BEHAVIOURAL AND PHARMACOLOGICAL ASSESSMENT OF ADDICTION IN PLANARIA

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Abstract

Animal models of learning and memory can provide useful insights into how humans learn and retain new information. This is important for understanding the roles of learning and memory in addiction. Animals repeatedly exposed to rewarding substances in the presence of environmental cues learn to associate such cues with the reward. Subsequent repeated exposure to these cues in the absence of the reward leads to extinction of the previously learned behaviour. However, re-exposure to the rewarding substance leads to the reinstatement of the previously extinguished conditioned response. The experiments reported in this thesis determined whether these effects, commonly observed in rodents, are evident in invertebrates, specifically, planaria. Planaria were exposed to sucrose in one context in alternation with trials in which they were exposed to water in a different context. Test trials in which animals chose between contexts indicated the development of conditioned place preference (CPP) for the context associated with sucrose. Repeated test trials without sucrose led to extinction of CPP. Re-exposure to sucrose in a novel context after extinction, reinstated CPP. Further data showed that the development of the appetitive response (e.g., CPP) depended on the dopamine reward system. Additional experiments investigated how repeated exposure to a reward with specific environmental cues led to the development of tolerance and the establishment of a conditioned compensatory response. The development of tolerance or the conditioned compensatory response was independent of the dopamine system. Following this basic finding, the role of the cholinergic system in these learning mechanisms, specifically the encoding and reconsolidation of learned information, were assessed. Treatment with atropine (a muscarinic acetylcholine receptor antagonist) prevented memory consolidation and interfered with memory reconsolidation. These results suggest that addiction to sucrose can be characterised as a learned response in planaria, one that depends upon the principles and mechanisms that rule associative learning in rodents. Such findings may provide the basis for pre-clinical models of learning and memory with future applications in the treatment of addiction and obesity.

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Rafat

Dedication

For my parents……

For my brothers and sister……

For my husband and my lovely kids ……..

I dedicate my work

Rafat

Declaration

The work presented was carried out by myself and has not previously been submitted for another degree. Experiment 4 in Chapter 3; and Experiments 6 and 7 in Chapter 4 have been published in:

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Table of Contents

List of Tables

List of Figures

List of Abbreviations

cAMP	Cyclic Adenine Monophosphate
VTA	Ventral Tegmental area
NAc	Nucleus Accumbens
Ach	Acetylcholine
nAchrs	Nicotinic Acetylcholine Receptors
mAchrs	Muscarinic Acetylcholine Receptors
CPP	Conditioned Place Preference
LICL	Lithium chloride
CCR	Conditioned Compensatory Response
ANOVA	Analysis of Variances
SEM	Standard Error of Mean
LSD	Least Statistical Difference
nor-BMI	nor Binaltorphimine
CTA	Conditioned Taste Aversion
Suc	10% Sucrose
SCH 23390	R(+)-7-Chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1H- 3-benzazepine hydrochloride, a dopamine D1 antagonist
Suc+D-	10% Sucrose and dopamine D1 antagonist
ATR	Atropine
Suc+ ATR	10% Sucrose and Atropine

Chapter 1: General Introduction

Addiction is a complex construct, characterised by the compulsive, uncontrollable engagement with rewarding stimuli despite negative consequences. The impact of addiction on addicts, their families and the wider society can be profound, making it a significant public health issue. Addiction can take many forms including addiction to food, sexual behaviour, natural plants (e.g., Opium poppy, Cannabis, Tobacco and Coca) and synthetic drugs (e.g., Amphetamine, Cocaine, Mephedrone and Heroin). Most addictive behaviours, irrespective of their specific form, hinge upon the pursuit of reward.

Addiction is a gradual, cyclic process that develops over time. An example in the context of drug use is given in Figure 1.1. Drug use leads to intoxication, creating a rewarding effect. Over time, tolerance develops. This is characterized as a gradual decline in the response to the effect of the reward agents (drugs or food) after repeated exposure; as a result, the body will need to consume a larger dose of the drug to gain the same initial effect (McSweeney, Murphy, & Kowal, 2005). In the event of the drug not been available, the individual typically displays withdrawal signs of distress/ anxiety that exacerbate the drug seeking behaviour.

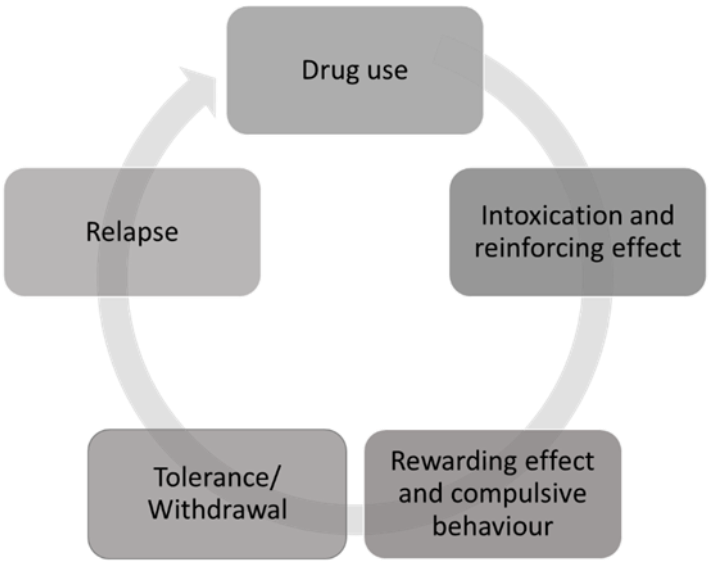


Figure 1.1. The basic cycle of addiction.

In general terms, addiction can be conceptualised as a combination of neurochemical and psychological responses to a rewarding substance or activity and the environmental cues associated with that reward. Exposure to a rewarding substance (e.g., drugs, food or drink) or engagement in a rewarding activity (e.g., winning money due to gambling) produces an impulsive behaviour such as activating the dopamine and opioid systems, increasing drugs intake and producing a feeling of pleasure; these symptoms represent the positive reinforcing side of addiction. An individual is likely to repeat this behaviour to achieve the positive feeling of reward and, as a result, different regions of the brain change to produce the addictive behaviour (e.g., within system and/or between system changes). One important example of the within system changes is the neuroadaptation of dopamine and opioid systems; they functionally become less effective (hypofunctional) to the effect of the rewarding substance. These changes transfer to other systems and result in between system alterations such as activation of the antireward system, Corticotropin-Releasing Factor (CRF), norepinephrine and dynorphin that lead to a development of compulsive behaviour and withdrawal signs (e.g., anxiety and stress); which reflect the negative reinforcement or the dark side of addiction. Therefore, addiction could develop as a shift from the positive reinforcement effects and intoxication to the negative reinforcement behaviour as a way to minimize or avoid the development of withdrawal symptoms after a period of obsession (Koob, 2013; Koob & Le Moal, 2008).

Furthermore, addiction known as a bad habit because it could happen as a result of the transition from an impulsive taking of addictive substance to a compulsive consumption habit and loss of control despite the negative consequences. The development of addiction involves different learning mechanisms/associations such as: Action-Outcome (A-O, a goal directed behaviour), Stimulus-Response learning (S-R, a habit learning); and Environmental cues—Reward associations (CS-US, Pavlovian conditioning). Initially, a learned association develops between the rewarding substance/activity and the environmental cues present at the time of reward. This association is followed by an action as a response to the presentation of the reward. Finally, with repeated exposure to the rewarding substance in specific environmental cues, this response becomes independent on the previous association and automatically continues as a habit. One way to dissociate between the A-O and R-S association is by a reinforcer devaluation

(making it less effective or valuable). For example, during A-O, rats learn that pressing the lever means the food obtained. Therefore, if these animals are fed to satiation before the training session or the food is paired with a drug that makes them feel sick, animals will stop responding to the lever. In comparison, the habitual learning (S-R association) is not affected by the reinforcer devaluation (Everitt & Robbins, 2005, 2016).

Associative learning is key to understand the process of addiction and relapse. Indeed, in this context, addiction can be conceptualised as a disorder of learning and memory in that, even after long periods of abstinence, exposure to the same addictive environment activates drug wanting, seeking and consumption (Hyman, Malenka, & Nestler, 2006). However, in many cases, addicts continue taking the drug of abuse not because they like it but because to avoid the negative consequences (e.g., withdrawal) and keep the balance. Thus, it is important to clarify the meaning of "wanting" and "liking" and understand the mechanism behind them. Wanting means that the addicts show a real desire, craving and incentive salience/motivation to get a rewarding substance. It expresses the compulsive consumption of an addictive substance to maintain the body's balance after the neurochemical and physiological changes occurred. It could develop as an example of Pavlovian learning, for example, the development of the conditioned compensatory response; although it is not pleasant, this mechanism increases the motivation to obtain he drug and counteract the effect of the conditioned compensatory responses. Different neurophysiological alterations occur such as neural sensitisation and modification of several neurotransmitter systems (e.g., dopamine, opioid and glutamate neurotransmitters (Berridge, Robinson, & Aldridge, 2009).

In contrast, liking could be defined as a response or an expression that followed the exposure to the reward; it could be the expression of pleasure after getting a desirable reward (liking) or bad expression after experiencing an aversive substance or a negative event (disliking). Liking/disliking seems to be highly associated with the hedonic or aversive impact (e.g., the development of conditioned place preference or conditioned place aversion) (Berridge et al., 2009). It seems that both wanting and liking of a rewarding agent are two closed mechanisms, however, they could be dissociated using

neurochemical manipulations, pharmacological and psychological methods. The dissociation between them offers a better understanding to the mechanism of addiction.

It is well known that addiction is a compulsive behaviour which negatively affects the addicts, their families and wider society. A large body of research using animal models such as rodents has been dedicated to understand the causes of addiction. However, the neurochemical and behavioural mechanisms behind the development of addictive behaviour is yet to be fully elucidated. This is particularly the case for understanding the relationship between the neurochemical changes in the brain and the role of environmental cues in addicts. Understanding this association is fundamental to developing robust protocols to control addictive behaviour, treat addicts and prevent serious outcomes such as relapse.

1.1. The neurochemical basis of drug addiction

The neurobiological basis of drug addiction lies in the brain's reward system, where dopaminergic neurotransmission has been consistently identified as a key candidate in the development and maintenance of addictive behaviour. Addiction develops as a result of direct alteration in the dopamine reward system (mesolimbic dopamine pathway) or via the effects of dopamine modulating the function of other neurotransmitters, for example, Gama-aminobutyric acid, opioid, acetylcholine and serotonin (Tomkins & Sellers, 2001). A brief overview of key neurotransmitters and their respective functions is given below. Further details, where relevant, are given in the experimental chapters that follow.

1.1.1. Dopamine

The dopamine system is one of the most important neurotransmitter systems in the mammalian brain. It controls a range of key human functions, including motor activity, emotion and cognitive behaviour (Wickens, Horvitz, Costa, & Killcross, 2007). Dysfunction of the dopamine system has been implicated in a number of neurodegenerative (e.g., Parkinson's and Alzheimer's) and neuropsychiatric disorders (e.g., schizophrenia; Hughes, Daniel, & Lees, 2001), as well as in addiction (Volkow & Morales, 2015). The dopamine system consists of the chemical neurotransmitter,

dopamine, and five different G protein-coupled dopamine receptor subtypes (D1, D2, D3, D4 and D5). D1 and D5 are similar and they belong to the D1-like subfamily. D1 receptors play a vital role in the production of the cyclic adenine monophosphate (cAMP) via the activation of adenylate cyclase; cAMP is involved in the phosphorylation of calcium channels. D2, D3 and D4 constitute a different group related to D2-like subfamily. Unlike D1 receptors, dopamine D2 receptors suppress the adenylate cyclase enzyme and control potassium channels (Missale, Nash, Robinson, Jaber, & Caron, 1998). Dopamine receptors are important in the context of addiction, where the dopamine D1 receptor is particularly significant because of its key role in reward and in the development of learning in various animals via their projections from the ventral tegmental area (VTA) to the nucleus accumbens (NAc) (Beninger, 2006; Beninger & Miller, 1998). The nerve terminations located in the ventral tegmental and substantia nigra are mainly responsible for the supply of the dopamine in the striatum. Blocking the dopamine receptors prevents the development of rewarding behaviour after the consumption of drugs of abuse (Berke & Hyman, 2000).

Consumption of rewarding substances such as food or drugs stimulates the nucleus accumbens to release dopamine which is essential for the development of reward associated learning. The dopamine system has an important role in different stages of addiction. It mediates the reinforcing effects of the addictive substance (Montague, Hyman, & Cohen, 2004), and contributes to the development of the association between the environmental cues and the drug effects which contribute to the maintenance of the addictive effect (Wise, 2008). It is also thought to be responsible for the relapse after abstention (Di Chiara & Bassareo, 2007). The dopamine system is also involved in food taking behaviour and the development of over feeding diseases such as obesity. Evidence from obese individuals shows a change in the density of dopamine receptors and an alteration in the dopamine level result in an elevation of food consumption that leads to obesity (Wang et al., 2001). The main pathway in addiction is that the addicted individual normally consumes the reward substance to get the pleasurable effect. The dopamine system plays an important role in encoding the learned information in this stage and associate the rewarding effects of the drug with environmental related cues. Continued exposure to the reward would result in consolidating the reward-cues association, leading to physiological adaptation, loss of control and addiction. Learning

of reward-cues association is supposed to be ruled by the same principles of Pavlovian conditioning (e.g., Siegel, 1975).

1.1.2. Acetylcholine (Ach)

Although dopamine has a central role in the development of addiction, there are other neurotransmitters involved in its development and maintenance. Acetylcholine (Ach), for example, plays an important role in the associative learning which leads to addiction to substances. Ach is synthesized by the choline acetyltransferase while it is broken down into a choline and acetic acid by the activity of acetylcholinesterase enzyme (Ribeiro, El-Shehabi, & Patocka, 2005). Several areas of the brain are responsible of Ach release include the ventral and dorsal striatum, substantia nigra, amygdala, hippocampus and prefrontal cortex. The cholinergic system involves two cholinergic receptors: nicotinic acetylcholine receptors (nAchrs) and the muscarinic acetylcholine receptors (mAchrs). The nAchrs are ligand-gated receptors while the mAchrs are G-protein-coupled receptors. There are different subtypes of each receptor which are identified both in vertebrates and in invertebrates (Gotti et al., 2009; Kruse et al., 2014; Nishimura, Kitamura, Taniguchi, & Agata, 2010).

Ach regulates different activities of the brain: attention, learning and memory, sleeping and dreaming (Hasselmo, 2006). It is an important neurotransmitter for the acquisition and retention of information during reward-related learning for food or drugs (Boccia, Blake, Acosta, & Baratti, 2003). Robinson, Platt, and Riedel (2011), for example, have shown that the blockage of either the muscarinic or the nicotinic acetylcholine receptors by an antagonist drug impairs the acquisition of conditioned learning and short term memories.

Via interactions with dopamine, acetylcholine is involved in the mechanisms of addiction for many natural and chemical rewards. This interaction occurs in different brain regions where are mostly involved in reward learning and memory processes in addiction. However, the specificity of this relationship remains unresolved. Imperato, Obinu, Dazzi, and Gessa (1994), for example, showed that dopamine levels directly affect acetylcholine levels in rats. Specifically, they found that treating rats with

dopamine D2 antagonist drugs leads to increased acetylcholine release in the treated rats; and this stimulating effect could be counteracted by the action of dopamine D1 antagonist drugs. This effect could be due to the endogenous effect of the dopamine system on the cholinergic system. De Parada, Parada, Rada, Hernandez, and Hoebel (2000) also reported evidence for the dopamine-acetylcholine interaction in the lateral hypothalamus in rats. They mainly concentrated on the effects of this interaction on animal's behaviour in terms of locomotor activity, feeding and reward behaviour. They found that injecting a muscarinic agonist drug (Carbachol) in the lateral hypothalamus enhanced locomotion as well as dopamine and its metabolites levels. In addition, they found that treating the animals with a dopamine D2 antagonist drug (Sulpiride) enhanced acetylcholine release and activated the locomotor activity; this effect was counteracted by the action of a muscarinic antagonist drug (Atropine). This effect could be dose dependent and vary in different brain regions.

Although there is evidence for a relationship between dopamine and acetylcholine in the development and maintenance of addiction, the precise relationship between them may depend upon a particular stage in the addictive cycle. For example, during initial exposure/s, dopamine appears to increase while the level of Ach is low. However, with repeated exposure, the level of dopamine decreases and Ach increases during the exposure of addicts to the addictive cues with the omission of the drug (producing withdrawal effects).

Rada, Jensen, and Hoebel (2001) have illustrated this negative relationship between dopamine and acetylcholine neurotransmitters using nicotine as an addictive substance in rats. They found that acute administration of nicotine elevated the level of both: dopamine and acetylcholine. However, administering a nicotine antagonist drug to create withdrawal symptoms after a chronic consumption of nicotine results in increasing acetylcholine level and decreased dopamine level in the extracellular fluids. This relationship was not only observed during the development of nicotine related withdrawal signs; it was also observed during sucrose binging, in depression and conditioned preference and aversion tasks (Avena & Rada, 2012; Hoebel, Avena, & Rada, 2007; Rada, Mark, Pothos, & Hoebel, 1991). For example, after ending with a meal, the level of acetylcholine increases leading to inhibit feeding behaviour through the stimulation of the cholinergic neurons in the ventral tegmental area (VTA).

However, during binge eating, the acetylcholine takes more time to reach the significant level. Research using microdialysis confirms that the level of acetylcholine increases during the development of condition taste aversion and that the treatment with a cholinergic agonist (neostigmine) stimulates the development of conditioned taste aversion in rats and reduce food taking behaviour (Mark, Weinberg, Rada, & Hoebel, 1995; Taylor, Mark, & Hoebel, 2011).

A study by Mark, Weinberg, Rada, & Hoebel (1995), illustrated the role of cholinergic system in feeding behaviour. There were two groups of rats in their experiment: experimental and control group; both groups had implanted microdialysis probes in both sides of the nucleus accumbens while they were feeding ad libitum along the night cycle. The control group was injected with a ringer solution throughout the probes while the experimental group was injected with neostigmine (a cholinergic agonist) after three hours from the feeding process. Data indicated that there was a significant decrease in food taking in the experimental group; the animals felt satiated and not motivated for obtaining food compared with the control group. However, treating the animals with ethylcholine azirdinium mustard (AF64A) (toxic to the cholinergic neurons) produced an opposite effect, doubling the food intake.

1.1.3. Other important neurotransmitters in addiction

Dopamine and acetylcholine neurotransmitters have a crucial role in the addiction pathway. However, other neurotransmitters are importantly involved in addiction. Initial administration of a rewarding substance produces its reinforcing effects through the dopamine reward system in the nucleus accumbens, by activation of the direct pathway through dopamine D1 receptors, and suppression of the indirect pathway through dopamine D2 receptors. However, repeated exposure to the addictive substances and their associated environmental cues results in chronic neurochemical changes in the brain and the dopamine reward system. One of the important changes is the modification of the glutamatergic neurons, dopamine neurotransmitters and neurons in the striatum and midbrain, which leads to the development of reward-cues associations, interference with self-control, stress and dysphoria (Volkow & Morales, 2015).

The opioid system also contributes to the development of addictive behaviour. It consists of opioid peptides (e.g., β-endorphin, enkephalins and dynorphins) and receptors such as mu, kappa, and delta. Opioid peptides and receptors distribute in different brain regions like the ventral tegmental area and the nucleus accumbens. They are actively involved in the addiction cycle and reward mechanism especially in the early stages of addiction such as binging and intoxication of opiate and non-opioid addictive substances. They have an important role in addiction for many drugs such as opioids (heroin and morphine) when the animals continue showing a place preference and addictive behaviour in case of a lesion of the dopamine system. This could highlight an important fact about the role of the opioid system in addiction for some drugs of abuse and that this system could work independently of the dopamine system (Koob & Le Moal, 2005). Opioid peptides are involved in the development of the reinforcing properties and sensitization after repeated consumption of drugs of abuse (Avena, Rada, & Hoebel, 2008; Le Merrer, Becker, Befort, & Kieffer, 2009).

Serotonin is suspected to play an important role in behavioural disorders such as anxiety, depression and also addiction. Serotonin mostly diffuses in the forebrain area of the brain. It could have an opposite effect to the dopamine so that it could be an inhibitory neurotransmitter involved in the development of inhibitory behaviour after the omission of addictive substances, or during aversive conditioning and punishment (Boureau & Dayan, 2010). However, others suggest that serotonin is involved in the reward mechanism and has a similar role as dopamine. Serotonin might help the development of addiction by enhancing the pleasant and rewarding effect of drugs of abuse (Fischer & Ullsperger, 2017).

There is clear evidence that different neurotransmitters are involved in the various stages/ aspects of the development of addiction. Dopamine is significantly involved during the first stages, modulating the positive motivational effects of reward substances. More important is the interaction and the balance between this neurotransmitter and other systems like the cholinergic system, known to play an important role in later stages. This interaction could have a significant function in shaping the addictive behaviour and, after a period of drug abstinence, during the development of withdrawal signs. Therefore, unveiling the neurochemistry of addiction, the role and the balanced interaction between different neurotransmitters, is key to

better understand the development of addiction and envisage therapeutic strategies to treat dependence disorders.

1.2. The role of environmental cues in learning and addiction

The environmental cues present at the time of reward are a key factor in shaping addictive behaviour. Exposure to the environment associated with the reward activates drug wanting, seeking and consumption (Hyman et al., 2006). This phenomenon is apparent in animal models of addiction, in which animals encode an association between the reward substances and the environmental cues presented during the consumption of reward substances. This learning is supposed to be ruled by the same principles that govern Pavlovian conditioning (Siegel, 1975) and it is stored in the brain as an implicit memory. A variety of neurotransmitters (e.g. dopamine, acetylcholine and serotonin) play an important role in the development of this learning and in the consolidation of long term memories. A number of experimental paradigms have been developed to examine the effects of learning in addiction on animal behaviour and these have primarily been employed mammalian (rodent) models.

1.2.1. Conditioned Place Preference (CPP)

Addiction develops according to different mechanisms. Once that the drugs of abuse produces the rewarding effect at the early stages of addiction that leads to increase self-administration intake of drugs of abuse. However, with the repeated exposure to the rewarding substances, addicts develop an association between the environmental cues (e.g., time and place) associated with the consumption of the drugs of abuse and the effect of the drugs. In this case, addicts prefer the environmental cues where the rewarding agents are presenting; a development of CPP. CPP is ruled by the same principles of Pavlovian conditioning controlled by the incentive motivation (Everitt & Robbins, 2005). It is used to assess the rewarding effect of a natural or a pharmacological agent. CPP can also help understanding the neurochemical mechanisms which underline some aspects of addictive behaviour (e.g., extinction and relapse) using pharmacological and genetic manipulations.

There are multiplicity of studies that show that animals (including humans) develop a clear preference for contexts associated with the rewarding effects of drugs/ foods. In experiments with rats, exposure to a particular context (environment) in the presence of drugs of abuse such as amphetamine, cocaine or nicotine results in conditioned place preference (CPP). When the animals are exposed to two different contexts, they show a preference for the one in which drugs have been consumed previously (Tzschentke, 2007a). Vastola, Douglas, Varlinskaya, and Spear (2002) studied the development of conditioned place preference in young rats treated with a low dose of nicotine (0.6 mg/kg). There were four stages in this experiment: habituation (day 1-3), pre-test (day 4), conditioning (day 5-12) and CPP test (day 13). During the habituation phase of the experiment, animals were given a chance to habituate to the subcutaneous injection procedure using saline. During pre-test, each animal received a subcutaneous injection of saline before the exposure to two contexts; the animal had a free access to the two sides of a box (CPP compartment). Time spent in each side of the CPP compartment was measured and the initial preference was calculated. Animals were equally exposed to the two sides of the CPP compartment during the conditioning. They were exposed to the less preferred context for 15 minutes after been injected with nicotine; and to the preferred context after receiving an injection of saline in the alternative day. During the CPP test, animals were placed again in the CPP compartment and they were allowed to freely move. The results of this experiment indicated that the animals showed a clear preference for the initially less preferred side of the CPP compartment paired with nicotine.

Galaj, Manuszak, Arastehmanesh, and Ranaldi (2014) studied the development of CPP in rats treated with 10 mg/kg of cocaine, assessing also the role of the dopamine D1 receptors in the development of CPP. They found that rats treated with cocaine during the conditioning trials showed a clear preference for the context in which they had been exposed to cocaine. In addition, a direct injection in the ventral tegmental area with different doses of a dopamine D1 antagonist drug (SCH23390) before the test sessions blocked the expression of cocaine CPP and this effect was dose dependent.

Pina and Cunningham (2014) examined the role of dopamine D1 and D2 receptors in the acquisition of ethanol mediated CPP using D1 and D2 antagonists (SCH-23390 and Raclopride) in mice. There were three stages in this experiment: habituation,

conditioning and test session. Animals were randomly assigned to different groups and all animals received 5 minutes of habituation before the conditioning. They used two distinctive surfaces and two different orders during the conditioning; animals were injected with the drug (ethanol or Lithium Chloride, LiCl) on one day and they were injected with saline on the alternative day before been exposed to the contexts. A CPP test took place 24 hours after the first two conditioning trials; animals then received two additional conditioning trials and were tested again after 24 hours. They also assessed the effect of different concentrations of Raclopride or SCH-23390 on the CPP acquisition. Finally, they examined the effect of SCH-23390 alone to assess whether it produces a condition place aversion. They found that SCH 23390 at high doses (0.1-0.3 mg/kg), but not Raclopride, blocked CPP acquisition produced by ethanol during the first CPP test. In addition, they found that SCH 23390 alone did not produce a conditioned place aversion.

Other studies have addressed the role of different dopamine receptors on the expression of CPP following training. For example, Dickinson, Lee, Rindal, and Cunningham (2003) studied the effect of different concentrations of dopamine D1, D2 or D3 antagonist on CPP expression induced by ethanol in mice. Using an unbiased CPP design, animals were conditioned with ethanol (2 g/kg). A first test trial took place 24 hours after the last conditioning trial; however, there were 48 hours gaps between the three remaining tests. Animals were tested with a D1 (0.015 or 0.03 mg/kg SCH-23390), D2 (0.3 mg/kg or 0.6 mg/ Kg Raclopride) or D3 (10 mg/kg or 20 mg/kg U99194A). It was found that the expression of the ethanol induced CPP was not affected by treatment with dopamine receptors antagonists. These data suggest that the dopamine reward system is important in the establishment of the association between the rewarding agent and the associated cues, but not its expression.

1.2.2. Tolerance as development of conditioned compensatory response (CCRs)

Tolerance develops after repeated exposure to the same amount of a drug in the same environmental cues; this leads to a decrease in the effects of the drug and several physiological systemic changes such as reduced susceptibility of receptors to the drug and enhanced metabolic rate of the administrated drug (Cochin, 1969). These

modifications are the physiological correlate of behavioural changes due to learning. According to the conditioning theory, tolerance occurs because the animal develops an association between the reward agent and the environmental cues, such as the context in which the reward is presented. This association becomes more obvious after a number of conditioning trials as can be expected in Pavlovian conditioning preparations (Pavlov, 1927). For example, exposing animals to a drug like adrenaline will produce tachycardia; regular exposure to the drug in the same contextual cues results in the development of tolerance to the drug (absence of tachycardia). However, injecting the animals with vehicle in the presence of the same contextual cues would result in bradycardia, a conditioned compensatory response (CCR) (Subkov & Zilov, 1937).

Evidence for the development of conditioned compensatory responses as the mechanism behind the development of tolerance to drugs has been reported in experiments with rats treated with opioids (Siegel, 1975). Siegel (1975) described an experiment assessing the mechanism of tolerance. There were three experimental groups in which animals were exposed to morphine in different environmental cues during the experimental sessions. In the first group, animals were treated with vehicle and exposed to a hot plate. These animals showed a base line latency of paw licking—the unconditioned response associated with the experience of pain in the hot plate. The second group was treated with morphine and exposed to the hot plate. Those animals showed longer latencies to paw licking, therefore demonstrating the analgesic properties of morphine. However, the latencies decreased over the three days of training suggesting the development of tolerance, hyperalgesia as a compensatory response. These two groups were then tested in exactly the same way on the fourth day and their latencies were identical, confirming the development of tolerance. The third group was given morphine in the home-cages, and was exposed to the hot plate for the first time during the test in day 4. Those animals, in spite of the fact that they had been exposed to morphine in the same way as the animals in the group exposed to the hot plate, showed high latencies to paw licking, suggesting that mere experience of morphine does not result in tolerance. This experiment suggests that the physiological processes cannot explain tolerance; it strongly supports the learning theory and suggests that the key element for the development of tolerance is an association between the context and the effects of the drug. In a context in which the animal expects the presentation of the drug, the CCR guarantees that the animal is exposed to the drug in an advantageous

position to counteract the effect of the drug (Siegel, 1984). This is an important phenomenon because it also explains why long-term addicts can die after exposure to drugs of abuse in a novel context: taking a drug in the presence of new environmental cues prevents or decreases the opportunity of the body to produce a conditioned compensatory response, and even consumption of the usual dosage of the drug the individual typically uses could be fatal (Siegel, 1976).

Siegel, Hinson, Krank, and McCully (1982) reported evidence that the environmental cues play a key role in the development of tolerance to heroin in rats. In their experiment, there were 15 sessions of exposure to gradually increased doses of heroin, from 1 mg/kg during the first session to 8 mg/kg in the last session. The drug was injected intravenously using a cannula previously fixed in the vein. Two different environmental cues were used in this experiment: the colony (home cage) and a distinctive environment (a room with a permanent white noise). During the exposure sessions, half the animals were exposed to heroin in their colony room and to dextrose in the distinctive room alternatively while the other half were exposed to heroin and dextrose in reverse order. Then, all animals were tested with a higher dosage of heroin (15 mg/kg) in one context. Animals were divided into two groups according to their previous experience with the context in which they were trained and tested with heroin: group same-tested (ST) and group different-tested (DT). Also, there was a third group of animals that received 30 injections of dextrose in the alternative contexts during the training phase and were tested with the high dose of heroin during the test (control group). The data showed that 67.6% of animals who were given tolerance training and tested with a fatal dose in the training context (group ST) survived. Animals trained but tested in a new context (group DT) had a survival rate of 35.7%. Finally, animals never trained (control group) showed a survival rate of 3.6% to the fatal dose of heroin. These data suggest that the previous experience with the environmental cues associated with the exposure to the drug of abuse; and the development of conditioned compensatory responses allowed animals tested in the same trained context to receive the potentially fatal dose of morphine in an advantageous position, reducing the effect of the drug.

The development of the conditioned compensatory response has also been studied in humans with alcohol. Remington, Roberts, and Glautier (1997) assessed the development of tolerance and the role of the environmental cues in cognitive

performance and self-reported levels of intoxication. There were two groups in their experiments which were matched in terms of alcohol consumption. The first group consumed alcohol in a familiar form (beer). The second group consumed the same amount of alcohol but in an unfamiliar form (blue peppermint mixture). They used electrocardiogram (ECG) electrodes to record the heart rate and a skin conductance and assessed hand-eye coordination and other cognitive functions. They found significant differences between the two groups in terms of motor and cognitive performance. The group that consumed the familiar form of alcohol could control the intoxicating effects of alcohol better than the group that consumed the unfamiliar form of alcohol. This evidence suggests that the CCR is naturally developed as an adaptation to protect the individuals against the effects of external factors. The cues typically associated with alcohol consumption (the beer) activated a CCR that reduced the intoxication and protected cognitive function.

1.2.3. Conditioned place aversion

Conditioned place aversion is another important type of Pavlovian conditioning. Conditioned place aversion occurs when animals show a change in their preference for a previously preferred context after pairing it with an aversive stimulus. Risinger and Oakes (1995) assessed the effect of different concentrations of nicotine (0.25–2.0 mg/kg, IP) in the development of conditioned place preference and conditioned place aversion in mice. They used an unbiased CPP design; there were four conditioning sessions (15 min each) in which animals were exposed to nicotine in one context and saline in the other context. Data of the conditioning sessions showed that acute exposure to a high dose of nicotine (2.0 mg/kg, IP) results in significant reduction in locomotion. However, repeated exposure led to the development of tolerance over the four sessions of conditioning. Data from the test showed that 0.5 mg/kg of nicotine produced a significant increase in the preference for the context associated with the drug. In contrast, the high dose of nicotine (2.0 mg/kg) had an aversive effect in that animals developed an aversion for the context. In addition, they found that other doses of nicotine did not have any effect on the animals. These data show that the effect of nicotine on the development of preference/ aversion is dose dependent.

Cunningham and Henderson (2000) examined the effect of different concentrations of ethanol in the development of conditioned place aversion in mice. They also tested the relationship between the time of drug exposure and the length of the experimental sessions. They found that 2 and 4 g/kg produced a significant aversive effect, and that exposing the animals to the ethanol before the experimental sessions significantly interfered with the establishment of condition place aversion. In addition, they illustrated that animals exposed to ethanol for short sessions (5, 15, 30 min) showed a significant shift in their preference for the initial preferred context. However, long sessions (60 or 90 min) had no aversive effect. Finally, they found that repeated exposure of animals to the context conditioned stimulus in the absence of the ethanol extinguished the aversive response.

1.2.4. Extinction and recovery effects: spontaneous recovery, renewal and reinstatement

Extinction is a gradual decline of the conditioned response (CR) that occurs after the exposure to the conditioned stimulus (CS) in the absence of reward (omitting the unconditioned stimulus, US) (Pavlov, 1927). Some learning theories claim that extinction is an example of unlearning or forgetting by which the associative link between the CS and US developed during acquisition simply disappears (e.g. Rescorla and Wagner, 1972). This seems, however, not to be the case. If following extinction of the CR, the animal is allowed a resting period, the CR spontaneously reappears when the CS is presented again, a case of spontaneous recovery (Robbins, 1990). The spontaneous recovery effect clearly shows that the association between the CS and the US developed during training was not erased or forgotten. Extinction is therefore now assumed to involve the development of a second association, inhibitory in nature, which competes with the excitatory association for the control of the behaviour of the animal (e.g., Bouton, 1994).

Extinction has been shown to be a key part of context dependent learning. In the renewal procedure (e.g., Bouton & Peck, 1989), animals trained in one context are then given extinction in a second context. Following successful extinction, testing the animals in the training context results in renewal of the CR, an ABA renewal effect

(where A and B refer to the training and extinction contexts; Bouton & King, 1983). An example of the renewal effect was reported by Bouton & Peck (1989), who assessed the renewal effect in rats using an ABA procedure. They used an appetitive conditioning task, paring a tone CS with a food US. Conditioning resulted in the development of a conditioned response (indicative of the establishment of an excitatory association between the CS and the US). During the extinction trials, animals in the renewal group were presented with the CS in context B; in the control group, the animals were trained, extinguished and tested always in the same context (AAA). In the renewal test, in the same context where the conditioning happened (A), there was a significant difference between the two groups: contrary to the control group, animals in the renewal condition displayed a strong conditioned response in the presence of the CS.

Moreover, re-exposure to the US leads to the reinstatement of the previously learned CR, a reinstatement effect (e.g., Delamater, 1997). Bouton & Peck (1989) assessed the reinstatement effects in rats. Animals given acquisition and extinction were divided into two groups, R and NO-R, according to whether they were re-exposed to the US or not. They found that animals re-exposed to the US after successful extinction reinstated the previously learned conditioned response compared to the group NO-R and the control group. These data suggest that during the reinstatement test, the excitatory association between the CS and the US was better retrieved than the inhibitory association that is supposed to develop during extinction.

The reinstatement effect can be used as a behavioural model of relapse. Reinstatement has been shown in vertebrates such as rats (Aguilar, Rodríguez-Arias, & Miñarro, 2009; Bouton and Peck, 1989) and in invertebrates such as the molluscs, *Aplysia* (Chen et al., 2014) and the garden snail (Alvarez, Morís, Luque, & Loy, 2014).

According to a learning model of addiction, addicts develop an excitatory association between environmental cues and the effects of the drug. Treatment with cue exposure techniques, based on extinction, involves exposure to these cues in the absence of the drug effects. This leads to new inhibitory learning: the environmental cues associate with the absence of the drug effects. This new learning competes with the previous one but it does not delete it, meaning that previous excitatory learning is still present

somewhere in the brain or the mind of the individual but is not retrieved. Exposure to the addictive drug, its associated cues or stress is likely to result in relapse.

1.2.5. Withdrawal

Withdrawal effects are a behavioural indicator used for the assessment of physical dependence in animals (Lu, Grimm, Hope, & Shaham, 2004). Withdrawal refers to the symptoms of stress observed when animals expect an abused drug. It has been studied in different animals such as rats (Avena et al., 2008; Grimm, Shaham, & Hope, 2002) and invertebrate animals such as planaria (Hutchinson, Prados, & Davidson, 2015; Raffa & Desai, 2005; Zhang, Tallarida, Raffa, & Rawls, 2013).

Research with rats has established that withdrawal effects are mediated by a learning mechanism through which animals develop an association between the rewarding agent and the environment. Therefore, if the expected drug is not present in the defined context (or is chemically blocked, e.g., Avena et al., 2008) animals will show withdrawal signs such as anxiety (File, Lippa, Beer, & Lippa, 2004) and behavioural depression (Porsolt, Anton, Blavet, & Jalfre, 1978). This also results in craving and active drug seeking behaviour (Lu et al., 2004; Rawls et al., 2011). Withdrawal signs are a symptom of addiction and a factor for relapse. There is good evidence to suggest that withdrawal is driven by the same learning mechanisms involved in the development of tolerance and conditioned place preference.

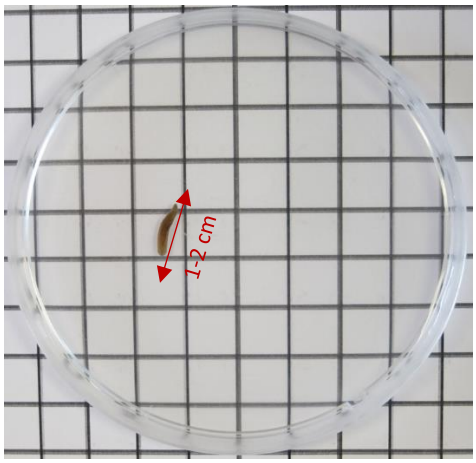
The effect of a drug in the organism leads to the display of a compensatory response (opposed to the drug's effect) which aims to restore the balance. This compensatory response can become controlled by environmental cues through a conditioning process. For example, if a drug like morphine has an analgesic effect in animals, exposure to the same environmental cues and morphine results in the development of a conditioned response of hyperalgesia as a compensatory response controlled by the environmental cues—the CS (Siegel, 1978). The conditioned compensatory response (CCR) of hyperalgesia in the absence of the expected morphine dose puts the individual out of balance. A way to restore the balance would be the consumption of morphine. This accounts for the drug seeking behaviour characteristic of addiction.

1.3. Invertebrate (planarian) models in addiction research

The majority of behavioural and pharmacological mechanisms of learning and addiction have been studied using vertebrate animals (e.g., rats and mice) as an experimental model for humans (Buttarelli, Pellicano, & Pontieri, 2008). However, there has been growing interest in using invertebrate animals in behavioural and pharmacological research. A number of studies have shown that the basic principles underlying associative learning (such as those outlined above) in rodent models can also be applied to invertebrate species including *Drosophila* (McClung & Hirsh, 1998), honey bees (Chandra, Wright, & Smith, 2010), *Aplysia* (Chen et al., 2014) and planaria (Hutchinson et al., 2015; Prados et al., 2013). In some respects, this may be unsurprising given that there are many similarities between the nervous system of most of invertebrate and vertebrate animals (Sarnat & Netsky, 2002). Invertebrates, for example, have very small, simple, centralized bilateral nervous systems and lateral nerve cords which are equivalent to the spinal cord in mammals (Denes et al., 2007). They also present neurotransmitters such as glutamate, gamma aminobutyric acid, acetylcholine and monoamines (dopamine and serotonin) that can be found in mammals (Søvik & Barron, 2013). They are, therefore, a suitable model for behavioural and psychopharmacological research especially that underlying the mechanisms of learning and memory in mammals (Kandel, 2007).

1.3.1. Planaria

Planaria have been heralded as one of the most interesting invertebrate species that could be used as a model for vertebrates in behavioural and pharmacological research (Buttarelli et al., 2008) They are free-living *Platyhelminthes*, the most distant phylum from chordate in the phylogenetic tree which still present a centralized nervous system. They have a bilateral centralised nervous system and two longitudinal lateral nervous cords similar to the spinal cord in vertebrates (Palladini et al., 1982). They are small in size (1-2 cm in long) and have a soft and flattened body covered by cilia from the ventral side (Figure 1.2).

(a) 

(b) 

Figure 1.2. (a) A planarian; (b) planaria colony in which around 100 animals are kept together.

The planarian nervous system consists of two cerebral ganglions located in the ventral part of their bodies and two lateral nerve cord equivalents to the spinal cord in the vertebrates; this structure forms a U-like shape. It also has two eyes located in the dorsal side of the head connected with the third branch cluster and the visual axon. The sixth-ninth branch clusters form the auricles which could work as a sensory organ for the taste (Agata et al., 1998) (Figure 1.3). In addition, it has motor and sensory nerves connected with muscular cells at different parts of their bodies (Sarnat & Netsky, 2002). Moreover, in comparison with other invertebrates the structure of the nervous cells in planaria is similar to the neuron structure in the nervous system of vertebrate animals: they are multipolar and have several dendrites and a single axon (Sarnat & Netsky, 1985). Planaria possess the same main neurotransmitters which are found in vertebrates, with a similar function (Table 1.1).

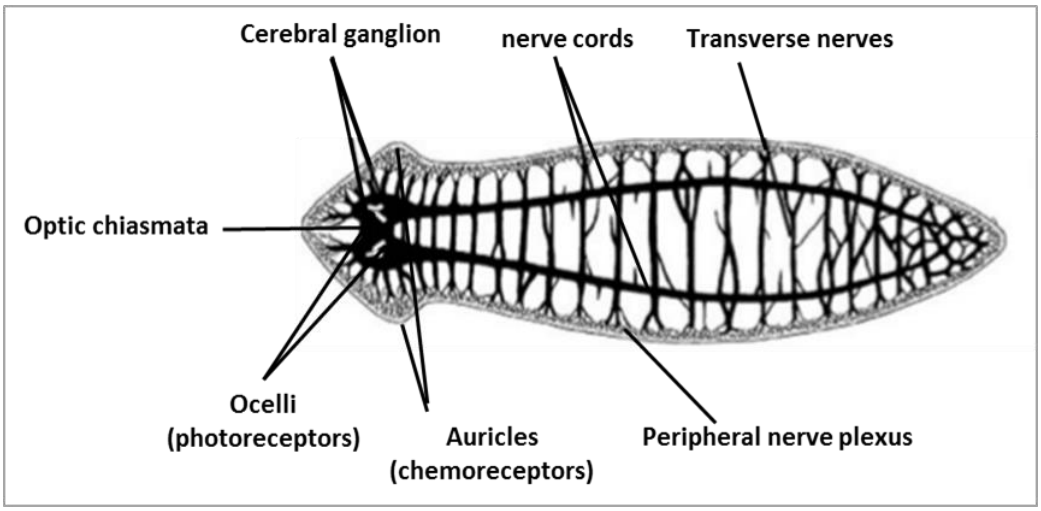


Figure 1.3. Nervous system of flatworm (planaria); shows the two cerebral ganglions, photoreceptors, chemoreceptors, nerve cords, transverse nerves and peripheral nerve plexus.

A number of studies, both behavioural and pharmacological, have provided evidence for the presence of dopamine (Palladini et al., 1996; Passarelli et al., 1999; Zhang et al., 2013), norepinephrine (Ness, Foley, Villar, & Hansen, 1996) and acetylcholine (Buttarelli, Pontieri, Margotta, & Palladini, 2002) in planaria. Dopamine and norepinephrine have been measured using High Performance Liquid Chromatography (HPLC) (Ness et al., 1996). Dopamine D1 and D2 receptors have been also illustrated by using pharmacological and biochemical procedures, such as using dopamine agonist and antagonist drugs to confirm their presence (Venturini et al., 1989). Recently, a learning study by Zhang et al. (2013) suggested that the dopamine system in flatworms could play a role in developing CPP. Animals developed CPP induced by sucrose; however, animals treated with a dopamine antagonist failed to develop CPP. Buttarelli, Pontieri, Margotta, and Palladini (2000) have shown that, like in vertebrates, the cholinergic and the dopaminergic receptors are involved in the motor activity of planaria. They demonstrated that animals exposed to physostigmine or nicotine (cholinergic agonist drugs) show hypomobility which can be reversed by atropine (a cholinergic antagonist). Also, animals treated with nomifensine or apomorphine (dopamine agonist drugs) showed hyperactivity which can be reversed by dopamine antagonist drugs such as SCH 23388 or sulpiride. Nishimura et al. (2010) demonstrated the role of the cholinergic system in the planaria using a pharmacological and RNA

interference approach. Animals exposed to physostigmine (Ach agonist) showed a muscle contraction as a result of increasing the Ach level. However, pre-treating the animals with either a muscarinic Ach receptor antagonist drug (atropine) or nicotinic Ach receptor antagonist (tubocurarine) prolong the latency time of the contraction produced by the physostigmine. In addition, they identified the gene of choline acetyltransferase (Djchat), which plays a crucial role in the synthesis of the Ach in planaria, using the immunofluorescence technique.

Neurotransmitter	Functions	References
Serotonin like compound and four G-protein coupled receptors similar to human 5-HTA and Drosophila (5-HT drol) receptors (Saitoh, Yuruzume, Watanabe, & Nakata, 1997; Umeda, Stagliano, Borenstein, & Raffa, 2005).	• Regeneration processes • Circadian rhythms • Regulation of muscle contraction and movement of the cilla	(Franquinet & Martelly, 1981) (Itoh & Igarashi, 2000) (Reuter, Maule, Halton, Gustafsson, & Shaw, 1995)
Acetylcholine and Acetylcholinesterase	• Acetylcholinesterase is the enzyme which degrades acetylcholine (Ach) • Could be involved in locomotion	(Li, 2008) (Buttarelli et al., 2002)
Gama amino butyric acid (GABA) (Eriksson & Panula, 1994).	• An essential inhibitory neurotransmitter in vertebrate and invertebrate animals Although its precise role remains unspecified in planaria, it may control movement through the neuromuscular junction.	(Akerman & Cline, 2007)
Glutamate and Aspartate	• They are present but their role in planaria is not clear	(Rawls, Gomez, Stagliano, & Raffa, 2006)

Dopamine and Norepinephrine Dopamine D1 and D2 receptors have also been detected in planaria	• Development of the reward-learning mechanism for the addictive substances • Development of CPP • Locomotion	(Ness et al., 1996) (Venturini et al., 1989; Zhang et al., 2013); (Palladini et al., 1996); (Passarelli et al., 1999); (Nishimura et al., 2007)
Opioids receptors and Cannabinoids receptors	• Play a role in developing addictive like behaviour • Involved in different stereotypical activity	(Raffa, Baron, & Tallarida, 2006); (Raffa, Stagliano, & Tallarida, 2007) and (Rawls, Gomez, & Raffa, 2007)

Table 1.1. Neurotransmitters found in planaria with their associated functions.

1.3.2. Suitability of planaria for studying addiction

In recent years, a literature has begun to emerge concerning the suitability of planaria in substance abuse research. Drugs can be easily introduced in planarian bodies by simply soaking them in a petri-dish containing a drug solution. This makes administration very simple compared with mammals which require the use of injections or mini-pumps to administer the drugs, or with other invertebrates such as drosophila or honey bees which need drug volatilization for drug administration. Planaria also have the ability to regenerate all parts of their bodies including their nervous system compared with other vertebrates and invertebrates such as drosophila (Saló, 2006; Wenemoser & Reddien, 2010), and they are a useful model in terms of genetics and molecular approaches (Oviedo, Nicolas, Adams, & Levin, 2008). For all these reasons, they may represent an alternative to vertebrate early in pre-clinical research.

1.3.2.1 Learning in Planaria

There is robust evidence that planaria can learn, remember and display a clear response to drugs commonly-abused by humans. As such, they represent a potentially useful model for studying learning mechanisms and the neuropharmacology of drugs of addiction. Planaria, for example, exhibit a number of conditioning phenomena commonly found in humans and rodents. Pavlovian conditioning has been observed in planaria using aversive and appetitive conditioning (e.g., Thompson & McConnell, 1955; Rawls et al., 2011). Other characteristics of Pavlovian conditioning have been also reported, including partial reinforcement (Kimmel & Yaremko, 1966); extinction (Baxter and Kimmel, 1963); and overshadowing and blocking (Prados et al., 2013).

An early experiment by Thompson and McConnell (1955) assessed aversive conditioning in planaria using a shock as the US. The unconditioned response to shock-US is longitudinal shrinking. They used light as a neutral CS and paired it with a shock during conditioning. The results suggested that animals developed an association between the light and shock. They measured the times of shrinking in the presence of the light-CS as an index of learning. There were four groups in their experiment: light-shock paired; light only; shock only and control group, which was not exposed to the light or the shock. They found that animals trained with light followed by shock during conditioning showed a significant increase in the number of shrinking response when they were tested with the light only compared with the other three groups. These data are taken as evidence for Pavlovian conditioning in planaria. However, in the absence of an adequate control group (exposed at random to the CS and the US, Rescorla, 1967) could lead to a misinterpretation of the results; one could be that the increase of shrinking in the presence of the CS was a case of sensitization rather than proper associative learning.

This procedure was improved by Baxter and Kimmel (1963), who counterbalanced the familiarity with the CS (light) and the US (shock) using two groups of animals. The first group received a paired CS-US conditioning and the second group of animals were exposed to the CS and the US but in unpaired form. Therefore, it was expected that the first group would associate the CS with the US, but not the unpaired group. They found

that animals in both groups showed some response when they were exposed to the light during the test. However, there was a significant increase in the number of the CRs in the paired group compared with the unpaired group. Unfortunately, in this experiment the authors did not consider the possibility that the animals in the unpaired group could develop an inhibitory association; the presentation of the CS means the absence of the US. Thus, the results of this experiment could be significant due to the reduction in the response in the unpaired group rather than the increase in the CR in the paired group. One way to solve this problem would be to compare the effect of light-shock conditioning between a paired group and a random control group. In this situation, animals should not develop a link between the presentation of the CS and the US (Rescorla, 1967). Following this suggestion, using a random control group, Levison and Gavurin (1979) reported evidence showing that previous results were due to an increase of the CR in the paired group compared to the control condition.

Jacobson, Horowitz, and Fried (1967) provided evidence that planaria could learn about the CS-US association during the conditioning rather than the development of pseudo-conditioning or sensitization in a simple discrimination task. In this experiment, they used light and vibration as CS and shock as a US. In the conditioning phase of the experiment, animals were exposed to the light followed by the shock, and a vibration but not paired with shock (the exposure to both CSs was counterbalanced). In this way, animals associated the presentation of the light, but not the vibration, with the shock over the conditioning trials. They then ran a reversed test in which the vibration was paired with the shock and the light with its absence. The animals successfully adapted their behaviour to the new reinforcing conditions.

More recently, Prados et al. (2013) provided strong evidence that planaria can learn the CS-US association during conditioning, and that the same principles that rule learning in vertebrates could control learning in this species. They reported three different experiments. The first experiment assessed the development of Pavlovian conditioning using light as a CS and shock as a US. During training, animals were exposed to the light for 10 seconds and the presentation of a shock during the final second of the presentation of the CS; there were 20 conditioning trials with 5 min intervals between each trial. The development of the CR was indicated as the percentage of how often animals shrink, suddenly turned or showed both shrinking and turning during the

presentation of the light. There were two groups in this experiment: experimental group (paired) and control group (unpaired, exposed to both light and shock but randomly). The data of this experiment indicated a significant increase in the CR in the experimental group compared with the control group. In addition, Prados et al. (2013) assessed whether cue competition could be observed in planaria. Animals in the experimental group were conditioned with a presentation of two CSs (light and vibration) at the same time. Animals in the control group were trained with a single cue, either light or vibration (counterbalanced). All groups received 20 conditioning trials. There were 10 test trials in which animals in groups light-vibration (LV-L) and light only (L-L) were tested with the light; and animals in groups vibration-light (VL-V) and vibration only (V-V) were tested with the vibration. Overall, the experimental groups showed a significant lower CR during the test than the control groups, an overshadowing effect. The researchers also showed that pre-training with one of the elements, e.g., A, before training with the compound AB, prevented learning about B, a blocking effect. These data strongly suggest that cue competition can be observed in planaria.

Avoidance behaviour has also been studied in planarian. Wisenden and Millard (2001) provided evidence that planaria could show avoidance learning after exposure to a threat stimulus. In their experiments, animals were tested with two stimuli, a fish odour and a risky stimulus, chemicals delivered from injured planaria. They found that exposing the animals to fish odour did not cause any behavioural changes compared with animals exposed to the risky stimulus that resulted in avoidance behaviour. However, presenting the two stimuli together led to the development of an association between them, and the animals showed avoidance behaviour in the presence of the fish odour during the final test.

As outlined above, the conditioned place preference protocol measures the preference for the environmental cues where a reward has been presented. Using different drugs as a rewarding agent, CPP has been reported in different vertebrate and invertebrate species (Tzschentke, 2007b), including the planaria. Rawls et al. (2011) examined the development of CPP in planaria after exposure to different concentrations of nicotine in the less preferred environment (with light) using a biased design. In the conditioning session (20 minutes), animals were exposed to water or nicotine in one context during

the first 10 minutes followed by another 10 minutes of exposure to water or nicotine in the alternative context; the order in which the animals were exposed to the contexts was, therefore, counterbalanced. Two hours after conditioning, animals were tested for the development of CPP in a 10-minute session. They found that planaria exposed to nicotine in the less preferred context (light place) showed a significant change in their preference for the light context and this effect did not depend on the sequence according to which the animals were exposed to the drug.

In another experiment, Raffa, Shah, Tallarida, and Rawls (2013) observed the development of CPP to different concentrations of amphetamine. Planaria treated with 0.1 and 1mM of amphetamine showed a significant change in the preference score for the light side of the petri-dish during the test session. Hutchinson et al. (2015) assessed the development of CPP in planaria using different concentrations of cocaine and mephedrone and two contexts, rough and smooth, as the conditioned stimuli. They found that animals develop a CPP for cocaine in two concentrations (1 and 10µM) but did not show any change in the preference score with mephedrone. Following conditioning, the animals were given several test trials in extinction. They run 4 test trials over 13 days; the CPP response was not extinguished with this particular procedure.

CPP has also been shown in planaria using natural substances such as sucrose (Zhang et al., 2013). Zhang et al. (2013) exposed the animals to different concentrations of sucrose (0.1, 1, and 10 %), glucose (0.1, 1%) and lactulose (0.1, 1%) solutions. Lactulose is a molecule very similar to sucrose but it cannot be metabolised by animals; it used as a control substance to compare the rewarding effect of the digestible molecules (sucrose and glucose) with the non-digestible form. In the experiment, light and dark sections of a petri-dish were used as the contexts to be discriminated by the animals. They found that animals trained with a 1% sucrose solution or glucose (at 0.1% and 1%) showed a significant shift in the preference score for the light side of the petri-dish. None of other groups showed a change in the preference score.

Zhang et al. (2013) also showed that the rewarding effect of sucrose is dependent upon the dopamine system in planarian. Animals exposed to sucrose and a dopamine antagonist (D1 or D2 antagonist: SCH23390 and Sulpiride) during conditioning did not

develop conditioned place preferences; also, treatment with the dopamine antagonists during the test did not prevent the expression of the CPP response.

More recently, Ouyang et al. (2017) illustrated the behavioural effect of different sweeteners using a CPP procedure and looking for the development of withdrawal signs in planaria. They used different concentrations of Splenda, which mainly consists of sucralose, maltodextrin and dextrose; and Equal, which has the same active ingredients as in Splenda except aspartame and acesulfame potassium instead of the sucralose. They found that 0.01 % of both Splenda and sucrose result in significant CPP. However, exposing the animals to Equal at the same concentration failed to change the preference score for the less preferred context. In this experiment, the researchers also tested the effect of maltodextrin and sucralose on the development of CPP. They found that 0.1% maltodextrin resulted in a significant CPP compared with sucralose. They suggested that the rewarding effects of different sweeteners depend on the active ingredients of each substance; and that the Splenda produced a significant CPP due to the effect of the maltodextrin which is similar to the rewarding effect of the dextrose. The development of a C-like movement and the absent of the CPP after the exposure to Equal, however, could be because the excitatory and the aversive effects of the aspartame that prevents the rewarding effect of other ingredients.

Taken together, these findings confirm that planaria develop CPP in the same way as vertebrate animals like rats.

1.3.2.2 Extinction in Planaria

If the development of CPP is equivalent to the development of conditioned responses in Pavlovian conditioning, repeated exposure to the CS with the absence of US after CPP should result in extinction of the conditioned place preference (Pavlov, 1927). It is well known that sometimes the extinguished conditioned responses can be reinstated after re exposure to the US (Bouton & Ricker, 1994; Pavlov, 1927). There are many studies that were conducted to clarify the mechanisms of extinction in vertebrates, for example, rats (Meil & See, 1996) and in invertebrates such as snails (Alvarez et al., 2014).

Extinction was studied in planaria in the 1960s. For example, Baxter and Kimmel (1963) reported that planaria exposed to a CS (light) afterward a US (shock) developed a CR to the CS. They also found that repeated exposure to the CS with the absence of the US extinguished the learned CR. However, if the animals were tested again after a period of time, they showed the CR again, a spontaneous recovery effect. Kimmel and Yaremko (1966) assessed the effect of a partial reinforcement on extinction in planaria. In this experiment, one group was repeatedly exposed to the US after the exposure to CS during the conditioning sessions. Animals in a second group were exposed to the CS followed by the US but just in 50% of the conditioning trials. A third group of animals was exposed to both stimuli but in an unpaired form. During the extinction test trials, animals in all groups were tested several times in the presence of CS alone. They found that animals received partial reinforcement during conditioning showed slower extinction of the CR than the group that received continuous reinforcement.

A recent study by Amaning-Kwarteng et al. (2017) reported the development of CPP, extinction and reinstatement to cocaine in planaria. They used the same method used by Hutchinson et al., (2015). During the pre-test, animals were individually tested with water in two different contexts (smooth and rough) for a 15 minutes session to determine which side was more preferred by the animals. During conditioning, animals were exposed to a 5 µM solution of cocaine in the less preferred context and to water in the preferred context in alternating days over 8 days of conditioning. CPP test took place 24 hours after the last conditioning. Animals showed a successful shift in the preference score for the less preferred context; they were—then repeatedly tested until the CPP extinguished. After extinction, animals were put again in the same concentration of cocaine, 5 µM methamphetamine or 5 µM JHW-007 (benztropine analogue) for 10 minutes followed by another 10 minutes of exposure in water in the same contexts used during the conditioning. A further test showed a recovery of the conditioned response; the authors claim that this would be an example of reinstatement. However, it is more appropriate to consider this particular result as evidence for reconditioning because the animals were exposed to the drugs in the same contexts used during conditioning (the CS and the US) whereas reinstatement requires exposure to the US alone.

1.3.2.3 Sensitization and Withdrawal in Planaria

Withdrawal symptoms are likely to be observed when a drug is omitted in a context in which the animals expect its presentation. Exposure to a drug typically induces changes in the organism which are compensated by an opposed body reaction. Repeated presentations of a drug in the presence of distinctive contextual cues result in the association of the context with the drug. Drug sensitization and withdrawal effects have been reported in planaria. Indeed, a number of key withdrawal signs have been reported in planaria. These include a reduction in locomotor activity and some abnormal movements (e.g. nodding, jerky, head swing, tail twist; and screw and scrunching movement).

Raffa, Stagliano and Umeda (2003) assessed the effect of different drugs on the locomotor velocity. The activity of each animal was measured individually; animals were put in the centre of a petri dish containing water or different drug solutions (0.1 µM saline, 0.001- 1 µM U-50,488H, 1 µM (+) U-50,488; 1-10 µM naloxone, or 20 µM nor-BNI) for 1 hour. The animals in all groups were then immediately tested in water or in drug solutions for a 5-minute session, and their activity was measured. They found that only animals exposed to U-50,488 during the exposure session and tested in water showed a significant decline in their locomotion. They also observed that treating the animals with a combination of 10 µM naloxone and 0.1 µM U-50,488H reversed the negative effect of U-50,488H. They suggested that the reduction of locomotor activity seen by animals treated with U-50,488H and tested directly in drug free water is a sign of withdrawal, and that effect could be reversed by the opioid antagonist drug (naloxone).

Raffa and Desai (2005) showed that different concentrations of cocaine produce withdrawal symptoms in planarian. They exposed the animals to cocaine (8×10^{-5} M) or water overnight, and tested the animals immediately in a cocaine solution or in drug free water over 1 hour. In this experiment, they assessed the development of abnormal stereotypical activity: nodding, jerky, head swing, tail twist, and screw movement as signs of withdrawal. They found that animals transferred from a cocaine solution to cocaine free water showed a significant number of the stereotypical behaviours

compared with animals in a control group (water-water) and animals tested with cocaine (cocaine-cocaine). Stereotypical behaviour is seen just for the first 5 minutes period of the test session, and significantly decreased by the end of the 1-hour session.

Rawls et al. (2011) reported the effect of nicotine in the development of different signs of addictive behaviour in planaria such as sensitization, tolerance, withdrawal and CPP. They used the same methods described above, and assessed the locomotor activity and the C-like movement as indexes of withdrawal. They found that the acute exposure to different concentration of nicotine (0, 0.01, 0.03, 0.1, 0.3, 1, 3, 10 mM) for 5 minutes significantly affected both the locomotor activity and the C-like movement. This effect was dose dependent, nicotine concentrations below 1mM significantly increased the locomotion but they did not produce a stereotypical movement. However, nicotine concentrations from (1-5 mM) resulted in a significant reduction in animal's activity and raised the development of C-like movement. In addition, they established that animals treated with 0.3 mM nicotine for 60 minutes and then tested for 5 minutes in the absence of the drug (plain water) showed a clear decline in their activity compared with animals in other groups (water-water, nicotine-nicotine, and water-nicotine). Also, they examined whether animals treated with 0.1, 0.3,1, and 3 mM solutions of nicotine show evidence of sensitization or tolerance after repeated exposures. In this experiment, animals were exposed to nicotine for a 5-minute session followed by a second session of drug exposure after 2 hours. Animals were then given another 5 minutes session of exposure to the drug after 4 days of drug free period. They showed that animals treated with a low nicotine concentration developed drug sensitization, while, high concentrations of nicotine resulted in tolerance.

Zhang et al. (2013) assessed the withdrawal /distress effect typically observed after discontinuation of access to a drug. Planaria were exposed to a sucrose solution for 60 minutes. Immediately after that, the animals were put in plain water and their behaviour was monitored for 5 minutes. Reduced motility was observed in the animals exposed to sucrose by comparison to a control group exposed to water. The reduced motility was interpreted as an instance of a withdrawal effect. The authors were also interested in other signs of distress, like the "head bops" described by Raffa and Dessai (2005) in animals previously exposed to cocaine. The results did not show evidence of distress signals like "head bops" after exposure to sucrose.

Although these studies have provided important findings, a key limitation is that animals were always tested when they were still under the effects of the drug (transferred immediately from drug solution to drug free water). Therefore, the observed behaviour could simply be a post effect of exposure to the drug rather than evidence for a withdrawal effect.

1.4. Summary

As outlined above, an emerging literature suggests that planaria represent a promising invertebrate model of the behavioural and pharmacological effects of substance abuse. Planaria exhibit complex learning in standard Pavlovian and instrumental conditioning tasks and display behavioural responses to drugs of abuse that are similar to those seen in mammals, including cocaine behavioural sensitization and CPP, changes in locomotor activity, withdrawal, development of tolerance and conditioned compensatory responses after repeated exposure to drugs of abuse or other rewards such as sucrose.

The purpose of the experiments that follow in this thesis was to assess the development of addictive behaviour and the neuropharmacological changes associated with drug of abuse in planaria. In doing so, a core objective was to further characterise conditioned place preference (CPP) and the development of tolerance as examples of Pavlovian conditioned responses (CR). One key feature of Pavlovian CRs is that they are subject to extinction. As such, the acquisition and extinction of CPP responses were assessed, as was the development of conditioned compensatory responses during the course of tolerance training. The role of different neurotransmitters (e.g. dopamine and acetylcholine) in the development of CPP and tolerance were also assessed. These important research questions are addressed across 3 experimental chapters (Thesis Chapters 3-5), as follows:

Chapter 3: The experiments outlined in Chapter 3 address whether the exposure to sucrose affects the behaviour of planaria. This includes food seeking behaviour and changes in locomotion after exposure to sucrose. In addition, the experiments in Chapter 3 assess the effects of repeated exposure to sucrose in a particular context on

the establishment of CPP using sucrose as a reward substance and the extinction of CPP, the post-effect of sucrose after one single exposure, and the development of tolerance as a conditioned compensatory response after repeated exposure to sucrose in a particular context.

Chapter 4: The experiments reported in Chapter 4 assess the role of the dopaminergic system in the development of CPP and its extinction, and whether the animals show a recovery of the previously extinguished CPP response by exposing them to the rewarding agent, a reinstatement effect. This chapter also reports the development of conditioned compensatory responses during the course of tolerance training. Finally, it provides evidence to dissociate CPP and tolerance by blocking the D1 dopamine receptors.

Chapter 5: The experiments reported in Chapter 5 assess the role of the cholinergic system in the development of CPP and its extinction. This chapter also assesses the role of acetylcholine in the development of conditioned compensatory responses during the course of tolerance training.

Chapter 2: General Methods

The following general methods apply to all experiments that follow in this thesis. Any changes therein are outlined in the relevant experimental chapters. The specific drugs/substances and their concentrations used for experimental groups are given in the relevant experimental chapters that follow.

2.1. Animals

2.1.1. Species

The experimental animals used in this thesis were brown planaria (*Dugesia*), sourced from Blades Biological Ltd, Cowden, Edenbridge, Kent, UK.

2.1.2. Animal husbandry

Planaria were housed in plastic containers filled with 2 litres of tap water treated with 1ml/L Aquasafe (Tetra, Germany). The colony was kept at a room temperature of 20±2 ^{o}C, with a light:dark cycle of 14:10 hours (08.00-22.00 light: 22.00-08.00 dark). Animals were fed raw chicken every 1-2 days for 1-2 hours, after which their water was changed. Given that we used a food as the rewarding agent in our experiments, the animals were food deprived for 2 days prior to the start of experiments in order to keep sucrose as an effective reward. This practice is common in rodent appetitive procedure using food as reward; it significantly increases the response of the animals to the experimental conditions and minimizes the experimental variation (Heiderstadt, McLaughlin, Wrighe, Walker, & Gomez-Sanchez, 2000).

2.1.3. 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.

2.2. Materials

During the experimental sessions, the planaria were exposed to either water or a sucrose solution or a drug solution in a 9 cm in diameter petri-dish; the drug could be a selective D1 dopamine receptor antagonist, SCH 23390, a non-selective muscarinic cholinergic receptors antagonist, atropine, or a combination of sucrose with the SCH 23390 or atropine. We used plastic petri-dishes which could provide two different surfaces, smooth (the plastic itself) or rough (in this case the petri dishes were covered with sand glued to the plastic using silicon). The dish could be filled with 5-7 ml of treated water, a sucrose solution or different drug solutions. The activity of the planaria (distance covered) and time spent in each side of the petri dishes were recorded using a Video-Track System (Viewpoint, France).

2.3. Experimental procedures

2.3.1. Measures of activity

The principal measures of planarian activity recorded in the experiments outlined in this thesis were locomotion (distance covered in a given time period) and the time spent in a particular context (e.g. different environmental surfaces, such as rough or smooth—further details of which are given below). During the experimental sessions, the animals' activity was recorded by using the Video-Track System.

2.3.2. Conditioned place preference (CPP)

Conditioned place preference **(**CPP) experiments were made up of three phases: pre-training test (day 1 of experiment), training (days 2-9) and post-training test (day 10; see Figure 2.1). During experimental sessions planaria were kept individually in a 9 cm diameter petri-dish. Animals were typically assigned to 1 of 2 groups, a drug group and a control group, matched by the time spent in the different areas during the pre-training tests.

The pre-training test took place on the first day of the experiment. All the animals were put in the centre of a petri-dish with two different contexts, rough (the sandy surface)

and smooth (the plastic surface) filled with 5-7 ml of treated water. The planaria were allowed to freely move around for the duration of the session, 30 minutes. The time spent by the planaria in each context was registered, and a preference score was calculated for the less preferred context (time spent in the less preferred context/total time). To establish whether animals showed conditioned place preference, indicated by a shift of preferences in relation to the pre-training test, we subtracted the preference score observed for the less preferred context in the pre-training test from the preference score observed during the post-training test. A difference score of (0) would indicate no change in preferences; any positive value would indicate a positive change in the preferences (a CPP response).

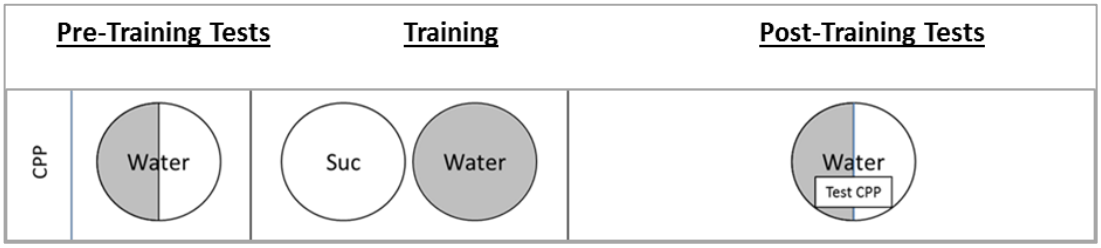


Figure 2.1. Schematic representation of the procedure of the conditioned place preference (CPP) experiments. The white and shadowed areas represent the two surfaces (contexts) used in the experiments (smooth and rough). Sucrose is given as an example drug/substance in the trained context (in CPP experiments, the less preferred context during the pre-training test).

The training stage of the experiments took place typically over 8 days (experimental days 2-9). During the training phase, planaria were exposed to the two surfaces (rough and smooth) in alternation every 24 hours. In the drug groups, planaria were exposed to the less preferred context in the presence of the drug solution; and to the preferred context in the presence of treated water. For example, in days 2, 4, 6 and 8 animals were exposed to the less-preferred context with the drug; in days 3, 5, 7, and 9 they were exposed to the preferred context with treated water. The control group underwent an identical procedure except that planaria were always exposed to both contexts in the presence of treated water. The experimental sessions always lasted 30 minutes.

During the post-training phase of the experiment, the planaria were again exposed to the two sided petri-dishes as during the pre-training test. For the Post-Training test, a

change in preference score was calculated by subtracting the preference score observed during the pre-training test from the preference score observed during the post-training test. Therefore, positive values would indicate the development of a conditioned preference for the context rewarded with the drug during the training. In the experiments reported in this thesis, we used a biased CPP procedure; the development of the CPP was compared between an experimental group and a control group. The results indicated a development of a significant CPP in the experimental group compared to the control group; this is strong evidence against the regression to the mean. The risk of using unbiased CPP is that animals could have a natural preference for the rewarded context. In this case there could be a *silence effect* and no change in preferences or CPP. Also, there is a chance of regression to the mean in the unbiased design.

2.3.3. Conditioned place aversion

The procedure was similar to the procedure described for CPP. As in the CPP experiments, there were three phases: pre-training test (day 1 of experiment), training (days 2-9) and post-training tests (day 10). In the pre-training phase, all the animals were placed in the centre of a petri-dish with two different contexts, rough and smooth and allowed to freely move around for the duration of the session, 30 minutes. The time spent by the planaria in each context was registered. As in the CPP experiments, a preference score was calculated for each animal. However, unlike the CPP experiments for which a score was calculated based on the non-preferred context, for the conditioned place aversion experiments it was calculated for the preferred surface (time spent in the preferred surface / total time). During the training phase (days 2-9) animals were exposed to the two surfaces (rough and smooth) on alternate days. However, planaria in the drug groups were exposed to the preferred context in the presence of the drug solution; and to the less-preferred context in the presence of treated water. Animals in group control, were exposed to water both in the less preferred and preferred contexts. In the post-training test (day 10), the planaria were again exposed to the two sided petri-dishes as during the pre-training test. For the Post-Training test, a change in preference score was calculated by subtracting the preference score observed during the pre-

training test from the preference score observed during the post-training test. Therefore, negative values would indicate the development of a conditioned aversion.

2.3.4. Extinction and reinstatement of CPP

For the assessment of extinction and reinstatement, the same 3-phase experimental protocol used for the CPP experiment was used. However, the experiment was extended after the post-training CPP test. Animals were given four daily post-training test trials (days 10-13 of the experiment) to monitor the extinction of any CPP response observed (CPP Extinction Tests). During the CPP Extinction test trials, the animals were exposed to the conditioned stimuli (contexts) in the absence of the unconditioned stimulus (the reward). In this way, the conditioned place preference that might have developed during training was expected to extinguish. Extinction was assumed to have taken place when the response to the conditioned stimulus declined to levels comparable to the baseline score (the one observed before conditioning). Once the extinction of the CPP was completed, all the animals were re-exposed for 30 minutes to the sucrose solution in a new surface (5 cm diameter glass petri dishes) one hour after the last extinction trial (day 13). The following day, the animals were tested again in the two-sided petri dish to assess the reinstatement of the CPP response (CPP Reinstatement Test).

2.3.5. Tolerance training

There were two phases in the experiments: the training and test phases. The training phase lasted eight days (days 1-8), during which planaria were exposed to two surfaces (rough and smooth) on alternate days. Half the animals were exposed to a drug in the smooth context for 30 minutes in the odd days (days 1, 3, 5 & 7) (trained context) and water in the rough context in the even days (days 2, 4, 6 & 8) (control context). For the other half of the animals this arrangement was reversed. The order in which the two surfaces were presented during each test was counterbalanced across animals. In our experiments, sucrose produced hypoactivity during the first training trials. We could expect the context to produce a conditioned compensatory response of hyperactivity.

Test trials took place on days 9, 10, 13 and 14 of the experiment (see Figure 2.2). In days 11-12 animals were given a retraining cycle following the procedure described for training. Animals were tested in the Trained and Control contexts under two conditions: in the presence of water (Test Water, for example on days 9 and 10 of the experiment) and in the presence of sucrose (Test Sucrose, Days 13 and 14). On day 9, half the animals were tested in the trained context used for exposure to the drug solution; the other half of the animals was tested in the alternative control context; on day 10, animals were again tested this time in the alternative context with water. The test with water assessed the development of hyperactivity conditioned compensatory responses in the trained context. The test with the drug (Test Sucrose) assessed the effectiveness of sucrose in reducing the activity of the animals in the trained and the control contexts. According to the conditioning theory of tolerance, it is believed that the effect of the drug is followed by a compensatory response of the organism to restore the balance. This compensatory response is opposite to the unconditioned effect of the drug and becomes a conditioned response controlled by the environmental cues.

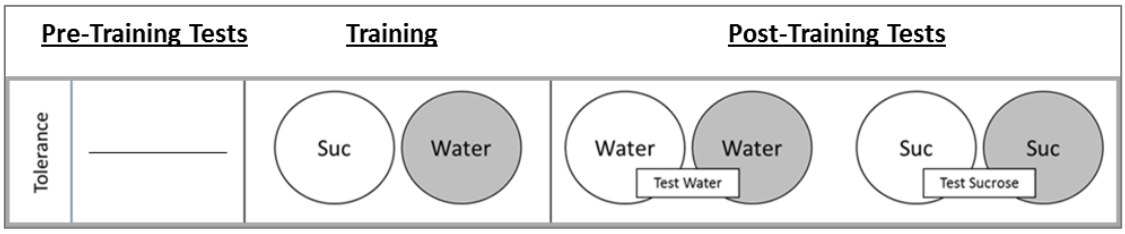


Figure 2.2. Schematic representation of the procedure of the tolerance experiment: tolerance as the development of a conditioned compensatory response. The white and shadowed areas represent the two surfaces used in the experiments (smooth and rough textures). Sucrose is given as an example drug/substance in the trained context.

Chapter 3: Assessment of the effects of sucrose on addictive-like behaviour in planaria

3.1. Introduction

3.1.1. The neuropharmacology of addiction: is sucrose an addictive substance?

Sucrose is an example of a natural substance that can cause addiction-like behaviour. Abuse of sucrose results in a number of different behavioural and neurochemical symptoms of drug addiction in rats. The abuse of sucrose and the development of sucrose addiction could play a relevant role in eating disorders like obesity and binge eating, which are important public health problems. Smith and Robbins (2013) stated that overeating could be determined as a class of addiction similar to the addiction to drugs of abuse; they both share the same physiological, neurochemical and psychological alterations. In particular, binge eating represents an example of addiction to food (e.g., sucrose) in terms of uncontrolled overconsumption of a large amount of food in a limited amount of time. In addition, individuals with overeating fail to control the amount of food that they eat despite the negative health and social consequences.

In terms of the neurochemical changes, sucrose results in neurochemical changes similar to these observed in the brain as a consequence of cocaine or opioids abuse such as alteration of different regions and receptors in the brain as well as the amount of different neurotransmitters such as dopamine and opioids (Avena et al., 2008). The dopamine and opioid systems play an important role in addiction, and their levels in the brain, mainly in the mesolimbic region and the nucleus accumbens, can be changed as a results of substance abuse (Avena et al., 2008). Several pharmacological studies indicate that dopamine and dopamine receptors (D1 and D2) have a significant effect on behaviour (Beninger, 2006) and increases the rewarding effect of drugs or natural reward substances (Kelley & Delfs, 1991). Rats treated with cocaine or morphine show an increase in D1 receptors and reduction in D2 receptors in the nucleus accumbens (Georges, Stinus, Bloch, & Moine, 1999; Unterwald, 2001). Similar alterations have been shown in animals that abused sugar. In experiments with rats, there seems to be a correlation between dopamine and opioids: daily consumption of glucose and chow

elevate the ability of the dopamine neurotransmitter to bind with D1 receptors in the nucleus accumbens. It also increases the affinity between opioid neurotransmitter and Mu-opioid receptors in different areas of the brain including the nucleus accumbens. However, it reduces the binding ability of the dopamine with D2 receptors in the striatum region (Colantuoni et al., 2001). Similarly, Avena et al. (2008) showed that consumption of large amounts of sucrose produces dependent-like symptoms and enhances the release of extracellular dopamine and opioids in the brain. Likewise, the levels of opioid and opioid receptors increase during the abuse of drugs, and this increase is positively related with the increase of dopamine and dopamine receptors (Unterwald, 2001).

Furthermore, overeating behaviour that develops in binge eaters is controlled by the associated cues; it has been found that the cues previously presented with food activate binge eating behaviour. Ågmo, Galvan, and Talamantes (1995) studied the changes in the dopamine and opioid systems and the interaction between them during the development of drug related behaviour in rats. In their experiment, they used sucrose as a rewarding agent and a biased CPP method to assess the development of CPP to sucrose. They also assessed the effect of the treatment with a dopamine D1/D2 antagonist (cis (z)-flupentixol HCl) and an opioid antagonist (naloxone) on the developed CPP response. The data of this experiment showed that rats developed a CPP response mediated by sucrose; but treatment with a dopamine D2 antagonist or an opioid antagonist prevented the development of this sucrose mediated CPP. This shows that the dopamine reward system (and the closely related opioid system) is involved in the CPP learning mechanism.

The cholinergic system is also actively involved in the modulation of behaviour that results from food abuse, and its action is related to the dopamine and opioid systems (Kelley, Baldo, & Pratt, 2005). Some studies have suggested a negative correlation between acetylcholine and dopamine during drug abuse, evidence showing that the level of acetylcholine increases as the level of dopamine is reduced in morphine withdrawal (Rada et al., 1991). Similar results have been observed in rats that had access to sucrose: giving a mixed muscarinic agonist injection inhibited the feeding mechanism while an injection of a muscarinic antagonist drug enhanced the feeding mechanism (Mark, Shabani, Dobbs, & Hansen, 2011).

These three neurotransmitters play a potential role in the regulation of the appetite and the progress of eating disorders such as binge eating and obesity. Sugar is a substance which produces dependent like behaviour such as withdrawal, craving, CPP and tolerance. Consumption of sucrose also alters the neurochemical balance in a way similar to drugs of abuse (like cocaine). The affinity of opioid receptors increased during sugar consumption can result in increased consumption of sugar and might contribute to the development of obesity (Fullerton, Getto, Swift, & Carlson, 1985).

In contrast, other studies argued against the idea that food can be an addictive substance and they showed some limitations using human studies. These studies indicated that animals under intermittent sugar consumption regime or animals that consumed a highly sweet tasted food develop an addictive-like behaviour to the sugar. However, they pointed that there is a lack of evidence from human studies (Westwater, Fletcher, and Ziauddeen, 2016; Ziauddeen, Farooqi, & Fletcher, 2012). It is known that obesity and binge eating represent public health problems, however, there is still no clear understanding about the neurochemical and behavioural changes that occur and whether food (e.g., high sugar or salt diets) can be classified as an addictive substance. Therefore, further studies need to be conducted to differentiate between the neurochemical and behavioural mechanisms that underline obesity, food addiction and binge eating; and establish whether food can be an example of the substances that elicit addictive behaviour.

The experiments reported below systematically assessed the effect of exposure to sucrose on planarian behaviour.

3.1.2. The effect of sucrose on behaviour in planarian

Zhang et al. (2013) assessed the effect of different concentrations of sucrose (0.1, 1 and 10%), glucose (0, 0.1, and 1%), and lactulose (0, 0.1, and 1%) on the behaviour of planarian. They focused on the effect of sucrose on the locomotor activity, stereotypical movements and the development of CPP. In their experiment to determine the effect of sucrose on locomotion, planaria were exposed to a 1% sucrose solution for 60 minutes. The animals were then immediately put in plain water and their behaviour monitored for

5 minutes. It was found that the motility of the planaria in this group was significantly reduced compared to other groups (Water-Water, Suc-Suc, and Water-Suc). However, 1% sucrose did not produce a stereotypical activity (e.g. head bops).

In the CPP experiment, light and dark sections of a petri dish were used as the contexts to be discriminated by the animals. They used a biased CPP design with three experimental phases: a 5-minute session as a pre-test, 30 min conditioning session with different concentrations of sucrose, glucose or lactulose in the less preferred context (light side); and 5min post-test session. They found that planaria exposed to a low concentration of sucrose (1%) or glucose (0.1, and 1%) during conditioning developed a significant CPP in comparison with animals in control groups.

Finally, they assessed the role of the dopamine system in the establishment of CPP and the rewarding effect of sucrose. In this experiment they tested the effect of dopamine D1antagonist, SCH 23390 (1 μM), or dopamine D2 antagonist, sulpiride (1 μM). Animals could be either exposed to these drugs during the conditioning or during the test session of the experiment. They found that animals treated with a 1 μM of SCH 23390 or sulpiride during the conditioning but not in the test failed to show a significant change in their preference for the light side.

This suggests that the dopamine system mediates the development of CPP in planaria. However, their study is not fully convincing for several reasons:

1- They did not explain the mechanism behind the development of the CPP and withdrawal effects, and the role of the environmental cues in withdrawal.
2- They assessed the development of CPP after a single exposure to the sucrose (60 minutes long); however, this might not be enough to develop CPP, or long term CPP.
3- Animals were not equally familiar with the test contexts because they spent more time during the training in the context which was paired with sucrose than in the other context. Mere habituation could be contributing to the CPP effect reported.
4- Evidence of withdrawal symptoms might be simply post- effects of sucrose because animals were tested immediately after the exposure phase.
5- Animals were always tested under the effect of sucrose. This means that there was no long-term assessment (at least 24 hours) of the development of CPP or withdrawal

effects. It would be useful to allow enough time before conducting the test to eliminate the sucrose from the body.

The five experiments that follow in this chapter addressed these points by assessing: 1) the effect of different concentrations of sucrose on locomotor activity in planaria; 2) the effect of sucrose on food seeking behaviour, comparing animals exposed to sucrose with animals not exposed to sucrose as a group control; 3) whether acute exposure to sucrose (30 minutes exposure) produces significant withdrawal effects (as suggested by Zhang et al., 2013); 4) the effects of repeated exposures to sucrose in a particular context: animals were trained with context A in the presence of sucrose, and with an alternative context B in the absence of sucrose (our main interest was to establish whether the effects of sucrose habituate and whether these changes were context dependent); and 5) whether long-term CPP develops using sucrose as a reward substance in planaria.

3.2. Experiment 1: Sucrose reduced the motor activity of planaria

The purpose of Experiment 1 was to compare the effect of different concentrations of sucrose solution on the locomotor activity of the animals and identify which concentrations are more effective in changing the activity of the planaria.

3.2.1. Method

3.2.1.1. Subjects and Materials

Thirty-two brown planaria (*Dugesia*) purchased from Blades Biological Ltd. (Kent, UK) served as the subjects in the present study. The flatworms were held in a plastic container filled with two litres of water treated with 1 ml/l Aquasafe (Aquasafe, Tetra, Germany). The planaria colony was kept at a room temperature of 20^0C (±2) with a light cycle of 14/10 hours. The animals were fed raw chicken meat every two days for

1-2 hours; the water of the aquarium was changed after feeding the animals. They were deprived of food, however, from two days before the start of the experiments.

During the experimental session, the animals were exposed to either water or a sucrose solution in a 9 cm in diameter petri-dish. We used plastic petri-dishes which could provide two different surfaces, smooth (the plastic itself) or rough (in this case the petri dishes were covered with sand glued to the plastic using silicon. The dish could be filled with 5-7 ml of treated water or a sucrose solution. We used three different concentrations of sucrose: 5%, 10% and 15% w/v. The activity of the planaria (distance covered) was recorded using the Video-Track System.

3.2.1.2. Procedure

The experiment consisted of one single session during which planaria were exposed to one of the surfaces (rough and smooth, counterbalanced), for 30 min. The animals were assigned, at random, to four experimental groups. The control group was exposed to treated water; each of the remaining three groups were exposed to sucrose at different concentrations: 5%, 10% and 15%. The planaria were allowed to freely move around for the duration of the session, and the distance covered by the animals was recorded over periods of 5 min.

3.2.2. Results and discussion

Figure 3.1 displays the distance covered by the different groups of animals at 5 min intervals when exposed to different concentrations of sucrose. Compared to the control group, animals exposed to sucrose showed slowed motor activity during the experimental session. Interestingly, animals exposed to the higher concentration of sucrose (15%) showed a marked reduction of motor activity from the onset of the experiment. Animals exposed to lower concentrations did not differ from the control group during the first 5 min; however, their level of activity were gradually reduced and by the end of the 30 min session all the groups exposed to sucrose showed similar levels of activity. An ANOVA with Group (5%, 10%, 15% and control) and Intervals (5, 10, 15, 20, 25 and 30 minutes) showed a significant effect of group, $F(3,28)=6.45$, $p=0.002$,

η_p^2=.40, and Intervals, F(5,140)=14.65, p<0.01, η_p^2=.34. The interaction between Groups x Intervals was also significant, F(15,140)=1.84, p=0.03, η_p^2=.16.

Further analysis using Univariate ANOVA and Post Hoc using Least Significant Differences (LSD) were conducted to identify group differences during the six 5-minutes intervals. Data showed a significant main effect of groups during the first 5 minutes interval, F(3,28)=3.29, p=0.03, η_p^2=.26, and that the group 15% sucrose showed significantly lower levels of activity compared to the other groups, ps<0.05. In addition, animals in this group (15% sucrose) maintained their low levels of activity compared to the control group in the remaining intervals. Data from the second interval (10 minutes of exposure) also showed a significant effect of groups, F(3,28)=4.00, p=0.01, η_p^2=.30, and that animals in group 10% sucrose showed a significant reduction in activity compared with the control group, p=0.02. During the third and fourth intervals (15 and 20 minutes of exposure) there was no significant main effect of group. In the last two intervals (25 and 30 minutes of exposure) there was a significant effect of group, minimum F(3,28)=3.61, p=0.02, η_p^2=.27. Also, animals in the sucrose groups showed a significant reduction in their activity compared to the group control (ps<0.05) in these two last intervals.

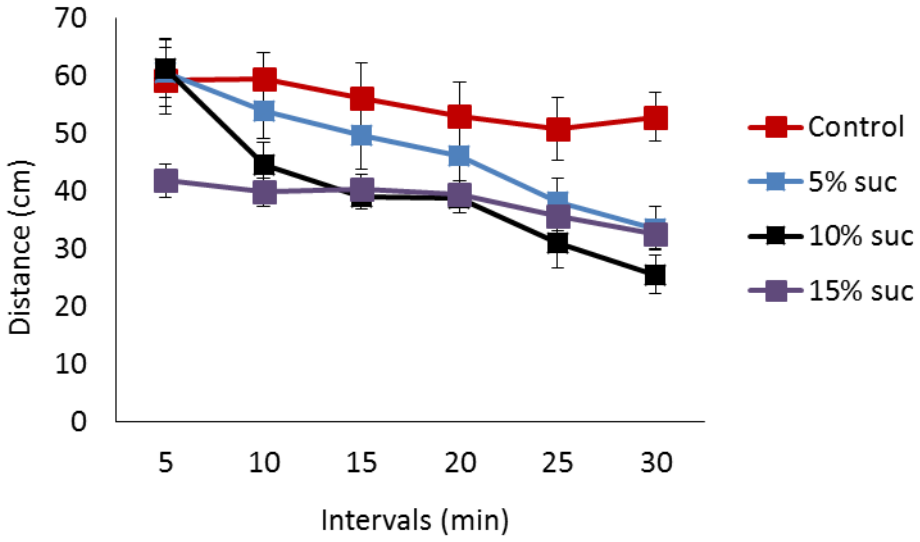


Figure 3.1. Mean distance (± 1 *SEM*) covered by the animals in different groups during the 30 minutes exposure phase of the experiment.

These data show the effects of exposure to different concentrations of sucrose on the locomotion (compared to exposure to plain water). Exposure to a high concentration, 15% sucrose, results in reduced activity from the onset of the session. Exposure to lower concentrations (5% and 10%) of sucrose results in a gradual reduction of the levels of activity. Animals exposed to 10% sucrose show the effect of sucrose 5 min in to the session, animals exposed to 5% sucrose show this reduction in their motor activity at a later stage, after about 15 minutes of exposure. Overall, over a 30-minute session it is clear that exposure to sucrose reduces the activity of the animals.

According to Butarelli et al. (2000), in planaria, increased levels of dopamine result in increased motor activity; also, increased levels of acetylcholine result in low motor activity. Given that sucrose seems to result in the release of dopamine it might be reasonable to expect increased motor activity. Following Butarelli et al. (2000), it has been suggested that excitation of D1 receptors might result in the release of acetylcholine, which would reduce the motor activity of the animals. According to this hypothesis, at least initially, dopamine release should increase the activity of the animals. In our experiment, there was a slight increase of activity during the first 5 min period in the animals exposed to 5% and 10% sucrose. However, this was non-significant. The reduced activity observed might be related to the release of Ach. Overall, this experiment strongly suggests that exposure to sucrose reduces motor activity in planarian. This information was used to help (with other preliminary studies reported below) the selection of the optimal concentration of sucrose solution (10%) which shows a clear effect in planaria.

3.3. Experiment 2: effect of sucrose on food searching behaviour

The purpose of Experiment 2 was to test the effects of sucrose in animals that had been deprived of food two days before the test. In this case the aim was to assess whether the sucrose solution has nutritious properties for the animals—if they, for example, satiated after exposure to sucrose.

3.3.1. Method

3.3.1.1. Subjects and Materials

Sixteen brown planaria (*Dugesia*) were used in this experiment. The flatworms were held in the same way described for the previous experiment. During the pre-test stage, animals were exposed to either water or sucrose; during the test stage of the experiment, planaria were put in a 9 cm diameter plastic petri dish filled with 5-7 ml treated water. For the purpose of the register, the petri- dishes were divided during the test into three areas: "far from food", "vicinity of food" and "food area" which contained a piece of chicken meat (see Figure 3.2). These virtual areas were drawn by using the Video-Track System. The activity of the animals (time spent and the distance covered in the different areas) was registered.

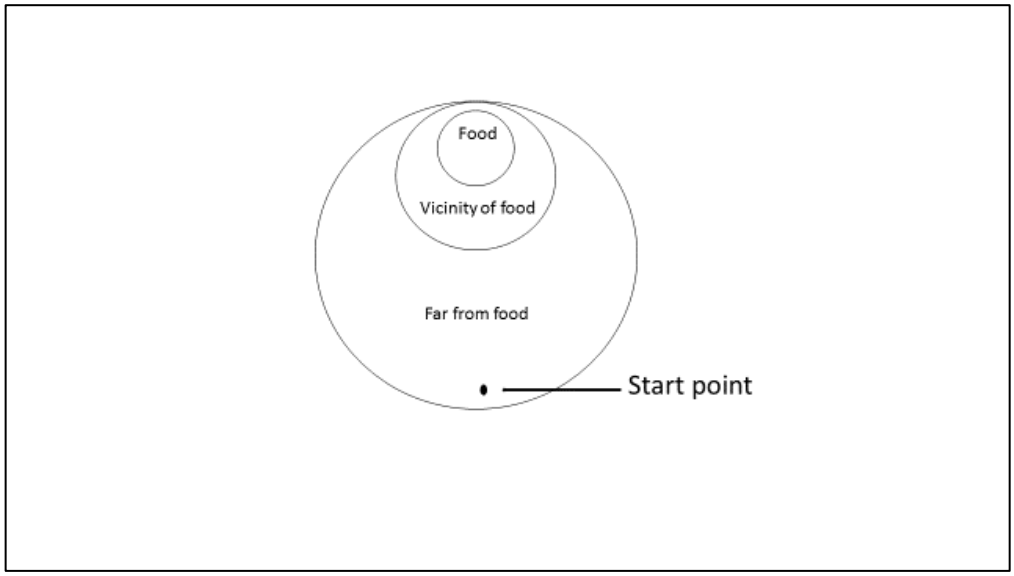


Figure 3.2. Diagram of the petri-dish areas used during the test phase of the experiment, circles in the petri-dish represent different identified areas during the test phase.

3.3.1.2. Procedure

There were two trials in this experiment: pre-test and test. In the pre-test trial, eight planaria were placed, individually, in a plastic petri- dish containing a 10% sucrose

solution for 30 minutes (group experimental). The other eight planaria (group control) were placed individually in a petri dish containing water for 30 minutes. The animals were then immediately transferred to the petri-dishes containing water and a piece of food (raw chicken). The planaria were put in the distal point of the away area in the petri-dish (start point; see Figure 3.2). The planaria were allowed to freely move around during the test session for 30 minutes. The time spent by the planaria in each area and the distance covered by them was registered.

3.3.2. Results and discussion

The time spent in each area ("far from food", "vicinity of food" and "food") during the test are displayed in Figure 3.3. Animals in the control group spent a similar amount of time in the three areas. However, animals exposed to sucrose during the pre-test trial spent most of the time in the area away from the food; that is, these animals showed no interest in the food. An ANOVA with Group (experimental *vs.* control) and Areas as factors showed a significant effect of Areas, $F(2,28)=5.81$, $p=0.008$, $\eta_p^2=.29$, and a significant interaction Group x Areas, $F(2,28)=6.71$, $p=0.004$, $\eta_p^2=.32$, but there was no significant effect of Group, $F<1$. Further analyses were carried out to analyse the Group x Areas interaction. Analysis of the simple main effects showed that the two groups differed in the area Away, $F(1,14)=11.97$, $p=0.004$, and Food, $F(1,14)=5.57$, $p=0.033$, but not in the Vicinity of food area.

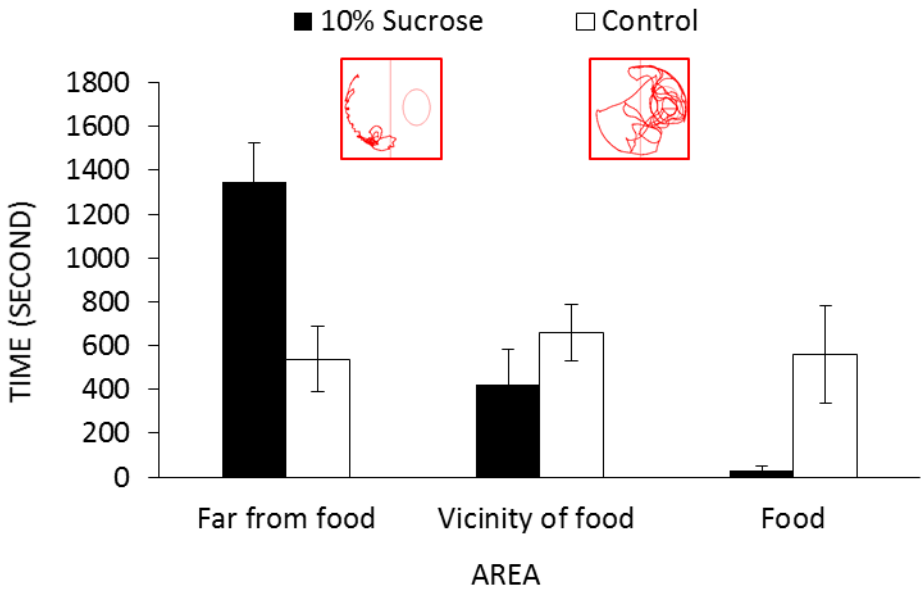


Figure 3.3. Mean total time (± 1 *SEM*) spent by the animals in groups 10% sucrose and control in each area during the test phase of the experiment. The diagrams show examples of the activity of animals in the two groups.

The distance covered in each area (far from food, vicinity of food and food) during the test are displayed in Figure 3.4. As in the previous experiment, animals in the group control showed higher levels of activity than the animals that had been exposed to sucrose during the pre-test trial. An ANOVA with Group (Experimental *vs.* Control) and Areas showed a significant effect of Group, $F(1,14)=10.91$, $p=0.005$, $\eta_p^2=.43$, but there was no significant effect of Areas $F(2,28)=2.006$ and no significant interaction Group x Areas, $F<1$.

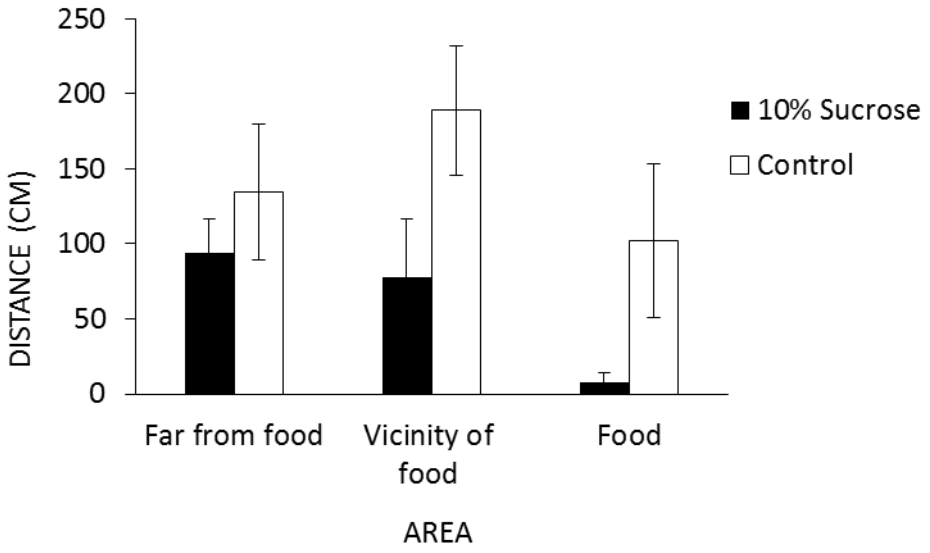


Figure 3.4. Mean total distance (± 1 *SEM*) covered by the animals in the groups 10% sucrose and control in each area by during the test phase of the experiment.

This experiment was conducted to show that sucrose has effects on animal's behaviour (e.g., the searching food behaviour). There are two interesting results from this experiment: animals exposed to 10% sucrose solution and subsequently to treated water with a piece of food keep away from the food area, while animals exposed to treated water are more likely to explore all the areas of the petri- dish and spend much more time in the food area. Secondly, animals exposed to sucrose showed a lower level of activity (cover less distance during the 30 minutes exposure period).

The results could suggest that planaria exposed to sucrose were satiated and did not engage in food searching behaviour due to the amount of sucrose absorbed into their bodies. Also, the overall activity levels of the animals exposed to sucrose are reduced, replicating the main effect observed in Experiment 1.

3.4. Experiment 3: the effect and post-effect of a single exposure to sucrose in developing withdrawal symptoms

Research with rats has established that withdrawal effects are mediated by a learning mechanism; animals develop an association between the rewarding agent and the environment. Therefore, if the expected drug is not present in the defined context or chemically blocked (Avena et al., 2008), animals will show withdrawal signs such as anxiety (File et al., 2004) and behavioural depression (Porsolt et al., 1978).

There are also other factors that can affect the development of withdrawal signs in different animals such as duration of animal exposure to the drug, type of drug and drug concentration (Sacavage et al., 2008).

Withdrawal effects have been reported by using planaria as an experimental model using cocaine (Hutchinson et al., 2015; Raffa & Desai, 2005). Also, Zhang et al. (2013) assessed withdrawal effects in planaria by using sucrose as a natural reinforcing agent. They exposed planaria to sucrose for 60 min and after that put them in sucrose free water for 5 min. They claim that withdrawal signs such as decrease in the locomotion in comparison with a control group were observed. However, their experiment does not clarify what is the nature of the mechanism behind this reduction in activity, and they also ignore any role played by the context in which sucrose is presented.

The purpose of Experiment 3 was to assess the effect of sucrose (single exposure for 30 min) and after effect of sucrose (test in water) in the same or a different context, and explore the potential role played by the context in which the animals are exposed to the sucrose solution in producing withdrawal symptoms. In this experiment we also assessed whether exposure to sucrose could reduce the mobility of the animals by sticking sucrose to their cilia (what could limit their movements). In order to avoid this confound, the animals were cleaned in free sugar water for 1 min before the crucial test.

3.4.1. Method

3.4.1.1. Subjects and Materials

Thirty-two brown planaria (*Dugesia*) were used in this experiment. The flatworms were held in the way described for previous experiments. The same petri- dishes used in the previous experiments were used in the present experiment. Animals could be exposed to a 10% sucrose solution or treated water.

3.4.1.2. Procedure

There were two phases in the experiment: exposure and test phase. During the exposure phase, planaria in the group sucrose were exposed to a 10% sucrose solution either in the smooth or the rough context for 30 minutes. Following that, the animals were immediately washed up in a cup containing treated water for 1 minute to wash out the sucrose attached to the cilia. Finally, the planaria were transferred to the petri-dishes again for 30 minutes in the presence of treated water. Half the animals (Group Suc-Same) were tested in the trained context used for exposure to sucrose; the other half of the animals was tested in the alternative control context (Group Suc-Different). Animals in two control groups (Control-Same) and (Control-Different) were treated in exactly the same way as described for group sucrose, except that the worms were always exposed to treated water (see Table 3.1). The level of activity (distance covered) was registered both for the exposure and the test phases of the experiment.

Group	Exposure	Test
Suc-Same	A-Suc	A-W
Suc-Diff	A-Suc	B-W
Control-Same	A-W	A-W
Control-Diff	A-W	B-W

Table 3.1. Experimental design of post effects of 10% sucrose compared with control group; (A/B: either rough or smooth context, counterbalanced across subjects; Suc= sucrose; W= water).

3.4.2. Results

The data of the exposure phase of the experiment are displayed in the left hand panel of Figure 3.5. Animals showed lower levels of activity (in terms of the distance covered) during the 30 minutes of exposure to the context in the presence of sucrose than in the presence of treated water (groups control). The differences between the groups seems to disappear during the test phase of the experiment (right hand panel, Figure 3.5). An ANOVA with Group (Sucrose *vs.* Control), Phase (Exposure *vs.* Test) and Context (Same *vs.* Different) showed no significant effect of any of the three factors, but the interaction Phase x Groups was significant $F(1,28)=7.37$, $p=0.01$, $\eta_p^2=.20$. Further analyses were carried out to analyse the Phase x Group interaction. Two Univariate ANOVAs were carried out with group as the factor for each of two phases. Results showed that the two groups differed in the Exposure phase $F(1,30)=14.44, p=0.001, \eta_p^2=.32$, but not in the Test phase of the experiment. This shows that groups Suc stay the same but the water ones show a significant decrease. Further analysis was conducted to compare the effect Exposure *vs.* Test for each group. The data of the groups control showed a significant difference between Exposure and Test phase of the experiment, $F(1,15)=5.98$, $p=0.02$, $\eta_p^2=.28$. However, there was no significant difference between both phases in groups Suc.

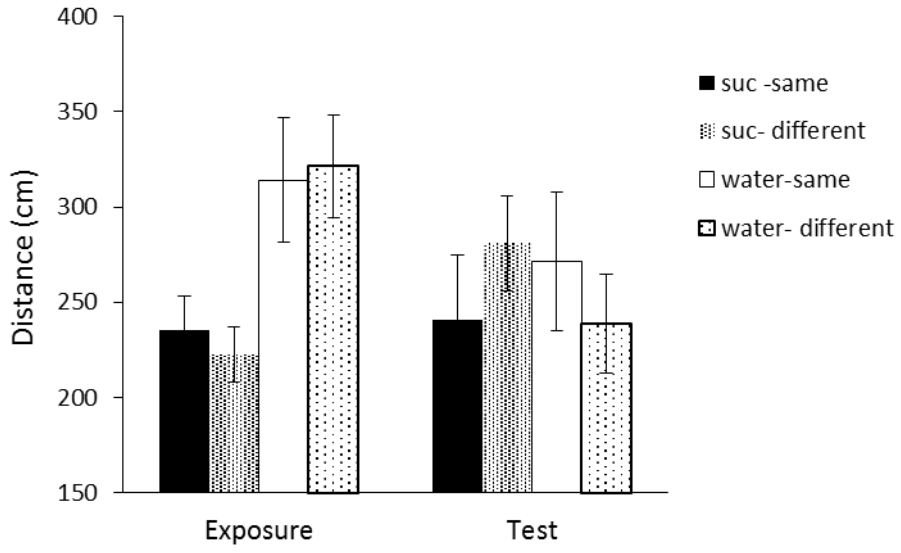


Figure 3.5. Mean distance (± 1 *SEM*) covered by the animals in the group sucrose and control in the Exposure and Test phases of the experiment.

3.4.3. Discussion

Animals exposed to 10% sucrose during the exposure phase showed reduced activity (distance covered by the animals in the petri- dish) compared with animals exposed to water in the group control. During the test, animals that were exposed to water reduced their activity; this is similar to other data produced recently in our laboratory in which the locomotor activity of the planaria was recorded during a long session (1 hour). The data showed that after 30 minutes, animals showed a decreased in their activity. This is fully consistent with the data reported in this experiment; and it still shows the effect of sucrose. In addition, animals exposed to sucrose did not show changes in the levels of activity during the 30 minutes of exposure to sucrose and the next 30 minutes of exposure to water. This experiment could suggest that sucrose results in reduction in locomotor activity of the animals. Also, the continued exposure to the test environments in the presence of water seems to reduce the activity after 30 minutes.

However, this finding conflicts with a finding by Zhang et al. (2013) who argue that animals exposed to 1% sucrose for 60 minutes followed by 5 minutes exposure to water in the same context showed slowed activity compared with animals in other groups (water-water; water-sucrose; and sucrose- sucrose).

Zhang et al. (2013) also argue that the acute exposure of planaria to 10% sucrose results in a reduction in the locomotor activity as a toxic sign of the high concentration. However, it could be suggested that the reduction in the locomotion occurs because animals absorbed the sucrose solution through their bodies and they might feel satiated and not seeking food. Also, it could reflect the slowing effect of the release of acetylcholine as discussed above.

However, this experiment did not clarify the effect of the tolerance in the development of withdrawal behaviour and there is no clear evidence about the effect of the context and the dopamine in withdrawal mechanism. This was addressed in Experiment 4, which included multiple exposures to sucrose to allow the development of tolerance and the clarification of the role of the environmental cues in conditioned responses in planaria.

3.5. Experiment 4: Tolerance development

Tolerance refers to the reduced response to a drug after repeated use. There are two theories about tolerance. The physiological theory suggests that animals develop tolerance due to a systematic change in their bodies, such as reduced susceptibility of receptors and enhanced metabolic rate of the administrated drug (Cochin, 1969). On the other hand, the conditioning theory suggests that tolerance occurs because the animal develops an association between the rewarding agent and the environmental cues, such as the context in which the reward is presented. This association becomes more obvious after a number of conditioning trials. The environmental cues counteract the effect of the drug by producing a compensatory response which is opposite to the unconditioned effect of the drug (e.g. Siegel, 1975).

The purpose of Experiment 4 was to study the effect of repeated exposures to sucrose. Animals were tested in the same or a different context in which sucrose was presented, to assess whether planaria develop a compensatory response which is under the control of the environmental cues as predicted by Siegel (1975).

3.5.1. Method

3.5.1.1. Subjects and Materials

Sixteen brown planaria (*Dugesia*) were used in this experiment. The flatworms were held in the way described for previous experiments. The same petri- dishes used in the previous experiments were used in the present experiment. Animals were exposed to either a 10% sucrose solution or treated water.

3.5.1.2. Procedure

There were two phases in the experiment: training and test. The training phase lasted eight days (days 1-8 of experiment); during this phase, planaria were exposed to the two surfaces (rough and smooth) in alternate days. Half of the animals were exposed to a

10% sucrose solution in the smooth context in days 1, 3, 5 and 7 of the experiment; in days 2, 4, 6 and 8 they were exposed to treated water in the alternative context. For the other half of the animals this arrangement was reversed. The two contexts were counterbalanced throughout subjects. The test phase took placed in days 9 to 14 of the experiment. In day 9, half the animals were tested, in the presence of water, in the same context used for exposure to sucrose; the other half of the animals were tested in the other context. In day 10, animals were tested in the alternative context, again with water. After that, the animals received one cycle of retraining before they were tested for another two days with a 10% sucrose solution in the two contexts.

3.5.2. Results

The data of the training phase of the experiment are displayed in Figure 3.6. Animals showed lower levels of activity in the presence of sucrose than in the presence of water during the first two cycles of training. However, the activity of the animals when they were exposed to sucrose increased over the training days. A within-subjects ANOVA with Treatment (sucrose *vs.* water) and Cycle of training showed significant effects of the Treatment, $F(1,15)=18.849$, $p=0.001$, $\eta_p^2=.55$, Cycle, $F(3,45)=5.224$, $p=0.004$, $\eta_p^2=.25$; the interaction Treatment x Cycle was also significant $F(3,45)=6.281$, $p=0.001$, $\eta_p^2=.29$. Further analyses were carried out to analyse the interaction, showing that animals showed a significant increase in activity when exposed to sucrose over the four training trials, $F(3,45)=18.086$, $p<0.001$, $\eta_p^2=.54$, whereas they showed no significant changes over the trials in which they were exposed to water, $F<1$. Also, the animals showed less activity in sucrose than in water in days 1 and 2, $F_s(1,15)\geq 18.434$, $ps<0.001$; in the days 3 and 4, however, the levels of activity in sucrose and water did not differ. During the Re-Training, animals showed a lower activity level in sucrose (301.70±12.125) than in water (342.96±10.22), $F(1,15)=4.732$, $p=0.04$, $\eta_p^2=.24$.

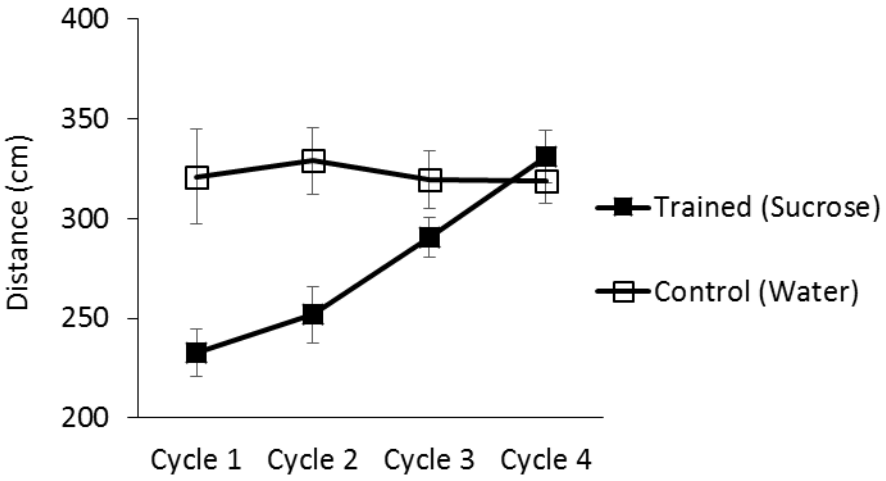


Figure 3.6. Mean distance (± 1 *SEM*) covered by the animals in the contexts were Sucrose (Trained) and Water (Control) were presented over four cycles of training during the tolerance training phase of the experiment.

The data from the test phase of the experiment are displayed in Figure 3.7. Animals showed a higher level of activity during the 30 minutes test in the context in which the sucrose was presented during the training phase than in the alternative context, both in the presence of water and sucrose. In addition, animals showed higher levels of activity in both contexts when they were tested with water than when they were tested with sucrose. An ANOVA with Treatment (sucrose *vs.* water), and Context (Trained *vs.* Control) confirmed these impressions, showing a significant effect of Treatment, $F(1,15)=35.75$, $p<0.01$, $\eta_p^2=.70$, and Context, $F(1,15)=5.50$, $p=0.03$, $\eta_p^2=.26$; the interaction Treatment x Context was not significant $F<1$.

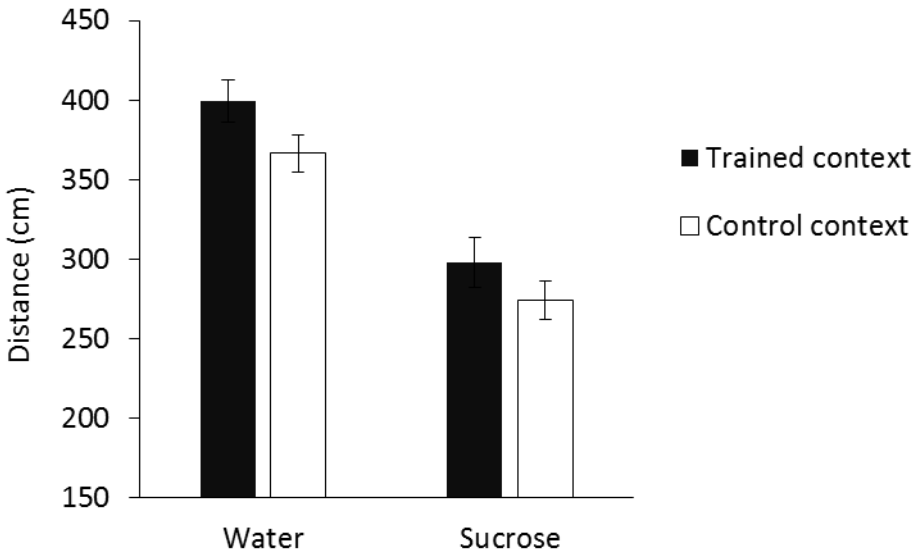


Figure 3.7. Mean distance (± 1 *SEM*) covered by the animals in the Trained and the Control contexts throughout the two cycles of test trials in the presence of Water and Sucrose.

3.5.3. Discussion

Animals exposed to 10% sucrose during the first cycle of the training phase showed a reduction in the activity compared with their activity when they were exposed to water. However, the activity of animals in sucrose clearly increased over the training cycle, suggesting the development of tolerance to sucrose.

According to the conditioning theory of tolerance, the context where sucrose is expected would activate a conditioned compensatory response which opposes the effect of the drug. Given that sucrose produced hypo-activity during the first training trials, we could expect the context to produce a conditioned compensatory response of hyperactivity. In both tests (with water and sucrose) the animals where more active in the context in which sucrose was presented during training than in the context in which the animals always experienced water.

These findings suggest that although sucrose reduces animals' activity, after several exposures to sucrose the animals developed hyperactivity as a compensatory response which results in the development of tolerance. Similar evidence was provide by Subkov

and Zilov (1937), who found that animals treated with adrenaline showed tachycardia as the unconditioned effect of the drug. However, regular presentation of adrenaline in the same environmental cues resulted in the development of tolerance and a reduction of tachycardia. Finally, injecting the animal with vehicle in the same contextual cues where adrenaline was injected produced bradycardia, a conditioned compensatory response (Siegel, 1975).

Similar results regarding the development of tolerance were obtained with planaria by Rawls et al. (2011) who found that planaria developed tolerance after continuous repeated exposure to a high concentration of nicotine. However, they did not address the role of the environmental cues which are key to understand the mechanism of tolerance.

Another interpretation for the results could be that the animals develop tolerance to the effect of a repeated exposure to sucrose as a part of instrumental processes. The hypothesis would be that animals have to apply more effort to move around during the exposure to sucrose in the training sessions. Therefore, during the test sessions, animals show increased activity in the context in which they expect the presentation of sucrose (Trained context) compared with the Control context. A study by Lê and Kalant (1992) examined the development of acute tolerance to ethanol intoxication and the role of intoxication practice in tolerance development in rats. Ethanol impairs the motor activity; thus, they assessed the reduction in the motor impairment as an indicator for the development of tolerance to the effect of ethanol. Animals were injected with ethanol and frequently tested along different time intervals under ethanol intoxication in one single test session. They found that there was a significant decrease in the level of motor impairment when the animals were tested after long time intervals following the injection. Importantly, they found that the frequent tests (intoxicated practice) significantly enhanced the development of acute tolerance to ethanol intoxication.

Similar evidence was found from human studies, Zack and Vogel-Sprott (1995) stated that tolerance and sensitisation to the effect of a repeated exposure to alcohol could be developed as a learned instrumental response. There were two groups in their experiment; one to assess the development of tolerance and the second to measure whether the participant develop sensitisation; the motor activity was used as a sign for the development of either tolerance or sensitisation. The data showed that the rewarding

effect of alcohol could increase the development of tolerance or sensitisation even in the absence of alcohol. Similarly, the outcome of the rewarding effect of sucrose in our experiment could rise the occurrence of the observed behaviour (hyperactivity).

In conclusion, this experiment provides strong evidence that exposure to sucrose results in the development of tolerance. It also highlights the role of the contextual cues in the mechanism of tolerance, by controlling the development of a conditioned compensatory response.

3.6. Experiment 5: Conditioned Place Preference (CPP)

Conditioned place preference (CPP) is a behavioural procedure in which animals are initially exposed to two sets of contextual cues. In the biased design, if they show a preference for one context, the alternative context is then paired with a reward. Typically, post-training tests show a shift in preferences: the animals, for example, spend more time in the initially less preferred context that had been paired with the reward.

There are different factors that need to be taken in to account when using a CPP procedure, such as the time of conditioning and test sessions, drug concentrations and the type of the reward agent. CPP has been reported in vertebrate animals (e.g., rats) by using different rewarding agents (drugs and natural substances).

If the development of CPP is equivalent to the development of conditioned responses in Pavlovian conditioning, exposure to the context after CPP should result in extinction of the conditioned place preference (Pavlov, 1927). The purpose of Experiment 5 was to assess whether animals develop CPP using sucrose as the rewarding agent, and to check whether CPP extinguishes when the animals are repeatedly exposed to the context in the absence of the rewarding agent (sucrose).

3.6.1. Method

3.6.1.1. Subjects and Materials

Thirty brown planaria (*Dugesia*) were used in this experiment. The flatworms were held in the same way described for previous experiments. During the experimental sessions, the planaria were kept, individually, in a 9 cm diameter petri-dish. The dish could be filled with 5-7 ml treated water or a sucrose solution (either a 1% or a 10% w/v sucrose solution). The planaria were exposed to two different contexts within the petri-dish, the rough and the smooth surfaces described for the previous experiments. We used petri-dishes with two contexts for pre-training and post-training tests (one half was covered with sand, the rough context; the other half was the smooth context). For the training sessions we used petri dishes with just one surface, either rough or smooth. Time spent in different contexts during Pre-Training and Post-Training tests was registered.

3.6.1.2. Procedure

There were three phases in this experiment: pre-training test, training and post-training tests. During the first day of the experiment, the pre-training test, all the animals were put in the centre of a petri-dish with two different contexts, rough (the sandy surface) and smooth (the plastic surface) filled with 5-7 ml of treated water. The planaria were allowed to freely move around for the duration of the session, 30 minutes. The time spent by the planaria in each context was registered, and a preference score was calculated for one of the contexts. In this experiment, the preference score was calculated for the smooth context, which was the less preferred by all the animals. Planaria were assigned to three groups (n=10) matched by their smooth context preference score: Control, 1% Sucrose; and 10% Sucrose.

During the training phase of the experiment, planaria were exposed to the two surfaces (rough and smooth) in alternating days. In the control group, planaria were always exposed to the context in the presence of treated water. Planaria in the 1% sucrose group were exposed to the less preferred context, the smooth context, in the presence of a 1% sucrose solution; and to the rough contexts in the presence of treated water.

Finally, planaria in the 10% sucrose group were exposed to the smooth context in the presence of a 10% sucrose solution and the rough context in the presence of treated water. The training lasted eight days (days 2-9 of experiment). In days 2, 4, 6 and 8 animals were exposed to the smooth context; in days 3, 5, 7, and 9 they were exposed to the rough context. The experimental sessions always lasted 30 minutes. In This experiment, the distance covered by the animals during the training sessions was not monitored.

During the post-training phase of the experiment, the planaria were again exposed to the two sided petri-dishes as during the pre-training test. The planaria were given three tests, in days 10, 13 and 16 of the experiment. The first post-training test took place 24 hours after the last training session; the post-training tests 2 and 3 took place at intervals of 72 hours. For the Post-Training tests, a change in preference score was calculated by subtracting the preference score observed during the Pre-Training Test from the preference score observed during the Post-Training Test. Therefore, positive values would indicate the development of a conditioned preference for the context rewarded with sucrose during the training.

3.6.2. Results

During the Pre-Training Test, animals in group 1% Suc showed a preference score (for the less preferred context) of 0.32 (*MSE*±0.32); the animals in group 10% Suc showed a preference score of 0.28 (*MSE*±0.58) and the animals in group control showed a preference score of 0.29 (*MSE*±0.68). A univariate ANOVA showed that there were no differences between the groups, $F<1$.

The data of Post-Training Tests corresponding to the change in preference scores for the initially less-preferred context are displayed in Figure 3.8. An ANOVA with Group (1% sucrose, 10% sucrose and Control) and Test Trials showed significant effect of Group, $F(2,27)=4.316$, $p=0.02$, $\eta_p^2=.24$, and Test Trials, $F(2,54)=4.64$, $p=0.014$, $\eta_p^2=.14$. The interaction Group x Test Trials was not significant $F<1$. Post Hoc tests using the Bonferroni correction showed that group 10% Sucrose significantly differed from the control group ($p=0.03$); none of the other comparisons was significant.

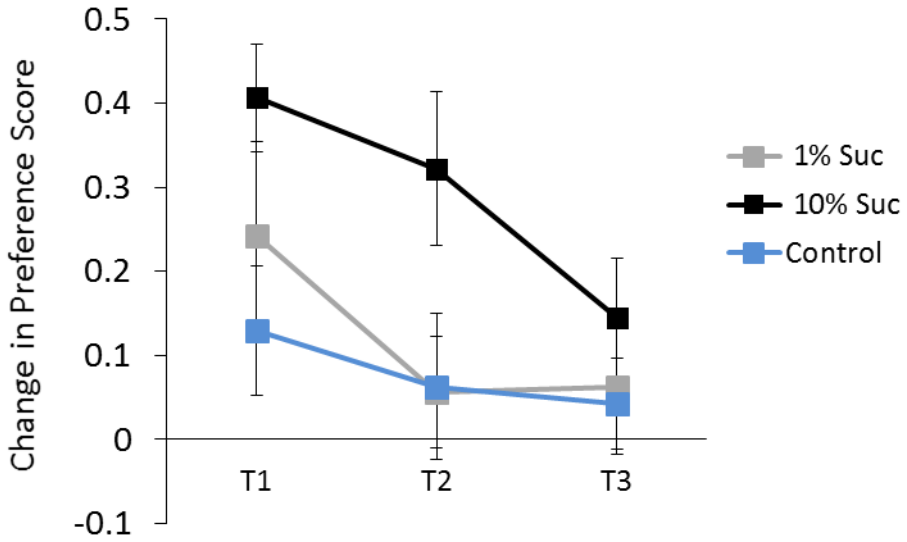


Figure 3.8. Mean change in preference score (± 1 *SEM*) to the target context during the extinction (T1-T3) test trials of the test phase of the CPP experiment.

3.6.3. Discussion

Sucrose was (a natural rewarding agent) was used to produce a conditioned place preference in planaria. The results of this experiment provide evidence that 10% sucrose causes a significant increase in preference score for the smooth context compared with 1% sucrose and the control group. Previous research with rats showed a similar result. The finding of this experiment is consisted with a finding by Ågmo et al. (1995); (see also Chaperon & Thiebot, 1996; Guyon et al., 1993) who found that rats treated with sucrose in drinking water showed a clear preference for the rewarded compartment after a conditioning cycle in the absence of the rewarding agent.

Raffa et al. (2013) examined the CPP in planaria conditioned with different concentrations of cocaine or amphetamine for 30 minutes training sessions in a light context (less preferred side in their experiment). They found that planaria showed a significant change in the preference score for the light side of the petri- dish during the test phase. Similarly, Zhang et al. (2013) studied the development of CPP by using sucrose as a natural reward agent and light and dark contexts. They found that planaria conditioned with 1 % sucrose revealed a significant increase in the preference score

(time spent in less preferred context, light area) compared with planaria were exposed to water in the control group.

However, one problem with Raffa et al. (2013) and Zhang et al. (2013) experiments is that the animals were exposed to the light context much more than to the dark context (animals were exposed for 30 minutes to the light context during the conditioning phase, but they were not given exposure to the other side). This would result in the habituation of the photophobic response. Evidence for that is found in their results in the control group exposed to water in the light context, which showed a positive shift in their preference score for the light. In the present experiment, this problem was controlled by equating the experience with both contexts (smooth and rough) to control for the habituation of the responses elicited by the contexts.

More recently, Ouyang et al. (2017) illustrated the behavioural effect of different sweeteners (Splenda, Equal and Sucrose) using a CPP procedure and the development of withdrawal signs. They also equated the time of exposure to the dark and light contexts in their experiment: 5 minutes exposure to two sides petri-dish in pre and post-test while animals were exposed for 30 minutes to the less-preferred context with the reward, followed by another 30 minutes of exposure to water in the preferred context during the conditioning phase. They found that 0.01 % of both Splenda and sucrose result in significant CPP; however, exposing the animals to Equal at the same concentration fails to change the preference score for the previous less preferred context. They suggested that the reward effects of different sweeteners depend on the active ingredient of each substance; and that maltodextrin has similar rewarding properties as dextrose; however, aspartame has an aversive effect that prevents the establishment of the CPP response.

However, another problematic aspect in Raffa et al. (2013), Zhang et al. (2013) and Ouyang et al. (2017) studies on CPP is that animals were tested when they were still under the effect of the drug (the test took place immediately after the conditioning phase). In the present experiment there was a 24─ hours gap between the last training trial and the test to establish whether animals have developed long term memory of the newly acquired preference for the context associated with the sucrose (see also

Hutchinson et al., 2015, for a similar procedure using cocaine as the rewarding agent). This suggests that, in our procedure, the animals develop long-term CPP.
In this experiment, the effect of 1% and 10% sucrose solution (experimental groups) on the development of CPP were compared with a control group (treated water only). However, in future studies it could be worth adding another two control groups; CS and US separately at random to show whether exposure to the US or CS by itself produces an effect.

In the data reported here, the preference scores for the smooth context significantly decreased along test trials, suggesting that the CPP responses extinguished as a result of exposing the conditioned stimulus several times in the absence of the rewarding agent (sucrose). This result suggests extinction of the conditioned response and it is unlikely to be forgetting; also, it is consistent with findings by Alvarez et al. (2014), who reported extinction of a conditioned response in an invertebrate model, the garden snail. However, future studies might include a better control group, for example, a group of animals that has a same training but no T1 or T2 just T3. If these animals show a reduction in the conditioned response during the T3; this means that the observed result is more likely due to forgetting rather extinction. If the opposite happens, so, this will be strong evidence of extinction.

In conclusion, this experiment illustrates that planaria conditioned with 10% sucrose displayed a significant change in the preference score for the smooth context compared with the group exposed to 1% sucrose and the group control. This CPP response seems to extinguish after three test trials without the rewarding agent.

3.7. General discussion and conclusion

Chapter 3 assessed the behavioural and pharmacological effects of repeated exposure to sucrose in planaria. The results of this chapter illustrated that sucrose affects planarian's food searching behaviour: animals exposed to a 10% sucrose solution and tested directly in a Petri dish that contained treated water with a piece of food were less motivated to explore the context searching for food; these animals also showed a decrease in their locomotor activity. This indicates that the absorbed amount of sucrose

interferes with the normal appetite and the animals were satiated; or it could be due to the reduction in their activity.

Our experiments have established that exposure to a 10% sucrose solution reduces the locomotor activity of planaria. We have considered different possibilities to account for this reduced activity. One possibility was that the viscosity of the sucrose solution made it difficult for the animals to move normally. However, in Experiment 3, we found that animals exposed to a sucrose solution and washed up in clean water still showed a lower locomotion. This suggests that the viscosity of the sucrose solution was not the reason behind the reduced activity.

An alternative hypothesis takes into account the dopamine-acetylcholine interaction. It is known that consumption of a rewarding agent activates the dopamine reward system and results in dopamine releases. The increase in dopamine levels is related with the increase of animal's activity at the early stages of consumption. However, at later stages, the level of acetylcholine elevates (Rada et al., 2001) resulting in declined motility (Butarelli et al., 2000). The reduced activity observed in our experiments could be attributed, therefore, to the release of acetylcholine that follows the increase of dopamine levels after exposure to sucrose.

Experiment 1 and 4 have shown that animals exposed to sucrose show a significant reduction in their locomotor activity. Interestingly, repeated exposure to sucrose in a particular context was found to increase the locomotor activity of the animals in Experiment 4; this is evidence for the development of tolerance to sucrose.

The results of the Test Water (Experiment 4) confirm that the animals have developed a hyperactivity conditioned compensatory response to the surface associated with sucrose. On the other hand, they could suggest that tolerance develop as a part of an instrumental mechanism. The observed hyperactivity shown by the planaria in the trained context could be an effect of the expectation of a sucrose solution in which they need to do more effort to move. The results of the Test Sucrose show that the animals are more tolerant to the effects of sucrose (reduced activity) in the context previously associated with sucrose; sucrose more effectively reduced the activity of the animals in the context in which it was never presented before. These data suggest that planaria could learn how

to control the effects of the drug and develop a conditioned compensatory responses in a similar way as vertebrates (e.g., Siegel, 1975).

An important key finding in this chapter is that planaria repeatedly exposed to sucrose and water in alternative contexts showed acquisition and extinction of a conditioned place preference (CPP) response. The effect or a subsequent exposure to sucrose was assessed in two experimental groups (1% and 10% sucrose) compared to a control group (treated water only). The data indicate that animals treated with 10% sucrose showed a significant development of CPP and extinction compared with the other groups; however, comparing these results with an unpaired control (CS and US separately at random), CS or US alone might be a better control for future studies. The fact that the learned conditioned response extinguishes when the animals are exposed to the target context in the absence of the reward confirms that CPP can be characterised as a standard Pavlovian conditioned response. Previous researches showed that planarian can develop a significant CPP using sucrose as a rewarding agent (Zhang et al., 2013). However, there were number of limitations in their studies. For example, animals were tested immediately after the conditioning session; therefore, they could simply be showing post-effects of sucrose exposure rather than the development of CPP. In addition, there was just a single session of conditioning, and animals were unequally exposed to the two contexts (rewarded and non-rewarded) which might lead to the development of habituation in the control group rather the development of CPP in the experimental group (Zhang et al., 2013). In our experiments the animals were equally exposed to the two surfaces during training and were tested 24 hours after the last training cycle thus demonstrating genuine long-term CPP.

Moreover, during the CPP extinction test trials, animals were tested several times in the presence of the CS alone (without the US); this led to extinction of the CPP mediated by sucrose rather than mere forgetting; however, we recognise that comparing this group with a control trained in the same way but tested just in the last test trial would offer a better demonstration of extinction. One of the key features of Pavlovian conditioned responses is that they are subject to extinction. When the animal is exposed to the conditioned stimulus (CS) in the absence of the rewarding unconditioned stimulus (US) the conditioned response gradually declines and may disappear. It is well established that extinction does not erase the original learning (the excitatory association between

the CS and the US, for example), but instead generates new learning (the CS now signals the absence of the US). The excitatory and the inhibitory associations coexist and compete for the control of the behaviour of the individual. In situations similar to the acquisition, the excitatory association is more likely to be retrieved resulting in the display of the conditioned response; in situations similar to the extinction phase, the inhibitory association would be preferentially retrieved and the conditioned response would be omitted (e.g., Bouton, 2004).

Chapter 4: The role of dopaminergic system in sucrose addiction in planaria

4.1. Introduction

4.1.1. Rodent psychopharmacological models of sucrose addiction

Sucrose has been characterised as a substance of abuse in animal models where rats exposed to sucrose display a number of characteristic neurophysiological and behavioural responses. Neurophysiological investigations have, for example, found evidence of sucrose-induced enhancement of extracellular dopamine in the nucleus accumbens (Bassareo & Di Chiara, 1997; Rada, Avena, & Hoebel, 2005), similar to those elicited by drugs of abuse like cocaine or amphetamine (Avena et al., 2008).

Rada et al. (2005) assessed the levels of dopamine and acetylcholine during 21 days of a daily intermittent consumption of 10% sucrose solution in 12h deprived rats. They found that there was a significant increase of sucrose consumption throughout the experiment. They also found that the levels of extracellular dopamine significantly increased during days 1, 2 and 21 of the experiment. This increase in dopamine levels occurred during the first hours of the access to sucrose while the level of acetylcholine increased during the last hours of sucrose binging.

Ågmo et al. (1995) studied the establishment of condition place preference in rats following consumption of a sucrose solution. They also clarified the role of the dopamine and opioid systems in the CPP mechanism and the development of sucrose's reward properties. They found that consumption of a sucrose solution produced a CPP in the experimental animals. In addition, they showed that treating the animals with 0.5mg/kg of a mixture of D1 and D2 dopamine antagonist drugs (flupentixol) or 16mg/kg of an opioids antagonist (naloxone) prevented the development of CPP. They also observed that treatment with naloxone at this dose or a lower dose, 1mg/kg, produces a significant decline in sucrose consumption. These data suggest that the rewarding effects of sucrose are mediated by the dopamine system. However, other neurotransmitters (e.g., opioids) play an important role in the maintenance of the rewarding properties of sucrose.

Cervo and Samanin (1995) examined the effect of a D1 antagonist (SCH23390 at 0.1-0.2 mg/kg i.p.), a D2 antagonist (sulpiride at 50-100 mg/kg i.p.), and a non-competitive NMDA receptor antagonist MK-801 (0.1–0.5 mg/kg i.p.) on the rewarding effects of cocaine and the development of CPP in rats. They found that treatment with a D1 antagonist or NMDA receptor antagonist during the exposure to the relevant context prevents the development of CPP. However, there was no significant effect when they were given following training, immediately before a test session. Also, treatment with a D2 antagonist did not prevent the development of CPP. These findings confirm that dopamine D1 receptors and NMDA receptors have a vital role in promoting the rewarding effect of cocaine and establishing cocaine induced CPP.

Studies that are more recent have addressed the interaction between the dopamine and other neurotransmitter systems in the development of CPP. Latagliata, Saccoccio, Milia, & Puglisi-Allegra, (2016) assessed the effect of amphetamine (a dopamine agonist) in producing CPP. They also addressed the role of the bilateral pre- and infra-limbic levels of norepinephrine in maintaining or attenuating the CPP response and extinction of amphetamine mediated CPP. They found that rats conditioned with amphetamine developed a CPP response. To assess the effect of the norepinephrine on the extinction of the CPP, a day after the CPP test—before starting the extinction trials, animals were injected with a drug that interferes with the level of norepinephrine in the pre-limbic and infra-limbic areas of the brain. They found that inhibiting norepinephrine in the pre-limbic area facilities the extinction of the conditioned preference to amphetamine, while, reduction of the norepinephrine in the infra-limbic have an opposite effect (blocks the extinction and prolongs the conditioned response). This study shows how the action of the dopamine system in producing CPP is modulated by other systems and brain's regions. However, more research is needed to further elucidate the way in which different neurotransmitter systems interact to produce CPP.

The aim of the experiments reported in this chapter was to assess the role of the dopamine reward system in the development of CPP and tolerance in planaria.

4.1.2. Planarian models of sucrose addiction

Dopamine and norepinephrine have been detected in planaria and their amount inside the planarian's body can be measured by using High Performance Liquid Chromatography (HPLC) (Ness et al., 1996). Dopamine D1 and D2 receptors have been also illustrated by using pharmacological and biochemical procedures, such as the use of dopamine agonist and antagonist drugs to confirm their role in planarian's behaviour (Venturini et al., 1989). A study by Zhang et al. (2013) suggests that the dopamine reward system could play a role in developing CPP in flatworms. Worms exposed to sucrose and a dopamine antagonist (a D1 or D2 antagonist: SCH23390 and Sulpiride) did not develop CPP.

It can be expected that the development of CPP is controlled by the dopamine reward system and depends on the rewarding properties of the sucrose-US. However, the establishment of a conditioned compensatory response (which is not necessarily a pleasant one) might be independent of this reward system. To assess this hypothesis CPP and tolerance training procedures were used to compare the conditioned responses developed by animals treated with a dopamine D1 antagonist with control animals.

This chapter aimed to characterise two examples of Pavlovian conditioned responses (CR): CPP and the development of tolerance. Extinction is a key element of Pavlovian CRs. Therefore, both the acquisition and extinction of CPP responses were monitored in a set of experiments. The development of conditioned compensatory responses during the course of tolerance training were also assessed in separate experiments. We also compared the effect of blocking the D1 dopamine receptors on CPP and tolerance; and finally, the effect of the D1 dopamine antagonist itself on the development of conditioned place aversion was assessed.

The four experiments that follow in this chapter assessed: 1) the role of the dopaminergic system in the development of CPP acquisition; 2) the role of the dopaminergic system in the development of CPP and tolerance; 3) the role of the dopaminergic system in the extinction of a CPP response; and 4) whether exposure to a dopamine antagonist results in the development of conditioned place aversion.

4.2. Experiment 6: treatment with dopamine antagonist impairs CPP development

The purpose of Experiment 6 was to assess the development of CPP in planaria using sucrose as the rewarding agent; and whether the development of CPP depends upon the dopaminergic system. It also assesses the extinction of CPP and whether exposure to the rewarding agent reinstates a previously extinguished CPP.

4.2.1. Method

4.2.1.1. Subjects and Materials

Forty-eight brown planaria *(Dugesia*) were used in this experiment. The flatworms were held as described in Chapter 2. Animals could be exposed to a 10% sucrose solution; treated water; a 1µM solution of a selective D1 dopamine receptor antagonist (SCH-23390 hydrochloride, Sigma-Aldrich, UK); or a mixture of 10% sucrose and a 1µM SCH 23390 solution. Previous pharmacological studies in rats showed that the effect of SCH 23390 could last for a very short period of time (20 min) after giving the animals an i.p injection (Kilts, Dew, Ely, & Mailman, 1985). However, another study in mice showed that the effect of SCH23390 peaked after 1h from an oral administration and it could persist from 2 to 4 hours after its administration (Iorio, Barnett, Leitz, Houser, & Korduba, 1983). In addition, both studies indicated that the effect of the drug and its duration of action depend on the route of administration, the dose and the time intervals. Thus, in the experiments reported in this chapter, animals were tested 24h after exposure to the dopamine antagonist; this period guarantees that the animals were no longer under the effect of the drug during the test. In addition to the petri-dishes described for the previous experiments, glass petri-dishes (5 cm in diameter) were used for the reinstatement treatment. During the experimental sessions, the animals' activity was tracked by using a Video-Track System.

4.2.1.2. Procedure

The procedure of this experiment was similar to the procedure described for the previous experiments of CPP with three phases: pre-training test (day 1 of experiment),

training (days 2-9) and post-training test (days 10-14). For the test trials (pre- and post-training), the animals were exposed to treated water in one of the two-sided (half smooth—half rough) petri dishes. They were allowed to freely move for 30 minutes; the time spent in each of the two surfaces of the petri dish was recorded and a preference score was calculated for the less preferred surface (time spent in the less preferred surface / total time). Animals were assigned to one of two experimental conditions: the group D-Ant (n=24), treated with the dopamine antagonist during all the sessions of the training phase; and the Control group (n=24) that was never presented with the dopamine antagonist. In this experiment, for each group, the preference score was calculated for the smooth context, which was the less preferred by half of animals (n=12); and to the rough context, which was the less preferred by the other half of animals (n=12).

The training phase took place after the pre-training test and lasted 8 days in which the animals were exposed to the two surfaces in alternation every 24 hours. The animals were exposed for 30 minutes to a 10% sucrose solution in the less preferred surface during, for example, the even days; and to treated water for 30 minutes in the preferred surface in the odd days; this cycle was repeated four times. During the post-training tests the animals were again exposed to the two-sided petri dishes. To establish whether animals showed conditioned place preference, indicated by a shift of preferences in relation to the pre-training test, a change of preference score was calculated. Animals were given four daily post-training test trials (days 10-13 of the experiment) to monitor the extinction of any CPP response observed (CPP Extinction Tests). Once the extinction of the CPP was completed, all the animals were re-exposed for 30 minutes to the sucrose solution in a distinct glass petri dish (5 cm in diameter) one hour after the last extinction trial (day 13). The following day, the animals were tested again in the two-sided petri dish to assess the reinstatement of the CPP response (CPP Reinstatement Test).

4.2.2. Results

The data of the Training phase of the experiment are displayed in Figure 4.1. Animals in the control group showed lower levels of activity in the presence of sucrose than in the

presence of water; similarly, the animals in the D-Ant group showed lower levels of activity than the group control both in the presence of sucrose and water. An ANOVA with Group (Control *vs.* D-Ant) and Stimulus (Sucrose *vs.* Water) showed a significant effect of Group, $F(1,46)=10.44$, $p=0.002$, $\eta_p^2=.18$, and Stimulus, $F(1,46)=30.70$, $p<0.01$, $\eta_p^2=.40$. There was no interaction.

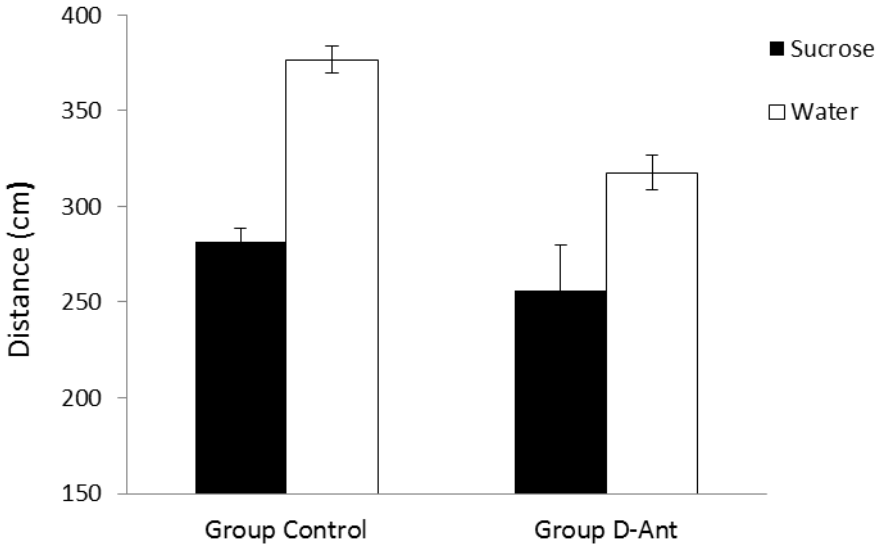


Figure 4.1. Mean distance (± 1 *SEM*) covered by the animals in each group (control and group D-Ant) in the presence of sucrose and water during the training trials of the experiment.

During the Pre-Training Test, animals in group Control showed a preference score (for the less preferred context) of 0.33 (±0.04 *SEM*); the animals in group D-Ant showed a preference score of 0.43 (±0.02). A One-Way ANOVA confirmed that there were no differences between the groups, $F(1,47)=3.53$, $p>0.05$.

The data for the Post-Training and Reinstatement Tests corresponding to the change in preference scores for the initially less-preferred context are displayed in Figure 4.2. An ANOVA with Group (Control and D-Ant) and Test Trials (T1-T4) showed a significant effect of Group, $F(1,46)=12.27$,$p<0.01$, $\eta_p^2=.21$ and Test trials, $F(3,138)=5.45$, $p<0.01$, $\eta_p^2=.10$. There was also a significant interaction Group x Test Trials, $F(3,138)=6.03$, $p<0.01$, $\eta_p^2=.11$. Further analyses were carried out to analyse the interaction, showing

that animals in the group Control showed a significant decrease in change of preference score over the four days of test, $F(3,69)=11.34$, $p<0.01$, $\eta_p^2=.33$, whereas the animals in the D-Ant group showed no significant changes over the test trials, $F<1$. Also, the two groups differed in test trials 1 and 2, $F_s(1,46)\geq17.47$, $p<0.01$, $\eta_p^2=.27$, but they did not differ in the tests 3 and 4. The data of the Reinstatement Test (RT) are displayed on the right hand part of Figure 4.2. A One-Way ANOVA confirmed that the difference between the groups was significant, $F(1,47)=18.74$, $p<0.01$.

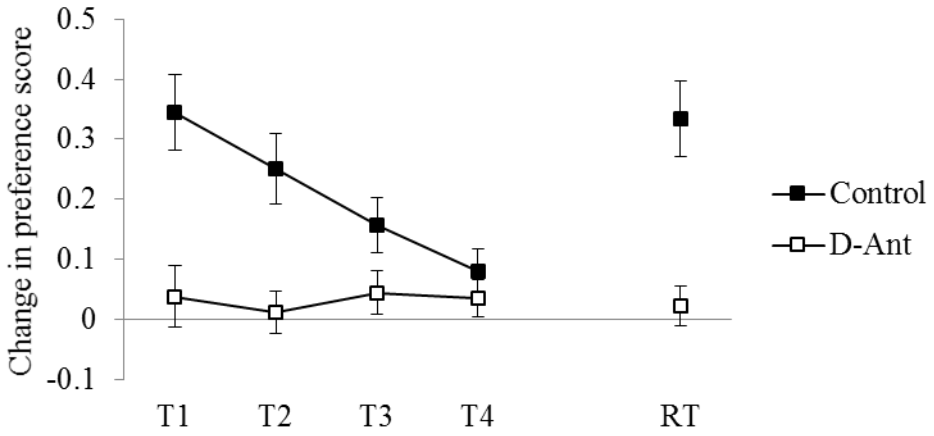


Figure 4.2. Mean change in preference score (± 1 *SEM*) to the target context during the extinction (T1-T4) and reinstatement test (RT) trials of the test phase of the experiment.

4.2.3. Discussion

During training, animals treated with sucrose showed significantly lower levels of activity than animals exposed to treated water; this replicated the findings of Experiments 1 and 4 (Chapter 3). Similarly, animals treated with the dopamine antagonist showed significantly lower levels of activity than animals treated with sucrose or water.

It is generally agreed that the dopamine system is involved in the locomotor activity in planaria (Nishimura et al., 2007; Palladini et al., 1996). The observed reduction in the locomotor activity in animals treated with the dopamine D1 antagonist is consistent with other studies in planaria such as Buttarelli et al., (2000), who found that treatment

of animals with a dopamine antagonist resulted in a hypomobility; however, treating the animals with a dopamine agonist would produce a reverse effect (hyperactivity).

Animals treated with SCH 23390 (1µM) solution during the training phase of the experiment failed to develop a CPP response compared with animals in the group control. These data suggest that the D1 antagonist drug (SCH 23390) blocks the acquisition of the learned conditioned response when it is given during the training cycles. In the control group, however, animals exposed to sucrose in the less preferred context developed a CPP response which extinguished during the non-reinforced test trials. Subsequent exposure to sucrose in a different context reinstated the CPP response. This suggests that CPP in planaria is an example of a standard Pavlovian response. This result suggests that CPP in planaria is an example of a standard Pavlovian response; and provide evidence that the observed effect is extinction rather than forgetting. However, future studies might include another control group, for instance, animals trained and tested same as animals in the experimental group but without the exposure to the US before the reinstatement test.

Similar results were reported by Alvarez et al. (2014) in snails using an appetitive conditioning task: repeated presentation of a conditioned stimulus without the presence of the unconditioned stimulus resulted in a clear decline in the conditioned response compared with a control group. They also found that re-exposure of the animals to the unconditioned stimulus used during conditioning reinstated the conditioned response.

Moreover, an experiment by Amaning-Kwarteng et al., (2017) used a procedure originally developed by Hutchinson et al. (2015) to show that a cocaine mediated CPP response can be reinstated after extinction. In their experiment, however, after the extinction of the conditioned response, animals were exposed again to the drug in the same context used during the conditioning. This could strengthen the CS-US association developed during the conditioning, an example of reconditioning. In the present experiments, a distinctive context (a 5 cm glass petri dish) was used during the re-exposure to sucrose before the reinstatement test. This context is different from the one that was used in the conditioning session avoiding additional CS-US pairings. Our results, therefore, strongly suggest that the recovery of the response following exposure to the sucrose-US is a genuine example of reinstatement, showing that the learning

acquired during training was not eliminated by extinction (Bouton & King, 1983). The present results confirm previous results reported with rats and planaria.

Ågmo et al. (1995) state that the dopamine system plays a vital role in the establishment CPP responses, which involve the development of an association between the unconditioned stimulus (reward agent) and the conditioned stimulus (environmental cues). Furthermore, Cervo and Samanin (1995) found that rats treated with a dopamine D1 antagonist (SCH 23390 0.1-0.2 mg/kg i.p) before the exposure to cocaine in the conditioning phase results in a significant blockage of the CPP. In planaria, Zhang et al. (2013) suggested that the rewarding effect of sucrose is dependent upon the dopamine reward system like in vertebrate animals. They found that animals exposed to sucrose and a dopamine antagonist (D1 and D2 antagonist drugs: SCH 23390 and sulpiride) did not develop CPP.

4.3. Experiment 7: role of dopaminergic system in CPP and tolerance

The findings of Experiment 6 suggest that, in planaria, dopamine plays an important role in movement control as well as in reward-related learning. Experiment 7 compared the effects of blocking the D1 dopamine receptors on the development of CPP and tolerance. Our hypothesis is that CPP but not tolerance development is dependent on the reward system; therefore, the development of tolerance should not be affected by treatment with a dopamine antagonist.

4.3.1. Method

4.3.1.1. Subjects and Materials

Thirty-two brown planaria (*Dugesia*) were used in this experiment. We used the same petri-dishes with two surfaces (smooth and rough) described in the previous experiments. During training, animals could be exposed to a 10% sucrose solution;

treated water; a 1μM SCH 23390 solution; or a 10% sucrose and a 1μM SCH 23390 solution

4.3.1.2. Procedure

Animals were assigned at random to one of two groups: the Control group (n=16) and the D-Ant (n=16) which was treated with a dopamine antagonist during the training. There were three phases in the experiment: pre-training test, training, and post-training test (see Figure 4.3.). The pre-training and training phases (days 1-13 of the experiment) followed the same procedure described for CPP above; the only change is that the animals were given six instead of four cycles of training. After the completion of the training phase all the animals were given one CPP Test (for example on Day 14) and a Water Test (days 15-16) to assess the development of the hyperactivity conditioned compensatory response. In this experiment, the preference score was calculated for the smooth context, which was the less preferred by all the animals. The order of the tests and the order in which animals were exposed to the Trained and Control contexts during the Test Water were counterbalanced across subjects. There was a one cycle of re-training between the first block of test trials and the second block of test trials.

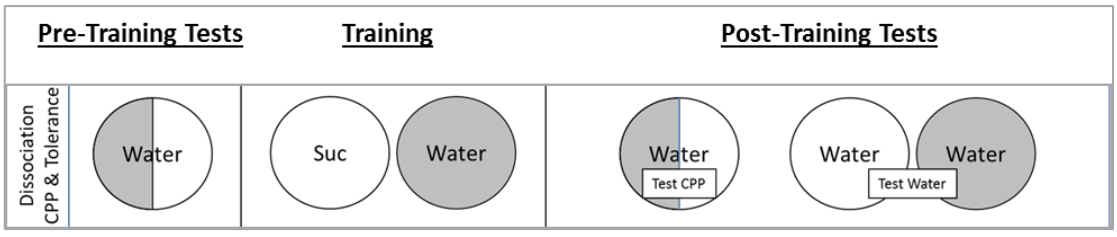


Figure 4.3. Schematic representation of the procedure of Experiment 7. The white and shadowed areas represent the two surfaces used in the experiment (plastic and sand textures). Sucrose refers to the presentation of a 10% sucrose solution in the trained context.

4.3.2. Results

During the Pre-Training Test, animals in group Control showed a preference score (for the less preferred context) of 0.33 (±0.02 *SEM*); the animals in group D-Ant showed a

preference score of 0.33 (±0.01 *SEM*). A One-Way ANOVA showed that there were no differences between the groups, $F<1$.

The data of the training phase of the experiment are displayed in Figure 4.4., which shows the activity of the two groups of animals across two blocks of three cycles of training trials in the contexts associated with sucrose and water. Animals showed lower levels of activity in the presence of sucrose than in the presence of water. Also, animals in the D-Ant group, exposed to the dopamine antagonist, showed lower levels of activity than the Control group. An ANOVA with Group (Control vs. D-Ant), Stimulus (Sucrose *vs.* Water) and Blocks (of Cycles of Training) showed a significant effect of Group, $F(1,30)=160.45$, $p<0.01$, $\eta_p^2=.84$, Stimulus, $F(1,30)=165.05$, $p<0.01$, $\eta_p^2=.84$, and Blocks, $F(1,30)=6.36$, $p<0.05$, $\eta_p^2=.17$. The interaction Stimulus x Group was also significant, $F(1,30)=35.89$, $p<0.01$, $\eta_p^2=.54$. Further analyses carried out to assess the Stimulus x Group interaction showed that the Stimulus factor was significant for the Control group, $F(1,15)=184.46$, $p<0.01$, $\eta_p^2=.92$, as well as for the D-Ant group—although to a lesser extent, $F(1,15)=22.60$, $p<0.01$, $\eta_p^2=.60$.

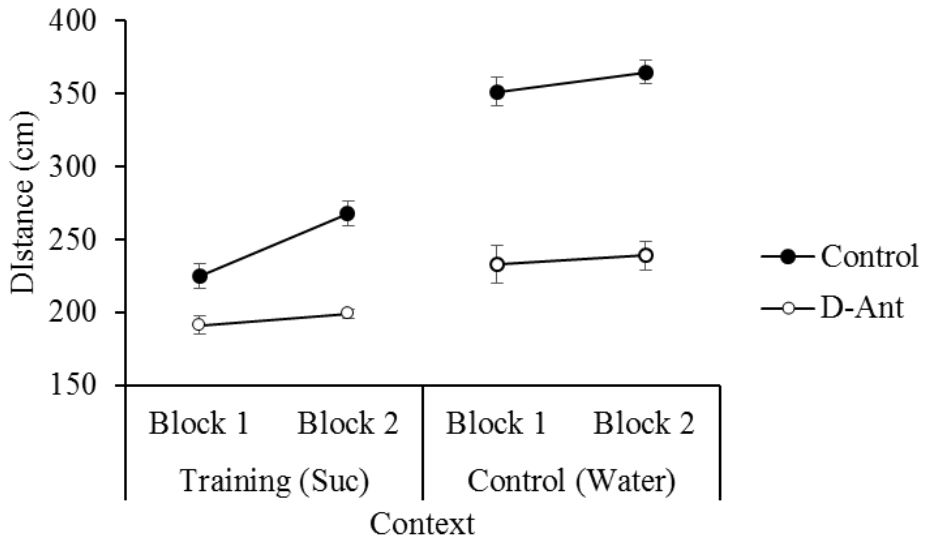


Figure 4.4. Mean distance (±1 *SEM*) covered by the animals in the contexts were Sucrose (Training) and Water (Control) were presented over two blocks of three cycles of training during the training phase of the experiment.

The significant effect of blocks suggested a general increase in the levels of activity throughout the training trials. However, as can be seen in Figure 4.4, it is the increase of activity in the Control group in the presence of sucrose what mainly contributes to the general increase of activity across the training cycles. Although the triple interaction Stimulus x Group x Blocks was not significant, $F(1,30)=2.20$, $p=0.10$, additional analyses were performed on the effect of the exposure to sucrose and water on the activity across blocks of trials for the two experimental groups separately. An ANOVA with Stimulus (Sucrose vs. Water) and Blocks carried out on the data of the group Control showed a significant effect of Stimulus, $F(1,15)=184.79$, $p<0.01$, $\eta_p^2=.92$, and Blocks, $F(1,15)=11.00$, $p=0.005$, $\eta_p^2=.42$, and a nearly significant Stimulus x Blocks interaction, $F(1,15)=3.87$, $p=0.06$, $\eta_p^2=.20$. Analysis of the main effects showed a significant increase in the levels of activity in the presence of sucrose across the two Blocks of Cycles of training, $F(1,15)=13.49$, $p=0.002$, $\eta_p^2=.47$, but not in the presence of water, $F(1,15)=1.52$, $p=0.23$; this suggests that animals in the Control group developed tolerance to the effects of the sucrose. The same analysis on the data of the D-Ant group showed a significant effect of Stimulus, $F(1,15)=22.60$, $p<0.01$, $\eta_p^2=.60$; however, neither the Blocks factor nor the Stimulus x Blocks interaction was significant, Fs<1.

The data of the water test, assessing the hyperactivity conditioned compensatory response, are displayed in Figure 4.5. Animals showed a hyperactivity conditioned response in the trained context (associated with sucrose during training) compared to the control context both in the groups Control and D-Ant. An ANOVA with Group (Control vs. D-Ant) and Context (Trained Context *vs.* Control Context) showed a significant effect of Group, $F(1,30)=4.198$, $p=0.04$, $\eta_p^2=.12$, and Context, $F(1,30)=11.96$, $p=0.002$, $\eta_p^2=.28$; the interaction Treatment x Context was not significant $F<1$.

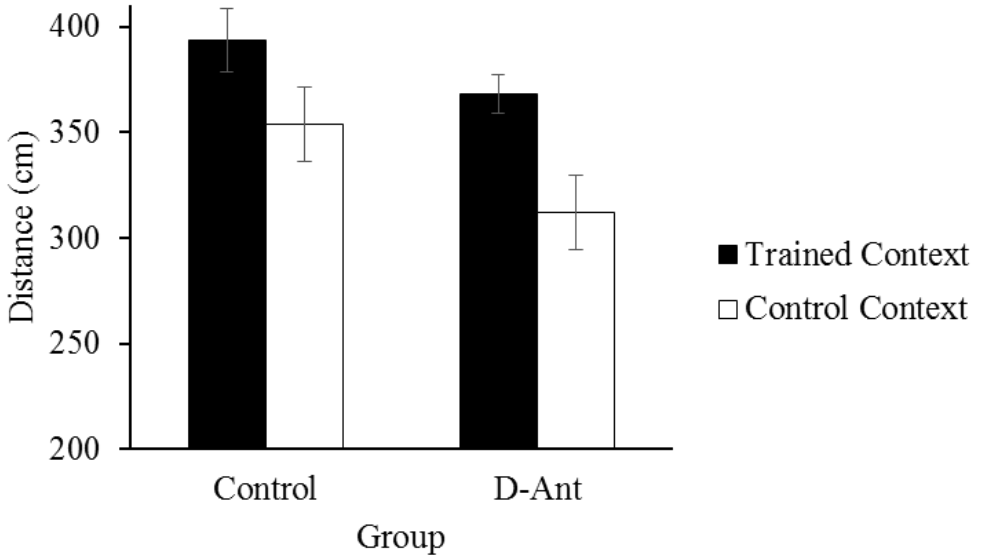


Figure 4.5. Mean distance (±1 *SEM*) covered by the animals in the groups Control and D-Ant in the Trained and the Control contexts in the presence of water during the conditioned compensatory response test.

The data for the Post-Training CPP Test corresponding to the change in preference scores for the initially less-preferred context are displayed in Figure 4.6. Animals in the Control group showed strong evidence of CPP whereas animals in the D-Ant group did not show any change in preference. A One-Way ANOVA showed a significant difference between the groups, $F(1,31)=51.57$, $p<0.01$.

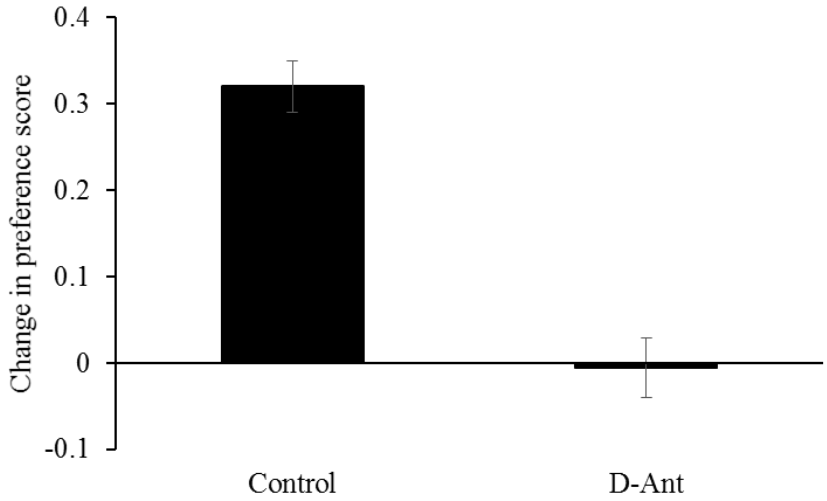


Figure 4.6. Mean change in preference score (± 1 *SEM*) to the target context during the CPP Test.

4.3.3. Discussion

During the training phase, animals in the group control showed lower levels of activity in the presence of sucrose than in the presence of water; similarly, the animals treated with the dopamine antagonist in group D-Ant showed lower levels in the presence of sucrose and water (this is similar to the data of Experiment 6 in this chapter). However, animals in group control showed a significant increase in their activity during the conditioning with sucrose over the training cycles. These data, indicative of the development of tolerance, are similar to what was found and discussed in the previous experiments (Experiment 4, Chapter 3).

The data from the tolerance test indicated that animals in both groups showed hyperactivity when they were tested in water in the trained context which was paired with sucrose during the training cycles compared with the control context in which the animals always experienced water. In addition, animals in group D-Ant always showed a lower level of activity in both contexts compared with animals in group control. These data suggest that the hyperactivity was shown by the animals in both groups in the sucrose-paired context, evidence that the context activated a conditioned compensatory response (CCR) to counteract the effect of the sucrose. This, again, replicates the findings discussed in the previous experiments (Experiment 4, Chapter 3). Interestingly, this experiment suggests that the dopamine system does not play a role in the development of tolerance; animals treated with the dopamine antagonist still show evidence of developing a CCR in the trained context despite the fact that these animals did not show an increase in their activity during the conditioning sessions when they were exposed to either sucrose or water in combination with the dopamine antagonist. Simply, this could be because the effect of the dopamine antagonist masks the development of tolerance during the training.

Finally, the data of the CPP test showed that animals in the group control, treated with 10% sucrose, developed a significant CPP response; however, animals in the group D-Ant, showed a complete failure to develop CPP to sucrose; this replicates the results of the previous experiments (Experiment 6 in this chapter). These data allow a dissociation of CPP and Tolerance strongly suggesting that they depend on independent learning mechanisms.

4.4. Experiment 8: the role of dopaminergic system in the CPP extinction

The purpose of Experiment 8 was to assess the role of the dopaminergic system on the CPP extinction using sucrose as a reward substance. This is important to establish the role of the dopamine system in the learning mechanism responsible for extinction.

4.4.1. Methods

4.4.1.1. Subjects and Material

Sixteen brown planaria (*Dugesia*) were used in this experiment. The same petri- dishes used in the previous CPP experiments were used here. Animals could be exposed to a 10% Sucrose solution; treated water; and a 1µM SCH 23390 solution.

4.4.1.2. Procedure

The procedure of this experiment was similar to the procedure described for the previous experiments of CPP with three phases: pre-training test, training and post-training tests. For the pre-training test, the animals were exposed to treated water in one of the two-sided (half smooth—half rough) petri dishes. They were allowed to freely move for 30 minutes; the time spent in each of the two surfaces of the petri dish was recorded and a preference score was calculated for the less preferred surface. The animals in this experiment were assigned to two groups (n=8) matched by the preference score: Group control and Group D-Ant. In this experiment, for each group, the preference score was calculated for the smooth context, which was the less preferred by half of animals (n=4); and to the rough context, which is the less preferred by the other half of animals (n=4).

In the training phase of this experiment, planaria were exposed to the two surfaces (rough and smooth) in alternating days. Planaria in both groups were exposed to the less preferred context in the presence of a 10% sucrose solution; and to the preferred context in alternate days in the presence of treated water during the conditioning phase.

During the post-training tests, planaria were exposed again to the two sided petri-dishes. Planaria were tested in consecutive days (days 10-13 of the experiment); the first test trial took place 24 hours after the training session; the interval between the different test trials was always 24 h. During the post-training test trials, the animals in group control were tested in the two-sides petri dishes in the presence of treated water, while animals in the group D-Ant were tested in the presence of a 1μM SCH 23390 solution. Twenty-four hours after the last extinction trial (Post-Training Test 4), all the animals were exposed for 30 minutes to a 10% sucrose solution in a different petri-dish (the 5-cm glass petri-dish), a reinstatement procedure. 24 hours later, all the animals were tested again by using the two-sided petri dishes to test the reinstatement of the conditioned place preference response.

4.4.2. Results

The data of the Training phase for all animals in the experiment, are displayed in Figure 4.7. Animals showed lower levels of activity in the presence of sucrose than in the presence of water. A repeated measures ANOVA with Treatment (Sucrose *vs.* Water showed a significant effect, $F(1,15)=133.7$, $p<0.01$, $\eta_p^2=.89$.

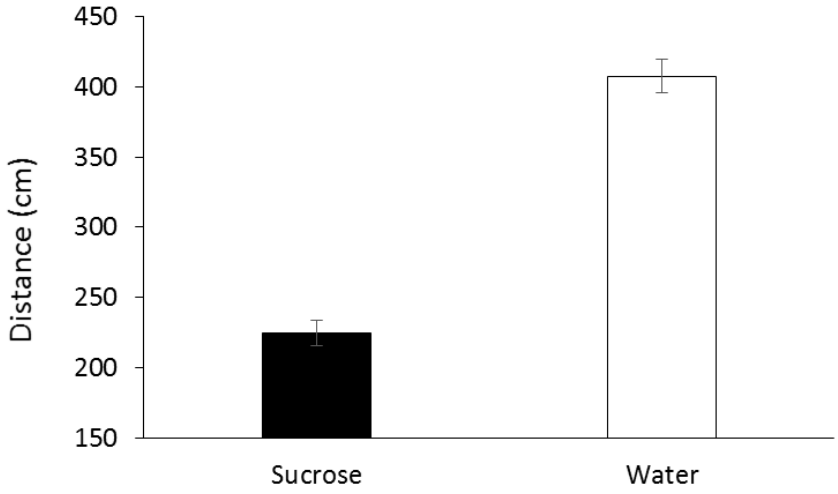


Figure 4.7. Mean distance (±1 *SEM*) covered by the animals in the contexts were Sucrose (Training) and Water (Control) were presented over training cycles during the training phase of the experiment.

During the Pre-Training Test, animals in group control showed a preference score (for the less preferred context) of 0.22 (±0.06 *SEM*); the animals in group D-Ant showed a preference score of 0.23 (±0.06 *SEM*). A One-Way ANOVA showed that there were no differences between the groups, $F<1$.

The data for the CPP Extinction and Reinstatement Tests corresponding to the change in preference scores for the initially less-preferred context are displayed in Figure 4.8. We observed an increase in the preference score in the first test (T1) after training and then a decrease in the preference score over the test trials (T1-T4). An ANOVA with Test Trials (T1-T4) and Group (Control vs. D-Ant) showed a significant effect of Test trials, $F(3,42)=6.16$, $p<0.01$, $\eta_p^2=.30$, neither the factor Group nor Group x Test Trials interaction was significant, $F<1$. The data of the Reinstatement Test are displayed on the right hand part of Figure 4.8. An ANOVA was carried out to compare the two groups over the last extinction trial and the reinstatement test trial, showing a significant reinstatement effect for both groups: the test trials factor was significant, $F(1,14)=34.09$, $p<0.01$, $\eta_p^2=.70$. There were no significant differences between the groups, and the Group x Test Trials interaction was also not significant, Fs<1.

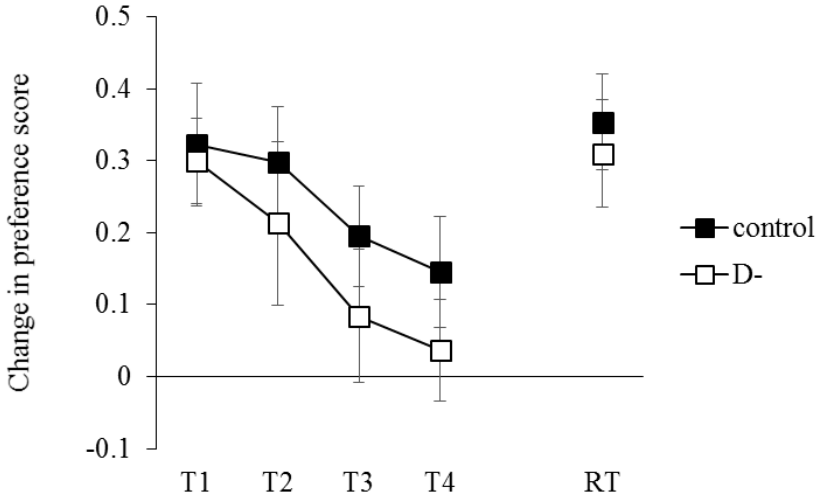


Figure 4.8. Mean change in preference score (±1 *SEM*) to the target context during the extinction (T1-T4) and the reinstatement test (RT) trials of the test phase of the experiment.

4.4.3. Discussion

Experiment 8 tested the role of the dopamine system on the expression and extinction of sucrose CPP. Animals tested with a SCH 23390 (1μM) solution during the extinction test trials showed a normal expression and extinction of CPP, the same as the animals tested with plain water in the control group. In addition, animals in both groups showed a significant reinstatement of the conditioned response after a single re-exposure to the 10% sucrose. These data show that the D1 antagonist drug (SCH 23390) did not affect the expression, extinction and reinstatement of the conditioned response during the test trials. This experiment had power > 80% to detect effect sizes of d or greater. In addition, in this thesis there are number of experiments that showed a significant main effect of group with n=8 animals per group. For example, Experiment 1 in Chapter3, $F(3,28)=6.45$, $p=0.002$, $\eta_p^2=.40$; and Experiment 12, Chapter 5, $F(1,15)=8.419$, $p=0.01$, $\eta_p^2=.12$ that showed a significant development of CPP.

Our results are similar to those reported in vertebrate species. Cervo and Samanin (1995) studied the effect of different dopaminergic (D1 and D2) receptor antagonists (SCH 23390 and sulpiride); and a glutamatergic NMDA (MK-801) on the acquisition and the expression of CPP to cocaine in rats. They demonstrated that treatment with either a dopamine antagonist or a glutamate antagonist significantly blocked the acquisition of CPP to cocaine but it did not affect its expression when it was given before the test session. In addition, Inoue, Izumi, Maki, Muraki, and Koyama (2000) found that rats treated with SCH 23390, a D1 antagonist, before the conditioning phase of fear conditioning showed significant reduction in the conditioned freezing behaviour. However, giving the same drug after conditioning or during the test did not prevent the expression of the fear conditioning. Moreover, Dickinson et al. (2003) assessed the effect of SCH23390 (a dopamine D1 antagonist), raclopride (a dopamine D2 antagonist) and U99194A (a dopamine D3 antagonist) on the CPP expression to ethanol in mice. They found that animals conditioned with ethanol developed a significant CPP; and that the treatment with either of the dopamine antagonists before the test phase of the experiment did not prevent the establishment of CPP. Zhang et al. (2013) reported similar results in planarian. They found that planaria developed a significant CPP to 1% sucrose, and this effect was prevented by exposing the animals to either a dopamine D1 antagonist, SCH 23390 (1 μM) or a dopamine D2 antagonist, sulpiride (1 μM) during

the conditioning phase of the experiment. However, they showed that both treatments did not affect the expression of CPP when they were presented during the test session of the experiment. These data suggest that both, dopamine D1 and D2 antagonists, interfere with the acquisition of CPP but not its expression.

4.5. Experiment 9: Conditioned Place Aversion

The main aim of Experiment 9 was to assess whether the dopamine D1 antagonist (SCH 23390) results in the development of a Conditioned Place Aversion. The importance of this experiment is to provide evidence about the role of the dopamine system in the previous experiments and to confirm that the reported effects are due to the effect of the dopamine antagonist drug in the prevention of CPP rather than the possibility of the development of an opposed aversive response.

4.5.1. Method

4.5.1.1. Subjects and Materials

Eight brown planaria *(Dugesia*) were used in this experiment. The same petri- dishes used in the previous experiment were used in the present experiment. Animals could be exposed to treated water or a 1µM SCH 23390 solution.

4.5.1.2. Procedure

The procedure of this experiment was similar to the procedure described for the previous CPP experiments with three phases: pre-training test, training and post-training tests. During the pre-training and post-training tests, animals were tested with water in the two-sided petri dishes (half smooth —half rough). Time spent in each side of the petri dishes was recorded and the preference score was calculated for the preferred context. In this experiment, the preference score was calculated for the smooth context, which was the preferred by half of animals (n=4); and to the rough context, which is the preferred by the other half of animals (n=4). During the training phase of this

experiment, planaria were exposed to water in the less preferred context and to 1µM SCH 23390 solution in the preferred context in alternating days.

4.5.2. Results

The data of the Training phase of the experiment are displayed in Figure 4.9. Animals showed a reduction in their activity in the presence of the dopamine antagonist. An ANOVA with Treatment (D- *vs.* Water) showed a marginally significant effect of Treatment, $F(1,7)=5.36$, $p=0.05$, $\eta_p^2=.43$.

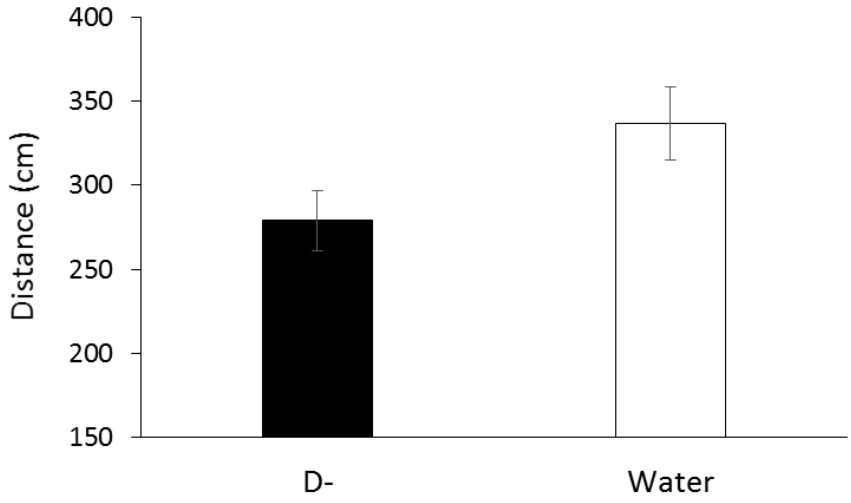


Figure 4.9. Distance (±1 *SEM*) covered by the animals in the context rewarded with the dopamine antagonist and the context treated with water during the training phase of the experiment.

The data for the Pre- and Post-Training Tests are displayed in Figure 4.10. A repeated measures ANOVA with Test (Pre- and Post-) showed no significant change in the preference scores, $F<1$.

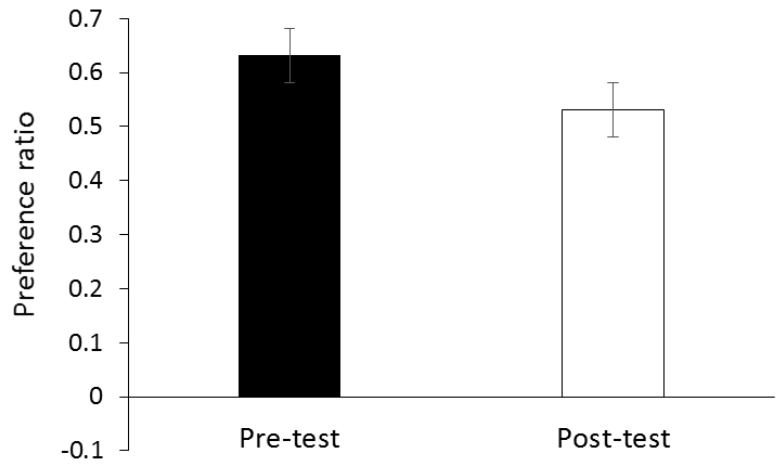


Figure 4.10. Change in preference ratio (±1 *SEM*) for the initially preferred context for the Pre-Test and Post-Test sessions of the experiment.

4.5.3. Discussion

In the present experiment, we tested the effect of the dopamine D1 antagonist SCH 23390 system on the development of place learning. Animals treated with SCH 23390 (1µM) solution during the training phase of the experiment showed a lower level of motor activity in the presence of D1 antagonist than in the presence of plain water. This replicates what we have previously observed in previous experiments (Experiments 6 and 7 in this Chapter).

During the test trial, the animals did not show any change in place preferences. The marginal reduction in the preference score from 0.6 to 0.5 observed was non-significant and can merely reflect the development of habituation after equal and repeated exposure to the two contexts throughout training. This experiment had power > 80% to detect effect sizes of d or greater. In addition, in this thesis there are number of experiments that showed a significant main effect of group with n=8 animals per group. This is consistent with the results reported by Risinger, Brown, Oakes, and Love (1999), who found that mice given pairings of a flavour-CS and ethanol-US developed a conditioned taste aversion (CTA). However, treatment with haloperidol (a D2 antagonist) or SCH 23390 (a D1 antagonist) reduced the action of ethanol in producing CTA. Moreover, neither haloperidol nor SCH 23390 were effective on their own in developing CTA. Similarly, Pina and Cunningham (2014) assessed the role of the

dopamine system in the acquisition of CPP produced by ethanol in mice. Also, they assessed whether treatment with SCH23390 interferes with the development of CPA to ethanol after pairing it with LiCl during conditioning; or whether SCH23390 by its own produces CPP or CPA. They found that the treatment with dopamine D1 antagonist (SCH23390), but not dopamine D2 antagonist (raclopride) before training blocks the CPP to ethanol. However, it did not interfere with the development of CPA; and did not result in a development of either CPP or CPA when it was tested by its own. The present data are important because they confirmed that the action of the dopamine antagonist in our experiments is the prevention of the development of CPP (appetitive learning mediated by sucrose) rather than the development of a conditioned place aversion.

4.6. General discussion and conclusions

The experiments outlined in the chapter aimed to assess the role of the dopamine system in the development of addictive-like behaviours in planaria. It has been often suggested that, in planaria, dopamine plays an important role in movement control (e.g., Buttarelli, et al., 2002) as well as in reward-related learning. In our experiments, animals treated with a dopamine D1 antagonist did not develop a CPP response. The present results confirm that the dopamine reward system mediates the establishment of appetitive Pavlovian conditioned responses like CPP.

The data of this chapter also showed that the sucrose-mediated CPP extinguishes in the absence of the reward and can be reinstated by exposure to the rewarding agent. Animals exposed to the conditioned stimulus (CS) in the absence of the rewarding unconditioned stimulus (US) showed a gradual decline in the learned conditioned response. It is well established that extinction does not delete the original learning (the excitatory association between the CS and the US); it results in an inhibitory association (the CS means the absence of the US). These two associations control the behaviour displayed by the animals (e.g., Bouton, 1994). Therefore, following CPP acquisition, animals display the conditioned response. However, in the extinction phase, the development of an inhibitory association will compete with the excitatory association, and the animals will not show the conditioned response. Moreover, one single exposure

to the rewarding substance facilitates the retrieval of the excitatory memory and reinstates the conditioned response in the presence of the CS alone (Bouton 2004; Bouton and King, 1983; Shaham, Shalev, Lu, De Wit, & Stewart, 2003). This confirms that CPP can be characterised as a standard Pavlovian conditioned response in planaria. Future studies, however, might include another control group (e.g., animals treated as same as the previous groups but without the exposure to the US before the reinstatement test). Treatment with the dopamine D1 antagonist does affect acquisition of CPP (the establishment of an excitatory association), but not its extinction (inhibitory learning) or expression. These data suggest that the dopamine system is important for the development of CPP, however, different neurochemical systems could be involved in the extinction or the expression of the conditioned response.

In this chapter, the effects of the dopamine antagonist itself in the development of conditioned place aversion was also assessed. Animals were exposed to the dopamine antagonist in the preferred surface and to water in the less preferred surface. The experiment showed that exposure to the dopamine antagonist did not altered the preferences shown by the animals in the pre-conditioning test. These evidence strongly suggest that treatment with the D1 antagonist SCH-23390 prevents the development of CPP rather than the development of a conditioned aversion.

The most significant result of this chapter is the dissociation between two learning mechanisms, CPP and tolerance, using a dopamine antagonist. During the development of CPP, animals show a clear preference to the cues associated with a reward substance. The CPP is an example of appetitive learning ruled by the same principle of Pavlovian conditioning and seems to be controlled by the dopamine reward system. However, the dopamine system did not play a role in the development of tolerance to the effect of sucrose; animals treated with D-Ant still show evidence of developing a conditioned compensatory response in the trained context with sucrose. It is suggested that different mechanisms are involved in these two phenomena; and this will be further discussed in Chapter 6.

Chapter 5: The role of the cholinergic system in sucrose addiction in planaria

5.1. Introduction

5.1.1. Short literature review and experimental rationale

Acetylcholine (Ach) can be found in the nervous system of both vertebrates and invertebrates, playing a range of different functions: it is involved in the excitatory mechanism at the neuromuscular conjunction; it is responsible for smooth and cardiac muscle contraction; stimulates muscles to initiate locomotor activity; and regulates glands secretion. It is also involved in the regulation of different activities including attention, sleeping, dreaming, learning and memory (Hasselmo, 2006).

Boccia et al. (2003) reported that Ach plays an important role in the encoding of memory and the retrieval of information. They found that mice treated with a cholinergic agonist drug (e.g. 70.0 or 150.0 μg/kg of Physostigmine) or a muscarinic acetylcholine antagonist drug (e.g. 1.0 mg/kg of Atropine) act differently during a task of inhibitory avoidance. It was observed that animals treated with Ach agonist showed a significant increase in the performance of the task, an effect that was prevented by previous treatment with atropine only when the Ach antagonist was given immediately before the agonist. A 10 minutes delay between the two injections eliminated the interfering effect of the antagonist. These data suggest that atropine, by blocking the cholinergic system, interfered with the retrieval of learned information. The retention of the inhibitory avoidance response during the test is modulated by the interaction between the cholinergic agonist and antagonist drugs. Similarly, Zangeneh and Bakhtiarian (2006) confirmed that Ach is necessary for memory processes, specially in the retrieval phase, and that animals treated with scopolamine (an Ach antagonist) fail to retrieve information previously acquired.

Ach is also involved in the modulation of the acquisition and encoding of information, as well as the extinction and expression of learned responses. Robinson et al. (2011) indicated that the blockage of either the muscarinic or the nicotinic acetylcholine receptors by antagonist drugs results in short term memory impairment, preventing the

acquisition of conditioning. Likewise, Rotella et al. (2015) assessed the effect of both muscarinic and nicotinic acetylcholine receptors antagonist on the acquisition and the expression of a conditioned flavour preference (CFP) responses using fructose as the reward, and a conditioned flavour aversion (CFA) using quinine in rats. In the CFP procedure, animals were exposed to 8% fructose + 0.2% saccharin in the present of a grape or cherry flavour (CS+) and 0.2 saccharin+ flavour without the fructose (CS-) in alternating days. In the CFP procedure animals were given pairings of the fructose and saccharine with quinine (CS+); and fructose and saccharine (CS-). To examine the role of the muscarinic antagonist (scopolamine) and the nicotinic antagonist (mecamylamine) on the expression of the CFP, animals in the different experimental groups received an injection of the drugs 30 min before either the beginning of the training or the test sessions. They found that the treatment with the muscarinic receptor antagonist but not with the nicotinic receptor antagonist impaired the acquisition of the CFP when the drugs were given before the training trials; and did not have a significant effect on the expression of the CFP when the drugs were given before the test sessions. Also, they found that blocking the nicotinic receptors by mecamylamine had a significant positive effect on the development of the CFA produced by quinine. These data suggested that different receptors play various roles in the different stages of the development of appetitive and aversive conditioning.

Nisanov, Galaj, and Ranaldi (2016) confirmed that blocking the muscarinic acetylcholine receptors by scopolamine prevents the acquisition of reward learning but has no effect on its expression in rats. During a pre-training phase, animals were exposed to two levers. Pressing one lever led to the presentation of a light; pressing the second lever led to the presentation of a tone. During the training, the animals were given pairings of the light with a food-US. The animals were then tested in the presence of the two levers: effective conditioning should result in a preference to press the lever associated with the presentation of the light. Animals in the experimental condition were treated with scopolamine whereas animals in the control group were given vehicle. The drug could be presented before every single training session (to assess the acquisition) or before each test session (to assess the expression). The authors found that animals treated with scopolamine before conditioning did not develop a significant increase in their preference compared with animals treated with the drug just before the test session

or animals in the control group. These data suggested that scopolamine blocks the acquisition of the development of the CR but not its expression.

Interestingly, research with planaria has shown the presence of dopamine (Palladini et al., 1996; Passarelli et al., 1999; Zhang et al., 2013) and acetylcholine (Buttarelli et al., 2002) in their bodies. In addition, the function of these neurotransmitters in planaria are related and equivalent to vertebrates. Buttarelli et al. (2000) revealed that there is an interaction between the cholinergic and the dopaminergic receptors in planaria (as in vertebrates). They showed that animals exposed to physostigmine or nicotine (cholinergic agonist drugs) established a hypomobility and an increase in abnormal movements such as bridge-like and walnut shape which were reversed by treatment with atropine, a cholinergic antagonist. However, animals treated with nomifensine or apomorphine (dopamine agonist drugs) showed hyperactivity and screw-like movements which were reversed by treatment with dopamine antagonist drugs such as SCH 23388 or sulpiride.

Nishimura et al. (2010) highlighted the role of the cholinergic system in the planaria using pharmacological and RNA interference techniques. They found that animals exposed to physostigmine (Ach+) showed a muscle contraction as a result of increasing the Ach level. However, pre-treating the animals with either a muscarinic Ach receptor antagonist drug (atropine) or nicotinic Ach receptor antagonist (tubocurarine) prolong the latency time of the contraction produced by the physostigmine. In addition, they identified the gene of choline acetyltransferase (Djchat) which plays a crucial role in the synthesis of the Ach in planaria using the immunofluorescence technique.

Ramakrishnan, Amatya, DeSaer, Dalhoff, and Eggerichs (2014) confirmed the involvement of the cholinergic system in the locomotor activity of planaria. They tested the effect of treatment with different concentration of a muscarinic cholinergic antagonist, scopolamine, at different doses (0.001, 0.01, 0.1, 0.5 and 1.0 mM) on animals' behaviour: locomotion and the development of stereotypical activity. They found that planaria exposed to a high concentrations of scopolamine (1mM) showed a significant reduction in the activity in comparison with animals in the other groups. They also found that animals treated with a mild concentration of scopolamine (0.1 and 0.5mM) showed c-like hyperkinesia.

Ramakrishnan et al. (2014) also assessed the effect of scopolamine on the development of associative learning using a Pavlovian conditioning paradigm in which a CS was paired with a shock (see Prados et al., 2013). They found that pre-conditioning exposure to scopolamine prevents the learning about the association CS-US in comparison with other three control groups (treated with water only, unpaired presentation of the CS-US, and random presentation of CS-US). In addition, they reported that the negative effects of the scopolamine on both the locomotion and the associative learning was significantly counteracted by the treatment with a low concentration of galantamine, an acetylcholinesterase inhibitor that reduces the hydrolysis of the Ach and increases its level. These data confirmed the significant role of the cholinergic system in different behaviour tasks and learning mechanisms, it also showed that the effect of the drugs depends on the concentration used.

Ach and dopamine play a role in the regulation of the appetite and the progress of eating disorders such as binge eating and obesity. Sucrose is a substance which produces dependent- like behaviour such as withdrawal, craving, CPP and tolerance. Consumption of sucrose also alters the neurochemical balance in a way similar to drugs of abuse (like cocaine). The cholinergic system is actively involved in the modulation of behaviour that results from food or drug abuse, and its action is related to the dopamine and opioid systems (Kelley et al., 2005). Some studies have suggested a negative correlation between acetylcholine and dopamine following drug consumption. For example, the level of acetylcholine increases while the level of dopamine is reduced in rats that were induced morphine withdrawal (Rada et al., 1991). Similar results have been observed in animals that had access to sucrose: treatment with a muscarinic agonist injection inhibited the feeding mechanism while an injection of a muscarinic antagonist drugs enhanced the feeding mechanism (Mark et al., 2011).

Taking in to account all these points, five experiments were designed to further our understanding of the effects of atropine in planaria given access to sucrose by assessing: 1) the effect of different concentration of atropine (a non-selective muscarinic acetylcholine antagonist) on the locomotor activity and determine the effective concentration of atropine that could be used in the following experiments; 2) the effect of the cholinergic system on the development of tolerance after repeated exposures to sucrose in a particular context in the presence of atropine using the same method which

was used previously with the dopamine antagonist (see Chapter 4); 3) the role of the cholinergic system in the development of CPP; 4) the role of the cholinergic system on CPP extinction; and finally, 5) whether the atropine by itself results in conditioned place aversion.

5.2. Experiment 10

5.2.1. Experiment 10 A: atropine effect on locomotion

The main purpose of experiment 10 A was to assess the effect of Atropine (0.2 mM) on locomotor activity in the planaria.

5.2.1.1. Method

5.2.1.1.1. Subjects and Materials

Twenty-four brown planaria (*Dugesia*) were used in this experiment. The flatworms were held in the same way described for previous experiments. The same petri- dishes used in the previous experiments were used in the present experiment. These dishes could be filled with 5-7 ml treated water (group Control, n=12) or 0.2 mM atropine solution, a non-selective muscarinic cholinergic receptors antagonist (group 0.2mM ATR, n=12). The half-life of atropine is around 2-4 hours; depending on the route of drug administration, the dose and the region where the sample is collected (Adams, Verma, Jackson, & Miller, 1982; Kanto, Virtanen, Iisalo, Mäenpää, & Liukko, 1981). Therefore, in the experiments reported in this chapter, animals were offered an adequate time (20+ hours) to clear out the drugs from their bodies before the test session start. The activity of the planaria (distance covered in different contexts) in a 30 minutes session was registered.

5.2.1.1.2. Procedure

Planaria were exposed to a petri dish with either a rough or a smooth surface (counterbalance) for 30 min. The animals were assigned at random, to two experimental groups. The control group was exposed to treated water; and 0.2 mM ATR group was exposed to 0.2 mM atropine solution. The planaria were allowed to freely move around for the duration of the session, and the distance covered by the animals was recorded over periods of 5 min; we also inspected the effect of the drugs during the first 10 min of the session.

5.2.1.2. Results and discussion

Figure 5.1 displays the distance covered in each 5-minute intervals during a 30 minutes exposure session by the different groups of animals. An ANOVA with Group (0.2 mM ATR *vs.* Control) and Intervals (5, 10, 15, 20, 25, 30 min) showed a significant effect of group, $F(1,22)=4.52$, $p=0.04$, $\eta_p^2=.17$. Neither the factor Intervals nor the Group x Intervals interaction was significant, Fs<1.

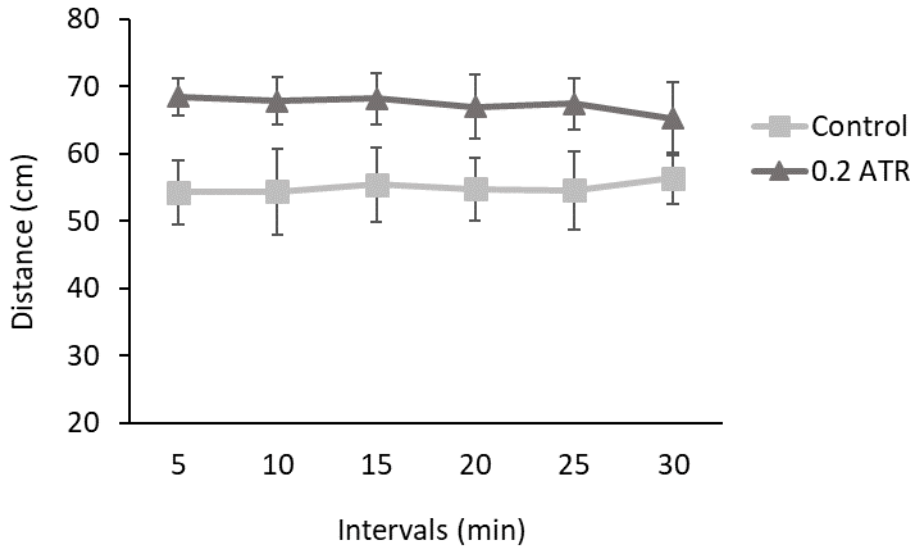


Figure 5.1. Mean distance (± 1 *SEM*) covered by the animals in different groups during the 30 minutes exposure phase of the experiment.

Figure 5.2 displays the distance covered in each 1-minute intervals during the first 10 minutes of the exposure session by the different groups. An ANOVA with Group (0.2 mM ATR *vs.* Control) and Intervals (1-10 min) also showed a significant effect of group, $F(1,22)=5.079$, $p=0.03$, $\eta_p^2=.18$. Neither the factor Intervals nor the Group x Intervals interaction was significant. The data of this experiment indicate that treatment with 0.2mM atropine increases the locomotor activity of planaria.

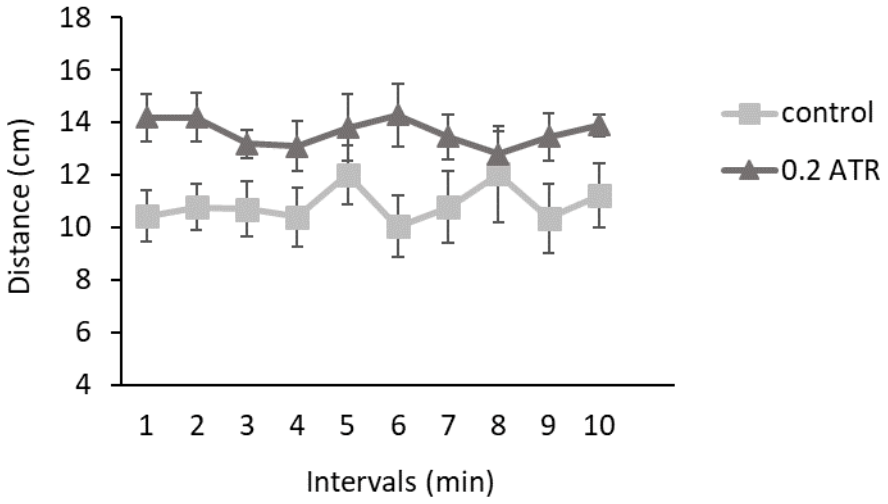


Figure 5.2. Mean distance (± 1 *SEM*) covered by the animals in different groups during the first 10 minutes exposure phase of the experiment.

The present data are similar to previous data reported in rats by Leaton and Rech (1972), who showed that relatively low dosages of atropine enhance the activity of the animals. In addition, Sipos, Burchnell, and Galbicka (1999) assessed the effect of different types of cholinergic antagonists on the locomotor activity during several time intervals in rats. Animals were assigned into 9 experimental groups treated with different types of cholinergic antagonists (aprophen hydrochloride, atropine sulfate, azaprophen hydrochloride, benactyzine hydrochloride, biperiden hydrochloride, diazepam, procyclidine hydrochloride, scopolamine hydrobromide, and trihexyphenidyl hydrochloride). Animals in each group were given a 1 ml/kg intraperitoneally injection of one drug 15 minutes before the experimental trial; this concentration was gradually increased until the animals showed significant changes in their behaviour. The activity of the animals was registered during 23 h after the exposure to the drug; and it was

compared with a baseline activity in two control groups (a group without injection and a group injected with a vehicle). They found that the activity of the animals significantly increased after a short period (1 h) from the exposure to atropine, azaprophen, biperiden, scopolamine, and trihexyphenidyl. However, this increased activity was reduced after 2-6 h from the exposure time. These data suggested that different drugs have a specific effect on animals; and the dosage of the drug, time of exposure and the duration significantly control the effect of the used drug.

5.2.2. Experiment 10 B: effect of treatment with sucrose and atropine on locomotion

The main purpose of experiment 10 B was to compare the effect of 10% sucrose, 0.2 mM atropine, and a mixture of 10% sucrose and 0.2 mM atropine solution with a group control tested in the presence of water on the locomotor activity.

5.2.2.1 Method

5.2.2.1.1. Subjects and Materials

Thirty-two brown planaria (*Dugesia*) were used in this experiment. The flatworms were held in the same way described for previous experiments. The same petri- dishes used in the previous experiments were used in the present experiment. These dishes could be filled with 5-7 ml treated water, a 10% sucrose, 0.2 mM atropine or a mixture of 10% sucrose+0.2 mM atropine. The activity of the planaria (distance covered in different contexts) in a 30 minutes session was registered.

5.2.2.1.2. Procedure

Planaria were exposed to one of the surfaces, rough or smooth (counterbalance) for 30 min. The animals were assigned, at random, to four experimental groups (n=8). The control group was exposed to treated water; the remaining three groups were exposed to

10% sucrose, 0.2 mM atropine and a mixture of 10% sucrose + 0.2 mM atropine solution respectively. The planaria were allowed to freely move around for the duration of the session, and the distance covered by the animals was recorded over periods of 5 minutes; we also inspected the effect of the drugs during the first 10 min of the session.

5.2.2.2. Results and discussion

Figure 5.3 displays the distance covered in 5-minute intervals during the 30 minutes exposure session by the different groups of animals. An ANOVA with Group (10% sucrose, 0.2 mM atropine, 10% sucrose+0.2 mM atropine, and Control) and Intervals (5-30 min) showed a significant effect of group, $F(3,28)=158.4$, $p<0.01$, $\eta_p^2=.95$, and Intervals, $F(5,140)=11.9$, $p<0.01$, $\eta_p^2=.28$. The interaction Group x Intervals was also significant, $F(15,140)=5.47$, $p<0.01$, $\eta_p^2=.37$. Post Hoc tests using the Bonferroni correction showed that group 10% sucrose and 10% sucrose+0.2 mM atropine were significantly slower than the group control (ps<0.01). In addition, the group 0.2 mM atropine was significantly more effective than the group control (p<0.01). There were no significant differences between the group 10% sucrose and the group 10% sucrose+0.2 mM atropine.

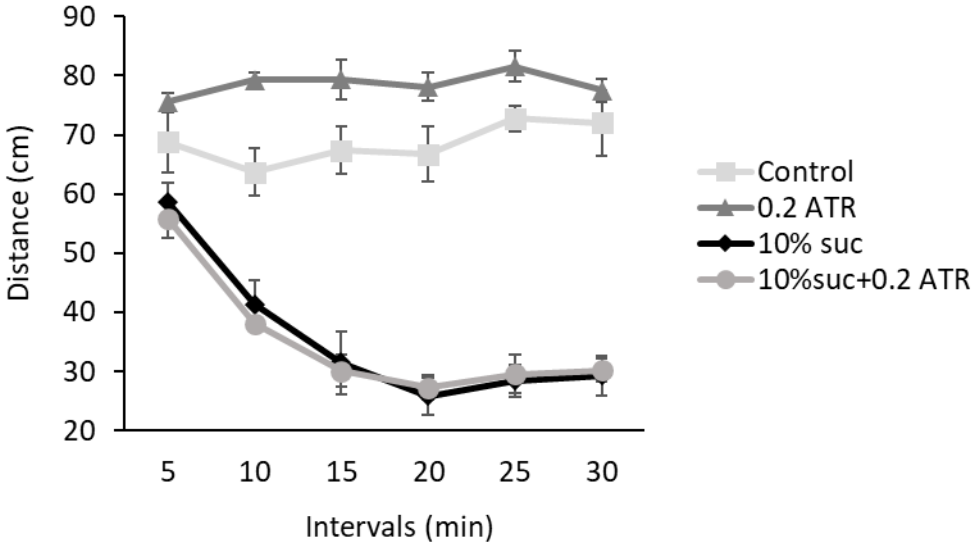


Figure 5.3. Mean distance (± 1 *SEM*) covered by the animals in different groups during the 30 minutes exposure phase of the experiment.

Figure 5.4 displays the distance covered in each 1-minute intervals during the first 10 minutes of the session by the different groups of animals. An ANOVA with Group (10% sucrose, 0.2 mM atropine, 10% sucrose+0.2 mM atropine and Control) and Intervals (1-10 min) showed a significant effect of Group, $F(3,28)=34.22$, $p<0.01$, $\eta_p^2=.78$, and Intervals, $F(9,252)=4.79$, $p<0.01$, $\eta_p^2=.14$. The interaction Group x Intervals was also significant, $F(27,252)=1.82$, $p=0.01$, $\eta_p^2=.16$. Post Hoc tests using the Bonferroni correction showed that groups 10% sucrose and 10% sucrose+0.2 mM atropine were significantly slower than the group control ($ps<0.01$). The group 0.2 mM atropine was significantly more active than the control group ($p<0.01$). There were no significant differences between the group 10% sucrose and the group10% sucrose+0.2 mM atropine in the level of locomotor activity. Further analysis were carried on to analyse the Group x Intervals interaction using a univariate ANOVA with two factors; Sucrose (yes *vs* no) and Atropine (yes *vs* no) during each 1 min intervals along a total 10 min session indicated that the two factors did not show significant differences during the 3 first minutes of the session, $Fs(1,28)\leq 2.715$, $p>0.05$. However, the differences became apparent from the minute 4, the animals exposed to a sucrose solution showed a significant reduction in their activity which started from the min 4 to the end of the session (min10), $Fs(1,28)\geq 10.93$, $ps\leq 0.01$, indicating that it takes few minutes for the drug to affect the behaviour of the animals.

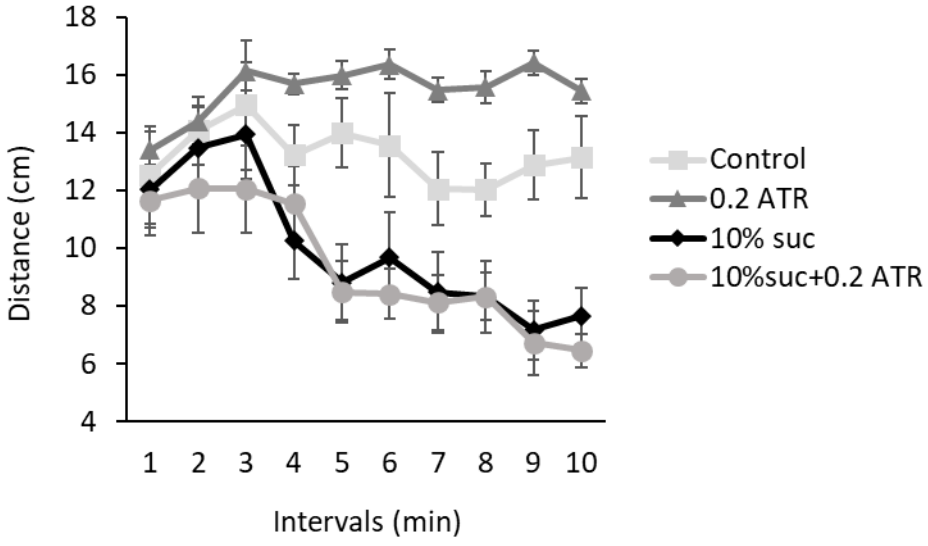


Figure 5.4. Mean distance (± 1 *SEM*) covered by the animals in different groups during the first 10 minutes exposure phase of the experiment.

5.2.3 Discussion

The data of this experiment show the effects of exposure to different concentrations of atropine on the locomotor activity (compared to exposure to plain water, 10% sucrose or a mixture of 10% sucrose+ 0.2mM atropine). Exposure to 0.02mM resulted in a slight increase in the locomotion compared to the control group. The activity of the animals in the group treated with 0.2mM atropine remained steady along the 30-minute session. Animals exposed to 10% sucrose showed a slight increase in their activity during the first 5 minutes of the exposure, however, a gradual reduction in their levels of activity appears after the first five minutes of the exposure; this is similar to previous data reported in Experiment 1 (Chapter 3). Also, animals exposed to a mixture of 10% sucrose +0.2mM atropine showed a similar reduction in their activity after 5-minute from the exposure as animals in the group 10% sucrose.

According to previous research, there is an interaction between dopaminergic and cholinergic system in planaria; high levels of dopamine increases the motor activity, however, excitation of the D1 dopamine receptors excites the release of acetylcholine which results in a reduction of the motor activity (Buttarelli et al., 2000). Our hypothesis was that exposing the animals to a cholinergic antagonist (atropine) that blocks the muscarinic receptors could attenuate the level of acetylcholine increasing the locomotor activity. The data of this experiment confirms this notion. However, this was not the case with the animals treated with a combination of 10% sucrose+0.2mM atropine, who showed a similar reduction in their activity as animals in the group 10% sucrose. It is known that glucose is the main compound of acetyl-coenzyme A and the later with choline synthesis the Ach by the action of choline acetyltransferase enzyme. Durkin, Messier, de Boer, and Westerink (1992) demonstrated that animals that received a combination of glucose and scopolamine showed a significant increase in the level of Ach in comparison with animals had an injection of glucose or choline alone or animals in the saline group. The increase of Ach level could be the reason behind the reduction in the activity of treated animals. This mechanism could account for the reduction in the activity of the animals in the group treated with sucrose and atropine.

5.3. Experiment 11: the role of cholinergic system in tolerance development

The purpose of Experiment 11 was to determine the role of the cholinergic system in the development of tolerance. The specific experimental aims were to demonstrate whether planaria develop a conditioned compensatory response (CCR) and tolerance after repeated exposure to a sucrose solution (as in Experiment 4, Chapter 3); and to assess the role of the cholinergic system in the development of tolerance and the CCR after repeated exposure to sucrose.

5.3.1. Method

5.3.1.1. Subjects and Materials

Sixteen brown planaria *(Dugesia*) were used in this experiment. The flatworms were held in the same way described for previous experiments. The same petri- dishes used in the previous experiments were used in the present experiment. Animals could be exposed to a 10% Sucrose solution; treated water; a 0.2 mM atropine; or a 10% sucrose and a 0.2 mM atropine solution.

5.3.1.2. Procedure

The procedure of this experiment was similar to the procedure described for the previous experiments of tolerance with two phases: training and test phase. During the training phase, planaria were exposed to the two surfaces (rough and smooth) on alternating sessions. Animals were exposed to the sucrose solution in one context (the trained context) and to treated water in the alternative context (control context). In contrast with previous experiments, rather than alternating daily sessions of exposure to the trained and control contexts, we exposed the animals to these contexts in alteration in am and pm sessions, shortening the length of the training phase from 8 to 4 days. There were two groups in this experiment: group control and group atropine. Group control was exposed to sucrose in the trained context and water in the control context.

Animals in group atropine were exposed to sucrose and atropine in the trained context and the atropine solution in the control context.

Following training, the animals received two tests: one with water and the other with 20% sucrose in both contexts (trained and control). The order in which the animals were tested with the trained and control contexts was counterbalanced. The locomotor activity of the animals was registered during the experimental sessions.

5.3.2. Results

The data of the training phase of the experiment are displayed in Figure 5.5. Animals showed lower levels of activity in the presence of sucrose than in the presence of water during the first cycle of training. However, the activity of the animals in sucrose was increased over training days in group control compared with group atropine. An ANOVA with Group (control *vs.* atropine), Stimulus (sucrose *vs.* water) and Training Cycles showed significant effects of Group, $F(1,14)=8.366$, $p=0.01$, $\eta_p^2=.37$, and Stimulus, $F(1,14)=84.878$, $p<0.01$, $\eta_p^2=.85$. There were significant interactions between Training Cycles x Group, $F(3,42)=3.766$, $p=0.01$, $\eta_p^2=.21$, Stimulus x Training Cycles, $F(3,42)=8.035$, $p<0.01$, $\eta_p^2=.36$, and Stimulus x Groups, $F(1,14)=5.306$, $p=0.03$, $\eta_p^2=.27$. All the other factors and interactions were non-significant, Fs<1.

Further analyses were carried out to analyse the interaction Stimulus x Training Cycles. An ANOVA with Training Cycles for the water data showed a non-significant effect of stimulus, $F(3,45)=2.49$, $p>0.05$.The data of training with sucrose showed a significant effect of stimulus over the four training cycles, $F(3,45)=8.61$, $p<0.01$, $\eta_p^2=.36$. Additional analyses were also carried out to analyse the interaction Stimulus x Group. An ANOVA with Stimulus showed a significant effect of Stimulus for the group control, $F(1,7)=86.84$, $p<0.01$, $\eta_p^2=.92$, and the group atropine, $F(1,7)=19.30$, $p=0.003$, $\eta_p^2=.73$. Finally, the interaction Training Cycles x Group was also analysed. An ANOVA with Training Cycles indicated that there was no significant effect of training cycle for neither of the groups, $Fs(3,21)\leq 2.65$, $ps>0.05$.

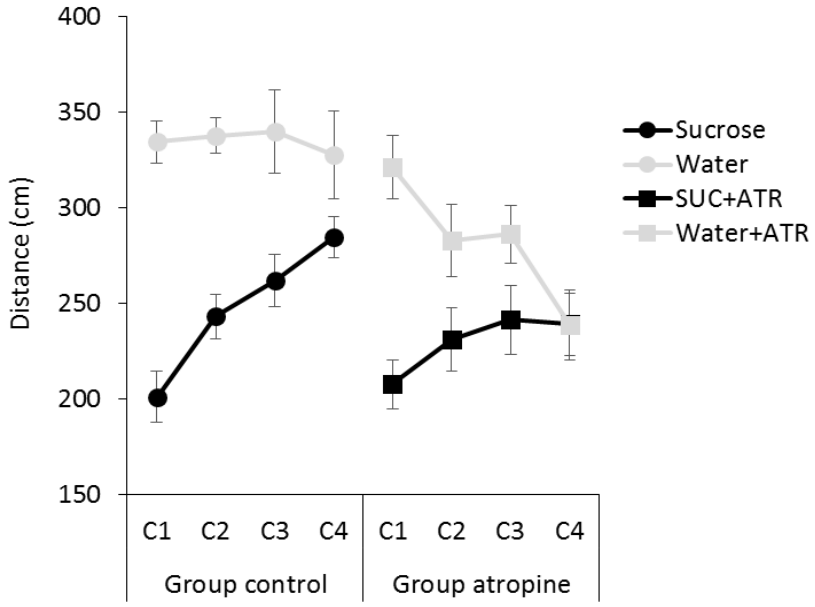


Figure 5.5. Mean distance (± 1 *SEM*) covered by the animals in the contexts were Sucrose (Trained) and Water (Control) were presented over four cycles of training during the tolerance training phase of the experiment.

The data of the test phase of the experiment are displayed in Figure 5.6. Animals in group control showed a higher level of activity during the 30 minutes test in the Trained context in which the sucrose was presented during the training phase than in the alternative Control context, in the presence of water. However, animals in both groups showed a low level of activity when they were tested with 20% sucrose both in the Trained and the Control context. In addition, animals showed higher levels of activity in both contexts when they were tested with water than when they were tested with sucrose.

An ANOVA with Group (control *vs* atropine), Stimulus (20% sucrose *vs.* water), and Context (Trained *vs.* Control) confirmed these impressions, showing a significant effect of Stimulus, $F(1,14)=31.360$, $p<0.01$, $\eta_p^2=.69$. There were no significant effects of Group and Context, $Fs<1$. However, there was a significant interaction Stimulus x Context, $F(1,14)=6.394$, $p=0.02$, $\eta_p^2=.31$, and between Stimulus x Context x Group, $F(1,14)=5.223$, $p=0.03$, $\eta_p^2=.27$. Further analyses were carried out to analyse the three-way interaction. An ANOVA with Stimulus (20% sucrose *vs.* water), and Context (Trained *vs.* Control) as factors was carried out for each group. The data analysis for the

group control showed a significant effect of Stimulus, $F(1,7)=28.15$, $p=0.001$, $\eta_p^2=.80$, and a significant Stimulus x Context interaction, $F(1,7)=8.54$, $p=0.02$, $\eta_p^2=.55$, but there was no significant effect of Context. Further analyses were carried out to analyse the interaction Stimulus x Context. An ANOVA showed a significant effect of context during the test with water, $F(1,7)=6.12$, $p=0.04$, $\eta_p^2=.46$; however, there was no significant effect to the context during the test with 20% sucrose, $p>0.05$.

Animals in the atropine group did not show differences in activity in the trained and control contexts neither in the presence of water nor in the presence of 20% sucrose. An ANOVA showed a significant effect of Stimulus, $F(1,7)=9.20$, $p=0.01$, $\eta_p^2=.56$. The factor Context, and the Stimulus x Context interaction were both non-significant, Fs<1.

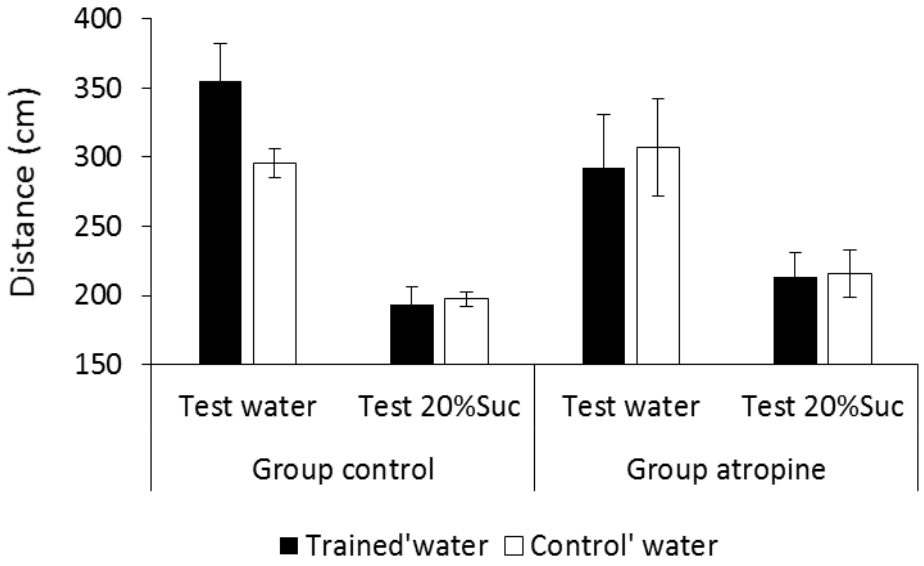


Figure 5.6. Mean distance (± 1 *SEM*) covered by the animals in the Trained and the Control contexts throughout the two cycles of test trials in the presence of Water and Sucrose.

5.3.3. Discussion

During the first training session, animals exposed to 10% sucrose in group control showed a decrease in their activity compared with their activity in water. However, the activity of animals in sucrose significantly increased over the training days, suggesting

the development of tolerance to sucrose. This replicates what we found in previous experiments (Experiment 4, Chapter 3) with a slightly different training procedure (alternating training trials in am and pm rather than daily sessions). Moreover, animals in the group atropine did not show a significant change in their activity over the training cycles when exposed to sucrose and atropine. However, these animals (in the group atropine) reduced their activity in the atropine solution compared with animals exposed to water in the group control; this might be indicative of the development of tolerance to the atropine.

Ramakrishnan et al. (2014) confirmed the function of cholinergic system in the locomotor activity in planaria. They identified that planaria treated with a high concentration of scopolamine showed a reduction in their locomotion and increase in the stereotypical activity and this effect is dose dependent: a low concentration of the drug did not reduce the activity. They also showed that the negative effect of scopolamine could be reversed by using a low concentration of a cholinesterase inhibitor drugs (Galantamine) but not a high concentration. Similarly, Bezerra da Silva et al. (2016) study the role of the cholinergic system on the activity of planaria. They found that animals treated with acetylcholinesterase inhibitor drugs (these drugs prevent the analysis of Ach and increase its level) showed a significant reduction in their activity and increase of convulsion as a result of the higher level of Ach in their bodies. This suggests that the cholinergic system is indeed involved in the locomotor activity of planaria.

One of the important mechanisms by which the animal develops a tolerance for the repeated exposure to the same dose of a drug is the development of a conditioned compensatory response. This compensatory response is produced by the body to counteract the unconditioned effect of the drug. As it is known, from our previous experiments, the effect of the sucrose is to reduce the locomotor activity of the animals. Therefore, the expected conditioned compensatory response is the increase of locomotion when the animals are tested in the trained context in the absence of the unconditioned stimulus.

The data of the Test Water showed that animals in group control were more active when they were tested with water in the trained context in which sucrose was presented

during training than the alternative control context in which the animals always experienced water. This confirms the results found in our previous experiments (Experiment 4, Chapter 3) about the role of the context in activating the CCR to counteract the effect of the drug. However, animals treated with atropine did not develop tolerance (or a CCR) to sucrose: when they were tested in water, they showed equivalent levels of activity in the trained and control contexts. These data suggest that treatment with a cholinergic antagonist interferes with the development of tolerance. Similarly, Nordberg and Wahlström (1982) assessed the role of cholinergic system in the mechanism of tolerance in rats after repeated exposure to ethanol. They found that treating the animals with atropine impairs the development of tolerance and reduces the signs of tolerance along test trials.

Interestingly, dopamine levels increase at the beginning of abusing of drugs or food binging; however, by the end of a meal taking, the level of dopamine is decreased and Ach is released inhibiting the feeding. Also, the level of acetylcholine significantly increase in response to the environmental cues associated with the use of addictive substances, a situation that results in withdrawal symptoms (Avena & Rada, 2012). Therefore, it is clear that the dopamine-acetylcholine balance is important in the mechanism of addiction and food consumption and abuse. Our data suggest that atropine prevents the development of tolerance and the CCR in comparison with the effect of the dopamine D1 antagonist, which did not affect the development of the tolerance or the CCR (Experiment 7, Chapter 4).

The data of Test 20% Sucrose showed that animals in both groups showed a significant reduction in their activity when they were tested in 20% sucrose in both the trained and the control contexts. This suggests that, although animals in the control group developed tolerance to 10% sucrose, exposure to a higher concentration (20%) resulted in unconditioned hypoactive response.

It is also important to highlight that we modified the experimental protocol by shortening the training phase of the experiment (using am and pm sessions) and we were still able to get robust results. This could have an important future implication.

In summary, the present results suggest that although sucrose reduces animals' activity, after several exposures to sucrose the animals develop hyperactivity as a conditioned compensatory response which indicates the development of tolerance. Treating the animals with atropine blocks the capacity of animals to develop a conditioned compensatory response.

5.4. Experiment 12: treatment with atropine blocks the CPP acquisition

The purpose of Experiment 12 was to determine the role of Ach in the development of CPP. The main objectives of this experiment were to assess CPP in planaria using sucrose using the same procedure described in Chapters 3 and 4; and assess whether the development of CPP depends upon the cholinergic system.

5.4.1. Method

5.4.1.1. Subjects and Materials

Sixteen brown planaria *(Dugesia*) were used in this experiment. The flatworms were held in the same way was described for previous experiments. The same petri- dishes used in the previous experiment were used in the present experiment. Animals could be exposed to a 10% sucrose solution; treated water; a 0.2 mM atropine solution; or a 10% sucrose and a 0.2 mM atropine solution.

5.4.1.2. Procedure

The procedure of this experiment was similar to the procedure described for the previous experiments of CPP with three phases: pre-training test, training and post-training tests. During pre and post-training test, animals were tested with water in the two-sided petri dishes (half smooth—half rough). Time spent in each side of the petri dishes was recorded and the preference score was calculated for the less preferred

context. In this experiment, the preference score was calculated for the smooth context, which was the less preferred by all the animals.

During the training phase of the experiment (days 2-9), animals in the control group were exposed to a 10% sucrose in the less preferred context and to treated water in the preferred context in alternative days; each session lasted 30 minutes and there were a 24-hour interval between the exposure sessions. The animals in the group atropine (n=8) was treated with 0.2mM atropine solution both in the less preferred context (in compound with sucrose) and in the preferred context.

During the post-training test, all animals were tested again in the two-sided petri dishes and the preference score calculated again to identify whether animals changed their preference for the previously less preferred context. The change in preference score was calculated in the same way described in the previous experiments on CPP.

5.4.2. Results and Discussion

The data of the Training phase of the experiment are displayed in Figure 5.7. Animals in both groups showed lower levels of activity in the presence of sucrose than in the presence of water. An ANOVA with Treatment (Sucrose *vs.* Water) and Group (Control *vs.* Atropine) showed a significant effect of Treatment, $F(1,14)=25.62$, $p<0.01$, $\eta_p^2=.64$. Neither the factor Group nor the Group x Treatment interaction was significant, Fs<1.

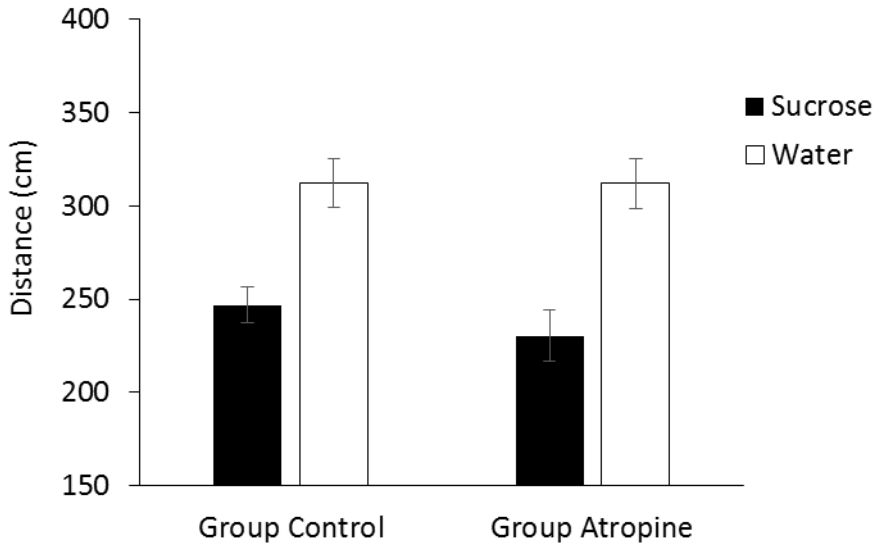


Figure 5.7. Mean distance (± 1 *SEM*) covered by the animals in groups control and atropine in the contexts were Sucrose (Training) and Water (Control) were presented over training cycles during the training phase of the experiment.

During the Pre-Training Test, animals in the group control showed a preference score for the less preferred context of 0.30 (*SEM*±0.04); the animals in the group atropine showed a preference score of 0.30 (*SEM*±0.04). A One-Way ANOVA showed that there were no differences between the groups, $F<1$.

The data for the Post-Training Tests corresponding to the change in preference scores for the initially non-preferred context are displayed in Figure 5.8. A One-Way ANOVA with Group (control and atropine) and Test showed a significant effect of Group, $F(1,15)=8.419$, $p=0.01$, $\eta_p^2=.12$.

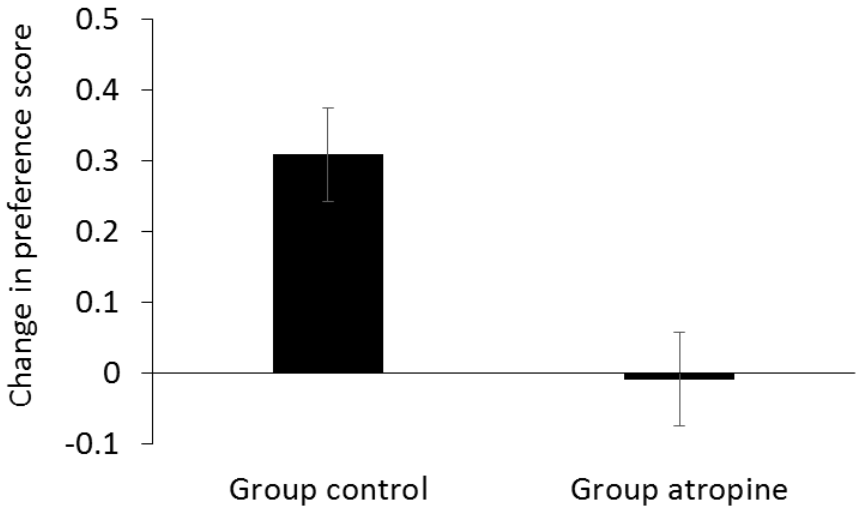


Figure 5.8. Mean change in preference score (± 1 *SEM*) to the target context during the CPP Test.

The data of the training phase of this experiment shows that exposure to sucrose significantly reduced the activity of the animals compared with their activity in water. This is similar to what we have observed in previous experiments.

The data of the CPP test session of the experiment shows that animals treated with 10% sucrose and 0.2mM atropine solution during the training phase of the experiment completely failed to develop CPP; animals in group control (treated with 10% sucrose only), however, developed a robust CPP response.

Rotella et al. (2015) found that scopolamine (a muscarinic cholinergic antagonist) significantly impairs the acquisition of fructose conditioned flavour preference (CFP) and attenuates the expression of CFP to fructose but it does not have an effect on the acquisition of quinine conditioned flavour avoidance (CFA). However, mecamylamine (a nicotinic cholinergic antagonist) did not affect the acquisition of fructose CFP but attenuated its expression during a test. However, it significantly affected the quinine avoidance acquisition.

Moreover, Nisanov et al. (2016) confirmed the role of the cholinergic system in an associative learning paradigm. They found that a systematic pre-conditioning treatment of rats with scopolamine blocks the acquisition of a conditioned response; however,

treating the animals systemically with the same drug but before the test phase did not affect the expression of the associative conditioned response.

Similar evidence was reported from planarian studies showing that treatment with scopolamine (a muscarinic antagonist) blocks the learning about the CS-US association in a classical conditioning task using light as a CS and shock as a US (Ramakrishnan et al., 2014).

Our data are consistent with this literature, and suggest that the cholinergic system plays an important role in CPP learning and that atropine affects the acquisition and blocks the development of CPP to sucrose when it was given during the training phase of the experiment.

5.5. Experiment 13: treatment with atropine interferes with the expression and extinction of CPP

The purpose of Experiment 13 was to determine the effect of atropine on the expression and the extinction of sucrose CPP. The main experimental objectives of this experiment were to assess CPP in planaria using sucrose and whether the cholinergic system plays a role in the expression and the extinction of sucrose CPP. We also checked whether exposure to the rewarding agent reinstates a previously extinguished CPP.

5.5.1. Method

5.5.1.1. Subjects and Materials

Thirty-two brown planaria *(Dugesia*) were used in this experiment. The flatworms were held in the same way described for previous experiments. The same petri- dishes used in the previous experiment were used in this experiment. Animals could be exposed to a 10% Sucrose solution; treated water; and a 0.2mM atropine solution. 5-cm diameter glass petri-dishes were also used for the reinstatement treatment as described in previous experiments.

5.5.1.2. Procedure

We followed the same general procedure described in previous experiments (Experiment 8, Chapter 4) which assessed the effect of a dopamine D1antagonist on the expression and the extinction of sucrose CPP. There were also three phases in this experiment: pre-training, training and post-training test. There were two groups in this experiment: group control (n=16) and group atropine (n=16). During the training sessions, animals in both groups were exposed to a 10% sucrose solution in the less preferred context and to water in the preferred context in alternative sessions; the preference score was calculated for the smooth context, which was the less preferred by all the animals.

In this experiment, the animals were given am and pm sessions during the training and post-training phases. Following CPP training, the animals received eight extinctions trials (T1-T8). Animals in group control were given all the extinction training trials in the presence of water. Animals in the group atropine were given four extinction training trials in a 0.2 mM atropine solution (trials T1-T4), followed by four extinction trials in water (trials T5-T8). Following extinction, the animals were re-exposed to the 10% sucrose solution in a distinctive 5 cm petri dishes made of glass. After 24 hours, animals were tested again in treated water in the two-sided petri dishes to assess the reinstatement of the learned conditioned response (CPP reinstatement). There were two test trials (am and pm) for the reinstatement. All sessions during the experiment were 30 minutes long.

5.5.2. Results

The data of the Training phase of the experiment are displayed in Figure 5.9. Animals showed lower levels of activity in the presence of sucrose than in the presence of water. An ANOVA with Treatment (Sucrose *vs.* Water) showed a significant effect of Treatment, $F(1,31)=415.89$, $p<0.01$, $\eta_p^2=.93$.

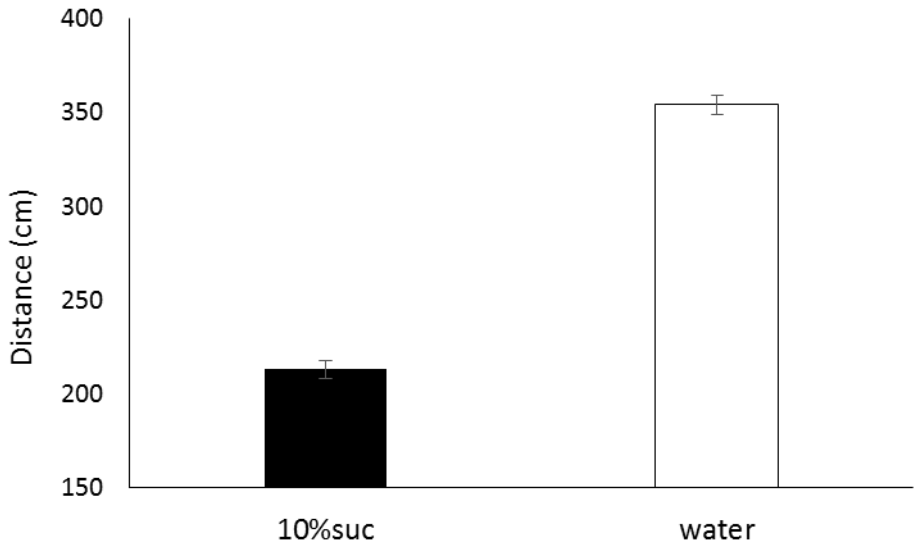


Figure 5.9. Mean distance (± 1 *SEM*) covered by the animals in the contexts were Sucrose (Training) and Water (Control) were presented over training cycles during the training phase of the experiment.

During the Pre-Training Test, animals in group control showed a preference score (for the less preferred context) of 0.27 *(SEM*±0.28); the animals in group atropine showed a preference score of 0.28 (*SEM*±0.24). A One-Way ANOVA showed that there were no differences between the groups, $F(1,31)=0.008$, $p>0.05$.

The data for the first four trials of extinction (T1-T4) are displayed on the left hand part of Figure 5.10. An ANOVA with Groups (control and atropine) and Test Trials (T1-T4) showed a significant effect of Test Trials, $F(3,90)=2.831$, $p=0.04$, $\eta_p^2=.08$, and a significant interaction between Groups x Test Trials, $F(3,90)=6.881$, $p<0.01$, $\eta_p^2=.18$. The factor Group was non-significant, $F<1$. Further analyses were carried out to analyse the interaction Test Trials x Group. A One-Way ANOVA with Group in each Test Trial showed a significant effect of Group for the first test, $F(1,31)=14.46$, $p=0.001$; the factor Group was not significant in the tests 2-4. This shows that animals treated with atropine at the time of extinction failed to express the CPP response.

The data of the second block of Test Trials (T5-T8), in which all animals were tested with water, are displayed in the middle part of the Figure 5.10. An ANOVA with Group (control and atropine) and Test Trials (T5-T8) showed a significant effect of Test Trials,

$F(3,90)=3.047$, p=0.03, $\eta_p^2=.92$. Neither the factor Groups nor the Group x Test Trials interaction was significant, maximum $F(1,30)=2.44$.

The data of the Reinstatement Test Trials (R1-R2) are displayed on the right hand part of Figure 5.10. An ANOVA with Groups (control and atropine) and Test Trials (R1-R2) showed a significant Groups x Test Trials interaction, $F(1,30)=3.99$, p=0.05, $\eta_p^2=.11$. Neither the factor Group nor Test Trials was significant, maximum $F(1,30)=3.49$. Further analysis were carried out to analyse the Group x Test Trials interaction. Two One-Way ANOVAs were carried out on the data of the Reinstatement Test Trials, showing a significant effect of Group in the first Reinstatement Test Trial, $F(1,31)=5.55$, p=0.02. The factor Group was non-significant in the second test, $F<1$.

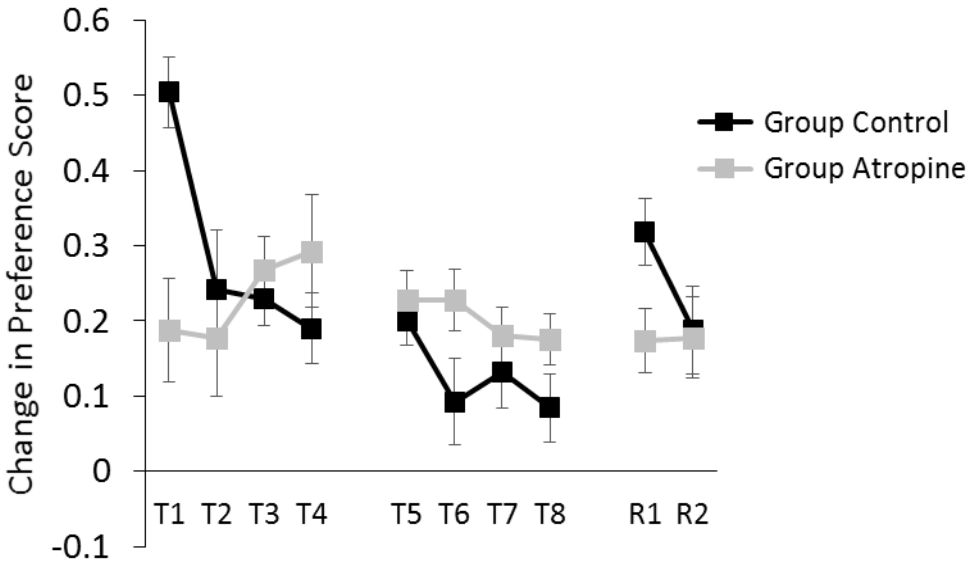


Figure 5.10. Mean change in preference score (± 1 *SEM*) to the target context during the extinction (T1-T8) reinstatement test (R1-R2) trials of the test phase of the CPP experiment.

5.5.3. Discussion

The data of this experiment indicate that sucrose slows down the activity of animals compared with their activity in water during the training phase, replicating once again what we observed previously. This experiment tested the role of the cholinergic system on the expression and extinction of sucrose CPP. The data of the CPP extinction test

trials (T1-T8) show that animals tested with 0.2mM atropine solution during the test trials did not express the CPP to sucrose compared with animals tested with water in the group control. In addition, animals in the group atropine did not show evidence of CPP when they were tested in water; and they also did not show reinstatement. This suggests that the exposure to atropine in T1-T4 erased the CPP response acquired during training.

Similarly, Schroeder and Packard (2004) assessed the role of the cholinergic system in memory consolidation during the extinction of a CPP in rats conditioned with amphetamine. They found that a peripheral or intra-amygdala direct injection of a mAchrs agonist (e.g. Oxotremorine) resulted in a rapid extinction of a previously tested CPP. This effect clearly observed when the drug was given directly after each extinction training session. However, injecting the drug two hours after extinction training failed to facilitate the extinction of the learned CPP. Zelikowsky et al. (2013) examined the effect of during- and post-extinction training administration of scopolamine on fear renewal in rats. They found that the fear renewal is significantly reduced when animals are treated with a low dose of scopolamine during the extinction trials. Treatment with scopolamine after extinction did not affect the fear renewal response.

It is well known that CPP extinguishes as a result of the exposure to the conditioned stimulus (context) alone. In the present experiment, re-exposure to the unconditioned stimulus (the 10% sucrose) in a different container in the same context, resulted in a reinstatement of the CPP in the control group. The results of the CPP reinstatement tests in the group atropine of this experiment show that atropine blocks the reinstatement after a single re-exposure to sucrose. Similarly, Gawel et al. (2016) studied the involvement of the cholinergic system in the CPP acquisition and reinstatement produced by ethanol in rats. They tested the effect of both an acetylcholinesterase and butyrycholinesterase inhibitors (Donepezil and Rivastigmine); these drugs increase the level of acetylcholine in the treated animals by preventing the analysis of Ach molecule. They found that treating the animals during the training with the ethanol with donepezil or rivastigmine similarly enhanced the CPP produced by the ethanol. In addition, they indicated that co administering of either of these two drugs enhanced the reinstatement of CPP to ethanol after a successful extinction. Moreover, they found that the effect of

these two inhibitors could be counteracted by the effect of nAchrs antagonist drug but not by the effect of a mAchrs antagonist.

The data of this experiment suggest that the cholinergic system could have an important role in different learning and memory processes such as memory consolidation, retrieval of the information and memory reconsolidation. The results suggest that the treatment with atropine impairs the expression of the CPP and/or it could interfere with the memory consolidation of extinction. In addition, the result that animals in the group atropine did not show evidence of reinstatement after the re-exposure to the sucrose, suggest that atropine interfered with the reconsolidation of the acquisition memory during the four first trials of extinction (T1-T4). This would open the possibility of, by targeting the cholinergic system, develop a pharmacological protocol to treat the addicts and control the negative outcomes of addiction.

An alternative to this view would be that atropine could have an aversive effect on the animals; and that the observed behaviour was due to the development of a conditioned place aversion. The aim of the following experiment is to assess this possibility.

5.6. Experiment 14: conditioned place aversion

The main aim of Experiment 14 was to assess whether the atropine (a muscarinic acetylcholine antagonist drug) results in a conditioned place aversion.

5.6.1. Method

5.6.1.1. Subjects and Materials

Sixteen brown planaria *(Dugesia*) were used in this experiment. The flatworms were held in the same way was described for previous experiments. Modified petri- dishes used in this experiment were made of white polyethylene plastic (the smooth surface is made of just the plain plastic, while the rough context is made of a rough texture of the

same white plastic). Animals could be exposed to a treated water or a 0.2mM atropine solution.

5.6.1.2. Procedure

The procedure of this experiment was similar to the procedure described for the previous CPP experiments with three phases: pre-training test, training and post-training tests. In the training phase of this experiment, planaria in the group control were exposed to water in the less preferred and preferred context. Animals in the atropine group, however, were exposed to a 2 mM atropine solution in their preferred context and to water in the less preferred context. In this experiment, for each group, the preference score was calculated for the smooth context, which was the preferred by half of animals (n=4); and to the rough context, which is the preferred by the other half of animals (n=4).

5.6.2. Results and Discussion

The data of the Training phase of the experiment are displayed in Figure 5.11. Animals in both groups showed a higher level of activity in the presence of water in the less preferred context compared with their activity in the preferred context with water for the group control or atropine solution in the group atropine. An ANOVA with Context (less preferred *vs.* preferred) and Group (control *vs.* atropine) showed a significant effect of context, $F(1,14)=6.696$, $p=0.02$, $\eta_p^2=.32$. However, there was no significant effect of group and no significant Group x Context interaction $F<1$.

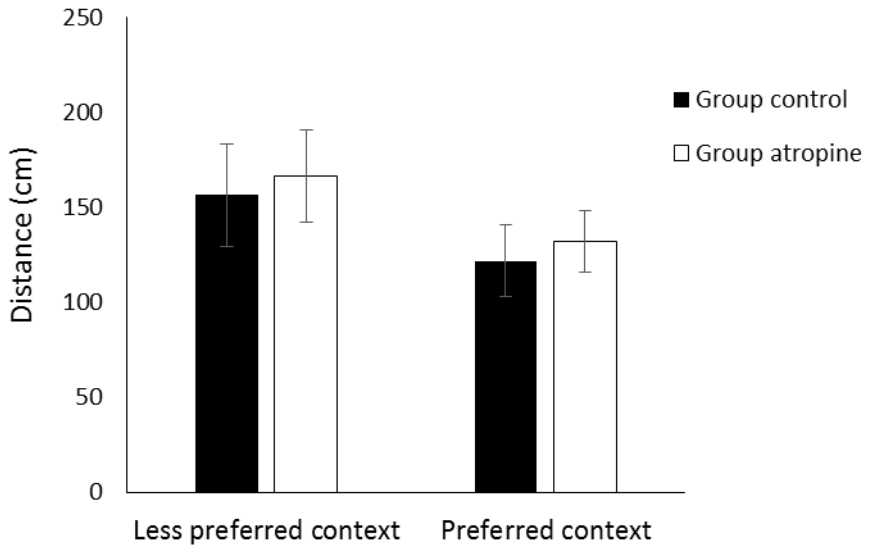


Figure 5.11. Distance (± 1 *SEM*) covered by the animals in groups control and group atropine in the less preferred context with water and the preferred context paired with water for the group control or atropine for the group atropine during the training phase of the experiment.

During the Pre-Training Test, animals showed a preference score (for the preferred context) of 0.71 (*SEM*±0.03) for the group control and 0.71 (*SEM*±0.04) for the group atropine. The data for the Post-Training Tests corresponding to the change in preference scores for the initially preferred context are displayed in Figure 5.12. A One Way ANOVA Test showed no significant difference between two groups $F<1$.

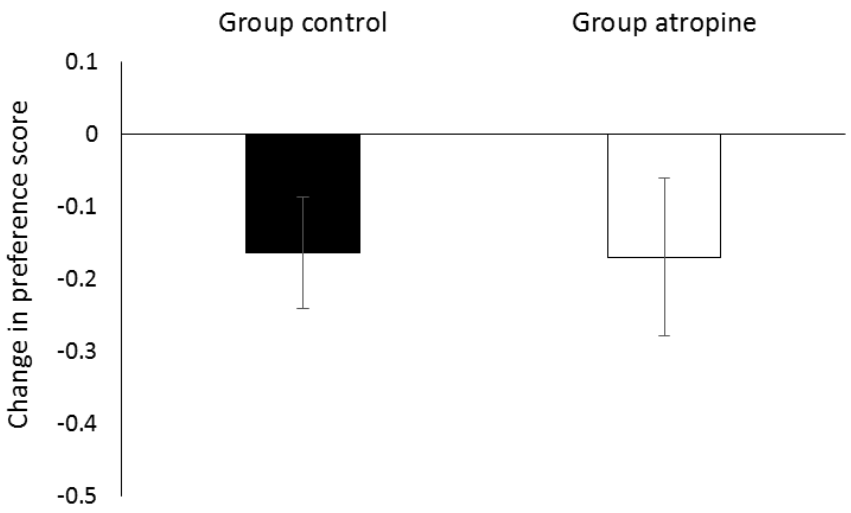


Figure 5.12. Change in preference score (± 1 *SEM*) for the initially preferred context for the groups control and group atropine for the test session of the experiment.

In this experiment, we tested whether atropine (a muscarinic acetylcholine antagonist drug) results in the development of a conditioned place aversion. Animals exposed to water in the less preferred context showed a hyperactivity compared to when they were exposed to water (group control) or an atropine solution (group atropine) in the preferred context. In this experiment, animals in both groups were always exposed to water in the less preferred context during the am session of the experiment and to water in group control or atropine solution in group atropine in the preferred context during the pm session. Therefore, the animals might be more active during the am session rather than the pm session; this could be a reason behind the reduction in the activity of the control animals during the pm session.

Moreover, the present experiment shows that animals treated with atropine did not develop a conditioned aversion. However, both group control and group atropine showed a mild reduction in their preference for the initial preferred context. This could be because animals familiarise with both contexts, which results in the development of habituation. These results confirm that atropine did not have an aversive effect on animals, suggesting that the results we observed in our previous experiments with atropine are due to the effect of atropine on learning and memory rather than the development of a conditioned aversion.

5.7. General discussion and conclusions

The experiments reported in this chapter aimed to assess the role of the cholinergic system in the development of addictive-like behaviours in planaria. It is well known that the cholinergic system controls various important functions like learning, the encoding of information, and memory processes in vertebrates. The dopamine-acetylcholine interaction plays an important role in cognitive behaviour and feeding behaviour. Research shows that the level of both dopamine elevates during the initial stages of the feeding process; this is followed by a decrease in the release of dopamine accompanied by an increase in the release of acetylcholine, which reduces the appetite (Rada et al., 2001). Research with planaria showed that they have the dopamine and acetylcholine neurotransmitters and there is a similar interaction between the dopaminergic and the cholinergic systems in planarian and rats (e.g., Buttarelli, et al., 2002; Nishimura et al., 2010).

The results of this chapter showed that animals in a group treated with atropine did not develop a hyperactivity conditioned compensatory response after repeated exposure to sucrose during tolerance training: the animals showed the same level of activity when tested with water in the context previously paired with sucrose (Trained Context) and the context always paired with water (Control Context; Test Water, Experiment 11). In addition, the results of the Test Sucrose showed that the effect of the sucrose was more obvious when the animals in both groups tested with a higher concentration of sucrose (20%). These data suggest that atropine impairs the development of tolerance and the hyperactivity conditioned compensatory response in the planaria.

Another significant finding in this chapter was that planaria treated with atropine during the conditioning cycles did not develop a significant CPP response (Experiment 12). Furthermore, the treatment of the animals with atropine during the CPP test sessions prevented the expression of CPP and CPP extinction. Animals treated with atropine during the first four extinction training trials did not show a reinstatement after the re-exposure to the sucrose-US in comparison with the animals in the control group, who showed a significant development of CPP, extinction and reinstatement (Experiment 13). These data confirm that the cholinergic system plays a role in different learning mechanisms; and strongly suggest that the treatment with atropine blocks the

acquisition and the expression of CPP; and it could interfere with memory reconsolidation.

It is well known that organisms, in a natural way, learn about the association between the presentation of a significant event (could be a rewarding or an aversive substance) and the cues where they were presented (CS); the information about this association stores in the brain as a short memory. With passage of time, this learned information becomes stable and fixes as a long-term memory by a process called memory consolidation. However, exposure to a reminder or retrieval of stored information results in a reactivation of the memory and it becomes unstable again. To be permanent, this activated information needs to enter a second process of memory fixation or memory reconsolidation (Nader, Schafe, & Le Doux, 2000). Both: memory consolidation and reconsolidation have a vital role in learning and storage of the information; any disruption in either mechanism would affect the memory and processing of the information.

There are different mechanisms could be involved in the process of memory consolidation and reconsolidation; one of these mechanisms suggested that memory consolidation or reconsolidation is a development of new learning mechanism which requires a protein synthesis process. Previous research (e.g., Nader et al., 2000) showed that rats exposed to a shock (US) after the presentation of a tone (CS) over 14 training sessions developed a fear conditioning response (freezing). Activation of the memory (re-exposure to the CS) resulted in successful expression of the previous conditioned response that developed during the training sessions. However, a second group of animals that received an immediate injection of a protein synthesis inhibitor (anisomycine) after the presentation of the reminder (CS), did not show a recovery of the fear response during the test. They also found that treating the animals with the protein synthesis inhibitor 6 hours after the exposure to the reminder did not produce a significant effect. This suggest that the anisomysine interferes with memory reconsolidation and prevents the expression of the fear response. It also highlighted the vital role of protein synthesis in forming the new learning.

Another mechanism suggested that the noradrenergic system could play a significant role in memory consolidation and reconsolidation. An experiment by Villain et al.

(2016) using different behavioural tasks demonstrated that the blockage of the β-noradrenergic receptors by propanol (a β-noradrenergic receptors blocker) interferes with both consolidation and reconsolidation of retrieved information in treated mice.

Furthermore, it is well known that the cholinergic system has a significant role in learning and memory processes (Hasselmo, 2006). Previous research has shown that the treatment of animals with a cholinergic antagonist (e.g., scopolamine or atropine) leads to memory impairment and inhibits learning in different tasks. On the other hand, animals treated with a cholinergic agonist show enhance in learning and memory (Boccia et al., 2003).

These data suggest that like consolidation, reconsolidation of the activated memory is an important mechanism for the maintenance of learning occurred during the training and for the performance of the conditioned response; and that any factor (e.g., amnestic agent or pathogen) that interferes with the reconsolidation of the activated memory results in a disruption of learning. Therefore, manipulating memory reconsolidation could be an effective method to treat addiction and maladaptive behaviours based on conditioned fear.

Experiment 14 showed that the atropine did not have an aversive effect on the animals; the prevention of the development and expression of CPP responses observed in Experiments 12 and 13 can be attributed to the interference with memory consolidation and reconsolidation.

It is clear that the effect of atropine in the outlined experiments was the interference with different learning mechanisms and memory processes involved in addiction. On one side, it prevents the encoding and the retrieval of the needed information during the development of CPP. On the other side, it impaired the learning about the negative effects of the drug and how the body can anticipate these effects, blocking the development of tolerance and the conditioned compensatory response.

Because addiction could be defined as a disease of learning and memory and the development of CPP and tolerance are two crucial components in the context of addiction, a potential way of treating addiction could be based upon using drugs that

attenuate the amount of acetylcholine and update the function of different receptors involved in this system.

Chapter 6: Summary, conclusions and future directions

Addiction is a chronic disease that involves the development of several complex behavioural and neurochemical time-dependent changes. Addiction can start as the result of a voluntary life-style behaviuor (e.g., smoking or drinking) or as the result of the normal biological need for natural rewards (e.g., food and sex). Initially, it develops as a controlled behavior; over time and with repeated exposure to the rewarding event it turns to compulsive uncontrolled behavior with a development of withdrawal symptoms when the access to the rewards is discontinued. Addiction can have a huge psychological and social negative impact. It develops as a gradual cycle: during the first stages of addiction, the rewarding substance activates the dopamine reward system and produces the positive reinforcement effect that promotes engaging in the addictive behavior and consequently repeat it. The repetition of the addictive behavior results in several within and between system adaptations such as decrease in the susceptibility of dopamine and opioid systems to the effect of the drugs of abuse; and activates the anti-reward system, producing the compulsive behavior and withdrawal signs after access to the rewarding substance is prevented or discontinued. These alterations represent the negative reinforcement side of addiction. Therefore, addiction could develop either to get the pleasant effect at the first stages or to avoid the unpleasant effect or withdrawal signs after abstention (Koob, 2013; Koob & Le Moal, 2008).

Moreover, addiction could develop as a habit; after repeated engagement in the addictive behaviour it converts from impulsive behaviour to compulsive uncontrolled behaviour despite the negative consequences of the addictive substance (Everitt & Robbins, 2005, 2016).

Furthermore, it is important to highlight that addiction does not always develop because addicts like a substance of abuse and they want to get pleasure, but because they want it to avoid the negative effects of abstinence and they show a real motivation to get it to maintain the balance (Berridge et al., 2009). The mechanisms behind wanting could be controlled by the same principles of Pavlovian condition. The development of a conditioned compensatory response, which is unpleasant for the individual, could be a good example to explain wanting.

The broad aim of this thesis was to determine the nature of the mechanisms that underlie the modulation of addictive-like behaviours in planaria and, in doing so, to provide evidence of the role of dopaminergic and cholinergic system in the development of addictive behaviour. Also, our work could contribute to develop promising pharmacological strategies for the treatment of addiction, basically, by assessing extinction, memory consolidation and reconsolidation. The main findings are summarised below.

The main findings outlined in chapter 3 illustrated that planaria exposed to 10% sucrose showed a clear reduction in food searching behaviour; and that these animals were satiated and less motivated to search for a food; or the reduction in their locomotion restricts them from moving around the context searching for food compared with animals in a control group. In addition, the data showed that a single exposure to sucrose produced a significant reduction in the locomotor activity of the planaria. This effect was observed after the first 5 minutes of exposure in a 30-minute session. The reduction in the activity was treated as an unconditioned response to sucrose. However, repeated exposure of animals to the sucrose in particular context resulted in a gradual increase in the activity. Testing the animals with water in the context in which sucrose was previously presented revealed a hyperactivity conditioned response that seems to modulate the development of tolerance. This conditioned compensatory response seems to counteract the unconditioned effect of sucrose and maintains the balance (e.g., Siegel, 1975). As an alternative, the hyperactivity shown by the animals when they were tested in the trained context could be controlled by an instrumental mechanism. This requires us to assume that animals have to exert a greater effort to swim in a sucrose solution than in plain water. Following training in a context paired with sucrose, the animals would exert a greater effort to move to compensate for the difficulty of moving in the presence of the expected sucrose solution. Both the instrumental (Lê and Kalant, 1992) and the Pavlovian reward learning (Zack & Vogel-Sprott, 1995) could have a significant role in tolerance development.

It is well known that repeated exposure to a rewarding event in specific environmental cues leads to the development of a positive association between the event and the associated cues (e.g., the development of conditioned place preference, CPP). On the other hand, subsequent exposure to the cues in the absence of the rewarding agent

results in the extinction of the learned conditioned response. During this stage, a new inhibitory association is supposed to develop; the presentation of the cues means the absence of the reward (CS-). This is not forgetting because the animals showed the conditioned response after a period of a time (a spontaneous recovery), after re-exposure to the US (reinstatement) or when they were tested in a new context (renewal effect). The data reported in Chapter 3 showed that planaria developed a significant CPP; this CPP response, however, extinguished after several exposures to the context in the absence of the rewarding agent, sucrose. These data suggest that planaria show an addictive-like behaviour to the effect of the repeated exposure to sucrose; both, in terms of the development of the positive association (e.g., CPP) and the development of tolerance or (a conditioned compensatory response) to protect themselves against the negative effects of the drugs.

Building on the idea that the dopamine system plays an important role in the development of abuse of the rewarding drugs, Chapter 4 assessed the role of the dopamine reward system in the establishment of CPP and in the development of tolerance.

It has been often suggested that dopamine plays an important role in movement control as well as in reward-related learning. The results reported in Chapter 4 showed that planaria treated with a dopamine D1 antagonist (SCH 23390) during the training phase did not show evidence of the development of a CPP response, extinction and reinstatement after re-exposure to sucrose compared with animals in a control group (Experiment 6). These data suggest that CPP is an example of appetitive Pavlovian conditioning controlled by the dopamine reward system. However, it could be argued that the treatment of animals with the dopamine D1 antagonist could be aversive and produce a conditioned place aversion. To control for this, the effect of D1 antagonist was tested alone in the preferred context in Experiment 9. The data showed that planarian treated with dopamine D1 antagonist in the preferred context did not develop conditioned place aversion. This strongly suggests that the effect of the D1 antagonist was to prevent the development of CPP rather than producing a conflicting place aversion response.

Interestingly, the treatment with the dopamine antagonist during extinction test trials did not affect extinction learning; and it did not interfere with recovery of the conditioned place preference response in a reinstatement test. The data suggested that the dopamine reward system plays a role in the acquisition but not the extinction of CPP response. This strongly suggests that acquisition and extinction depend on different learning mechanisms.

Chapter 4 provides strong evidence to dissociate between the learning mechanisms that rule the development of CPP and tolerance. The results of Experiment 7 show that animals in the Control group showed a significant CPP compared to animals treated with D1 antagonist that failed to show any evidence of CPP. In contrast, the data of the tolerance test showed that animals in both groups developed a hyperactivity compensatory conditioned response to the surface that was consistently associated with sucrose. These data strongly suggest that the development of the CPP and the conditioned compensatory response are controlled by independent learning mechanisms: the first one (CPP) is dependent on the dopamine reward system, however, the second one (tolerance, which involves the development of the conditioned compensatory responses) is not. It is known that animals develop a positive association (appetitive association) between the pleasant effect of the rewarding agent and the environmental cues which are presented at the time of conditioning. This mechanism is controlled by the dopamine reward system. In contrast, animals develop tolerance after continuous exposure to a drug in a particular context as a way to counteract the undesired effects of the drug and maintain the balance. Addicts tend to increase the dosage of the addictive substance to get the pleasant effects abused drug. This mechanism seems not to depend on the dopamine reward system in planaria.

The previous data reported in Chapter 4 suggest that different neurotransmitters could be involved in the mechanisms of reward learning and addiction. Therefore, chapter 5 aimed to assess the role of cholinergic system on the development of CPP and tolerance to sucrose. We used atropine (a muscarinic acetylcholine receptor antagonist, mAchrs).

Experiment 11 in Chapter 5 demonstrates that planaria treated with atropine do not develop a hyperactivity compensatory conditioned response following tolerance training.

It is well established that the cholinergic system plays a significant role in different learning mechanisms and memory processes. Therefore, Experiment 12 in (Chapter 5) provided strong evidence that atropine blocks the development of the acquisition of the CPP to sucrose. These data suggest that treatment with atropine could impair the consolidation of the information learned during the training sessions.

Another key finding in this chapter was that treatment with atropine during the test sessions significantly prevents the expression of the CPP and the extinction over subsequent test sessions (Experiment 13). In addition, it was found that animals treated with atropine during the extinction training failed to show a significant reinstatement of the CPP response after a single re-exposure to sucrose in comparison with the control group. These data strongly suggest that atropine blocks the consolidation of the learned information and the expression of the CPP. Also, it could suggest that atropine interferes with memory reconsolidation during the retrieval of the previous learned information. On the other hand, it could be proposed that atropine has an aversive effect on the animals and that the observed results was a case of the development of conditioned place aversion. However, the data of Experiment 14 in Chapter 5 disregarded this possibility: animals repeatedly exposed to atropine in their preferred context did not develop a conditioned place aversion. These data confirm that the observed effect of atropine is due to its interference with the learning and memory processes rather than the development of competing conditioned responses.

In conclusion, the data reported in this Thesis strongly suggest that sucrose elicits addictive-like behaviours in planaria such as the development of tolerance and the association with cues present at the time of consumption similar to those observed in vertebrates. It also results in number of neurochemical changes, analogous to those observed with the repeated consumption of the drugs of abuse. The important broader implication of these results is that sucrose can produce an addictive behaviour; and this could play a relevant role in eating disorders like obesity and binge eating, which are also important public health problem.

The data presented here also provide robust evidence that addiction to sucrose can be characterised as a learned response in planaria, one that depends upon the similar principles and mechanisms that rule associative learning in rodents controlled by the

dopamine reward system. In addition, the findings of Chapter 5 strongly suggest that the cholinergic system in planaria has a significant role in learning and memory processes; and that treatment with atropine prevents the acquisition of CPP/ consolidation process of the conditioned response and the memory reconsolidation when the acquisition memories are activated during the extinction training (exposure to the context-CS). To our knowledge this is the first evidence of the interference with memory consolidation and reconsolidation in planaria. These data could have strong future implications for the treatment of addiction and obesity based on the impairment of memory reconsolidation.

Planaria share similarities in the structure and neurochemistry of the nervous system with other vertebrates. They show equivalent responses to drugs of abuse and have the ability to learn and remember. As such, planaria are a good behavioural and pharmacological pre-clinical model of addiction. Therefore, planaria could represent a potential replacement for rodents in the pre-clinical model of addiction, obesity and neurodegenerative (e.g., Parkinson's and Alzheimer's) and neuropsychiatric disorders (e.g., schizophrenia). In comparison with other invertebrates (e.g., *c. elegance*), planaria have a similar structure of the nervous cells as in developed mammals, present the opioids and cannabinoids and their genetic analysis show that they have a high percentage of similarity in the genes that responsible on different neurotransmitters and receptors. Planaria also have the ability to regenerate their whole body including the nervous system in a short period of time, thus, they could be a valuable model to study the regeneration of the nervous system or to understand the aging and aging related diseases. Of all the invertebrate species that have been used and that have been put forward as candidates for invertebrate models of the pre-clinical research, planaria are arguably the most useful. It is important to highlight that the acknowledgment about the mechanisms behind the establishment of different types of the associative learning (e.g., CPP, extinction and the development of the CCR), would significantly help to develop a therapeutic protocol combining behavioural and pharmacological approached to reduce the maladaptive behaviours and that aims to control the outcomes of the addictive behaviours and prevent relapse.

The data reported in this thesis was concerned with the assessment of different behavioural and neurochemical changes produced by the subsequent repeated exposure

to sucrose. Specifically, the experiments assessed the development of the conditioned place preference, extinction, reinstatement and the change in the locomotor activity as an indicator for the development of tolerance and the conditioned compensatory response. In addition, a pharmacological protocol was used to assess the mechanism underlying these behavioural changes and the role of the dopaminergic and the cholinergic systems in these mechanisms; using a dopamine D1 antagonist (e.g., SCH23009) and a muscarinic cholinergic antagonist (Atropine). However, it could be useful for future experiments to target other neurotransmitters which have a significant role in addiction such as the opioids system. This could be supported by measuring the amount of different neurotransmitters released along different stages of the addiction cycle; and the changes in the density of the receptors using other pharmacological procedures such as the High Performance Liquid Chromatography (HPLC) and/or immunohistochemistry detection. Also, we could further our understanding of the addiction phenomenon by assessing phenomena characteristic of standard associative learning like the spontaneous recovery, the renewal effect; or the partial reinforcement extinction effect (PREE) usually observed in vertebrates. In addition, we could further investigate the effect of cues manipulation in improving the consolidation of information during the extinction or interfering with memory reconsolidation to prevent relapse.

Finally, planaria may represent a useful pre-clinical model to assess the effect of other sweeteners (e.g., glucose) or drugs of abuse (e.g., nicotine and cocaine) in the development of appetitive Pavlovian conditioning; or to assess the aversive learning and anxiety-like behaviour using an aversive substance (e.g., shock).

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