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Nicotine chronic tolerance development and withdrawal in the planaria (*Schmidtea mediterranea)*

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Abstract

Chronic nicotine exposure reduces sensitivity to the effects of nicotine, which then results in behavioural changes and tolerance development. In the planaria, a valuable first-stage preclinical model for addictive behaviour, acute nicotine administration has been shown to steadily alter the motility of the animals, a result that has been interpreted as evidence of tolerance and withdrawal effects; however, chronic exposure - typically regarded as a condition for the development of tolerance - and the role of the contextual cues have not been systematically assessed. The present study assessed the acute and chronic effects of nicotine on the motility of planarians (*Schmidtea mediterranea*). The animals in the experimental groups received long chronic exposure to nicotine (ten daily 30 min exposures); a control group was exposed to water in the same context but in the absence of the drug. The motility of the animals was closely monitored on every exposure. Following this phase, all the animals were subject to three different tests: in the presence of the exposure context (without the drug, Test 1); in the presence of nicotine in the exposure context (Test 2); and in the presence of the drug in a novel context (Test 3). Exposure to nicotine consistently reduced motility; the motility in the presence of nicotine increased with repeated exposures to the drug, an instance of tolerance development. Tolerance development was dependent on nicotinic receptor activation, because it was blocked by the co-administration of mecamylamine. However, this tolerance was found to be independent of the contextual cues where the effects of the drug had been experienced. The results are discussed by reference to the existent theories of tolerance development to drugs.

Key words: planaria; nicotine; tolerance development; addictive behaviour; motility

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1 Introduction

Nicotine addiction is a major preventable cause of death in humans and is characterized by multiple unsuccessful attempts to quit smoking cigarettes. As with addiction to other drugs of abuse, nicotine addiction seems to be driven by a combination of a) the rewarding effects of nicotine; b) tolerance development; and c) the presence of withdrawal symptoms following chronic exposure to the drug. Wikler (1973) was among the first to identify withdrawal and negative reinforcement as mechanisms driving the development of addiction (see also Solomon & Corbit, 1973). Also, it has been shown that nicotine tolerance correlates with the severity of nicotine addiction (Fagerström, 1978). Tolerance is characterised by a decrease in the physiological effects of a drug, so that a) larger doses are needed in order to achieve similar effects (Kalant, 1998); or b) the initial dose produces less effects with repeated administration. In particular, three different kinds of tolerance have been identified, on the basis of the number of exposures to the drug. Acute tolerance happens within the administration of a single dose of the drug: the physiological effects of the drug at a given concentration are smaller when looking at the descending portion of the drug's blood concentration —relative to the same concentration in the ascending portion of the curve (e.g., Perkins et al., 1991). Rapid tolerance is observed as less effect of the drug during a second administration of the drug, usually given between 8 to 24 hours after the first; in contrast, chronic tolerance is that observed after multiple—usually 3 or more—administrations of the drug (e.g., Stolerman et al., 1973). It is this chronic tolerance which is the focus of the present study.

Classic theories of addiction assume tolerance and withdrawal to develop in parallel; consequently, the magnitude of the withdrawal response would be related to the degree of tolerance development. This is consistent with the idea that both are manifestations of physiological dependence (Kalant et al., 1971), and that learning mechanisms (triggered by experience with the drug) are involved in the manifestation of tolerance and withdrawal (Solomon & Corbit, 1973). In humans, this observation

has been confirmed in nicotine addicts. For example, Pomerleau et al. (1983), monitored the changes in heart rate per plasma nicotine increments following smoking, and found evidence of higher levels of tolerance in heavy smokers than in light smokers. In addition, heavy smokers showed more abstinence signs following an overnight deprivation period. The relationship between tolerance and withdrawal was established at the individual level in a study by Hughes & Hatsukami (1986) in which tolerance to the effects of nicotine was found to correlate with signs of withdrawal discomfort (subjectively assessed by the smokers themselves as well as by independent observers).

The relationship between tolerance and withdrawal is well captured by psychophysiological theories of drug tolerance. A central tenet of these theories is that drugs such as nicotine produce homeostatic challenges and that environmental or contextual cues (hereafter called conditioned stimuli, or CS) become associated with the homeostatic challenge (Siegel, 1983; 2008; Solomon & Corbit, 1973). That is, drug presentation disturbs the homeostasis of the organism, and the organism produces a compensatory response to counteract the homeostatic imbalance produced by the disruptive effect of the drug. Following chronic drug administration in the presence of distinctive CSs, the compensatory responses that restore homeostatic balance come under the control of CSs through conditioning and result in a conditioned response typically referred to as Conditioned Compensatory Responses (CCRs). With sufficient experience, in the presence of the CS (contextual cues where the drug has been administered) the animals express CCRs which counteract and weaken the effects of the drug; in other words, they develop tolerance to the effects of the drug (Siegel, 1975).

The conditioning model of drug tolerance anticipates that chronic tolerance is under the control of CSs, and hence after an organism has had extensive experience with a drug, the observation of tolerance would be stronger in the presence of drug-predicting CSs (the contextual cues where the drug effects were experienced) than in their absence. Similarly, following tolerance development, presentation of drug-paired CSs in the absence of the drug should reveal CCRs. A number of studies have found that cue-induced compensatory responses (CCRs) are opposite to drug-induced unconditioned responses, and these are observed following discontinuation of the drug (e.g., Larson & Siegel, 1998; Rozin et al.,

1984; Siegel, 1975). Therefore, CCRs have been interpreted as withdrawal symptoms that arise as a consequence of the omission of the expected effects of the drug; in this context, the CCR per se would result in a homeostatic imbalance. In summary, there is abundant evidence observed in humans and nonhuman animals for the presence of CCRs following chronic exposure to drugs and for the claim that the development of tolerance and withdrawal follows similar principles as other basic learning processes (Siegel et al., 2000; Siegel & Allan, 1998; see Siegel, 2001, for a comprehensive review).

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

In the present study, we assessed the conditioning and habituation theories of tolerance development by monitoring the locomotor responses of planarians during chronic nicotine exposure. The planarians nervous system presents structural and physiological similarities to the nervous system of vertebrates: centralized and bilateral with similar neural networks, transmitters, and neuromodulators (Buttarelli et al., 2008; Rawls et al., 2011; Sandmann et al., 2011). They are suitable for the observation of conditioned place preference (CPP, Hutchinson et al., 2015; Mohammed Jawad et al., 2018.; Turel et

al., 2020), a canonical test for the rewarding effect of drugs of abuse and natural reinforcers (Tzschentke, 2007). In the study of basic learning processes, planarians show blocking and overshadowing (Prados et al., 2013), two phenomena suggesting the operation of selective processes as seen in rodents and humans. Planarians express cholinergic receptors and are sensitive to cholinergic agonists and antagonists including nicotine (Buttarelli et al., 2000). A previous report has suggested the observation of tolerance in planarians after three exposures to nicotine (Rawls et al., 2011). Tolerance in smokers, however, is likely to reflect the operation of adaptations that occur after repeated, chronic experience with nicotine. To model the development of tolerance and nicotine dependence, in the Experiment 1 reported below, we monitored the hypo-locomotor effects of nicotine in planarians following a regimen of nicotine treatment that better resembles the process of interest in humans. We measured the motility using an automated equipment that neutralizes observer bias. We also assessed the development of CCRs following chronic exposure to nicotine, and whether tolerance to the effects of nicotine diminishes in the absence of drug-paired CSs. Experiment 2 tested whether the development of tolerance to nicotine depends on nicotinic receptor activation. Experiments 3 and 4, assessed withdrawal responses following chronic nicotine exposure with higher doses.

2 Method

2.1 Animals

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

located in an incubator with conditions similar to the colony. All methods in this research were performed in accordance with the Policy on Research Involving the Use of Animals (University of Leicester, UK).

2.2 Materials

Animals were tested in 10 cm in diameter watch glass soda lime dishes; the surface of the dishes had been grooved by hand with a dental drill. These dishes served as the exposure context used during the chronic exposure to nicotine, and the Tests 1 and 2; similar dishes covered with a rough sandy surface were used as the alternative context in Test 3. The dishes could be filled with 20 ml of treated water or a nicotine solution (nicotine hydrogen tartrate salt, Sigma-Aldrich, UK, dissolved in autoclaved distilled water); we used three different concentrations of nicotine: 0.025 mM (Experiments 1 and 2), 0.05 mM (Experiment 3), and 0.1 mM (Experiment 4).

Animals were tested in groups of up to sixteen by using four wooden boxes (26 x 26 x 36 cm), that would each hold four dishes. These boxes were illuminated by dimmable LED panel lights (Model: 15-24 x 1W) placed at the bottom of the box; the light was set at 39 lux. The dishes were placed directly on top of the LED panel (see Figure 1). A camera on the top center of the wooden box could simultaneously record the activity of the four animals using *SharpCap* capture software; these videos were subsequently analyzed using a video-track system (*ViewPoint*, Lyon, France) allowing us to register the activity of the four animals in each box during the experimental sessions (see Prados et al., 2020).

2.3 Procedure

There were two phases in the experiments reported here: chronic exposure took place over ten consecutive days, followed by the test phase of the experiment over three additional days (see Figure 1).

2.3.1 Chronic exposure

The animals were placed on the grooved dishes for 30 minutes in the experimental context for them to habituate to the experimental setting on the day before the start of the chronic exposure. The following day, the chronic exposure started at a rate of one exposure session per day, over ten days. A chronic exposure session started by placing the animal in one of the grooved dishes containing either a

nicotine solution (the experimental condition, Group Nicotine) or treated water (the control condition, Group Water); the animals were allowed to freely move during thirty minutes in each exposure trial — receiving, therefore, a total of five hours of exposure to nicotine (or treated water) in the grooved dish. The animals' motility was recorded over the thirty minutes of each session and we compared the activity of the experimental and control groups in bins of ten minutes.

2.3.2 Test

Following chronic exposure, all the animals were given three test trials over three consecutive days. Tests 1 and 2 took place 24 and 48 hours after chronic exposure (the order of Tests 1 and 2 was counterbalanced across animals); Test 3 took place 72 hours after the completion of the chronic exposure phase of the experiment. During Test 1, the planarians were placed on the exposure surface with water for 30 minutes; exposure to the context in the absence of any drug was aimed to reveal any conditioned responses elicited by the contextual cues—that is, the conditioned compensatory responses or CCR which, according to the conditioning theory, underlie the development of tolerance.

During Test 2, all the planarians were exposed to nicotine in the exposure context to compare its acute (in the Group Water, exposed for the first time to nicotine) and chronic (in the Group Nicotine, exposed to nicotine during the chronic exposure phase) effects. As noted above, the motility of the animals' was recorded and analysed in 10 min bins. If tolerance to nicotine's effects developed, we expect more activity in Group Nicotine (i.e., less sensitivity to the effects of nicotine) relative to Group Water.

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

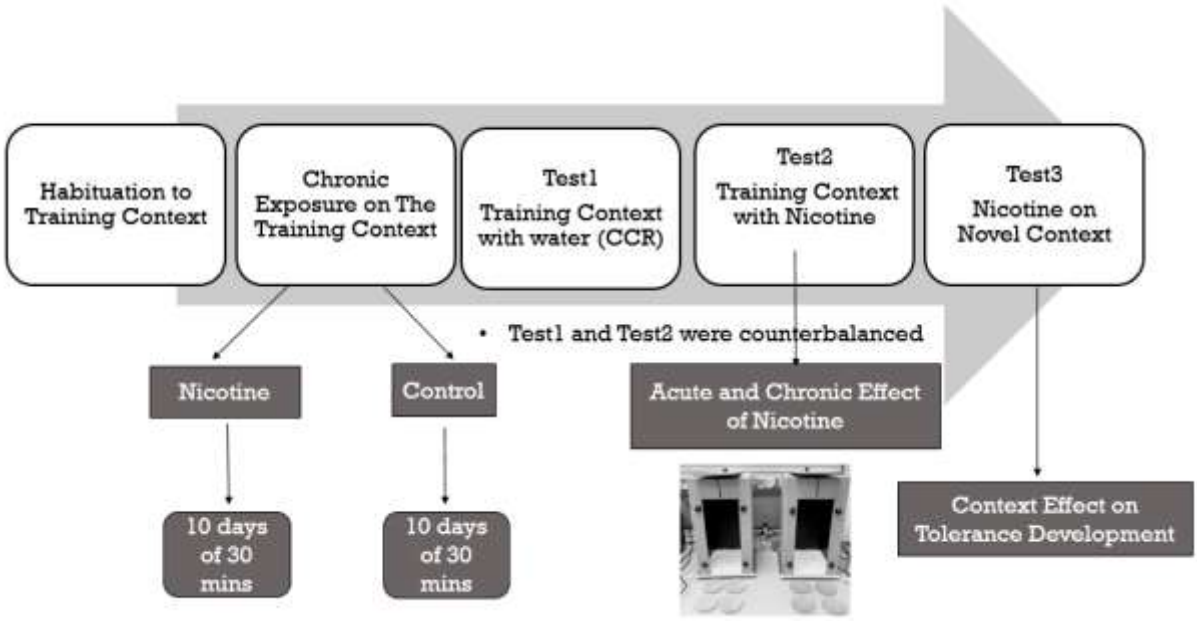

Figure 1. Summary of Experimental design

2.3.3 Data Analysis

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

3 Experiment 1. Nicotine-induced Tolerance Development with Low concentration (0.025 mM)

Preliminary experiments carried out in our laboratory had established that the unconditioned response to the exposure to nicotine (at different concentrations) was reduced motility—comparison made to control animals exposed to treated water; these preliminary studies also suggested that the motility of the animals tend to increase with repeated exposure to nicotine (an instance of tolerance development). The goal of Experiment 1 was to assess the development of tolerance to the hypo-locomotive effects of nicotine using a chronic exposure procedure that mimics the chronic exposure regimens used in other animals such as rodents—and indeed chronic self-administration in humans. We allocated a total of 32 animals to two groups, Nicotine and Water. One animal in group Water died over the course of the experiment, resulting in n = 16 for Group Nicotine, and n = 15 in Group Water. We used a relatively low concentration of nicotine (0.025 mM) which pilot experiments had indicated produces reliable hypo-locomotion in planarians.

3.1 Chronic Exposure

As expected (based on pilot data), exposure to nicotine reduced the motility of the animals: the planarians exposed to nicotine showed on average a 50% reduction in motility (M = 62.5 cm, SE = 3.2) relative to planarians exposed to treated water (M = 124.6 cm, SE = 3.3 cm) on the first day of exposure. The activity of nicotine treated animals gradually increased during the chronic exposure phase and their motility on the last exposure day was % 35 higher from the first day (M = 84.6 cm, SE = 4.1 cm). The data of the chronic exposure phase of the experiment is displayed in the Figure 2A. A visual inspection of the data suggests an increase level of motility in the Group Nicotine whereas the animals in the Group Water tend to maintain a consistent level of activity. These impressions were confirmed with a 2 (Group: Nicotine *vs*. Water) x 10 (Days: 1 – 10) x 3 (Bins: 1-3) mixed ANOVA that revealed a main effect of Group, $F(1, 29) = 106.98$, $p < .001$, $\eta_p^2 = .79$, Days, $F(9, 261) = 3.18$, $p = .001$, $\eta_p^2 = .10$, Bins, $F(2, 58) = 40.31$, $p < .001$, $\eta_p^2 = .58$, as well as significant interactions Group x Days, $F(9, 261) = 3.68$, $p < .001$, $\eta_p^2 = .11$, Group x Bins, $F(2, 58) = 61.475$, $p < .001$, $\eta_p^2 = .68$, and Days x Bins, $F(18, 522) = 1.66$, $p = .042$,

η_p^2 = .05. The remaining three-way interaction Group x Days x Bins was non-significant, $F(18, 522)$ = 0.68, p = .82, η_p^2 = .02. Within-subjects linear contrasts revealed an effect of Day in Group Nicotine, $F(1, 15)$ = 33.01, p < .001, η_p^2 = .68, but not in Group Water, $F(1, 14)$ = 0.003, p = .95, η_p^2 < .01, suggesting an increase in motility in Group Nicotine but not Water. These results confirm that the chronic exposure procedure used in the present experiment is effective in developing long-term tolerance to the effects of nicotine in the planaria.

3.2 Test 1, Conditioned Compensatory Responses (CCRs)

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

3.3 Test 2 and Test 3

These tests were conducted to assess the development of tolerance to the unconditioned (hypo locomotion) effects of nicotine, as well as to assess the context dependence of the tolerance developed to nicotine during the chronic exposure. Group Nicotine received the drug for the eleventh time whilst animals in the Group Water received it for the first time in the exposure context in Test 2. The animals in both groups were tested in the presence of nicotine again, but in a novel distinctive context, during Test 3. Figure 2B (central and right panels, T2 and T3) shows that planarians in Group Nicotine displayed more motility (*M* = 73.1 cm, *SE* = 3.2) than planarians in Group Water (*M* = 56.5 cm, *SE* = 3.3) both during the Test 2 (in the exposure context) and Test 3 (in the new context), suggesting the expression of tolerance to nicotine independent of context. These impressions were confirmed by a 2 (Group: Nicotine *vs*. Water) x 2 (Tests: Test 2 and Test 3) x 3 (Bins: 1-3) mixed ANOVA, which revealed significant main effects of Group $F(1, 29)$ = 12.48, p = .001, η_p^2 = .30, Tests, $F(1, 29)$ = 5.87, p = .02, η_p^2 = .17, and Bins, $F(2, 58)$ = 73.25, p < .001, η_p^2 = .72, as well as a significant interaction between Tests x Bins, $F(2, 58)$ =

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

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

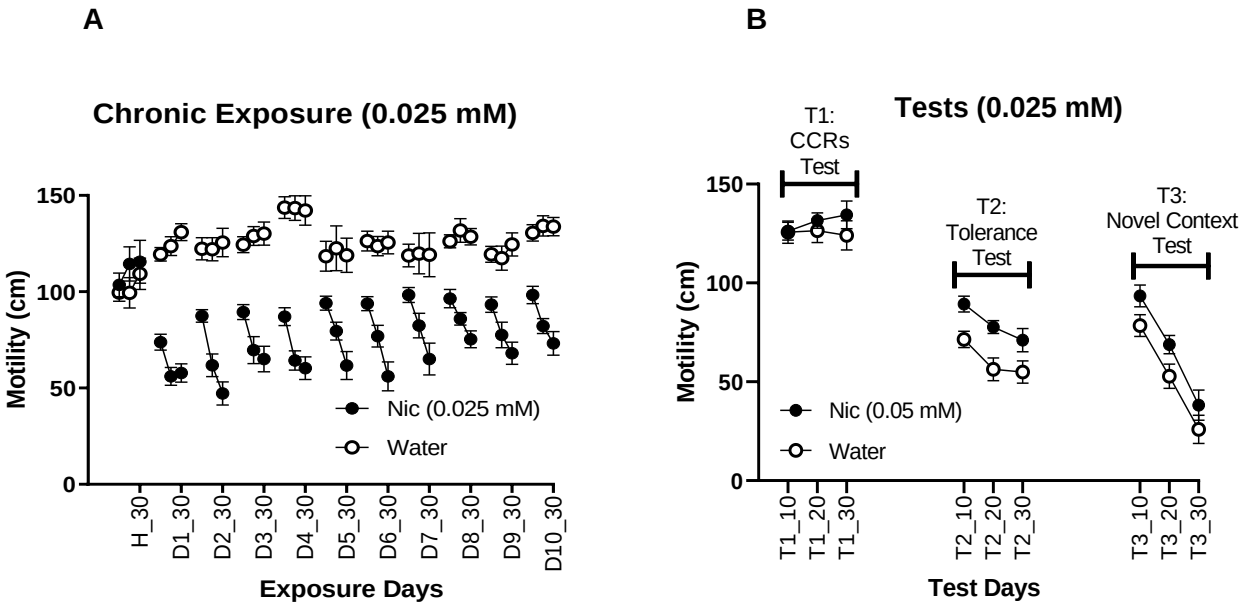

Figure 2. Experiment 1. (A) Mean distance covered by planarians in the exposure context throughout 1 session of habituation (H_30) and 10 sessions of 30 min in the presence of nicotine or water (D1_30 to D10-30). (B) Mean distance covered during the 30 min of CCRs test in the presence of only water (T1), 30 min of tolerance test in the presence of nicotine (T2), and 30 min of novel context test in the presence of nicotine with an alternative context (T3). Results represented in 10 minutes bins. Bars represent standard errors. n=15-16 planarians in each group.

4 Experiment 2: Nicotine-induced Tolerance Development with Low concentration (0.025 mM) and assessment of the effect of Mecamylamine

In Experiment 1, we observed that initial nicotine exposure decreased motility, and chronic exposure to nicotine resulted in the development of tolerance. Clarke and Kumar (1983) observed similar results with rats. Acute nicotine exposure reduced motility; however, tolerance to the initial effects

of nicotine was observed over the course of repeated exposure to nicotine. They also observed that pre-treatment with mecamylamine (a non-competitive antagonist of the nicotinic acetylcholine receptors) blocked the initial (acute) effect of nicotine. In another study (McCallum et al., 1999) observed that mecamylamine blocked the acute action of nicotine, and the development of tolerance. These two studies were conducted on rats, and suggest that both the acute effects of nicotine and the development of tolerance following chronic exposure depend on activation of nicotinic receptors. Therefore, the purpose of this experiment was to assess in planaria whether mecamylamine, a nAChRs antagonist, blocks (or attenuates) the decreased motility caused by acute nicotine exposure and the development of tolerance caused by chronic nicotine exposure. We used a 2 (Drug 1: nicotine vs water) x 2 (Drug 2: mecamylamine vs water) factorial design for this experiment. We allocated a total 112 animals to four groups, Nic, Nic+Mec, Water and Mec. One animal in Group Nic and two animals in Group Nic+Mec died over the course of the experiment, resulting in n = 27 for Group Nic, n = 26 in Group Nic+Mec, n = 28 for Groups Water and Mec. We used the same concentration of nicotine as in Experiment 1, and 0.05 mM mecamylamine (as used in Raffa et al., 2013). Test sessions were 60 minutes long, instead of the 30 mins used in Experiment 1. The flatworms were held in the same way as described in Experiment 1.

4.1 Chronic Exposure

Nicotine administration reduced motility of planaria, and mecamylamine administration partially blocked the effects of nicotine during the chronic exposure. The data of chronic exposure phase of the experiment is displayed in Figure 3A. Planaria that experienced nicotine showed significantly less motility (M = 66.3 cm, SE = 3) than the planaria that experienced nicotine plus mecamylamine (M = 81.6 cm, SE = 3). However, planaria exposed to water (M = 113.7 cm, SE = 3) behaved a similar way than planaria exposed to mecamylamine alone (M = 117 cm, SE = 3), suggesting mecamylamine did not have any effect when administered alone. This impression was confirmed by a 2 (Drug 1 [Nicotine vs Water]) x 2 (Drug 2 [Mecamylamine vs Water]) x 10 (Days: 1-10) x 3 (Bins: 1-3) ANOVA that revealed a significant effect of Drug 1, $F(1, 108) = 186.5$, $p < .001$, $\eta_p^2 = .63$, as well as significant of Drug 1 x Bins interaction, $F(1.6, 178.4) = 119.10$, $p < .001$, $\eta_p^2 = .52$, but no main interaction effect of Drug 1 x Days,

$F(8.7, 941.7) = 1.14$, $p = .33$, $\eta_p^2 = .01$, Drug 1 x Days x Bins, $F(15, 1617.3) = 0.877$, $p = .59$, $\eta_p^2 = .008$. There is also a main effect of Drug 2 $F(1, 108) = .31$, $p = .003$, $\eta_p^2 = .08$, Drug 2 x Bins interaction, $F(1.6, 178.4) = 8.35$, $p = .001$, $\eta_p^2 = .07$, but the remaining interactions were non-significant: Drug 2 x Days, $F(8.7, 941.7) = 1.58$, $p = .12$, $\eta_p^2 = .01$, Drug 2 x Days x Bins, $F(15, 1617.3) = 1.31$, $p = .19$, $\eta_p^2 = .012$. Furthermore, we also found a marginal interaction between Drug 1 and Drug 2, $F(1, 108) = 3.91$, $p = .050$, $\eta_p^2 = .03$, but the remaining interactions were not significant: Drug 1 x Drug 2 x Bins, $F(1.6, 178.4) = 0.50$, $p = .57$, $\eta_p^2 = .005$, Drug 1 x Drug 2 x Days, $F(8.7, 941.7) = 055$, $p = .84$, $\eta_p^2 = .005$, Drug 1 x Drug 2 x Days x Bins, $F(15, 1617.3) = 0.90$, $p = .56$, $\eta_p^2 = .008$. The (marginally) significant Drug 1 x Drug 2 interaction suggested the effect of nicotine was attenuated by co-treatment with mecamylamine. Further analysis of this interaction confirmed that mecamylamine attenuated the effect of nicotine, because Group Nic displayed less motility than Group Nic+Mec, $F(1, 54) = 15.01$, $p < .001$, $\eta_p^2 = .22$. Group Mec did not differ from Group Water, $F(1, 54) = 0.49$, $p = .48$, $\eta_p^2 = .01$, revealing that mecamylamine did not cause any changes in motility when given alone (see Figure 3A, left panel).

4.2 Test 1, Conditioned Compensatory Responses (CCRs)

The Test 1 was conducted in the exposure context with treated water to assess the development of CCRs and the effect of mecamylamine on the CCRs. As it can be observed in Figure 3B (left panel, T1), both nicotine ($M = 111.4$ cm, $SE = 5.6$ cm) and water ($M = 114.4$ cm, $SE = 5.8$ cm) groups covered similar amounts during the test, and the history of nicotine (Drug 1) or mecamylamine (Drug 2) exposure did not have any significant effects on the CCR test. This impression was confirmed by a 2 (Drug 1 [Nicotine vs Water]) x 2 (Drug 2 [Mecamylamine vs Water]) x 6 (Bins: 1-6), which revealed no main effect of Drug 1 (nicotine), $F(1, 105) = 1.42$, $p = .23$, $\eta_p^2 = .01$, no main interaction effect of Drug 1 x Bins, $F(3.1, 318.9) = 0.77$, $p = .51$, $\eta_p^2 = .01$, Drug 1 x Drug 2, $F(1, 102) = 0.36$, $p = .55$, $\eta_p^2 = .003$.

4.3 Test 2 and Test 3

These tests were conducted to assess the effect of mecamylamine on the development of tolerance to chronic nicotine exposure. We expected that animals previously exposed to nicotine would cover more distance than the control group that was not exposed to nicotine before (i.e., to replicate the

findings of Experiment 1), and that the administration of mecamylamine would block this effect. Figure 3B (left panel, T2 and T3) shows that planarians in Group Nic displayed more motility (*M* = 55.5 cm, *SE* = 3) than planarians in Group Water (*M* = 45.5 cm, *SE* = 2.9) both during the Test 2 (in the exposure context) and Test 3 (in the new context), suggesting the expression of tolerance to nicotine. However, Group Nic+Mec (*M* = 42.9 cm, *SE* = 3.1) showed similar levels of motility as Group Water (see above), suggesting that mecamylamine attenuated the development of tolerance to the effects of nicotine, but did not cause any changes alone (Group Mec [*M* = 45.9 cm, *SE* = 2.9] behaved a similar way as Group Water). A mixed ANOVA on Test 2 and Test 3 data (Drug 1 [Nicotine vs Water) x Drug 2 [Mecamylamine vs Water] x Tests [Test 2 vs Test 3] and Bin [6] as factors) revealed no effect of Drug 1, $F(1, 105) = 1.05$, $p = .31$, $\eta_p^2 = .01$, a marginal effect of Drug 2, $F(1, 105) = 3.55$, $p = .06$, $\eta_p^2 = .033$, and importantly a Drug 1 x Drug 2 interaction, $F(1, 105) = 4.06$, $p = .04$, $\eta_p^2 = .04$. We also observed a significant effect of Bins, $F(3.3, 344.9) = 146.05$, $p < .001$, $\eta_p^2 = .58$, and an interaction between Tests x Bins, $F(3.9, 405.9) = 3.16$, $p = .015$, $\eta_p^2 = .03$. None of the remaining effects or interactions were significant (largest $F = 3$). The significant Drug 1 x Drug 2 interaction suggested that nicotine induced tolerance development was sensitive to mecamylamine blockade. We followed up that interaction with a 2 (Drug 1 : Nicotine *vs*. Water) x 2 (Tests: Test 2 and Test 3) x 3 (Bins : 1-3) mixed ANOVA, to assess whether we observed tolerance in the absence of mecamylamine. The analysis revealed significant effects of Drug 1, $F (1, 53) = 5.73$, $p = .02$, $\eta_p^2 = .09$, Bins, $F (3, 157.6) = 6.59$, $p = .02$, $\eta_p^2 = .098$, and Tests x Bins interaction $F (3.6, 189.7) = 3.42$, $p = .01$, $\eta_p^2 = .06$, but no effect of Tests, $F (1, 53) = 0.004$, $p = .95$, $\eta_p^2 = .001$. The remaining interactions were all non-significant (all *F*s <1). These results suggested that tolerance to nicotine across the tests was significant. Moreover, a similar analysis with the groups that received mecamylamine revealed no effect of Drug 1, $F(1, 52) = 0.41$, $p = .52$, $\eta_p^2 = .008$, Tests, $F(1, 52) = 2.67$, $p = .11$, $\eta_p^2 = .05$, but a significant effect of Bins, $F(3.7, 191.4) = 73.1$, $p < .001$, $\eta_p^2 = .58$. None of the remaining interactions was significant (largest $F = 2.46$). These results suggest that animals treated with chronic nicotine showed tolerance development, and that mecamylamine blocked that effect across both tolerance tests.

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

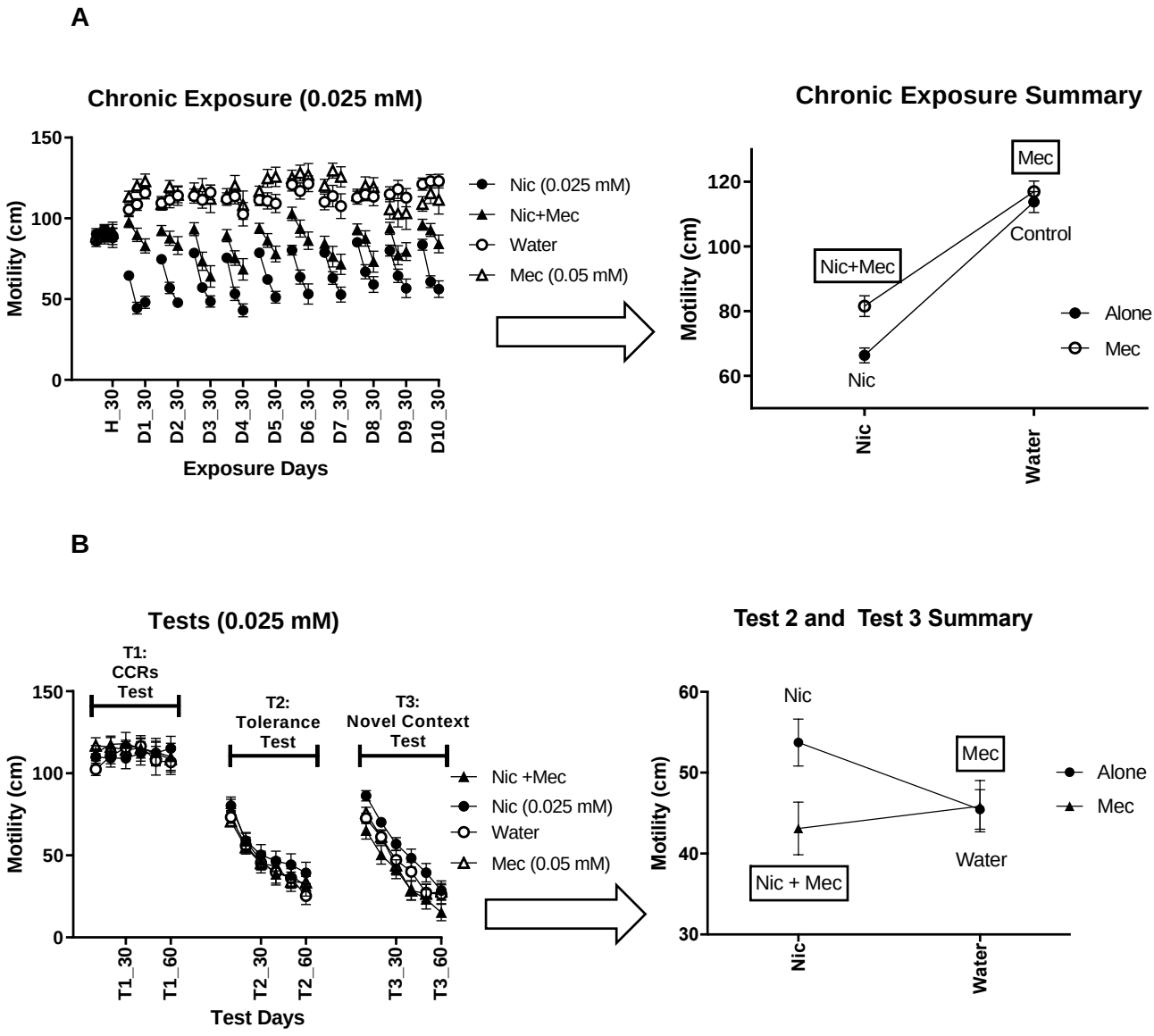

Figure 3. Experiment 2. (A, left panel) Mean distance covered by planarians in the exposure context throughout 1 day of habituation (H_30) and 10 days of 30 min in the presence of nicotine, water, mecamylamine or mecamylamine plus nicotine (D1_30 to D10_30). (A, right

panel) Summary of the chronic exposure results collapsing the data of the four groups across the ten days of training. (B, left panel) Mean distance covered during the 60 min of CCRs test in the presence of only water (T1); 60 min of tolerance test in the presence of nicotine (T2); and 60 min of novel context test in the presence of nicotine with an alternative context (T3). (B, right panel) Summary of the effect of nicotine following chronic exposure collapsing the data of the four groups across Tests 2 and 3 (in the presence of nicotine in the exposure and novel contexts). Results represented in 10 minutes bins (left hand panels). Bars represent standard errors. n = 26-28 planarians in each group.

5 Experiment 3. Abstinence-induced Behaviour and Tolerance Development with a medium dose (0.05 mM).

The goal of Experiment 3 was to investigate the after-effects of nicotine using the chronic exposure procedure used in Experiments 1 and 2 (10 daily exposure sessions); this would complement and expand the analysis by Rawls et al. (2011) who used an acute exposure procedure (a single exposure to nicotine). The procedure of Experiment 3 replicates the one described for previous experiments; however, following each daily exposure session throughout the experiment, the animals were given an additional 30 min in the exposure context but in the absence of nicotine to monitor the after effects of nicotine; also, following all test sessions, the animals were given an additional 30 min session with treated water in the exposure context (Tests 1 and 2) and in the novel context (after the Test 3). We allocated a total 48 animals to two groups, Nicotine and Water. Two animals in Group Nicotine died over the course of the experiment, resulting in n = 22 for Group Nicotine, and n = 24 in Group Water. We used a higher concentration of nicotine (0.05 mM) than the one used in the previous experiments because it would better approximate the dose used in previous planaria studies (Pagan et al., 2009; Rawls et al., 2011).

5.1 Chronic exposure

The data of the chronic exposure phase of the experiment is displayed in Figure 4A. As expected based on our previous findings, planarians exposed to nicotine showed less motility (M = 25.4 cm, SE = 3.6) than the planarians in the control condition, exposed to treated water (M = 82.1 cm, SE = 3.5) on the first day of the chronic exposure. Although some variability was observed across days, there did not seem to be a development of tolerance because there was an increase in the motility of both

groups across days. A 2 (Group: Nicotine *vs*. Water) x 10 (Days: 1 – 10) x 3 (Bins: 1-3) mixed ANOVA revealed main effects of Group, $F(1, 44) = 619.9$ $p < .001$, $\eta_p^2 = .93$, Days, $F(7.2, 321.1) = 4.89$, $p < .001$, $\eta_p^2 = .10$, and Bins, $F(1.9, 4.7) = 141.6$ $p < .001$, $\eta_p^2 = .76$, as well as significant interactions Group x Days, $F(7.2, 321.1) = 3.4$, $p = .001$, $\eta_p^2 = .07$, and Group x Bins, $F(1.9, 4.7) = 160.6$, $p < .001$, $\eta_p^2 = .78$, and a significant three-way interaction Group x Days x Bins, $F(12.2, 536.5) = 2.16$, $p = .01$, $\eta_p^2 = .05$. The remaining Days x Bins interaction was non-significant, $F(12.2, 536.5) = 1.31$, $p = .21$, $\eta_p^2 = .03$. Within-subjects linear contrasts revealed an effect of Day in Group Nicotine, $F(1, 21) = 22.33$, $p < .001$, $\eta_p^2 = .51$, but not in Group Water, $F(1, 23) = 2.27$, $p = .14$, $\eta_p^2 = .09$, suggesting an increase in motility in Group Nicotine but not Water. These results confirm the findings of Experiment 1, but with a higher dose, in that we observed development of tolerance to nicotine with a chronic exposure procedure in the planaria.

5.2 Test 1

Test 1 was conducted on the exposure context with water to assess the development of CCRs. As can be observed in Figure 4B (left panel, T1), planarians previously exposed to nicotine ($M = 88.9$ cm, $SE = 3.6$ cm) behaved in a similar way to animals in the Group Water ($M = 93.5$ cm, $SE = 3.5$ cm). This impression was confirmed by a 2 (Group: Nicotine *vs*. Water) x 3 (Bins: 1-3) mixed ANOVA, which revealed a significant effect of Bins, $F(2, 88) = 4.1$, $p = .02$, $\eta_p^2 = .08$, but no effect of Group, $F(1, 44) = 0.86$, $p = .35$, $\eta_p^2 = .02$, and no interaction between these factors, $F(2, 88) = 2.57$, $p = .08$, $\eta_p^2 = .05$.

5.3 Test 2 and Test 3

These tests were conducted to assess the development of tolerance to the unconditioned effects of nicotine (the hypo-locomotion response). Group Nicotine received the drug for the eleventh time whilst animals in Group Water received it for the first time in the exposure context (Test 2). Additionally, animals in both groups were tested with nicotine again, but in a novel distinctive context in Test 3. Figure 4B (central and right panels, T2 and T3) shows that planarians in Group Nicotine displayed higher levels of motility ($M = 41.1$ cm, $SE = 2.2$ cm) than the animals in the Group Water ($M = 29.2$ cm, $SE = 2.1$ cm) in both contexts, suggesting a context independent development of tolerance to the effects of nicotine.

These impressions were confirmed by a 2 (Group: Nicotine *vs*. Water) x 2 (Tests: Test 2 and Test 3) x 3 (Bins: 1-3) mixed ANOVA, which revealed main effects of Group $F(1, 44) = 15.70$, $p < .001$, $\eta_p^2 = .26$, and Bins, $F(2, 58) = 187.05$, $p < .001$, $\eta_p^2 = .81$, but no effect of Tests, $F(1, 44) = 3.27$, $p = .08$, $\eta_p^2 = .07$. There was a significant Group x Bins interaction, $F(2, 88) = 4.23$, $p = 0.02$, $\eta_p^2 = .09$. The remaining interactions were all non-significant: Group x Tests, $F(1,44) = 1.19$, $p = .28$, $\eta_p^2 = .03$, Tests x Bins, $F(2, 88) = 1.24$, $p = .29$, $\eta_p^2 = .03$, and the three-way Group x Tests x Bins interaction, $F(2, 88) = 0.22$, $p = .80$, $\eta_p^2 = .005$. These results suggest development of tolerance in the absence of context dependence.

5.4 Nicotine after-effect during chronic exposure

The data of after-effect sessions during the chronic exposure phase of the experiment is displayed in Figure 4C. Animals pre-treated with nicotine showed lower motility than planarians pre-treated with water during the added 30 min exposure to treated water across the chronic exposure phase. However, the motility of animals pre-treated with nicotine gradually increased across the days, consistent with the notion of tolerance development (in that case of the after-effect of exposure to nicotine). These impressions were confirmed with a 2 (Group: Nicotine *vs*. Water) x 10 (Days: 1-10) x 3 (Bins: 1-3) mixed ANOVA that revealed main effects of Group, $F(1, 44) = 25.11$, $p< .001$, $\eta_p^2 = .36$, Days, $F(6.9, 304.4) = 7.23$, $p < .001$, $\eta_p^2 = 0.14$, and Bins, $F(1.8, 80.4) = 28.27$, $p < .001$, $\eta_p^2 = .39$, as well as a significant interaction of Group x Bins $F(1.8, 80.4) = 26.62$, $p < .001$, $\eta_p^2 = .38$. The remaining interaction were all non-significant: Group x Days, $F(6.9, 304.4) = 1.64$, $p = .12$, $\eta_p^2 = .04$, Days x Bins, $F(14.5, 638.4) = 1.34$, $p = .17$, $\eta_p^2 = .03$, and the three-way Group x Days x Bins interaction, $F(14.5, 638.4) = 1.15$, $p = .31$, $\eta_p^2 = .03$. Within-subjects linear contrasts revealed an effect of Day in Group Nicotine, $F(1, 21) = 15.37$, $p < .01$, $\eta_p^2 = .42$, but not in Group Water, $F(1, 23) = 1,33$, $p = .26$, $\eta_p^2 = .05$, suggesting an increase in motility in Group Nicotine but not Water. This pattern of results suggests an after-effect of the nicotine treatment on the day of chronic exposure that progressively weakens by the end of the chronic exposure phase—indicating the development of tolerance of the nicotine after-effect.

5.5 Nicotine after-effect following Test 1

The after-effect responses were assessed following Test 1 (CCR test in the absence of the drug) by monitoring the animals during an additional 30 min period. The results of this additional 30 min period are displayed in Figure 4D (left panel, T1); planarians in the Groups Nicotine and Water behaved in a very similar way. A 2 (Group: Nicotine v*s.* Water) x 3 (Bins: 1-3) mixed ANOVA, revealed no significant effects of Group, $F(1, 44) = 0.02$, $p = .88$, $\eta_p^2 = .001$, and Bins, $F(1.7, 73.2) = 0.83$, $p = .41$, $\eta_p^2 = .02$; the interaction between these factors was also non-significant, $F(1.7, 73.2) = 1.73$, $p = 0.19$, $\eta_p^2 = .04$

5.6 Nicotine after-effect following Test 2 and 3

These tests were conducted to investigate the after-effect of nicotine following acute (Group Water) and chronic nicotine exposure (Group Nicotine): animals in the Group Water were exposed for the first time to nicotine in the Test 2, and only for the second time during Test 3 in a new environment (the animals in Group Nicotine were exposed to the drug for the eleventh and twelfth time). The results of the additional 30 min exposure to treated water in the exposure context after Test 2, and in the novel context in Test 3 are displayed in Figure 4D (central and right panels, T2 and T3). A visual inspection of the results suggest that both groups behaved in a very similar way. A 2 (Group: Nicotine *vs*. Water) x 2 (Tests: Test 2 and Test 3) x 3 (Bins: 1-3) mixed ANOVA, revealed a main effect of Tests, $F(1, 44) = 7.76$, $p = .01$, $\eta_p^2 = .15$, a significant Group x Bins interaction, $F(1.8, 81.9) = 5.88$, $p = .005$, $\eta_p^2 = .02$, and a significant Tests x Bins interaction, $F(2, 88) = 4.94$, $p = .01$, $\eta_p^2 = .10$. The remaining main factors and interactions were all non-significant: Group, $F(1, 44) = 0.53$, $p = .82$, $\eta_p^2 = .001$; Bins, $F(1.8, 81.9) = 22.10$, $p < .001$, $\eta_p^2 = .33$; Group x Tests interaction, $F(1, 44) = 0.54$, $p = .46$, $\eta_p^2 = .01$; and the three-way Group x Test x Bins interaction, $F(2, 88) = 0.43$, $p = .65$, $\eta_p^2 = .01$.

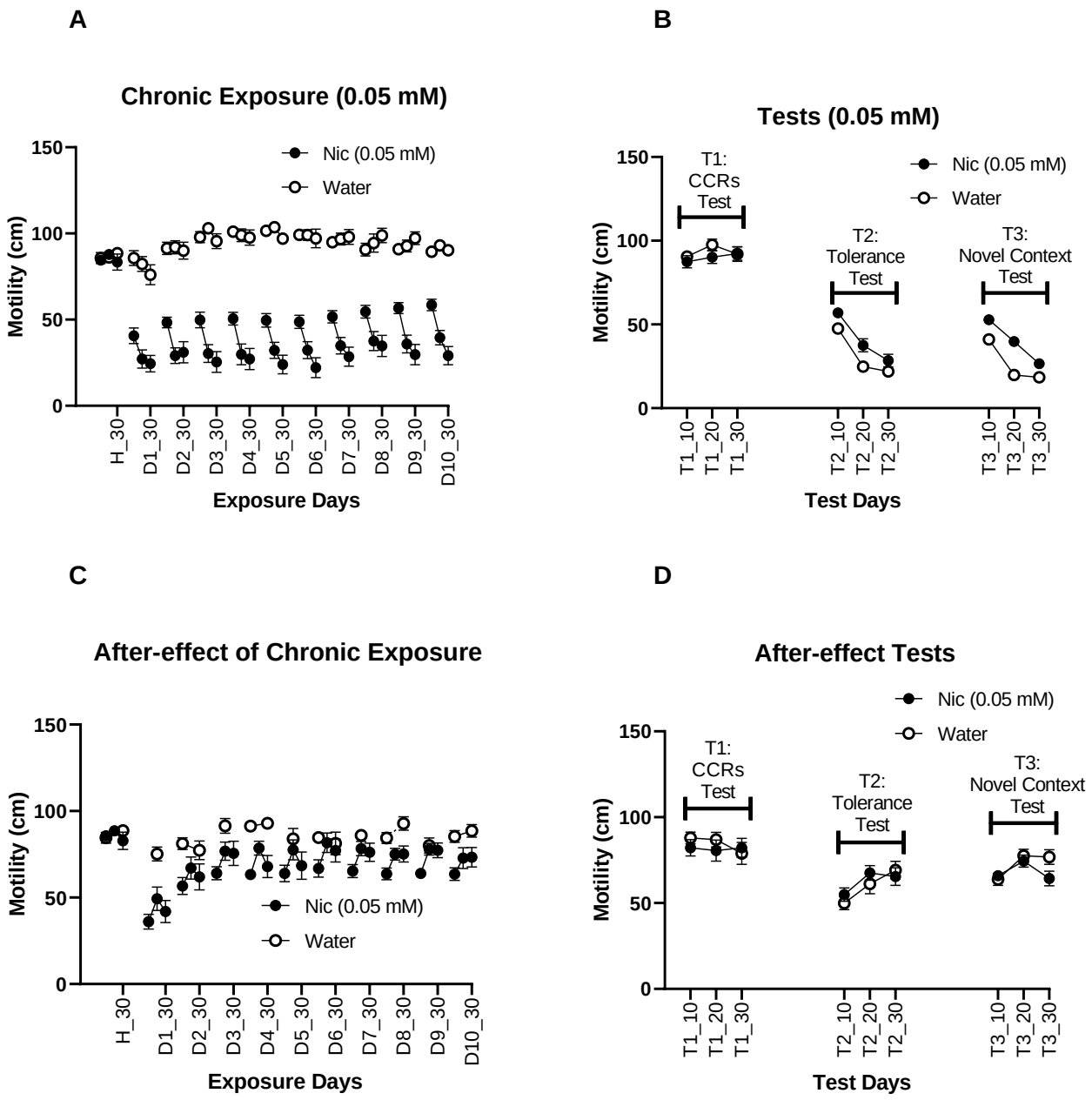

Figure 4. Experiment 3. (A) Mean distance covered by planarians in the exposure context throughout 1 session of habituation (H_30) and 10 sessions of 30 min in the presence of nicotine or water (D1_30 to D10-30). (B) Mean distance covered during the 30 min of CCRs test in the presence of only water (T1), 30 min of tolerance test in the presence of nicotine (T2), and 30 min of novel context test in the presence of nicotine with an alternative context (T3). Results represented in 10 minutes bins. Bars represent standard errors. n=15-16 planarians in each group. (C) Nicotine after effect assessed following exposure sessions: mean distance covered on the training context throughout 10 days of 30 min in the absence of nicotine after nicotine administration. (D) Nicotine after effect assessed following test: mean distance covered during 30 min abstinence test after the CCRs test (T1), 30 min of abstinence test after tolerance test (T2), and 30 min of abstinence test after novel context test (T3). Results represented in 10 minutes bins. Bars represent standard errors. n=22-24 planarians in each group.

6 Experiment 4. Abstinence-induced Behaviour and Tolerance Development with a high nicotine dose.

The goal of Experiment 4 was twofold: first, to replicate Experiment 3 with a higher nicotine concentration (0.1 mM); this was the nicotine concentration used by Pagan et al., (2009) in their study of withdrawal-like behaviour, and the lowest dose used by Rawls et al. (2011) used in their study of withdrawal-like behaviour and on the development of tolerance to nicotine. In addition, as we have observed, acute nicotine exposure of planaria causes a decrease in motility, and (in particular at high doses) others have also seen an increase in C-shaped responses (Rawls et al, 2011), which may be similar to stereotypies such as rearing or head twitching in rats. Rawls and colleagues' (2011) data suggest that C-shaped responses and motility are inversely related (*i.e.*, as C-shaped responses increase, the corresponding motility decreases). Therefore, a second goal was to quantify C-shaped responses to assess whether chronic exposure results in any changes in C-shaped behaviours. Following the results by Rawls and colleagues (2011), we did not expect a high rate of C-shaped behaviours because they did not observe that in their report with a similar dose (0.1 mM) as we used here. We allocated a total 48 animals to two groups, Nicotine and Water, resulting in n = 24 for Group Nicotine, and n = 24 in Group Water. Other than that, the experimental procedure replicates the one described in Experiment 3.

6.1 Chronic exposure

Over the course of the chronic exposure, the animals treated with nicotine showed lower levels of motility than the animals in the control group, exposed to treated water, replicating the results of previous experiments but with a higher nicotine concentration. Although some variability was observed across days, there did not seem to be a decrease in the effects of nicotine across days. The data of the chronic exposure phase of the experiment in Figure 5A suggest that motility in Group Nicotine was actually higher on the first day of exposure than on the last day of the exposure phase: the animals covered 33.8 (± 2.7) cm on Day 1, and 21.03 (± 2.6) cm on Day 10. These results do not suggest the development of tolerance during the chronic exposure to a relatively high concentration of nicotine; quite the opposite,

this pattern resembles the development of sensitization to the effects of the drug. These impressions were confirmed with a 2 (Group: Nicotine *vs*. Water) x 10 (Days: 1 – 10) x 3 (Bins: 1-3) mixed ANOVA that revealed a main effect of Group, $F(1, 46) = 858.9$, $p < .001$, $\eta_p^2 = .95$, and Bins, $F(2, 92) = 67.3$, $p < .001$, $\eta_p^2 = .59$, as well as significant Group x Days, $F(9, 414) = 2.58$ $p = .007$ $\eta_p^2 = .05$, and Group x Bins interactions, $F(2, 92) = 62.6$, $p < .001$, $\eta_p^2 = .58$. The remaining factor and interactions were all non-significant: Days, $F(9, 414) = 1.025$ $p = .42$, $\eta_p^2 = .02$; Days x Bins, $F(18, 828) = 0.69$, $p = .82$, $\eta_p^2 = .015$, and the three way Group x Days x Bins interaction, $F(18, 828) = 0.82$, $p = .67$, $\eta_p^2 = .02$. Within-subjects linear contrasts revealed an effect of Day in Group Nicotine, $F(1, 23) = 6.77$, $p = .01$, $\eta_p^2 = .22$, but not in Group Water, $F(1, 23) = 0.71$, $p = .40$, $\eta_p^2 = .03$. However, it should be noted that with this high dose, motility in Group Nicotine *decreased* rather than increased, revealing no tolerance development whilst the animals were under the effects of nicotine.

6.2 Test 1

Test 1 was conducted on the exposure context with water to assess the development of CCRs. As can be observed in Figure 5B (left panel, T1), the two groups performed in similar ways. A 2 (Group: Nicotine *vs*. Water) x 3 (Bins: 1-3) mixed ANOVA, revealed a significant effect of Bins, $F(2, 92) = 0.39$, $p = .68$, $\eta_p^2 = .008$. However, neither the main factor Group, $F(1, 46) = 0.68$, $p = .41$, $\eta_p^2 = .015$, nor the Group x Bins interaction, $F(2, 92) = 7.51$, $p = .001$, $\eta_p^2 = .14$, was significant.

6.3 Test 2 and Test 3

Figure 5B (central and right panels, T2 and T3) displays the results of Tests 2 and 3. The Group Nicotine displayed higher levels of motility than the Group Water in both contexts, suggesting the development of a context independent tolerance to nicotine. This impression was confirmed by a 2 (Group: Nicotine *vs*. Water) x 2 (Tests: Test 2 and Test 3) x 3 (Bins: 1-3) mixed ANOVA, which revealed significant main effects of Group $F(1, 46) = 6.57$, $p = .01$, $\eta_p^2 = .12$, and Bins, $F(2, 92) = 116.3$, $p < .001$, $\eta_p^2 = .72$; the factor Tests, however, was not significant , $F(1, 46) = 0.14$, $p = .71$, $\eta_p^2 = .003$. The main effect of Group, together with the absence of effect of the Tests factor suggest the development of context independent chronic tolerance to nicotine. The analysis also revealed a significant Test x Bins

interaction, $F(2, 92) = 5.31$, $p = 007$, $\eta_p^2 = .10$; the remaining interactions were all non-significant: Group x Tests, $F(1, 46) = 0.15$, $p = .70$, $\eta_p^2 = .003$; Group x Bins, $F(2, 92) = 2.31$, $p = .11$, $\eta_p^2 = .05$; and the three-way Group x Tests x Bins interaction, $F(1, 46) = 0.66$, $p = .52$, $\eta_p^2 = .014$.

6.4 Nicotine after-effect during tolerance training

The data corresponding to the additional 30 min of exposure to treated water in the exposure context following each of the chronic exposure trials is displayed in Figure 5C. Although we did not observe the development of tolerance (increased motility as the animal acquires experience with the drug) during the actual exposure trials, we observed the development of tolerance to the after-effects of the drug during the additional 30 min exposure to water. A 2 (Group: Nicotine *vs*. Water) x 10 (Days: 1 – 10) x 3 (Bins: 1-3) mixed ANOVA revealed main effects of Group, $F(1, 46) = 99.31$ $p < .001$, $\eta_p^2 = .68$, Days, $F(9, 414) = 10.22$, $p < .001$ $\eta_p^2 = .18$, and Bins, $F(1.5, 68.9) = 25.15$, $p < .001$, $\eta_p^2 = .35$, as well as significant Group x Days interaction, $F(9, 414) = 3.67$, $p < .001$, $\eta_p^2 = .07$, and Group x Bins interaction, $F(1.5, 68.9) =59.63$, $p < .001$, $\eta_p^2 = .56$. The remaining interactions were non-significant: Days x Bins, $F(11.8, 542.4) = 1.29$, $p = .19$, $\eta_p^2 = .03$, and the three-way Group x Days x Bins, $F(11.8, 542.4) = 1.17$, $p = .28$, $\eta_p^2 = .02$. Within-subjects linear contrasts revealed an effect of Day in Group Nicotine, $F(1, 23) = 79.51$, $p < .001$, $\eta_p^2 = .77$, but a marginally significant in Group Water, $F(1, 23) = 4.04$, $p = .056$, $\eta_p^2 = .15$. Thus, the assessment of the after effect of nicotine revealed tolerance development as was observed in previous experiments in this study.

6.5 Nicotine after-effect following Test 1

The after-effect responses were assessed following Test 1 (CCR test in the absence of the drug) by monitoring the animals during an additional 30 min period. The results of this additional 30 min period are displayed in Figure 5D (left panel, T1); planarians in the Groups Nicotine and Water behaved in a very similar way. A 2 (Group: Nicotine *vs*. Water) x 3 (Bins: 1-3) mixed ANOVA, revealed no effects of Group, $F(1, 46) = 0.24$, $p = .62$, $\eta_p^2 = .005$, Bins, $F(1.7, 76.7) = 0.18$, $p = .83$, $\eta_p^2 = .004$, and no interaction between these factors, $F(1.7,76.7) =0.13$, $p = .88$, $\eta_p^2 = .003$.

6.6 After-effect following Test 2 and Test 3

Figure 5D (central and right panels, T2 and T3) shows that the Group Nicotine displays higher levels of motility than Group Water during the additional 30 min that followed the Test 2 and Test 3. A 2 (Group: Nicotine *vs*. Water) x 2 (Tests: Test 2 *vs.* Test 3) x 3 (Bins: 1-3) mixed ANOVA, revealed significant main effects of Group, $F(1, 46) = 9.19$, $p = .004$, $\eta_p^2 = .17$, Tests, $F(1, 46) = 8.51$, $p = .005$, $\eta_p^2 = .16$, and Bins, $F(1.7, 79.1) = 75.9$, $p < .001$, $\eta_p^2 = .62$. The interactions between these factors were all non-significant: Group x Tests, $F(1, 46) = 1.35$, $p = .25$, $\eta_p^2 = .03$; Group x Bins, $F(1.7, 79.1) = 2.43$, $p = .10$, $\eta_p^2 = .05$; Test x Bins, $F(1.8, 82.8) = 0.61$, $p = .81$, $\eta_p^2 = .001$; and the three-way Group x Test x Bins, $F(1.8, 82.8) = 1.35$, $p = .26$, $\eta_p^2 = .03$.

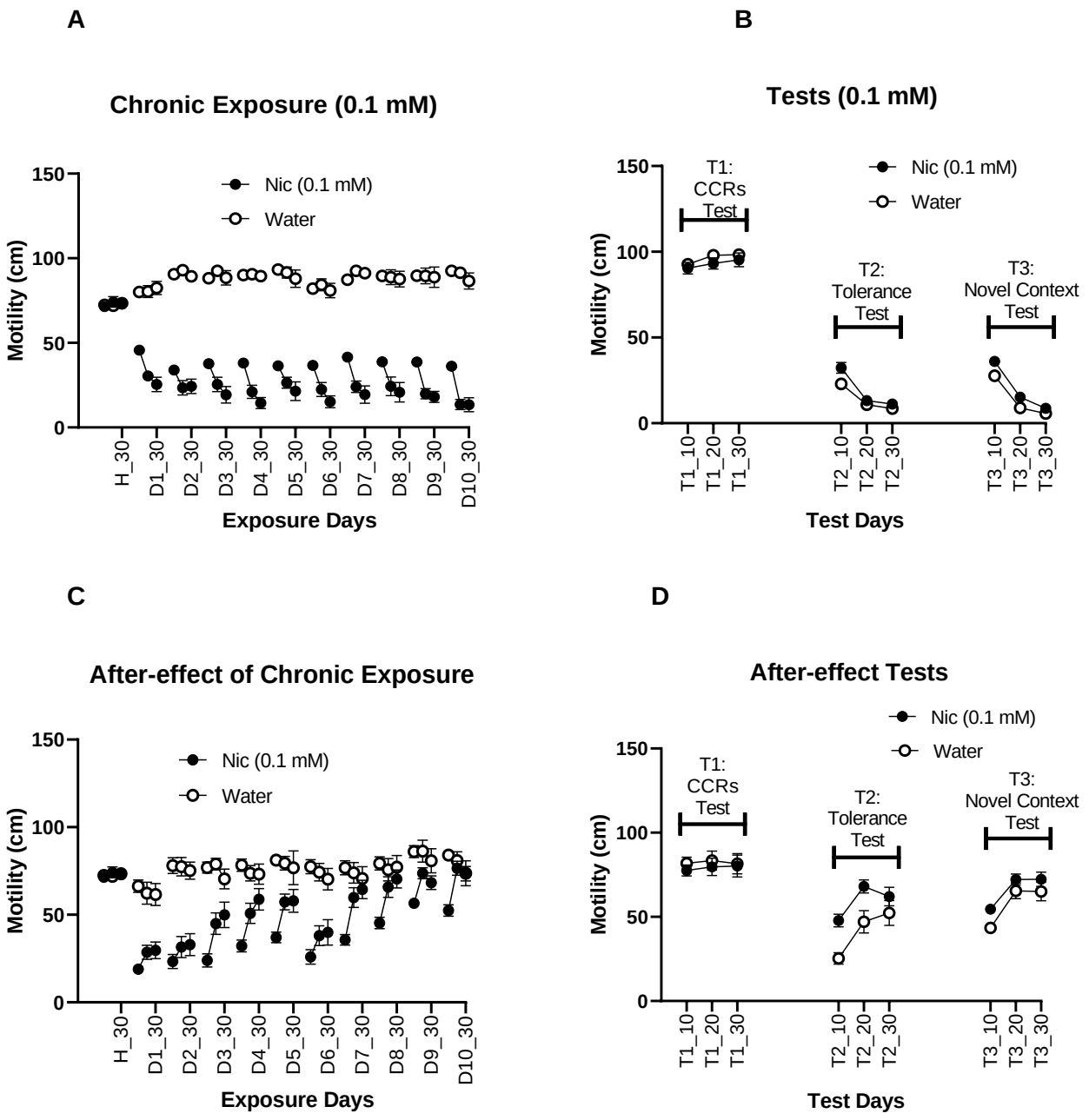

Figure 5. Experiment 4. (A) Mean distance covered by planarians in the exposure context throughout 1 session of habituation (H_30) and 10 sessions of 30 min in the presence of nicotine or water (D1_30 to D10-30). (B) Mean distance covered during the 30 min of CCRs test in the presence of only water (T1), 30 min of tolerance test in the presence of nicotine (T2), and 30 min of novel context test in the presence of nicotine with an alternative context (T3). Results represented in 10 minutes bins. Bars represent standard errors. n=15-16 planarians in each group. (C) Nicotine after effect assessed following exposure sessions: mean distance covered on the training context throughout 10 days of 30 min in the absence of nicotine after nicotine administration. (D) Nicotine after effect assessed following test: mean distance covered during 30 min abstinence test after the CCRs test (T1), 30 min of abstinence test after tolerance test (T2), and 30 min of abstinence test after novel

context test (T3). Results represented in 10 minutes bins. Bars represent standard errors. n=22-24 planarians in each group. Results represented in 10 minutes bins. Bars represent standard errors. n=24 planarians in each group.

6.7 C-shaped behaviours and the development of tolerance

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

7 General Discussion

The present study was aimed to assess 1) the development of tolerance to nicotine during repeated nicotine exposure in a specific context; 2) the expression of CCRs to nicotine-associated CS in the absence of nicotine; 3) the expression of nicotine tolerance in the presence of nicotine-associated cues; and 4) the role of a novel context on the expression of nicotine tolerance. We investigated the development of tolerance to the hypo-locomotor effects of nicotine using a long, 10-day chronic exposure regimen because it better resembles chronic exposure in humans. Across all experiments, we observed during the chronic exposure clear effects of increasing doses of nicotine suggesting that this paradigm and the dependent measure are sensitive to the effects of nicotine in the planaria *Schmidtea mediterranea*.

In Experiments 1, 2 and 3 using lower doses, we observed development of tolerance during chronic exposure, expressed as less effect of the drug during the last day of exposure (Day 10) relative to Day 1, and a linear effect during chronic exposure. The fact that this was not observed in Experiment 4 may be due to the large effect of the drug in suppressing motility. In none of the experiments we observed evidence for compensatory responses during Test 1. Nor did we observe any effect of changing the context from Test 2 to Test 3. However, all experiments revealed an effect of chronic exposure during Tests 2 and 3 (Comparison of Groups Nicotine *vs*. Water), suggesting the development of tolerance to the effects of nicotine. Experiments 3 and 4 also tested for evidence of withdrawal after nicotine removal, and both Experiments revealed an effect of chronic exposure on motility after nicotine was withdrawn, during chronic exposure and during Tests 2 and 3. Whilst variations in dose were paralleled by systematic changes in behaviour, the effect of chronic exposure was smaller during tests with increasing doses. This is likely due to the fact that we tested with the same dose as used during chronic exposure, and higher doses lead to larger unconditioned effects that may mitigate against the observation of tolerance. However, this is not surprising. Previous studies in rodents using nicotine (Stolerman et al., 1974) and morphine (Dafter & Odber, 1989) have also observed absence of tolerance development with high doses. Below we discuss the implications of these results.

These results are consistent with previous observations in planaria. For example, Rawls and colleagues (Rawls et al., 2011) observed a decrease in stereotypical activity following two administrations of high doses of nicotine (1 and 3 mM) on a third (5-min Test) exposure, suggesting tolerance development. Our results extend those previous findings to a chronic regimen of exposure (10 days) that better resembles chronic exposure on humans (also see Feng et al., 2006; and Polli et al., 2015, for similar results in *C elegans*). The results of these experiments also resemble observations in rodents. For example, Stolerman et al. (1973) observed a dose-dependent decrease in motility after different doses of nicotine (acute). In addition, chronic administration (3 times daily for 8 days) resulted in the development of tolerance to the effects of nicotine on motility, similar to what was found in the present experiments (see also Domino & Lutz, 1973 for similar results on bar-pressing behaviour). In

addition, in Experiment 2 we assessed whether mecamylamine, a nonselective nicotinic receptor antagonist, had an effect on the effect of nicotine and the development of tolerance. Consistent with previous observations in rodents, mecamylamine attenuated the unconditioned effects of nicotine and blocked the development of tolerance. Although mecamylamine did not completely block the acute effects of nicotine, this is likely due to the fact that we co-administered mecamylamine and nicotine, which may result in receptor binding by nicotine despite the administration of mecamylamine. The fact that mecamylamine blocked the development of chronic tolerance suggests that the latter depends on nicotinic receptor activity. Overall, the results of the present experiments are consistent with observations in other invertebrates and rodents, thus revealing that the mechanisms under study are evolutionarily conserved across vertebrate and invertebrate species.

Based on results obtained with other drugs of abuse in rodents and humans, it has been suggested that tolerance development (in particular learned tolerance) is context-dependent in that a novel context presentation eliminates tolerance to the unconditioned effect of drugs (Siegel, 1975). In all three experiments reported here, animals that received chronic nicotine exposure were tolerant to the suppressive effects of nicotine on the novel context, as suggested by a lack of interaction between Group and Test during Tests 2 and 3. It is possible that animals showed generalization from the exposure context to the novel context, although in other experiments we have observed good discrimination between the surfaces used here (e.g., Prados et al., 2020). Similarly, in the present experiments we did not observe the presence of CCRs when animals were tested in the presence of contextual cues but in the absence of nicotine. We did, however, use different concentrations, and observed that larger concentrations resulted in less motility, which in turn should result in more CCRs (if it is the case that CCRs result from homeostatic challenges). The presence of CCRs to nicotine-paired cues has not been widely observed in rodents, and some reports have failed to observe CCRs (Hakan & Ksir, 1988). However, experiments by Bevins et al. (2001; also see Walter & Kuchinsky, 1989) observed increased motility in rodents to context cues previously paired with nicotine effects. Although this effect was interpreted as a form of sensitization, the initial effect of nicotine was to supress motility and in that

sense these could be considered compensatory responses. Whether the lack of an effect in the present experiments represents a limitation of planarians or the incorrect choice of parameters is an open question at the moment. Finally, it could be possible that CCRs did manifest in the present parameters, but were not captured by motility as a dependent variable (DV). We chose to measure motility because this can be done automatically and therefore is bias-free, but it could be possible that the absence of CCRs is associated with our choice of DV, and that other DVs may reveal the presence of CCRs. Further research should shed light on this.

In Experiments 3 and 4, we investigated the after-effects of nicotine exposure to shed light on behaviour when nicotine has been removed (i.e., withdrawal). In Experiment 3, we observed during chronic exposure that the effect of nicotine decreased with training, so that the difference between Nicotine and Water Groups in Day 1 was no longer present on Day 10, although there were no differences between groups on the after-effect analyses conducted during Tests 2 and 3. A similar finding was observed during the exposure phase of Experiment 4 using a higher dose; however, we also observed an after-effect on Tests 2 and 3. That is, animals that had received chronic exposure to nicotine showed more motility relative to animals that experienced water, a finding that is similar to that of Pagan et al. (2009). We interpret this difference as indicative of withdrawal associated with tolerance development, for we observed *more* motility rather than less—which was observed by Rawls and colleagues (2011). Rawls et al. (2011) findings likely reflect after-effects of nicotine rather than withdrawal symptoms because they measured changes in the motility after a single (and short) exposure to nicotine, and any effects of drug-associated cues were not considered. The effect we observed was evident in the exposure (T2) and novel (T3) contexts. One intriguing possibility to explain these findings is that the interoceptive effects of nicotine acted as a conditioned stimulus, and this enabled both the observation of tolerance during nicotine exposure in Tests 2 and 3, and also the observation of a difference between groups in the after-effect period. Whilst this interpretation is speculative, there is convincing evidence in rodents (Murray & Bevins, 2007) and humans (Clemens et al., 1996) that nicotine can act as a conditioned stimulus. When nicotine is trained as a conditioned stimulus it can overshadow

and block performance about other associated environmental stimuli (Murray et al., 2011), and this may explain why tolerance was only observed during or after nicotine presentation, but not in the presence of nicotine-paired cues alone (Test 1).

In the introduction, we discussed two theories that explain tolerance following similar principles as those governing associative learning (Siegel, 1975; Solomon, 1980). Briefly, these models suggest that stimuli presented along with drug administration become associated with the unconditioned effects of drugs, and when presented in the absence of the drug elicit conditioned responses which are opposite to the unconditioned effect of the drug (CCRs). In addition, these theories predict that tolerance should be better observed in the presence of drug-associated cues than in their absence. In none of the experiments reported here we observed CCRs during Test 1. Similarly, we observed that tolerance to the hypo-locomotive effects of nicotine was similarly observed in the context where animals received chronic exposure and in a novel environment. The absence of differences during Test 1, given the large (dose-dependent) unconditioned effects we observed during chronic exposure, together with the insensitivity to context changes (Tests 2 and 3) are problematic for an associative account of tolerance. Rather, these results, and in particular the after-effect observed in Experiment 4, are consistent with a habituation explanation of tolerance as that put forward by Baker and Tiffany (1985). They suggested that the bulk of data available at the moment was more consistent with a habituation explanation of tolerance, and in particular with the basic tenets of habituation suggested by Wagner (1976). According to Wagner's model, habituation (and hence tolerance) occurs due to the action of either of two mechanisms: associative priming and self-generated priming (see Wagner, 1976 and Prados et al., 2020 for a detailed explanation). Associatively generated priming enables environmental cues associated with drug effects to attenuate, in the long-term, the unconditioned effects of drugs, resembling the well-known diminution of unconditioned effects observed in basic learning procedures (Kimmel, 1966). Self-generated priming allows a representation of the drug effects to be primed in short-term memory by a previous drug exposure, and reduces the unconditioned effects of drugs. Self-generated priming explains quite well the findings of Tests 2 and 3 in all experiments, and the after-effect observed in Tests

2 and 3 in Experiment 3, where planarians in Group Nicotine showed less effect of nicotine (i.e., tolerance) following discontinuation of the drug. According to the habituation explanation of chronic exposure to nicotine during these tests, the prior presentation of the drug during the test resulted in less responding to the drug after-effects, an explanation which is also consistent with the above speculations of nicotine acting as a CS.

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

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