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Cotinine antagonizes the behavioral effects of nicotine exposure in the planarian *Girardia tigrina*

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Abstract

Nicotine is one of the most addictive drugs abused by humans. Our laboratory and others have demonstrated that nicotine decreases motility and induces seizure-like behavior in planarians (pSLM, which are vigorous writhing and bending of the body) in a concentration-dependent manner. Nicotine also induces withdrawal-like behaviors in these worms. Cotinine is the major nicotine metabolite in humans, although it is not the final product of nicotine metabolism. Cotinine is mostly inactive in vertebrate nervous systems and is currently being explored as a molecule which possess most of nicotine's beneficial effects and few of its undesirable ones. It is not known whether cotinine is a product of nicotine metabolism in planarians. We found that cotinine by itself does not seem to elicit any behavioral effects in planarians up to a concentration of 1 mM. We also show that cotinine antagonizes the aforementioned nicotine-induced motility decrease and also decreases the expression of nicotine-induced pSLMs in a concentration-dependent manner. Also cotinine prevents the manifestation of some of the withdrawal-like behaviors induced by nicotine in our experimental organism. Thus, we obtained evidence supporting that cotinine antagonizes nicotine in this planarian species. Possible explanations include competitive binding of both compounds at overlapping binding sites, at different nicotinic receptor subtypes, or maybe allosteric interactions.

Keywords

planaria; cotinine; nicotine; motility; seizure-like movements; withdrawal; artificial pond water

1. Introduction

Planarians are freshwater flatworms and are excellent model organisms in the field of neurobiology, regeneration and pharmacology research. Their multipolar neurons are more similar to vertebrates than to more evolved invertebrates, displaying a primitive bilateral

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brain and featuring many of the neurotransmitters found in vertebrates [1,2]. Recent pharmacological research shows planarians being used as models for abused compounds such as cocaine, amphetamines, and nicotine among others [3-6].

Nicotine is one of the most commonly abused substances in the world that leads to addiction [7]. It is best-known as a component of tobacco products [8] and its main molecular target are nicotinic acetylcholine receptors [9]. In planarians, nicotine displays an increase in motility at lower concentrations, a decrease in motility at higher concentrations, and an increase in planarian seizure like movements (pSLM) [10,11]. Planarians exposed to nicotine for a period of about 24 hours display a series of withdrawal-like behaviors [5].

Cotinine is the major metabolite of nicotine in humans and both molecules are very similar structurally, the only difference being a carbonyl group. Despite this close structural similarity, cotinine does not display the same effects as nicotine [12]. For example, upon consumption of tobacco products, cotinine is found in the blood at higher levels than nicotine and is metabolized at a slower rate. Moreover, cotinine is not the final step in nicotine metabolism. In bovine adrenal chromafin cells, cotinine has shown to desensitize nicotinic responses and inhibit protein kinase C activation [13,14]). In rats, cotinine was found to have a binding affinity approximately three orders of magnitude lower than nicotine [15]. To the best of our knowledge, this is the first work describing nicotine-cotinine interactions in planarians. This work will contribute to the characterization of nicotinic cholinergic systems in our experimental organism.

2. Materials and methods

Brown planarians (*Girardia tigrina*) were purchased from Ward's (Rochester, NY). Cotinine and nicotine were purchased from Tocris, (Bristol, UK). Other laboratory materials and supplies were purchased from Fisher scientific (Suwanee, GA) or Sigma-Aldrich (St. Louis, MO). The graphs/statistical analyses were done with the Prism software package (GraphPad Inc., San Diego, CA).

Planarians were transferred to artificial pond water (APW, NaCl, 6mM; NaHCO3, 0.1 mM; CaCl2, 0.6 mM) upon arrival, and were acclimated to these conditions for at least 24 hours before use. All planarians received were used within three weeks of arrival. The APW was changed at least once every day except during weekends and always before experiments. All petri dishes, ceramic wells, graduated cylinders and microcentrifuge tubes were rinsed with APW before experiments. All experiments were performed at room temperature in APW. All reagents were prepared as APW solutions.

Planarian motility and seizure like movements were studied using established laboratory protocols [11,16,17]. To determine the planarian motility, a planarian (1.0-1.5 cm long) was placed in a clear APW-rinsed 6 cm polystyrene petri dish and placed over a $1cm^2$ grid. 5mL of experimental solutions (APW, nicotine 10-1000 μM, cotinine 10- 1000 μM, and combination 10-1000 μM nicotine/1000 μM cotinine) were added and the cumulative crosses were counted over a period of 8 minutes [16]. For pSLM, measurements a planarian was placed in an APW rinsed ceramic well. The ceramic well was filled with experimental

solution (APW, nicotine 10-1000μM, cotinine 10-1000 μM, combination nicotine 1000 μM/ cotinine 100-1000 μM) and pSLMs were observed and counted over a period of 10 minutes. The use of these ceramic containers allowed us to minimize the amount of reagents used. Withdrawal-like behavior experiments were adapted from established protocols [5]. Planarians were placed individually in 1.5 mL microcentrifuge tubes with experimental solutions of either APW, 100 μM nicotine, 100 μM cotinine, or a combination of 100 μM nicotine/100 μM cotinine and were closed for 21-26 hours. After this time, planarians were transferred to a ceramic well with APW in the absence of any other compounds and measured for the following movements: 'headbop' (bobbing head while moving), 'corkscrew' (spiral movement while in motion), 'tailtwist'(twisting of the tail), 'headswing' (head movement while tail is anchored), and 'squirming'(jerking or scrunching of the body) as described in [4]. Measurements occurred at three time periods, 0-5 minutes, 30-35 minutes, and 60-65 minutes.

3. Results

When measuring motility of the planarians exposed to 10 μM-1000 μM nicotine, they experienced a concentration-dependent decrease in velocity (Figure 1), with an IC_{50} of 162 μM. However, when exposed to the same amount of cotinine (10 μM-1000 μM), no significant motility decrease was observed. On the other hand, the presence of 1 mM cotinine significantly shifted the apparent nicotine IC_{50} to 490 μM, a threefold difference (Figure 1, p-value = 0.002, F-test).

When planarians were exposed to nicotine in solution with concentrations ranging from 10 μM-2000 μM, they experienced a concentration-dependent increase in pSLMs (For an example of such a concentration-response curve please see [18]). Cotinine, however, at ranges from 10 μM-1000 μM, caused no changes in pSLM when compared with controls of APW (data not shown). However, when the worms were co-exposed to a single concentration of nicotine (1 mM) concurrently with various concentrations of cotinine (100 μM - 1 mM), nicotine displayed a significant concentration-dependent cotinine-induced decrease in pSLMs (Figure 2).

Planarians exposed to nicotine for a period close to 24 hours expressed withdrawal-like behaviors, while those exposed to cotinine did not show any significant difference from control worms. However, when nicotine and cotinine were combined (Figure 3) although a qualitative decrease in the number of withdrawal-like behaviors were observed in some cases, these were not statistically significant as plotted. On the other hand, when the data was expressed, not as the number of events over time but rather, as the cumulative number of events in the presence of the compound combinations, we were able to observe a significant cotinine-induced decrease in some of the nicotine-induced withdrawal behaviors (Figure 4).

4. Discussion

Our experiments show several intriguing results in regards to nicotine/cotinine interaction in planarians. The first point is that these results confirm previous experiments in planarians using nicotine; namely, our results confirm that nicotine decreases motility, induces seizure-

like movements, and still causes withdrawal-like behavioral effects after an approximately 24 hour incubation [4,5,10,11]. Second, cotinine, a nicotine metabolite, elicits no statistically significant behavioral changes in our experimental organism, despite it being very structurally similar to nicotine.

The third and most important finding however, is that when these two compounds are in the same solution, the motility decrease and pSLM behaviors are significantly reduced when compared to the exposure of nicotine alone (Figures 1,2) which mimic acute exposure to nicotine. Cotinine also reduces the expression of three out of five nicotine-induced withdrawal-like behaviors that mimic chronic nicotine exposure (Figures 3,4).

Moreover, our work suggests that the molecular targets or maybe the mechanisms of nicotine that induce motility decrease, pSLMs and withdrawal-like behaviors may be independent of each other. Several points support this assertion. Cotinine does not antagonize all the nicotine-induced behaviors in all experiments (see Figures 3,4; withdrawal-like behaviors). In contrast, cotinine antagonizes both the motility decrease and the pSLMs induced by nicotine, albeit with different potencies. For example, in the motility studies, 1 mM cotinine is able to reduce the apparent nicotine potency threefold (Figure 1), while at the same concentration cotinine is only able to reduce nicotine's potency to induce pSLMs by approximately 50 % (Figure 2).

We speculate that cotinine is binding to the same or overlapping binding site of nicotine or that cotinine is binding at an allosteric site changing the conformation of the receptor target. Moreover, it is known that there is a variety of nicotinic acetylcholine receptors described in vertebrates. Several of these have also been identified in certain planarian species [19,20]. It is quite possible that different types of nicotinic receptors may modulate different behavioral responses. Future experiments will include work using other cholinergic compounds to elucidate the various mechanisms through which nicotine induces its planarian-related behaviors.

Author's contributions: D.J.B. wrote the first version of this manuscript and contributed to the design and performance of the experiments and to the data analyses/interpretation. M.T., D.L.B., S.D., and E.M. contributed to the performance of the experiments and to the data analyses/interpretation. O.R.P. originated the concept of testing cotinine and nicotine in planarians, designed and performed experiments, compiled, analyzed, and interpreted the data, designed and prepared the figures and edited the original manuscript. All authors proofread the paper.

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Highlights

- Our experimental animal is the planarian.
- Cotinine antagonizes the nicotine-induced motility decrease.
- Cotinine antagonizes the nicotine-induced seizure-like movements.
- Cotinine antagonizes three types of nicotine-induced withdrawal behaviors.

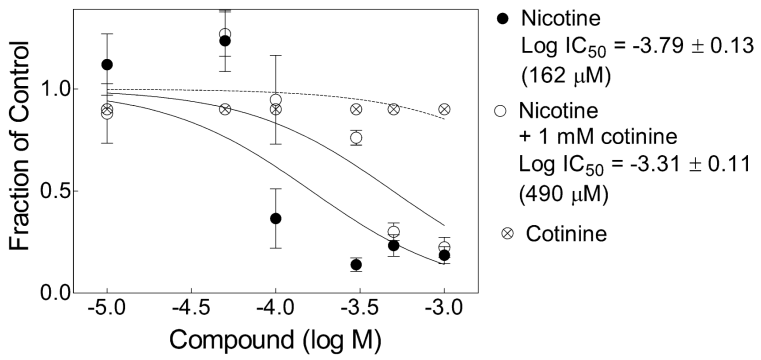

Figure 1.

Concentration-response curve of the fraction of control of planarian motility vs. either nicotine alone, cotinine alone or the combination of both. Cotitine did not reduce planarian motility, while nicotine did, in agreement to previous work. Moreover, the presence of cotinine significantly shifted the apparent IC_{50} of nicotine threefoldwise to the right (162 μM to 490 μM, p = 0.0002, see text). The symbols represent the average response of at least six worms. The data points were fit to the equation: Fraction of control = Bottom + (Top-Bottom) / $(1+10^{(\log M \text{ Nicotine} - \text{LogIC50})})$. The error bars represent the standard error of the mean.

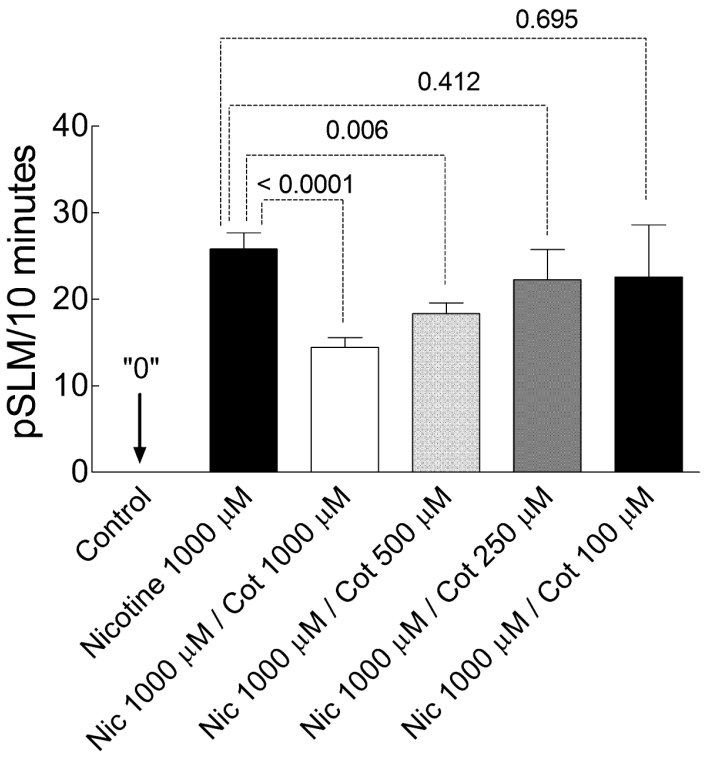

Figure 2.

Cotinine antagonizes the induction of pSMLs by nicotine in a concentration-dependent manner. The error bars represent the standard error of the mean. The bars are different from each other by ANOVA ($P < 0.0001$), and each bar represents the average of 4-16 worms. The individual p-values between bars shown above were calculated by the non-parametric Mann-Whitney test.

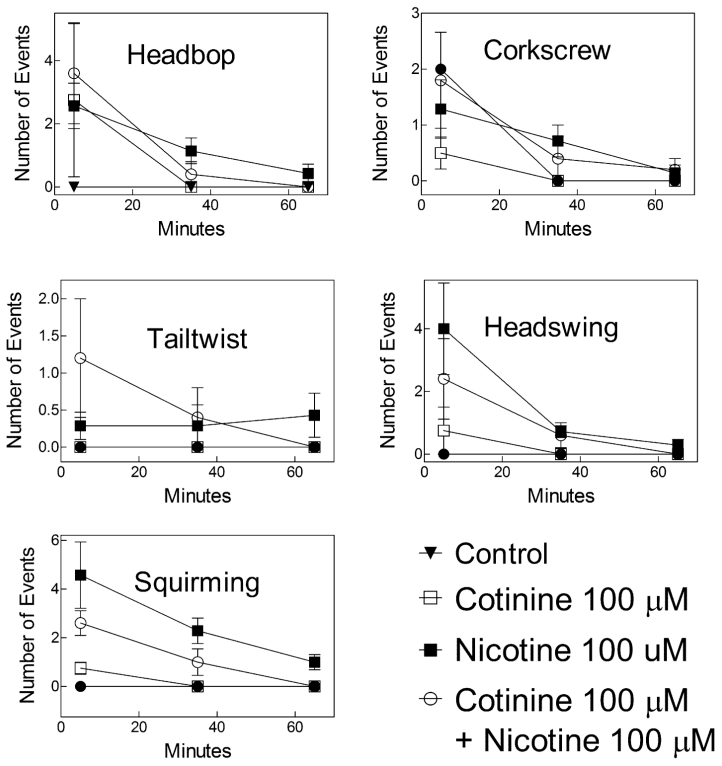

Figure 3.

Cotinine fails to antagonize the induction of withdrawal-like behaviors induced by nicotine as a function of time, as indicated. The error bars represent the standard error of the mean. Each symbol represents the average of 4 to 8 worms. In all behaviors, the results (control, nicotine, cotinine, and nicotine/cotinine, as indicated) were not statistically significant from each other (Kuskal-Wallis test followed by a Dunns post-test). Whenever a symbol is not visible it means that it's being overlapped by other symbols.

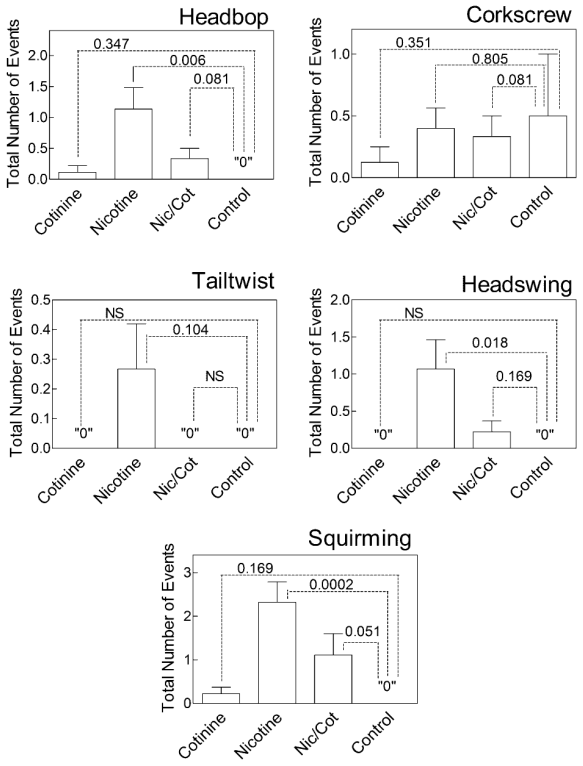

Figure 4.

Alternate analysis of the data in Figure 3. Cotinine antagonizes three out of the five nicotine- or cotinine-induced, withdrawal-like behaviors when analyzing them based on the cumulative number of events over the 65 minutes of each experiment. In none of the five cases cotinine induced the withdrawal-like behaviors, determined by comparing it to the control (APW; NS = Not Significant). Moreover, with the exception of the corkscrew and tail twist behaviors, the consistently observed pattern was: Cotinine vs Control = NS; Nicotine vs Control = Significant, Nicotine/Cotinine vs Control = back to NS. Thus, we interpret this data as the cotinine “alleviation” of the nicotine-induced withdrawal behaviors in three out of the five observed behaviors. P values were obtained by two-tailed, unpaired t-tests. The error bars represent the standard error of the mean. Each bar represents the average of 4 to 8 worms.