CCRs test in the presence of only water (T1), 30 min of tolerance test in the presence of nicotine (T2), and 30 min of novel context test in the presence of nicotine with an alternative context (T3) (B). **After-Effect.** Mean distance covered by planarians on the exposure context throughout 10 days of 30 min in the absence of nicotine after nicotine administration (C), 30 min abstinence test after the CCRs test (T1), 30 min of abstinence test after tolerance test (T2), and 30 min of abstinence test after novel context test (T3) (D). Results represented in 10 minutes bins. Data normalized to the first training session and represented in 30 min bins for the chronic exposure (E) and the after-effect (F). Bars represent standard errors. n=24 planarians in each group.

8.6 Discussion

The present study was aimed to assess 1) the development of tolerance to nicotine during repeated nicotine exposure in a specific context; 2) the expression of CCRs to nicotine-associated CS in the absence of nicotine; 3) the expression of nicotine tolerance in the presence of nicotine-associated cues; and 4) the role of a novel context on the expression of nicotine tolerance. We investigated the development of tolerance to the hypo-locomotor effects of nicotine using a long, 10-day chronic exposure regimen

because it better resembles chronic exposure in humans. Across all experiments, we observed during the chronic exposure clear effects of increasing doses of nicotine suggesting that this paradigm and the dependent measure are sensitive to the effects of nicotine in the planaria *Schmidtea mediterranea*.

In Experiments 14, 15 and 16 using lower doses, we observed development of tolerance during chronic exposure, expressed as less effect of the drug during the last day of exposure (Day 10) relative to Day 1, and a linear effect during chronic exposure. The fact that this was not observed in Experiment 17 may be due to the large effect of the drug in suppressing motility. In none of the experiments we observed evidence for compensatory responses during Test 1. Nor did we observe any effect of changing the context from Test 2 to Test 3. However, all experiments revealed an effect of chronic exposure during Tests 2 and 3 (Comparison of Groups Nicotine *vs*. Water), suggesting the development of tolerance to the effects of nicotine. Experiments 16 and 17 also tested for evidence of withdrawal after nicotine removal, and both Experiments revealed an effect of chronic exposure on motility after nicotine was withdrawn, during chronic exposure and during Tests 2 and 3. Whilst variations in dose were paralleled by systematic changes in behaviour, the effect of chronic exposure was smaller during tests with increasing doses (which refer tolerance). This is likely due to the fact that we tested with the same dose as used during chronic exposure, and higher doses lead to larger unconditioned effects that may mitigate against the observation of tolerance. However, this is not surprising. Previous studies in rodents using nicotine (Stolerman et al., 1974) and morphine (Dafter & Odber, 1989) have also observed absence of tolerance development with high doses. Below we discuss the implications of these results.

These results are consistent with previous observations in planaria. For example, Rawls and colleagues (Rawls et al., 2011) observed a decrease in stereotypical activity

following two administrations of high doses of nicotine (1 and 3 mM) on a third (5-min Test) exposure, suggesting tolerance development. Our results extend those previous findings to a chronic regimen of exposure (10 days) that better resembles chronic exposure on humans (also see Feng et al., 2006; and Polli et al., 2015, for similar results in *C elegans*). The results of these experiments also resemble observations in rodents. For example, Stolerman et al. (1973) observed a dose-dependent decrease in motility after different doses of nicotine (acute). In addition, chronic administration (3 times daily for 8 days) resulted in the development of tolerance to the effects of nicotine on motility, similar to what was found in the present experiments (see also Domino & Lutz, 1973 for similar results on bar-pressing behaviour). In addition, in Experiment 15 we assessed whether mecamylamine, a nonselective nicotinic receptor antagonist, had an effect on the effect of nicotine and the development of tolerance. Consistent with previous observations in rodents, mecamylamine attenuated the unconditioned effects of nicotine and blocked the development of tolerance. Although mecamylamine did not complete block the acute effects of nicotine, this is likely due to the fact that we co-administered mecamylamine and nicotine, which may result in receptor binding by nicotine despite the administration of mecamylamine. The fact that mecamylamine blocked the development of chronic tolerance suggests that the latter depends on nicotinic receptor activity. Overall, the results of the present experiments are consistent with observations in other invertebrates and rodents, thus revealing that the mechanisms under study are evolutionarily conserved across vertebrate and invertebrate species.

Based on results obtained with other drugs of abuse in rodents and humans, it has been suggested that tolerance development (in particular learned tolerance) is context-dependent in that a novel context presentation eliminates tolerance to the unconditioned effect of drugs (Siegel, 1975). In all three experiments reported here, animals that

received chronic nicotine exposure were tolerant to the suppressive effects of nicotine on the novel context, as suggested by a lack of interaction between Group and Test during Tests 2 and 3. It is possible that animals showed generalization from the exposure context to the novel context, although in other experiments we have observed good discrimination between the surfaces used here (e.g., Prados et al., 2020). Similarly, in the present experiments we did not observe the presence of CCRs when animals were tested in the presence of contextual cues but in the absence of nicotine. We did, however, use different concentrations, and observed that larger concentrations resulted in less motility, which in turn should result in more CCRs (if it is the case that CCRs result from homeostatic challenges). The presence of CCRs to nicotine-paired cues has not been widely observed in rodents, and some reports have failed to observe CCRs (Hakan & Ksir, 1988). However, experiments by Bevins et al. (2001; also see Walter & Kuchinsky, 1989) observed increased motility in rodents to context cues previously paired with nicotine effects. Although this effect was interpreted as a form of sensitization, the initial effect of nicotine was to supress motility and in that sense these could be considered compensatory responses. Whether the lack of an effect in the present experiments represents a limitation of planarians or the incorrect choice of parameters is an open question at the moment. Finally, it could be possible that CCRs did manifest in the present parameters, but were not captured by motility as a dependent variable (DV). We chose to measure motility because this can be done automatically and therefore is bias-free, but it could be possible that the absence of CCRs is associated with our choice of DV, and that other DVs may reveal the presence of CCRs. Further research should shed light on this.

In Experiments 16 and 17, we investigated the after-effects of nicotine exposure to shed light on behaviour when nicotine has been removed (i.e., withdrawal). In Experiment 16, we observed during chronic exposure that the effect of nicotine decreased

with chronic exposure, so that the difference between Nicotine and Water Groups in Day 1 was no longer present on Day 10, although there were no differences between groups on the after-effect analyses conducted during Tests 2 and 3. A similar finding was observed in Experiment 17 when using a higher dose, but in Experiment 17, we also observed an after-effect on Tests 2 and 3. That is, animals that had received chronic exposure to nicotine showed more motility relative to animals that experienced water, a finding that is similar to that of Pagan et al. (2009). We interpret this difference as indicative of withdrawal associated with tolerance development, for we observed *more* motility rather than less—which was observed by Rawls and colleagues (2011). Rawls et al. (2011) findings likely reflect after-effects of nicotine rather than withdrawal symptoms because they measured changes in the motility after a single (and short) exposure to nicotine, and any effects of drug-associated cues were not considered. The effect we observed was evident in the exposure (T2) and novel (T3) contexts. One intriguing possibility to explain these findings is that the interoceptive effects of nicotine acted as a conditioned stimulus, and this enabled both the observation of tolerance during nicotine exposure in Tests 2 and 3, and also the observation of a difference between groups in the after-effect period. Whilst this interpretation is speculative, there is convincing evidence in rodents (Murray & Bevins, 2007) and humans (Clemens et al., 1996) that nicotine can act as a conditioned stimulus. When nicotine is trained as a conditioned stimulus it can overshadow and block performance about other associated environmental stimuli (Murray et al., 2011), and this may explain why tolerance was only observed during or after nicotine presentation, but not in the presence of nicotine-paired cues alone (Test 1).

In the introduction, we discussed two theories that explain tolerance following similar principles as those governing associative learning (Siegel, 1975; Solomon, 1980). Briefly, these models suggest that stimuli presented along with drug administration

become associated with the unconditioned effects of drugs, and when presented in the absence of the drug elicit conditioned responses which are opposite to the unconditioned effect of the drug (CCRs). In addition, these theories predict that tolerance should be better observed in the presence of drug-associated cues than in their absence. In none of the experiments reported here we observed CCRs during Test 1. Similarly, we observed that tolerance to the hypo-locomotive effects of nicotine was similarly observed in the context where animals received chronic exposure and in a novel environment. The absence of differences during Test 1, given the large (dose-dependent) unconditioned effects we observed during chronic exposure, together with the insensitivity to context changes (Tests 2 and 3) are problematic for an associative account of tolerance. Rather, these results, and in particular the after-effect observed in Experiment 17, are consistent with a habituation explanation of tolerance as that put forward by Baker and Tiffany (1985). They suggested that the bulk of data available at the moment was more consistent with a habituation explanation of tolerance, and in particular with the basic tenets of habituation suggested by Wagner (1976). According to Wagner's model, habituation (and hence tolerance) occurs due to the action of either of two mechanisms: associative priming and self-generated priming (see Wagner, 1976; and Prados et al., 2020, for a detailed explanation). Associatively generated priming enables environmental cues associated with drug effects to attenuate, in the long-term, the unconditioned effects of drugs, resembling the well-known diminution of unconditioned effects observed in basic learning procedures (Kimmel, 1966). Self-generated priming allows a representation of the drug effects to be primed in short-term memory by a previous drug exposure, and reduces the unconditioned effects of drugs. Self-generated priming explains quite well the findings of Tests 2 and 3 in all experiments, and the after-effect observed in Tests 2 and 3 in Experiment 16, where planarians in Group Nicotine showed less effect of

nicotine (i.e., tolerance) following discontinuation of the drug. According to the habituation explanation of chronic exposure to nicotine during these tests, the prior presentation of the drug during the test resulted in less responding to the drug after-effects, an explanation which is also consistent with the above speculations of nicotine acting as a CS.

Overall, the present study suggests that planarians show tolerance to the unconditioned effects of nicotine, and that this tolerance did not show context dependency nor did stimuli associated with the unconditioned effect of the drug elicit compensatory responses. Taken as a whole, these results are consistent with a model of tolerance that captures it as following similar principles to those of habituation (Baker & Tiffany, 1985). In addition, these results are, by and large consistent with other findings in planaria and rodents, suggesting that the planaria is a useful preclinical model for the study of tolerance development following chronic exposure to drugs of abuse.

9 Chapter 9: A Meta-analysis of tolerance studies

Drug tolerance is the reduced behavioural responsiveness to unconditioned drug effects that results from repeated exposure. Associative learning plays an important role in development of drug tolerance as a consequence of the incremental developmental of homeostatic compensatory responses to drug-associated cues (Siegel, 1975). Therefore, development of drug tolerance is observed if chronic exposure and test take place in a similar, distinctive environment. Otherwise, tolerance is not observed, that is when the tolerance test is conducted in a context different from that of drug-paired CS (Siegel, 2001). However, Habituation theory (Baker & Tiffany, 1985) suggested that tolerance can result from both associative and non-associative routes, as an alternative to Siegel's (2001) Pavlovian Conditioning Tolerance Theory. Habituation theory (Baker & Tiffany, 1985) minimizes the contribution of homeostatic regulatory responses on tolerance responses and proposes that opponent responses are not essential for tolerance development.

We investigated the development of chronic tolerance to nicotine in *Schmidtea mediterranea* by testing the animals in nicotine-associated and non-associated (i.e., novel) contexts after conditioning them in a distinctive environment. Some experiments showed the development of tolerance only in the drug-paired context (see Experiments 10 and 12 in Chapter 7), one individual study showed only in the novel context (see Experiment 15 in Chapter 8), and other studies demonstrated it in both contexts (see Experiments 9 and 13 in Chapter 7; Experiment 14, 16 and 17 in Chapter 8). In order to obtain a more accurate estimation regarding the effect of context (nicotine paired vs. non-paired) on the development of tolerance to nicotine, we run three Meta-analyses across all experiments in *Schmidtea mediterranea,* assessing the evidence for the expression of CCRs (Test 1), and the context dependence of tolerance observed under the effects of

nicotine in the drug-paired context (Test 2) and in a novel context (Test 3). These experiments were presented in Chapters 7 and 8.

9.1 Methods and Results

See Chapter 5.4.2

9.2 Results

First, we evaluated the association between CCRs and chronic nicotine exposure when they were tested with water in the nicotine-paired context with a total of 8 experiments. The Forest plot in Figure 9.1 shows the effect size of each individual study (shown by square symbols) their 95% confidence intervals (CI; represented through horizontal lines). The summary analysis results for Test 1 indicated that hyper locomotion (i.e., CCRs) to nicotine-paired context was not associated with the chronic nicotine exposure (averaged effect size of Group, Cohen's d = -0.12; 95%CI: -0.33-(0.10); P = 0.281). Additionally, the value of I^2 is 0%, verifying the absence of considerable heterogeneity amongst the studies.

To address the question of whether tolerance to the chronic effects of nicotine are context-dependent, we run a meta-analysis on the results of each Tests 2 and 3, across all 8 experiments. In other words, 8 experiments evaluated the association of chronic nicotine exposure with tolerance development with nicotine paired and unpaired (i.e., novel) environments (Tests 2 and 3). The Forest plot in Figure 9.1 shows the effect size of each individual study (shown by square symbols) their 95% confidence intervals (CI; represented through horizontal lines). The effect summary indicates that chronic administration of nicotine is positively associated with tolerance development in Test 2 when animals were tested in the drug-paired context (averaged effect size of Group, Cohen's d = 0.78; 95%CI 0.50–1.06; P<.001; Fig 9.1 A). Additionally, there was also positive correlation between chronic nicotine administration and tolerance in Test 3 when

animals were tested in a novel context (averaged effect size of Group, Cohen's d = 0.65; 95%CI 0.39–0.90; P<.001; Fig 9.1 B). These results reveal that across experiments, the experimental group having history of ten days of chronic nicotine exposure were less sensitive to the effect of nicotine relative to the control group which had no prior history of nicotine exposure in either the nicotine-paired (Test 2) nor in novel context (Test 3).

However, chronic nicotine exposure had a relatively large impact on tolerance development with the nicotine-paired context (d = .78); and this impact was medium with novel environment (d = .65) (Cohen, 1988). However, chronic exposure in a distinctive context did not result in the development of CCRs (Test 1; d = -.12). Therefore, these meta-analysis of tolerance findings of across the experiments suggested that tolerance can be acquired with or without reliable/distinctive contextual cues. However, rate or magnitude of acquisition of tolerance are generally greater with reliable contextual cues (Baker & Tiffany, 1985)

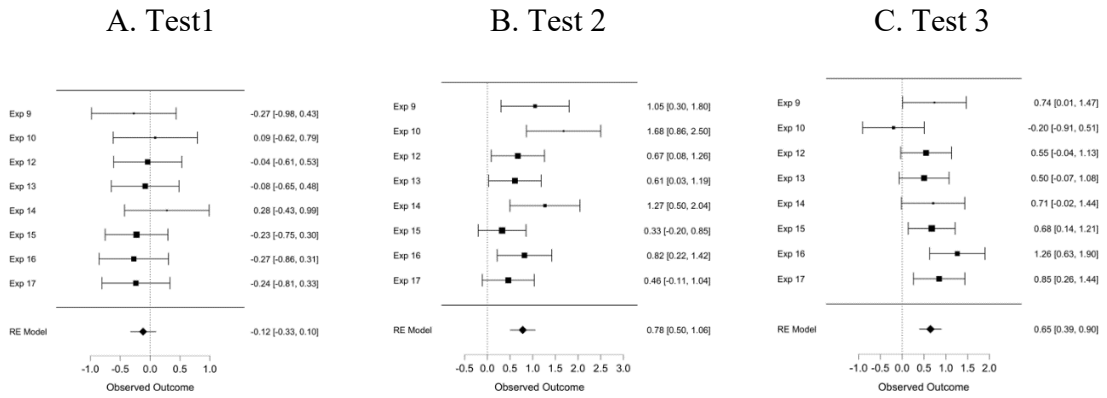


Figure 9. 1. Association of chronic treatment with the development of CCRs to drug-paired context (A) and the development of tolerance in nicotine drug-paired (B) and novel context (C).

9.3 Discussion

Overall, we investigated the effectiveness of chronic nicotine exposure to the development of tolerance with Meta-analysis. We can conclude, based on where the

diamond is situated on the forest plot, that animals having the history of chronic exposure of nicotine were less sensitive to unconditioned effect of nicotine than those exposed for the first time. Additionally, the magnitude of tolerance is greater when the test was carried out in the nicotine-paired context (d = .78) than in a novel environment (d = .65). These results are consistent with the habituation theory of tolerance put forward by Baker and Tiffany (1985), according to which tolerance simply reflects a process of habituation; from this perspective, homeostatic CCRs (Siegel, 1975; Solomon, 1980) are not necessary for the development of these particular instances of tolerance.

Chapter 10: Summary, conclusions and future directions

The present study was aimed to assess (i) the development of tolerance to nicotine during repeated nicotine exposure in a specific context; (ii) the expression of CCRs to nicotine-associated CS in the absence of nicotine; (iii) the expression of nicotine tolerance in the presence of nicotine-associated cues; and (IV) the role of a novel context on the expression of nicotine tolerance. To achieve these goals, we have conducted 17 experiments. Over the 17 experiments, we have manipulated different variables as follows: (a) different concentrations of nicotine (b) various amounts and regimens of exposure (c) different strains of planaria (e) pharmacological manipulations (i.e., blockade with nicotinic antagonists) (e) tests of associative and novel contexts. In general, our results showed that, across all experiments, the acute effect of nicotine is concentration-dependent, while the chronic effect of nicotine was dependent to the strain of planaria and regimen of exposure (5x and 10x). In particular, the chronic effect of nicotine on tolerance development was observed *Schmidtea mediterranea* but not *Dugesia sp*. Below we discuss the main findings in more detail:

Chapter 6: The development of tolerance and CCRs in Dugesia sp. with different nicotine concentrations and time of exposure

In chapter 6, we aimed to assess the chronic tolerance to nicotine exposure with planaria across eight experiments. In particular, these experiments were designed to examine whether chronic exposure to nicotine results in less effect of nicotine (i.e., tolerance to the unconditioned effects of nicotine) and also on the assessment of learned tolerance (i.e., context-dependency and CCRs) using commercially available *Dugesia sp*. For the assessment of chronic tolerance to nicotine, we manipulated three important variables: (i) concentration and (ii) amount of exposure and (iii) regimen of exposure.

The main findings outlined in chapter 6 illustrated nicotine cause concentration-dependent depressant locomotor effects during acute exposure. As expected, the higher the concertation, the lower the locomotor activity. However, there was neither development tolerance during or after nicotine exposure nor the development of CCRs. Although different exposure time (for example, 5h for Experiment 3, 4, 5 and 8; 10 hours for Experiment 6; and 16 hour in Experiment 7) and regimens (30 min x 10 days for Experiment 3, 4 and 8; 2.5 h x 2 days in Experiment 5; 2.5 h x 4 days in Experiment 6, and single 16 h overnight exposure in Experiment 7) and different concentrations (0.01, 0.025 and 0.1 mM) were used, the animals that received chronic nicotine exposure and those that received nicotine for the first time behaved in similar way. This suggests that the acute and chronic effect of nicotine was similar with *Dugesia sp.*, and hence that there was no tolerance development.

Following the examination of the behavioural changes after chronic nicotine exposure, we also investigated whether acute and chronic nicotine exposure caused changes in neurotransmitters and their metabolites. In particular, we examined the effect of acute and chronic nicotine exposure on the changes in neurotransmitter levels in Dopamine and its metabolites, DOPAC and HVA, and the serotonin metabolite 5HIIA, using the HPLC technique. We observed increased concentrations of DA and 5-HT neurotransmitters after both acute and chronic nicotine exposure; however, we failed to observe the difference in neurotransmitter levels between acute and chronic nicotine exposure regimens. This result is consistent with our behavioural results where we observed that chronic exposure did not results in tolerance development (hence, locomotor activity following a challenge with nicotine was similar in acute and chronic groups.

Drug associated environmental context is very important component for the development and expression of tolerance and CCRs so that presentation of alternative context eliminates CCRs that lead to failure of tolerance (Siegel, 2008). Drug treatment with reliable drug-cues signals (or contextual cues) result in the establishment of associations between the drug-cues and the effects of the drug (in Siegel's theory, for example, the compensatory response of the organism to the drug insult). These cues therefore can reduce the unconditioned drug effects, and drug presentation in the presence of the drug-cues elicits smaller URs compared to drug without reliable cues. Furthermore, associatively generated priming of tolerance (Baker & Tiffany, 1985) enables environmental cues associated with drug effects to attenuate, in the long-term, the unconditioned effects of drugs, resembling the well-known diminution of unconditioned effects observed in basic learning procedures (Kimmel, 1966). On the other hand, self-generated priming of tolerance (Baker & Tiffany, 1985) suggests tolerance can be seen with non-associative routes and describes tolerance as adaptation/ familiarisation to the characteristics of stimuli.

Therefore, the effect of context on the motility of planaria which is the main dependent variable for measuring tolerance was systematically assessed in this study. Initially, we assessed whether the changes in motility of the planaria (*Dugesia sp.*) was caused by pharmacological action of nicotine rather than the textures (smooth and rough) where nicotine was given. The results revealed a main difference between nicotine and water groups (hypo-active response to nicotine), but there was an absence of a Group by Texture interaction effect (p = .39), suggesting that nicotine reduced their locomotor activity, and it was not dependent of the texture. For the further experiments, the animals (*Dugesia sp.*) were tested on the nicotine-associated (Test 2) and non-associative context (Test 3). The results revealed the absence of tolerance to initial hypoactive effect of

nicotine. There was also an absence of a Group by Tests interaction across the experiments, suggesting that response of *Dugesia sp.* to nicotine was similar in both contexts (not modulated by different contextual surfaces). Furthermore, we applied the same experimental manipulations (Test 2 and Test 3) with a different strain of planaria, *Schmidtea mediterranea*. We observed the display of (test) tolerance to nicotine after chronic exposure, and it was significant in both contexts (both in nicotine-associated and non-associated novel context) as we observed with *Dugesia sp*. These results suggests that context is not an important variable for the assessment of nicotine tolerance both in *Dugesia sp.* and *Schmidtea mediterranea*. Animals might show generalisation between two surfaces.

Contrary to the absence of context effect, we found a difference in chronic tolerance development between two strains of planaria (*Dugesia sp.* vs. *Schmidtea mediterranea*). For example, Experiment 8 compared two different strains of planaria, and the results provided evidence of the development of chronic tolerance in *Schmidtea mediterranea*, but not in *Dugesia sp.* That results suggested chronic exposure to nicotine elicits tolerance with one strain but not another strain of planaria. This result is parallels findings in planaria (Ireland et al., 2020) and mice (Grieve & Littleton, 1979). For example, Grieve and Littleton (1979) compared the function of tolerance development to ethanol with different strains of mice (C57BL, TO Swiss and DBA2), and found that that the development of tolerance was very rapid with C57BL mice but slower with TO Swiss mice; also, little evidence of tolerance was observed with DBA2 mice. In previous studies, which used similar approach of chronic tolerance development, Mohammed-Jawad and colleagues (2018) reported the development of chronic tolerance to sucrose with *Dugesia sp*. We failed to observe chronic tolerance to nicotine with *Dugesia sp.*; while, we observed chronic tolerance to nicotine with *Schmidtea mediterranea*. These

results suggest that the development of chronic tolerance can vary depending on the strain of planaria and the type of agent used to study tolerance development. It could be possible, as suggested by Eikelboom and Stewart (1982) that nicotine and sucrose have different sites of action and these are responsible for the different effects of these agents (sucrose vs nicotine) on the development of tolerance.

Chapters 7 and 8: Chronic effect of Nicotine in Planaria (Schmidtea mediterranea): Tolerance and Compensatory Responses

After we observed the development of tolerance with *Schmidtea mediterranea*, to achieve the aforementioned hypothesis with *Schmidtea mediterranea*, we conducted further experiments (8 experiments) with three important manipulations in DVs: different regimens of chronic nicotine exposure, various nicotine concertations and pre-exposure or post-exposure to the specific context during repeated nicotine exposure and test sessions. We assumed that if animals receive a longer nicotine exposure with 5x (Experiment 9) and, higher nicotine exposure (Experiment 12, 13, 16, 17) or the development of anticipatory responses with prior presentation of nicotine contingent-cues (Experiment 13) would enhance the possibility to observe the engagement of Solomon's B-process (Solomon, 1980) that would result in 1) less effect of nicotine (i.e., tolerance to unconditioned effect of nicotine) and 2) learned tolerance (i.e., context dependency and CCRs) using *Schmidtea mediterranea*. Below we discuss the main findings in detail:

Manipulation of regimen of chronic nicotine exposure: 5x and 10x

We assessed the effect of chronic nicotine exposure in planaria with the different regimen of exposure; one with 10x, ten days of exposure with 30 min of exposure; another protocol was 5x, five days of exposure with 1h exposure. The results of this manipulation

demonstrated that (Experiment 9 and 10) animals treated with nicotine with 5x schedule had developed a hyperactivity CCRs to nicotine associated surface but not developed tolerance. On the other hand, animals trained with 10x schedule have developed tolerance but not CCRs. These data suggest that different schedule of chronic nicotine treatment/exposure produced different behavioural responses to stimuli associated with the nicotine administration. Solomon's *Opponent Process Theory* (1980) suggested that initial presentation of unconditioned stimuli elicits both a hedonistic drug effect, A-process and compensatory B-process. These two processes counteract with each other, and the intensity/ strength of A -proposes might result in a stronger B-process which would reveal the development of CCRs. Previous nicotine studies with rats (Clarke and Kumar, 1983) and planaria (Rawls et al., 2011) showed that increasing the concentration of nicotine (i.e., toxicity) cause more impairment in animals motor behaviours, as we found. Therefore, increasing the toxic effect of nicotine (A-process) with a higher concentration might results in a stronger B-process which would reveal the development of CCRs. In order to get a stronger CCRs as we observed in 5x schedule, we used higher nicotine concentrations with 10x experimental schedule.

Exposure to various nicotine concentrations

Concentration was one of the main independent variables changed throughout this thesis. Across these 17 experiments we used various nicotine concertations (0.01, 0.025, 0,05 and 0.1 mM). We observed that acute nicotine administration produced hypoactivity in a consistent concertation-dependent manner for *Dugesia sp.*, similar to other rats (Clarke & Kumar, 1983), and planaria (Rawls et al., 2011). However, we observed similar hypoactive responses following acute and chronic administration. Along with the absence of tolerance, we did not observe systematic conditioned responses in *Dugesia sp*.

However, we observed the expression of tolerance after chronic nicotine administration with different nicotine concentrations in a different strain, *Schmidtea mediterranea*.

The magnitude of test tolerance was stronger with lower concertation, and it became weaker with increased concentration for the experiments reported in Chapter 7. Furthermore, in Experiments 14, 15 and 16 using lower doses, we observed development of tolerance during chronic exposure, expressed as less effect of the drug during the last day of exposure (Day 10) relative to Day 1, and a linear effect during chronic exposure. This was not observed in Experiment 17 may be due to the large effect of the drug in suppressing motility. Furthermore, in none of the experiments we observed evidence for compensatory responses during Test 1. Nor did we observe any effect of changing the context from Test 2 to Test 3. However, all experiments revealed an effect of chronic exposure during Tests 2 and 3 (Comparison of Groups Nicotine *vs*. Water), suggesting the development of tolerance to the effects of nicotine. Experiments 16 and 17 also tested for evidence of withdrawal after nicotine removal, and both Experiments revealed an effect of chronic exposure on motility after nicotine was withdrawn, during chronic exposure and during Tests 2 and 3. Whilst variations in dose were paralleled by systematic changes in behaviour, the effect of chronic exposure was smaller during tests with increasing doses (which refer tolerance). This is likely due to the fact that we tested with the same dose as used during chronic exposure, and higher doses lead to larger unconditioned effects that may mitigate against the observation of tolerance. However, this is not surprising. Previous studies in rodents using nicotine (Stolerman et al., 1974) and morphine (Dafter & Odber, 1989) have also observed absence of tolerance development with high doses.

These results are consistent with previous observations in planaria. For example, Rawls and colleagues (2011) observed a decrease in stereotypical activity following two

administrations of high doses of nicotine (1 and 3 mM) on a third (5-min Test) exposure, suggesting tolerance development. Our results extend those previous findings to a chronic regimen of exposure (10 days) that better resembles chronic exposure on humans (also see Feng et al., 2006; and Polli et al., 2015, for similar results in *C elegans*). The results of these experiments also resemble observations in rodents. For example, Stolerman et al. (1973) observed a dose-dependent decrease in motility after different doses of nicotine (acute). In addition, chronic administration (3 times daily for 8 days) resulted in the development of tolerance to the effects of nicotine on motility, similar to what was found in the present experiments (see also Domino & Lutz, 1973 for similar results on bar-pressing behaviour).

Different drug applications with various concentration caused various distinctive stereotypical behavioural responses in planaria such as C-like, screw-like, and bridge-like positions. These hyperkinesia responses were varied with the manipulations of different receptor systems such as dopaminergic (Palladini et al., 1996; Ramoz et al., 2012), cholinergic (Nishimura et al., 2010; Buttarelli et al., 2000; Rawls et al., 2011) and serotonergic and opioid systems (Farrell et al.,2008). For example, Rawls et al., (2011) investigated chronic tolerance to nicotine by exposing animals to nicotine for 5 min of 3 times. The interval between each exposure was not consistent (twice on day1 and final exposure on day4). They also assessed tolerance by counting C-shape response which is based on researcher's subjective assessment. Therefore, it is difficult and takes a long time to count these hyperkinesia behaviours for each animal. Thus, most of the pharmacological studies with planaria focused on acute drug administration; however, chronic exposure - typically regarded as a condition for the development of tolerance - and the role of the contextual cues have not been systematically assessed. Therefore, in this study, we evaluated the acute and chronic effect of nicotine on planaria motility. We

used motility as a dependent variable. The locomotor activity of planaria was recorded with one camera system. Data were analysed using software, which allowed us to assess planaria behaviours with standardised testing conditions with more extended screening time and compare these results across the experiments. We observed acute nicotine exposure reduce the motility of planaria in concentration-dependent manner and chronic nicotine exposure expressed the development of test tolerance (less effect of nicotine for Group Nicotine than Group Water).

Additionally, we counted hyperkinesia behaviours of planaria alongside with their motility (reported in Experiment 17). C-shaped hyperkinesia was counted every three minutes (starting at mins 0, 3, 6, 9, 12, 15, 18, 21, 24, 27) using 30-sec samples (total, 300 seconds) during Test 2 (nicotine test with the nicotine associated environment). We found that that the number of C-shaped hyperkinesia were similar between groups, contrary to what we found with motility results (tolerance to nicotine). These results suggest that the higher motility observed in Group Nicotine is not due to a decrease in the number of C-shaped behaviours if any these were descriptively higher in Group Nicotine relative to Group Water. Thus, the development of test tolerance after chronic exposure does not seem to be driven by a decrease in C-shaped behaviours. Although the data suggests that there was no difference in hyperkinesia responses between groups, measurement of hyperkinesia behaviours by standardised testing conditions with longer screening time would be useful in the measurement of drug tolerance. For example, Ireland and colleagues (2020) measured hypokinesia responses (i.e., C-shape, corkscrew, contraction) in planaria for the assessment of chronic effect of common solvents (i.e., ethanol and methanol). Behavioural responses were recorded by a camera system which produced high-resolution images (5 fps), which were automatically analysed with custom scripts in MATLAB and Phyton. Therefore, measurement of abnormal behaviours with

standardised testing conditions would be useful have a second output measure for the measurement of nicotine tolerance.

Drug signalling with the contingent cue

Furthermore, we assessed the role of drug signalling in the development of tolerance and the observation of CCRs. Solomon posited that environmental stimuli could be associated with either A- or B-process with repeated exposure. If drug contingent cue activates the A-process, iso-directional response which is followed by drug-antagonistic response is observed; however, if the B-process is activated, conditioned compensatory responses (CCRs) are observed. Therefore, the failure to observe CCRs may result from the use of a simultaneous procedure, in which CS and US are presented always at the same time. Simultaneous conditioning does not always get reflected in performance, so it may be possible that by using an arrangement where presentations of the CS predict the later drug, that may better allow for the observation of CCR, if animals were exposed to the contextual cues alone for 30 min before the nicotine administration. For example, morphine (Grisel et al., 1994; Sherman, Strub & Lewis, 1984; Siegel, 1999) and ethanol (Larson & Siegel, 1998; Siegel & Larson, 1996; Siegel & Sdao-Jarvie, 1986) studies with Siegel's Pavlovian Tolerance Model found the development of tolerance when cue and drug repeatedly presented and observed the expression of compensatory responses when the drug-paired cue was presented alone (cue- no drug) before the joint presentations of the cue and drug events. Therefore, we assumed that establishing a predictive relationship between the surface and the effects of nicotine would allow animals to develop a CCRs to the hypo locomotive effects of nicotine. In Experiment 13, Animals received 30 min pre-exposure to the environmental stimuli before nicotine presentation in the same context so that the context would signal later drug delivery that leads to the development

of preparatory/ anticipatory response to nicotine delivery. Although we observed the development of chronic tolerance, but absence of anticipatory responses and CCRs.

We also assessed after-effect of nicotine to distinguish between after-effect and withdrawal responses (Experiment 16 and 17). Several planaria studies reported withdrawal responses immediately after single drug exposure (Pagan et al., 2009; Raffa and Rawls, 2008; Rawls et al., 2011). However, withdrawal would be more about drug craving or imbalance in the absence of drug exposure when the drug is expected but omitted. Therefore, animals received 30 minutes of water test in the same environment following nicotine presentation during the days of chronic exposure and over test days. The first after-effect response was similar to the unconditioned effect of nicotine, suggesting animals were still under nicotine effect; however, animals were developed tolerance to the after-effect of nicotine over the days of chronic exposure. Additionally, we observed chronic tolerance to after-effect response in Test 2 and Test 3 following exposure to high nicotine concentration (1.0 mM). Our results suggest that water test immediately after drug exposure might not be an appropriate way of assessing withdrawal response because animals were still under drug effect.

Chapter 9: Context effect on tolerance

Across eight experiments with *Schmidtea mediterranea*, we tested chronic tolerance with both nicotine associated and non-associated nicotine context. We did not obtain conclusive evidence for a role of context on the effect of chronic tolerance to nicotine and its association of with CCRs. Although by and large tolerance was observed in both contexts, and hence was interpreted as being context-independent, some experiments showed chronic tolerance only in the nicotine-paired surface (i.e., context), whilst some experiments only showed in both contexts, but others showed only in the

novel context. To derive a robust conclusion about the effect of context on chronic nicotine tolerance, in Chapter 9, we used the meta-analysis technique. We observed that chronic tolerance was significant in both contexts, hence chronic tolerance was not independent of context because it was somewhat more potent with nicotine associated context than a novel context.

Additionally, across all experiments there was conclusive evidence for the absence of CCRs. Overall, the drug-associated context increases the magnitude of chronic tolerance, but this effect is not sufficient to trigger CCRs in the absence of nicotine. These results are problematic for two main behavioural theories of tolerance: Solomon's Opponent-process (1980) and Siegel's Pavlovian Conditioning of tolerance (Siegel, 1975; 2001; 2008), because we did not observe the development of CCRs to nicotine associated context following the discontinuation of nicotine administration; however, consistent with Habituation model of tolerance (Baker & Tiffany, 1985), which suggests that compensatory responses are not essential for the expression of tolerance.

Solomon (1980) and Siegel (1975) claimed unexpected discontinuation drug presentation result in opponent/compensatory responses which were defined as a sign of distress from the drug's withdrawal. Tolerance and withdrawal symptoms are thought to be controlled by similar homeostatic mechanisms, and hence the severity and duration of withdrawal symptoms are linked to the magnitude of tolerance. For example, the opponent-process theory (Solomon, 1980) proposes that initial drug administration produce a hedonic effect (reward, pleasure, joy), called as A-process, and the body produces an opponent/compensatory response (B-process) to maintain homeostatic balance. This hedonic A-process is strong and fast, but the opponent B-process is and slow (“sluggish”, as Solomon stated) after a few stimulations. However, with repeated (i.e., chronic) drug stimulation, the opponent-process become faster and of a larger

magnitude, and this leads to an attenuation of the unconditioned effect of drug (A-process), which lead to the observation of tolerance. Therefore, opponent response to drug stimulation is suggested as the main reason for the development of tolerance and drug withdrawal responses. Additionally, Siegel's (1975) Pavlovian conditioning tolerance theory carried Solomon's opponent-process theory (Solomon, 1980) one step ahead and suggested that tolerance is context-dependent. Drug-associated cues lead to an environmental control of the homeostatic mechanisms. Therefore, alternation of drug-associated cue inhibits compensatory responses, and that leads to the failed drug tolerance. This theory has been supported by several drug addiction studies, and we already listed these studies in Table 2.1. It appears that the failure to observe CCRs in our results is at odds with these two behavioural theories of tolerance.

However, the habituation model of tolerance (Baker & Tiffany, 1985) suggests that tolerance can be expressed through both associative and non-associative routes and proposes that opponent responses are not essential for tolerance development. This theory is derived from Wagner's priming theory of habituation (Wagner, 1976; 1981), and tolerance – the attenuation of responding to drug – is observed if the properties of stimulus are retrieved from short term memory (STM) (see 2.2.3). According to this theory, habituation relates to how surprising and expected the drug administration is. If the presentation of a stimulus is surprising for the organism, the processing of its features in the short-term memory (STM) is effective and leads to strong unconditioned responses (URs). On the other hand, if the stimulus is expected/primed, the organism is familiar with it, and the stimulus becomes less surprising for the organism. In that case, its features are not processed in the STM, and as a result, it produces diminished, weakened URs. Tiffany et al. (1983) assessed conditioned tolerance to the analgesic effect of morphine in rats measuring the jumping response (as an index of sensitivity to electric shock). Rats

received an electric shock after saline or morphine injection in a distinctive context or home cage during drug-paired sessions. Then, they were all tested with morphine in the distinctive context to test context-specific tolerance and after administration of vehicle to measure CCRs. Tolerance results replicated previous findings of Siegel's (1975) study regarding conditioned context-specific tolerance to the analgesic effect of morphine. However, they did not observe any CCRs, which is in agreement with our findings.

The data reported in this thesis strongly suggest that repeated chronic nicotine exposure elicits tolerance to nicotine in *Schmidtea mediterranea*. Additionally, co-administration of mecamylamine, a nAChRs antagonist, partially attenuated the acute depressant effect of nicotine and blocked the expression of chronic nicotine tolerance. Although we tested pharmacological effect of mecamylamine in this study, we could further investigate the role of different subtypes of nicotine and test what nicotinic receptor subtypes planaria have.

In the introduction, we discussed two theories that explain tolerance following similar principles as those governing associative learning (Siegel, 1975; Solomon, 1980). Briefly, these models suggest that stimuli presented along with drug administration become associated with the unconditioned effects of drugs, and when presented in the absence of the drug elicit conditioned responses which are opposite to the unconditioned effect of the drug (CCRs). In addition, these theories predict that tolerance should be better observed in the presence of drug-associated cues than in their absence. In none of the experiments reported here we observed CCRs during Test 1. Similarly, we observed that tolerance to the hypo-locomotive effects of nicotine was similarly observed in the context where animals received chronic exposure and in a novel environment. The absence of differences during Test 1, given the large (dose-dependent) unconditioned effects we observed during chronic exposure, together with the insensitivity to context

changes (Tests 2 and 3) are problematic for an associative account of tolerance. Rather, these results, and in particular the after-effect observed in Experiment 17, are consistent with a habituation explanation of tolerance as that put forward by Baker and Tiffany (1985). They suggested that the bulk of data available at the moment was more consistent with a habituation explanation of tolerance, and in particular with the basic tenets of habituation suggested by Wagner (1976). According to Wagner's model, habituation (and hence tolerance) occurs due to the action of either of two mechanisms: associative priming and self-generated priming (see Wagner, 1976; and Prados et al., 2020, for a detailed explanation). Associatively generated priming enables environmental cues associated with drug effects to attenuate, in the long-term, the unconditioned effects of drugs, resembling the well-known diminution of unconditioned effects observed in basic learning procedures (Kimmel, 1966). Self-generated priming allows a representation of the drug effects to be primed in short-term memory by a previous drug exposure, and reduces the unconditioned effects of drugs. Self-generated priming explains quite well the findings of Tests 2 and 3 in all experiments, and the after-effect observed in Tests 2 and 3 in Experiment 16, where planarians in Group Nicotine showed less effect of nicotine (i.e., tolerance) following discontinuation of the drug. According to the habituation explanation of chronic exposure to nicotine during these tests, the prior presentation of the drug during the test resulted in less responding to the drug after-effects, an explanation which is also consistent with the above speculations of nicotine acting as a CS.

Based on results obtained with other drugs of abuse in rodents and humans, it has been suggested that tolerance development (in particular learned tolerance) is context-dependent in that a novel context presentation eliminates tolerance to the unconditioned effect of drugs (Siegel, 1975; Larson & Siegel, 1998). In these context-specific drug

tolerance studies; more than one contextual cue was used (i.e., distinctive environmental cue and home-cage). However, no invertebrate study systematically investigated drug tolerance by using different contextual cues because planaria studies mainly focused on the acute effect of drugs (Palladini et al., 1996; Buttarelli et al., 2000). Few invertebrate studies used chronic drug exposure protocol with planaria (Rawls et al., 2011; Pagan et al., 2009) and *C. elegans* (Feng 2006); however, these invertebrates were conditioned and tested in the same environmental condition (Rawls et al.,2011) so that the role of the contextual cues has not been systematically assessed in invertebrates. This present study assessed the acute and chronic effects of nicotine on the motility of planarians (*Schmidtea mediterranea and Dugesia sp*). We found the development of (test) tolerance after chronic nicotine exposure in *Schmidtea Mediterranea* which is independent of the contextual cues where the effects of the drug had been experienced. These results consistent with a habituation explanation of tolerance as that put forward by Baker and Tiffany (1985).

In this study, we highlighted the significance of planaria as a model system for studying long-term effect of drug and addiction. However, our results reflect the evidence of behavioural tolerance to nicotine driven by nicotinic acetylcholine receptors. However, CPP is a commonly used and important paradigm to assess the addictive and rewarding properties of drug in vertebrates (Childs and de Wit, 2013; Vastola et al., 2002) and invertebrates (Hutchinson et al., 2015; Rawls et al., 2011). A recent study assessed the rewarding properties of nicotine in planaria but after single nicotine training. It is important to note that the chronic effect of nicotine was not systematically assessed in planaria literature. Therefore, future experiments would extend our research forward within the context of addiction to nicotine using CPP paradigm with chronic exposure.

In summary, we observed pharmacological dynamics of nicotine plays an important role in the development of tolerance after repeated exposure, and

environmental factors augment the effect of chronic tolerance. In other words, there is an interaction between the pharmacological effect of nicotine and environmental factors in the development of tolerance to nicotine after chronic exposure. This result is consistent to rats and human studies in drug addiction literature. Therefore, for the development of a better therapeutic approach for the cessation of smoking, researchers should consider both pharmacological and environmental dynamics of nicotine addiction because environmental conditions may augment or reduce the pharmacological properties of nicotine.

References

Abramson, C. I., Squire, J., Sheridan, A., & Mulder Jr, P. G. (2004). The effect of insecticides considered harmless to honey bees (apis mellifera): Proboscis conditioning studies by using the insect growth regulators tebufenozide and diflubenzuron. *Environmental Entomology, 33*(2), 378-388.

Badiani, A., Caprioli, D., & De Pirro, S. (2019). Opposite environmental gating of the experienced utility ('liking') and decision utility ('wanting') of heroin versus cocaine in animals and humans: implications for computational neuroscience. *Psychopharmacology*, *236*(8), 2451–2471. https://doi.org/10.1007/s00213-019-05318-9

Baker, T. B., & Tiffany, S. T. (1985). Morphine tolerance as habituation. *Psychological review*, *92*(1), 78–108.Baxter, R., & Kimmel, H. D. (1963). Conditioning and extinction in the planarian. *The American Journal of Psychology, 76*(4), 665-669.

Berridge, K. C., & Robinson, T. E. (2016). Liking, wanting, and the incentive-sensitization theory of addiction. *The American psychologist*, *71*(8), 670–679. https://doi.org/10.1037/amp0000059

Bevins, R. A., Besheer, J., & Pickett, K. S. (2001). Nicotine-conditioned locomotor activity in rats: dopaminergic and GABAergic influences on conditioned expression. *Pharmacology, biochemistry, and behavior*, *68*(1), 135–145. https://doi.org/10.1016/s0091-3057(00)00451-2

Brubacher, J. L., Vieira, A. P., & Newmark, P. A. (2014). Preparation of the planarian Schmidtea mediterranea for high-resolution histology and transmission electron microscopy. *Nature protocols*, *9*(3), 661–673. https://doi.org/10.1038/nprot.2014.041

Buttarelli, F. R., Pellicano, C., & Pontieri, F. E. (2008). Neuropharmacology and behavior in planarians: Translations to mammals. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology, 147*(4), 399-408.

Buttarelli, F. R., Pontieri, F. E., Margotta, V., & Palladini, G. (2000). Acetylcholine/dopamine interaction in planaria. *Comparative Biochemistry and Physiology Part C: Pharmacology, Toxicology and Endocrinology, 125*(2), 225-231.

Buttarelli, F. R., Pontieri, F. E., Margotta, V., & Palladini, G. (2002). Cannabinoid-induced stimulation of motor activity in planaria through an opioid receptor-mediated mechanism. *Progress in Neuro-Psychopharmacology and Biological Psychiatry, 26*(1), 65-68.

Chen, S., Cai, D., Pearce, K., Sun, P. Y., Roberts, A. C., & Glanzman, D. L. (2014). Reinstatement of long-term memory following erasure of its behavioral and synaptic expression in Aplysia. *eLife*, *3*, e03896. https://doi.org/10.7554/eLife.03896

Childs, E., & de Wit, H. (2013). Contextual conditioning enhances the psychostimulant and incentive properties of d-amphetamine in humans. *Addiction Biology, 18*(6), 985-992.

Clarke, P., & Kumar, R. (1983). The effects of nicotine on locomotor activity in non-tolerant and tolerant rats. *British Journal of Pharmacology, 78*(2), 329-337.

Corringer, P. J., Le Novère, N., & Changeux, J. P. (2000). Nicotinic receptors at the amino acid level. Annual review of pharmacology and toxicology, 40, 431–458. https://doi.org/10.1146/annurev.pharmtox.40.1.431

Dafters, R., & Odber, J. (1989). Effects of dose, interdose interval, and drug-signal parameters on morphine analgesic tolerance: implications for current theories of tolerance. *Behavioral neuroscience*, *103*(5), 1082–1090. https://doi.org/10.1037//0735-7044.103.5.1082

Devineni, A. V., & Heberlein, U. (2009). Preferential ethanol consumption in drosophila models features of addiction. *Current Biology, 19*(24), 2126-2132.

DiFranza, J. R., & Wellman, R. J. (2005). A sensitization—homeostasis model of nicotine craving, withdrawal, and tolerance: Integrating the clinical and basic science literature. *Nicotine & Tobacco Research, 7*(1), 9-26.

Domino, E. F., & Lutz, M. P. (1973). Tolerance to the effects of daily nicotine on rat bar pressing behavior for water reinforcement. *Pharmacology Biochemistry and Behavior, 1*(4), 445-448.

Eikelboom, R., & Stewart, J. (1982). Conditioning of drug-induced physiological responses. *Psychological review*, *89*(5), 507–528.

Fagerström K. O. (1978). Measuring degree of physical dependence to tobacco smoking with reference to individualization of treatment. *Addictive behaviors*, *3*(3-4), 235–241. https://doi.org/10.1016/0306-4603(78)90024-2

Farrell, M. S., Gilmore, K., Raffa, R. B., & Walker, E. A. (2008a). Behavioral characterization of serotonergic activation in the flatworm planaria. *Behavioural Pharmacology, 19*(3), 177-182.

Feng, Z., Li, W., Ward, A., Piggott, B. J., Larkspur, E. R., Sternberg, P. W., et al. (2006). A C. elegans model of nicotine-dependent behavior: Regulation by TRP-family channels. *Cell, 127*(3), 621-633.

Finch, G. (1938). Salivary conditioning in atropinized dogs. *American Journal of Physiology-Legacy Content, 124*(1), 136-141.

Glautier, S., Clements, K., White, J. A., Taylor, C., & Stolerman, I. P. (1996). Alcohol and the reward value of cigarette smoking. *Behavioural pharmacology*, *7*(2), 144–154.

Grieve, S. J., & Littleton, J. M. (1979). Age and strain differences in the rat of development of functional tolerance to ethanol by mice. *The Journal of pharmacy and pharmacology*, *31*(10), 696–700. https://doi.org/10.1111/j.2042-7158.1979.tb13631.x

Grisel, J. E., Wiertelak, E. P., Watkins, L. R., & Maier, S. F. (1994). Route of morphine administration modulates conditioned analgesic tolerance and hyperalgesia. *Pharmacology, biochemistry, and behavior*, *49*(4), 1029–1035. https://doi.org/10.1016/0091-3057(94)90260-7

Hakan, R. L., & Ksir, C. J. (1988). Nicotine induced locomotor activity in rats: the role of Pavlovian conditioning. *Pharmacology, biochemistry, and behavior*, *29*(4), 661–665. https://doi.org/10.1016/0091-3057(88)90184-0

Hughes, J. R., Gust, S. W., Skoog, K., Keenan, R. M., & Fenwick, J. W. (1991). Symptoms of tobacco withdrawal: A replication and extension. *Archives of General Psychiatry, 48*(1), 52-59.

Hughes, J. R., & Hatsukami, D. (1986). Signs and symptoms of tobacco withdrawal. *Archives of general psychiatry*, *43*(3), 289–294. https://doi.org/10.1001/archpsyc.1986.01800030107013

Hutchinson, C. V., Prados, J., & Davidson, C. (2015). Persistent conditioned place preference to cocaine and withdrawal hypo-locomotion to mephedrone in the flatworm planaria. *Neuroscience Letters, 593*, 19-23.

Ireland, D., Bochenek, V., Chaiken, D., Rabeler, C., Onoe, S., Soni, A., & Collins, E. S. (2020). Dugesia japonica is the best suited of three planarian species for high-throughput toxicology screening. Chemosphere, 253, 126718. https://doi.org/10.1016/j.chemosphere.2020.126718

Irvine, E. E., Cheeta, S., & File, S. E. (2001). Tolerance to nicotine's effects in the elevated plus-maze and increased anxiety during withdrawal. *Pharmacology Biochemistry and Behavior, 68*(2), 319-325.

Itoh, M. T., & Igarashi, J. (2000). Circadian rhythm of serotonin levels in planarians. *Neuroreport*, *11*(3), 473–476. https://doi.org/10.1097/00001756-200002280-00009

Kalant, H. (1998). Research on tolerance: What can we learn from history? *Alcoholism: Clinical and Experimental Research, 22*(1), 67-76.

Kalant, H., LeBlanc, A. E., & Gibbins, R. J. (1971). Tolerance to, and dependence on, some non-opiate psychotropic drugs. *Pharmacological reviews*, *23*(3), 135–191.Kamin, L. J. (1969). Predictability, surprise, attention and cognition. *BA Campbell, RM Church New York: Appleton-Century-Crofts*.

Kimmel, H. D., & Garrigan, H. A. (1973). Resistance to extinction in planaria. *Journal of Experimental Psychology, 101*(2), 343.

Koob, G. F., & Le Moal, M. (2001). Drug addiction, dysregulation of reward, and allostasis. *Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology*, *24*(2), 97–129. https://doi.org/10.1016/S0893-133X(00)00195-0

Larson SJ, Siegel S. Learning and tolerance to the ataxic effect of ethanol. Pharmacol Biochem Behav. 1998 Sep;61(1):131-42. doi: 10.1016/s0091-3057(98)00072-0. PMID: 9715815.

Lê, A. D., Khanna, J. M., & Kalant, H. (1987). Role of Pavlovian conditioning in the development of tolerance and cross-tolerance to the hypothermic effect of ethanol and hydralazine. *Psychopharmacology*, *92*(2), 210–214. https://doi.org/10.1007/BF00177917

Lewis M. (2017). Addiction and the Brain: Development, Not Disease. *Neuroethics*, *10*(1), 7–18. https://doi.org/10.1007/s12152-016-9293-4

MacRae, J. R., Scoles, M. T., & Siegel, S. (1987). The contribution of Pavlovian conditioning to drug tolerance and dependence. *British journal of addiction*, *82*(4), 371–380. https://doi.org/10.1111/j.1360-0443.1987.tb01493.x

Millar, N. S., & Gotti, C. (2009). Diversity of vertebrate nicotinic acetylcholine receptors. Neuropharmacology, 56(1), 237–246. https://doi.org/10.1016/j.neuropharm.2008.07.041

McCallum, S. E., Caggiula, A. R., Epstein, L. H., Saylor, S., Ploskina, T., & Sved, A. F. (1999). Mecamylamine blocks the development of tolerance to nicotine in rats: implications for the mechanisms of tolerance. *Psychopharmacology*, *141*(3), 332–338. https://doi.org/10.1007/s002130050842

McCusker, C. G., & Brown, K. (1990). Alcohol-predictive cues enhance tolerance to and precipitate" craving" for alcohol in social drinkers. *Journal of Studies on Alcohol, 51*(6), 494-499.

Mohammed Jawad, R. A., Hutchinson, C. V., & Prados, J. (2018). Dissociation of place preference and tolerance responses to sucrose using a dopamine antagonist in the planarian. *Psychopharmacology*, *235*(3), 829–836. https://doi.org/10.1007/s00213-017-4801-8

Murray, J. E., & Bevins, R. A. (2007). The conditional stimulus effects of nicotine vary as a function of training dose. *Behavioural pharmacology*, *18*(8), 707–716. https://doi.org/10.1097/FBP.0b013e3282f14ec6

Murray, J. E., Wells, N. R., & Bevins, R. A. (2011). Nicotine competes with a visual stimulus for control of conditioned responding. *Addiction biology*, *16*(1), 152–162. https://doi.org/10.1111/j.1369-1600.2010.00228.x

Nathaniel, T. I., Panksepp, J., & Huber, R. (2010). Effects of a single and repeated morphine treatment on conditioned and unconditioned behavioral sensitization in crayfish. *Behavioural Brain Research, 207*(2), 310-320.

Nishimura, K., Kitamura, Y., Taniguchi, T., & Agata, K. (2010). Analysis of motor function modulated by cholinergic neurons in planarian dugesia japonica. *Neuroscience, 168*(1), 18-30.

Pagán, O. R., Rowlands, A. L., Fattore, A. L., Coudron, T., Urban, K. R., Bidja, A. H., & Eterović, V. A. (2009). A cembranoid from tobacco prevents the expression of nicotine-induced withdrawal behavior in planarian worms. *European journal of pharmacology*, *615*(1-3), 118–124. https://doi.org/10.1016/j.ejphar.2009.05.022

Palladini, G., Ruggeri, S., Stocchi, F., De Pandis, M. F., Venturini, G., & Margotta, V. (1996). A pharmacological study of cocaine activity in planaria. *Comparative Biochemistry and Physiology Part C: Pharmacology, Toxicology and Endocrinology, 115*(1), 41-45.

Passarelli, F., Merante, A., Pontieri, F. E., Margotta, V., Venturini, G., & Palladini, G. (1999). Opioid–dopamine interaction in planaria: A behavioral study. *Comparative Biochemistry and Physiology Part C: Pharmacology, Toxicology and Endocrinology, 124*(1), 51-55.

Pauly, J. R., Grun, E. U., & Collins, A. C. (1992). Tolerance to nicotine following chronic treatment by injections: A potential role for corticosterone. *Psychopharmacology, 108*(1-2), 33-39.

Perkins, K. A., Stiller, R. L., & Jennings, J. R. (1991). Acute tolerance to the cardiovascular effects of nicotine. *Drug and alcohol dependence*, *29*(1), 77–85. https://doi.org/10.1016/0376-8716(91)90024-s

Phelps, B. J., Miller, T. M., Arens, H., Hutchinson, T., Lang, K. A., Muckey, L. M., et al. (2019). Preliminary evidence from planarians that cotinine establishes a conditioned place preference. *Neuroscience Letters, 703*, 145-148.

Polli, J. R., Dobbins, D. L., Kobet, R. A., Farwell, M. A., Zhang, B., Lee, M. H., & Pan, X. (2015). Drug-dependent behaviors and nicotinic acetylcholine receptor expressions in Caenorhabditis elegans following chronic nicotine exposure. *Neurotoxicology*, *47*, 27–36. https://doi.org/10.1016/j.neuro.2014.12.005

Pomerleau, O. F., Fertig, J. B., Seyler, L. E., & Jaffe, J. (1983). Neuroendocrine reactivity to nicotine in smokers. *Psychopharmacology, 81*(1), 61-67.

Prados, J., Alvarez, B., Howarth, J., Stewart, K., Gibson, C. L., Hutchinson, C. V., et al. (2013). Cue competition effects in the planarian. *Animal Cognition, 16*(2), 177-186.

Prados, J., Fisher, C., Moreno-Fernández, M. M., Tazumi, T., & Urcelay, G. P. (2020). Short- and long-term habituation of photonegative and exploratory responses in the flatworm planaria (Dugesia). *Journal of experimental psychology. Animal learning and cognition*, *46*(3), 354–365. https://doi.org/10.1037/xan0000256

Raffa, R. B., & Desai, P. (2005). Description and quantification of cocaine withdrawal signs in planaria. *Brain Research, 1032*(1-2), 200-202.

Raffa, R. B., Shah, S., Tallarida, C. S., & Rawls, S. M. (2013). Amphetamine conditioned place preference in planarians. *Journal of Behavioral and Brain Science, 3*(01), 131.

Ramoz, L., Lodi, S., Bhatt, P., Reitz, A. B., Tallarida, C., Tallarida, R. J., et al. (2012a). Mephedrone ("bath salt") pharmacology: Insights from invertebrates. *Neuroscience, 208*, 79-84.

Rawls, S. M., Patil, T., Tallarida, C. S., Baron, S., Kim, M., Song, K., et al. (2011). Nicotine behavioral pharmacology: Clues from planarians. *Drug and Alcohol Dependence, 118*(2-3), 274-279.

Rawls, S. M., Patil, T., Yuvasheva, E., & Raffa, R. B. (2010). First evidence that drugs of abuse produce behavioral sensitization and cross-sensitization in planarians. *Behavioural Pharmacology, 21*(4), 301.

Remington, B., Roberts, P., & Steven, G. (1997). The effect of drink familiarity on tolerance to alcohol. *Addictive Behaviors, 22*(1), 45-53.

Rescorla, R. A., & Heth, C. D. (1975). Reinstatement of fear to an extinguished conditioned stimulus. *Journal of Experimental Psychology: Animal Behavior Processes, 1*(1), 88.

Robinson, T. E., & Berridge, K. C. (1993). The neural basis of drug craving: an incentive-sensitization theory of addiction. *Brain research. Brain research reviews*, *18*(3), 247–291. https://doi.org/10.1016/0165-0173(93)90013-p

Robinson, T. E., & Berridge, K. C. (2000). The psychology and neurobiology of addiction: An incentive–sensitization view. *Addiction, 95*(8s2), 91-117.

Rozin, P., Reff, D., Mark, M., & Schull, J. (1984). Conditioned opponent responses in human tolerance to caffeine. *Bulletin of the Psychonomic Society, 22*(2), 117-120.

Sal, F., Prados, J., & Urcelay, G. P. (2020). Nicotine chronic tolerance development and withdrawal in the planaria (Schmidtea mediterranea). *Pharmacology, biochemistry, and behavior*, *200*, 173075. Advance online publication. https://doi.org/10.1016/j.pbb.2020.173075

Sandmann, T., Vogg, M. C., Owlarn, S., Boutros, M., & Bartscherer, K. (2011). The head-regeneration transcriptome of the planarian Schmidtea mediterranea. *Genome biology*, *12*(8), R76. https://doi.org/10.1186/gb-2011-12-8-r76

Sarnat, H. B., & Netsky, M. G. (1985). The brain of the planarian as the ancestor of the human brain. *The Canadian journal of neurological sciences. Le journal canadien des sciences neurologiques*, *12*(4), 296–302. https://doi.org/10.1017/s031716710003537x

Sherman, J. E., Strub, H., & Lewis, J. W. (1984). Morphine analgesia: Enhancement by shock-associated cues. *Behavioral Neuroscience, 98*(2), 293.

Shomrat, T., & Levin, M. (2013). An automated training paradigm reveals long-term memory in planarians and its persistence through head regeneration. *Journal of Experimental Biology, 216*(20), 3799-3810.

Siegel S. (1972). Conditioning of insulin-induced glycemia. *Journal of comparative and physiological psychology*, *78*(2), 233–241. https://doi.org/10.1037/h0032180Siegel S. (1975). Evidence from rats that morphine tolerance is a learned response. *Journal of comparative and physiological psychology*, *89*(5), 498–506. https://doi.org/10.1037/h0077058

Siegel S. (1999). Drug anticipation and drug addiction. The 1998 H. David Archibald Lecture. *Addiction (Abingdon, England)*, *94*(8), 1113–1124. https://doi.org/10.1046/j.1360-0443.1999.94811132.x

Siegel, S. (1999b). Glucose enhancement of tolerance to morphine and ethanol in rats. *Psychobiology, 27*(3), 372-376.

Siegel, S. (2001). Pavlovian conditioning and drug overdose: When tolerance fails. *Addiction Research & Theory, 9*(5), 503-513.

Siegel, S. (2008). Learning and the wisdom of the body. *Learning & Behavior, 36*(3), 242-252.

Siegel, S., & Allan, L. G. (1998). Learning and homeostasis: drug addiction and the McCollough effect. *Psychological bulletin*, *124*(2), 230–239. https://doi.org/10.1037/0033-2909.124.2.230

Siegel, S., Baptista, M. A., Kim, J. A., McDonald, R. V., & Weise-Kelly, L. (2000). Pavlovian psychopharmacology: The associative basis of tolerance. *Experimental and Clinical Psychopharmacology, 8*(3), 276.

Siegel, S., & Larson, S. J. (1996). Disruption of tolerance to the ataxic effect of ethanol by an extraneous stimulus. *Pharmacology Biochemistry and Behavior, 55*(1), 125-130.

Siegel, S., & Sdao-Jarvie, K. (1986). Attenuation of ethanol tolerance by a novel stimulus. *Psychopharmacology, 88*(2), 258-261.

Solomon, R. L. (1980). The opponent-process theory of acquired motivation: The costs of pleasure and the benefits of pain. *American Psychologist, 35*(8), 691.

Solomon, R. L., & Corbit, J. D. (1973). An opponent-process theory of motivation: II. cigarette addiction. *Journal of Abnormal Psychology, 81*(2), 158.

Solomon, R. L., & Corbit, J. D. (1974). An opponent-process theory of motivation. I. Temporal dynamics of affect. *Psychological review*, *81*(2), 119–145. https://doi.org/10.1037/h0036128

Søvik, E., & Barron, A. B. (2013). Invertebrate models in addiction research. *Brain, Behavior and Evolution, 82*(3), 153-165.

Stewart, J., & Badiani, A. (1993). Tolerance and sensitization to the behavioral effects of drugs. *Behavioural Pharmacology,*

Stolerman, I. P., Fink, R., & Jarvik, M. E. (1973). Acute and chronic tolerance to nicotine measured by activity in rats. *Psychopharmacologia, 30*(4), 329-342.

Stolerman, I. P., Bunker, P., & Jarvik, M. E. (1974). Nicotine tolerance in rats; role of dose and dose interval. *Psychopharmacologia*, *34*(4), 317–324. https://doi.org/10.1007/BF00422555

Stolerman, I. P., Mirza, N. R., Hahn, B., & Shoaib, M. (2000). Nicotine in an animal model of attention. *European Journal of Pharmacology, 393*(1-3), 147-154.

Subkov, A. A., & Zilov, G. N. (1937). The role of conditioned reflex adaptation in the origin of hyperergic reactions. *Bulletin De Biologie Et De Médicine Expérimentale, 4*, 294-296.

Thompson, R., & McConnell, J. (1955). Classical conditioning in the planarian, dugesia dorotocephala. *Journal of Comparative and Physiological Psychology, 48*(1), 65.

Tiffany, S. T., Petrie, E. C., Baker, T. B., & Dahl, J. L. (1983). Conditioned morphine tolerance in the rat: Absence of a compensatory response and cross-tolerance with stress. *Behavioral Neuroscience, 97*(3), 335.

Turel, Z. B., Prados, J., & Urcelay, G. P. (2020). Heat shock disrupts expression of excitatory and extinction memories in planaria: Interaction with amount of exposure. *Behavioural processes*, *179*, 104197. https://doi.org/10.1016/j.beproc.2020.104197

Tzschentke, T. M. (1998). Measuring reward with the conditioned place preference paradigm: A comprehensive review of drug effects, recent progress and new issues. *Progress in Neurobiology, 56*(6), 613-672.

Tzschentke, T. M. (2007). Review on CPP: Measuring reward with the conditioned place preference (CPP) paradigm: Update of the last decade. *Addiction Biology, 12*(3-4), 227-462.

Umeda, S., Stagliano, G. W., Borenstein, M. R., & Raffa, R. B. (2005). A reverse-phase HPLC and fluorescence detection method for measurement of 5-hydroxytryptamine (serotonin) in planaria. *Journal of Pharmacological and Toxicological Methods, 51*(1), 73-76.

van der Kooy, D. (1987). Place conditioning: A simple and effective method for assessing the motivational properties of drugs. *Methods of assessing the reinforcing properties of abused drugs* (pp. 229-240) Springer.

Wagner, A. R. (1976). Priming in STM: An information-processing mechanism for self-generated or retrieval-generated depression in performance. *Habituation: Perspectives from Child Development, Animal Behavior, and Neurophysiology,* , 95-128.

Wagner, A. R. (1981). SOP: A model of automatic memory processing in animal behavior. *Information Processing in Animals: Memory Mechanisms, 85*, 5-47.

Walter, S., Kuschinsky, K., 1989 Jan-Feb. Conditioning of nicotine effects on motility and behaviour in rats. Naunyn Schmiedeberg's Arch. Pharmacol. 339 (1-2), 208–213. https://doi.org/10.1007/BF00165145, 2725697.

Wikler, A. (1973). Dynamics of drug dependence: Implications of a conditioning theory for research and treatment. *Archives of General Psychiatry, 28*(5), 611-616.