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Commentary

Investigating putative psychoactive compounds using planarians as an animal model using the environmental place conditioning protocol

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Abstract

There is a seemingly endless list of compounds used by humans that may have psychoactive reinforcing properties underlying their repeated use; many have never been adequately studied. In a 2019 paper, the potential reinforcing properties of cotinine, a major nicotine metabolite, were investigated for the first time. To pursue this line of inquiry, cotinine was assessed using the planarian as an animal model in environmental place conditioning (EPC) also known as conditioned place preference (CPP). In this 2019 study, planarians demonstrated that the compound cotinine, which is present in tobacco smoke, and is also the principal nicotine metabolite, establishes a conditioned place preference. These data represent the first ever demonstration that cotinine will establish a conditioned place preference in planarians and possibly contribute to the addictive properties of nicotine. In the present paper, arguments will be made for the merits of the use of the planarian as a useful experimental animal and in addition, a review of the utility of the conditioned place preference to assess psychoactive effects of drugs of abuse.

Keywords: Conditioned place preference, Environmental place conditioning, Planarians, Cotinine, Psychoactive effects, Incentive salience

Introduction

One way to study the effects of known or putative psychoactive compounds is to use an animal model such as the planaria. These fresh-water invertebrates have been widely used as an animal model to assess the effects of a variety of drugs, including ethanol, nicotine, and cocaine [1]. At first glance, this invertebrate might be considered as far too distal to humans or even mammals to be a useful animal model. There are, however, several lines of evidence to support the use of this invertebrate in basic research of the effects of drugs upon the nervous system. In flatworms such the planarians, the first evidence of cephalization, sequestering of polarized neurons in the anterior portion of the body, as well as bilateral symmetry are first seen [2]. The human brain and the planarian's primitive encephalization have enough commonalities such that the cephalic structures composing the planarian "brain" has been termed an ascendant of the human brain [3]; the planarian brain consists of bi-lobed cephalic ganglia consisting of approximately 20,000 to 30,000 neurons [4]. Genomic analyses of the genes responsible for the nervous system in one species of planarian isolated 116 genes, with 110 of these or more than 95% of these genes have their homologs in humans, *Drosophila melanogaster* and *Caenorhabditis elegans*. These genes were found to be involved in nervous system morphology as well as the formation of neural circuits [5]. In more recent research, glial cells have been identified in the planarian nervous system; Glia are increasingly recognized as a key participant in nervous system development and function. The glial cell types identified in the planarian are predicted to be involved in reuptake of GABA as well as the uptake of glutamate, similar to how astrocytes function in other animals [6]. Furthermore, planarians employ many of the same neurotransmitters as mammals, including serotonin,

dopamine, norepinephrine, acetylcholine, GABA, the excitatory amino acids glutamate and aspartate, as well as endogenous opioids and endocannabinoids [7]. In addition, some authors have argued that planaria can engage in behavior that reflect complex "decision making" in response to abrupt environmental changes and signals, indicating the invertebrate has behavioral commonalities with more complex organisms [4].

When planarians have been used as an animal model to study the effects of drugs with addiction potential, the primary dependent variables were measures of an acquired preference for a distinctive setting paired with a psychoactive drug. An acquired preference to seek out and remain localized in a distinct setting paired with a psychoactive drug is an outcome known as a conditioned place preference (CPP) or environmental place conditioning [8]. The conditioned place preference protocol is widely employed as a means of assessing a compound's potential rewarding or addictive properties [9]. Drugs with abuse potential will function as a positive reinforcer for self-administration by laboratory animals, and with a small number of outlier-exceptions, drugs that maintain self-administration also produce a conditioned place preference [10].

One of the simpler means to utilize planarians in a conditioned place preference procedure is by way of the biased conditioned place preference protocol which takes advantage of planarian's light phobic, or negative phototaxic behavior [11]. The majority of these flatworms display a strong preference to stay in a darkened environment [9]. While the relevant literature clearly states the planarians exhibit such light phobic behavior, this conclusion requires some qualifications. The degree of light avoidance can vary considerably as a function of the wavelengths of light used [11]. In a 2019 paper, Phelps and colleagues found that if light phobic behavior was defined as spending 70% of a specified time interval in a darkened half of a Petri dish, a considerable minority percentage of the planarians we observed did not meet this criterion [12]. In this study, 3.0w white LED lamps were used, and the precise wavelength spectrum of light emitted by these bulbs was not known. A typical means of defining light phobic behavior in the relevant literature using planarians in the CPP procedure takes a more liberal criterion; a majority, i.e., more than half of the time spent in a darkened half of a Petri dish indicates light phobic behavior [8].

In a biased CPP, the preference for different contexts or environments is assessed by placing the organism in the Petri dish and measuring the time an organism spends in the distinctive places when it can move about freely in either of the compartments. The environment in which the organism spent the least amount of time in is then paired with a reinforcer or compound with reinforcing properties [13]. If an illuminated environment which was originally a neutral stimulus (a NS) is paired with a compound with reinforcing properties, an unconditioned stimulus (a US), and the light-phobic behavior is reversed, this is considered a conditioned place preference for the drug-paired environment. The drug-paired context comes to function as a conditioned stimulus (a CS) which has acquired incentive salience that reflect the reinforcing properties of the drug [14].

A number of studies have documented the occurrence of CPP in the planarian and in a range of vertebrate species, including humans [14]. Relatively little research, however, in any animal model has assessed the behavioral effects of cotinine [12,15-20]. Prior researchers had raised the question of whether compounds in tobacco smoke or nicotine metabolites, such as cotinine, have putative roles in tobacco dependence [15,19]. One interesting question is the possibility that these compounds may alter the properties of nicotine or which might possess their own incentive salience effects. These compounds have been the subject of far less research, but some have proposed these compounds were worthy of investigation because they may alter or potentiate the properties of nicotine [15,19].

Cotinine poses interesting questions that have been neglected or given sparse attention [19]; cotinine is a minor alkaloid found in tobacco and tobacco smoke [15,19]. In addition, cotinine is the major metabolite of nicotine [20] and has been used as a biomarker of nicotine exposure or consumption in humans [21]. Cotinine bears a very close structural resemblance to nicotine, the sole difference being a carbonyl group and like nicotine, cotinine binds with nicotinic acetylcholine receptors and likely functions as a partial agonist upon nicotinic receptors in the mesolimbic system [21,22]. The two compounds produce similar interoceptive and potentially, similar behavioral effects [17,23]. Cotinine has a significantly longer half-life than that of nicotine, which entails that this compound is present as nicotine levels are becoming negligible [21,24]. Cotinine has also been shown to stimulate the release of dopamine in midbrain structures in some studies; other studies failed to find the same outcome [16,22]. These differences were possibly due to methodological differences. In drug discrimination studies, cotinine was shown to function as a discriminative antecedent stimulus that could alter the response rates on operant schedules of reinforcement with some degree of generalization to nicotine as a discriminative antecedent stimulus [17].

Bach and colleagues examined cotinine's effects in combination with nicotine on planarian motility [18] and only one study assessed the effects of cotinine on CPP, but in a rodent model [16]. Exposure to nicotine reduced planarian motility and was observed to antagonize the occurrences of seizure-like-movements elicited by nicotine [18]. Bach and colleagues concluded that cotinine in isolation did not elicit any statistically significant changes in the behavior of planarians [18]. With the question of assessing cotinine's effects on planarian behavior, Phelps et al. presented data that demonstrated cotinine established a CPP in planarians [12]. The significance of the 2019 paper were that unbeknownst reinforcing properties of cotinine were identified, a novel finding. This commentary seeks to point out that the planaria are an underutilized animal model and that the CPP is a procedure with wide-ranging and diverse application that is remarkably utilitarian in terms of apparatus, technique and outcomes. Some of the methodology from the 2019 paper on the effects of cotinine are presented below.

Materials and Methods

Brown planarians (*Dugesia dorotocephala*) were obtained from Carolina Biological Supply Company (Burlington, NC) and the use of these invertebrates were exempt from IACUC regulation. Cotinine was purchased from Toronto Research Chemicals (Toronto, Ontario, CA). The molecular weight of cotinine is 176.22. We made a 10 mM stock solution (1.76 mg/ml) using spring water. From the 10 mM stock solution, we prepared the diluted concentrations (0.01, 0.02, and 0.04 mM) by adding spring water. The solutions were kept under refrigeration until needed. The concentrations were brought to room temperature before the planarians were exposed

to the concentrations. All other laboratory supplies were obtained from Fisher Scientific (Suwanee, GA) or Carolina Biological Supply Company. The planarians were maintained in a small aerated 0.5-gallon aquarium with tap water prepared for the planaria with a water-treatment product (AmQuel ®), 1 ml of AmQuel per gallon of water. The flatworms were housed in a room with 12 h/12 h dark-light environmental controls and were acclimated to the laboratory environment for at least 24 h prior to any use. The water was changed approximately once a week, the worms were fed once every seven days, with overnight (12 h) access to a source of protein (a small amount of beef-calf liver).

The data collection for the CPP measures consisted of observations of the behavior of the planaria during pre-test, conditioning and post-test. In pre-test, planarians were transferred individually to a Petri dish (5.5 cm diameter) filled with water that was covered with opaque black plastic to the midline of the dish, creating a Petri dish that was half darkened, half illuminated. In addition to the overhead lighting during the 12 hour (h) light period, increased illumination came from a 3.0 watts (w) white LED lamp, approximately 30 cm above the Petri dish. All data collection was conducted during the light period of the day. The worms were introduced to the midline of the Petri dish with a 3 ml pipette. During a 10 min interval, trained research assistants timed the duration of the time the worms spent in the dark half of the dish compared to the time spent in the illuminated half, following procedures described in other studies [8]. Immediately following the pre-test period, the worms were transferred to a separate Petri dish containing either water as a control condition or a solution of cotinine in water (0.01 mM, 0.02 mM and a 0.04 mM) in a one-time, acute exposure. These concentrations were chosen based on the findings that nicotine in a 0.01mM concentration would establish a CPP using planarians [13] and we then extrapolated beyond that data to investigate the effects of the 0.02mM and the 0.04 mM cotinine solutions. The 10 min conditioning drug-solution exposure interval was also under LED illumination. Following the conditioning drug-solution exposure, the worms were relocated to a separate Petri dish containing water for a 120 min interval [8]. Finally, the worms were re-introduced to the half-dark/half-illuminated water-filled Petri dish for post-test observations. The durations of the time spent in the dark relative to the time in the illuminated portion of the dish were again measured for a 10 min interval; these intervals are based on relevant studies of planarians being exposed to nicotine [8].

In all, there were 160 light-phobic planaria, n=40 for each concentration (or control), that were exposed to the CPP protocol. In the present study, light phobic behavior was defined as a worm spending 7 min or longer in the dark-half of the Petri dish in the 10 min pre-test interval; flatworms that were not light phobic in the pre-test interval were excluded from the analysis. The CPP was calculated as a difference score between the amount of time spent in the illumination (i.e., post-test minus pre-test). Positive values indicate a CPP.

Results

A one-way ANOVA comparing mean preference scores indicated a significant main effect of concentration ($F(3, 159) = 37.14$, $p<0.001$, $\eta^2_p=0.42$). The effects of cotinine (or control) on preference scores are shown in Figure 1. Tukey post-hoc analyses revealed flatworms in each cotinine solution displayed a significantly

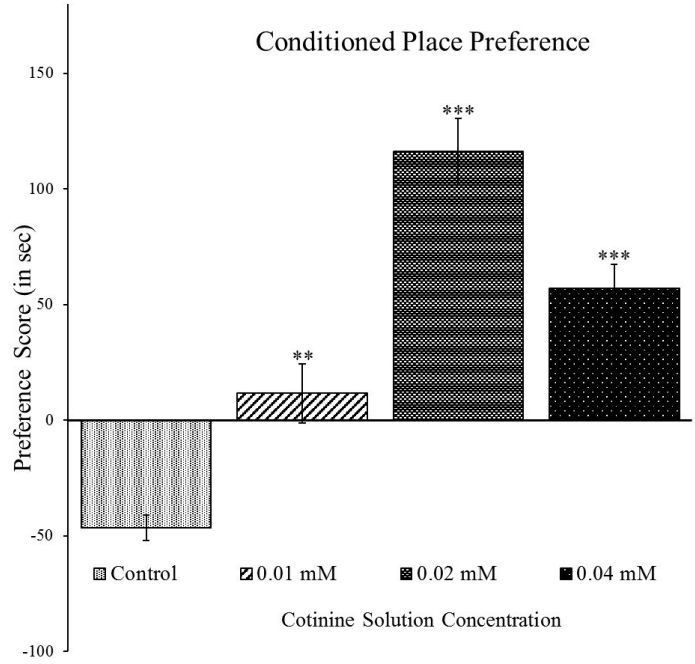

Figure 1: Preference score for the time in illuminated section for the planarians exposed to either water as Control or the 0.01 mM, 0.02 mM or 0.04 mM concentration of cotinine. Positive values indicate a conditioned place preference. Error bars indicate standard error. ***p<0.001 or **p<0.01 compared to control.

greater amount of time in the light compared to control, a likely demonstration of a CPP. Specifically, worms in the 0.01 mM condition spent significantly more time in the illuminated section compared to control (p=0.002, SE=16.01), 0.02 mM spent significantly more time in the illuminated section compared to control (p<0.001, SE=16.01). Finally, the 0.04 mM spent significantly more time in the illuminated section compared to control (p<0.001, SE=16.01); also see Figure 1.

Discussion

Cotinine has properties that make it worthy of analysis, but few studies have investigated the behavioral effects of cotinine in any animal model. The present results suggest that cotinine has reinforcing properties; furthermore, one possible conclusion is that cotinine may contribute to the effects of nicotine. The results of the 2019 study are comparable to a nicotine CPP study using planarians in a biased CPP design [8]; the results in figure 1 approximated a typical inverted U-shaped dose response curve.

The results are noteworthy because prior research has been decidedly mixed as to whether cotinine has significant effects on behavior. Marusich and colleagues reported cotinine would not establish CPP using rodents as subjects [16]. Another study by Bach et al., reported that cotinine antagonized the stimulant effects of nicotine with measures of planarian motility. This study also concluded that cotinine, by itself, produced no statistically significant effects on the behavior of planarians [18]. Goldberg et al., presented data that cotinine showed some degree of generalization as nicotine when functioning as an antecedent-discriminative stimulus; cotinine altered response rates on fixed interval schedules of reinforcement while having no effect on fixed ratio schedules of reinforcement performances, with responding by rats maintained by food as reinforcer [17]. Hatsukami, Lexau, Nelson, Pentel, Sofuoglu and Goldman concluded that cotinine had no effect on cigarette consumption [24]. While Grizzell and Echeverria concluded that cotinine was not likely to be a contributor to the addictive properties of nicotine, cotinine was demonstrated to facilitate the release of dopamine in the striatum via its interaction with nicotinic receptors [20,23]. However, Marusich et al. [16] reported that cotinine did not affect dopamine release in the nucleus accumbens.

Other literature may be relevant to a consideration of the effects of cotinine. On operant schedules of reinforcement, Clemens, Caille, Stinus and Cador [25] examined the reinforcing effectiveness of nicotine versus nicotine combined with a "cocktail" of nicotine plus the alkaloids anabasine, cotinine, myosmine, and nornicotine at the same proportions that occur in tobacco smoke. The cocktail was prepared to have the same proportions of these compounds as is typically found in tobacco smoke, and this entails that cotinine was a major component in this cocktail [19]. This study found that rats would respond significantly more for the nicotine with the alkaloid cocktail than for nicotine alone, with higher break points observed on progressive ratio schedules of reinforcement with the cocktail as a reinforcer. Furthermore, these researchers concluded that the minor alkaloids may contribute to nicotine's effects, establishing nicotine to function as a more powerful reinforcer [25]. To our knowledge, no published papers have examined the effects of cotinine delivered as a consequence for responding in any operant reinforcement procedure. Hoffman and Evans stated that no published studies had examined whether cotinine could function as a reinforcer [19]. That is to say, there are no data to address the question of whether cotinine in isolation will maintain operant self-administration.

Because cotinine exposure only occurs as an outcome of nicotine delivery, the relevance of the present findings would at first appear to be limited to basic researchers. Cotinine, however, may have more of a role in another translational research. At least two recent papers reviewed findings that cotinine may have significant therapeutic applications as a neuroprotective agent in rodent models of Parkinson's disease as well as Alzheimer's disease [26,27]. If these translational research programs do lead to clinical applications of cotinine as a preventative or a therapeutic mechanism, cotinine will be the subject of considerably increased research interest.

Other questions to be addressed are whether the present findings can inform public policy regarding tobacco use and regulation. The question of cotinine serving a role in tobacco cessation treatments has not been supported. Hatsukami et al. reported that cotinine had no effect on cigarette self-administration [24]. The 2019 findings suggested that cotinine is contributing to the addictive properties of nicotine [12]; as long as tobacco is a source of nicotine, cotinine production is an inevitable outcome. The question of cotinine serving a role in tobacco cessation treatments has not been supported. Some researchers have posited that tobacco products should be altered to have reduced nicotine levels and consequently reduced cotinine production, such that dependence would be less likely [28]. Any such change has never been successfully implemented in the marketplace.

These results have to be qualified as being a biased CPP protocol. The CPP protocol can be implemented in a biased or an unbiased design. In the former, the preference for different contexts or environments is assessed by placing the organism in the apparatus and measuring the time an organism spends in the distinctive places when it can move about freely in either of the environments or compartments. The environment in which the organism spent the least amount of time in is subsequently paired with a reinforcer [13]. In an unbiased CPP study, the particular environment assigned for pairing with a drug is determined by the researcher. The pairing of a specific context-environment with the drug is done irrespective of the subjects' initial preferences for any environment. Different CPP results have been observed as a function of the use of the different designs [13]. Interpretation of the results of a biased CPP design such as presented here are problematic according to some researchers, who argued that the shift in preference could either represent reinforcing effects or anxiety reduction [9]. The use of the phrase "light phobic" to describe the planarian's baseline behavior in the relevant literature seems to point to this "anxiety reduction" interpretation [1]. This explanation itself raises questions. The presence of anxiety-like behavior, which is therefore argued to be reduced, is merely an inference; the behavior argued to be anxiety-like cannot be observed independent of behavior. An inference drawn from merely observing behavior cannot be offered as an explanation for the same behavior.

The data from any CPP protocol are typically conceptualized as being the result of classical or respondent conditioning processes, and the pairing of neutral stimuli with a stimulus that functions as an unconditioned stimulus is explicit [9]. Conversely, it has been argued that CPP outcomes may be the result of voluntary behavior (i.e., emitted behavior that comes to be controlled by encountering reinforcing stimuli associated with distinctive antecedent events) [14]. The CPP protocol has been demonstrated to have a high degree

of generalizability across different organisms, with demonstrated place conditioning in planarians, [12,29], fruit flies, [30], nematodes [31,32], rodents, [16,33,34], primates, [35,36] and humans [37-39]. Furthermore, the CPP has been used to assess an extensive range of psychoactive compounds including cotinine, histamine, opiates, ethanol, nicotine, cocaine, amphetamines, caffeine, guarana, and bath salts [12,29,30,34-44]. As for novel applications, two recent publications referred to a compound described as having binge-mitigating properties, referring to ethanol binge drinking in humans. The articles in question made this reference yet only presented toxicological analyses as well as basic data on pharmacokinetics and pharmacodynamics analyses indicating rats could tolerate the compound MEAI also known as 5-methoxy-2-aminoindane [45,46]. The question of MEAI actually having behavioral-psychoactive properties that resemble those of ethanol has not been investigated. Such a question could be addressed using the planarian and the CPP protocol, both of which represent useful tools for basic and applied researchers.

Conflicts of Interest

The sole author of this paper has no conflicts of interest to declare.

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Brady J. Phelps is the sole author and is solely responsible for this manuscript.

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