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Levamisole and cocaine synergism: A prevalent adulterant enhances cocaine's action *in vivo*

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ABSTRACT

Levamisole is estimated by the Drug Enforcement Agency (DEA) to be present in about 80% of cocaine seized in the United States and linked to debilitating, and sometimes fatal, immunologic effects in cocaine abusers. One explanation for the addition of levamisole to cocaine is that it increases the amount of product and enhances profits. An alternative possibility, and one investigated here, is that levamisole alters cocaine's action *in vivo*. We specifically investigated effects of levamisole on cocaine's stereotypical and place-conditioning effects in an established invertebrate (planarian) assay. Acute exposure to levamisole or cocaine produced concentration-dependent increases in stereotyped movements. For combined administration of the two agents, isobolographic analysis revealed that the observed stereotypical response was enhanced relative to the predicted effect, indicating synergism for the interaction. In conditioned place preference (CPP) experiments, cocaine produced a significant preference shift; in contrast, levamisole was ineffective at all concentrations tested. For combination experiments, a submaximal concentration of cocaine produced CPP that was enhanced by inactive concentrations of levamisole, indicating synergism. The present results provide the first experimental evidence that levamisole enhances cocaine's action *in vivo*. Most important is the identification of synergism for the levamisole/cocaine interaction, which now requires further study in mammals.

1. Introduction

An old drug called levamisole (Ergamisol) that was once used to treat parasitic worm infections in humans is exacerbating health risks for an estimated 2 million cocaine users in the United States (Auffenberg et al., 2013). The news media (*e.g.* Time Magazine), scientific publications, and government agencies are alerting the general public, health officials, and physicians about potentially life-threatening effects of cocaine laced with levamisole (Zhu et al., 2009; Chang et al., 2010; Ullrich et al., 2011). An example of such a warning is the public alert issued in September of 2009 by the U.S. Department of Health and Human Services Substance Abuse and Mental Health Services Administration warning that "a dangerous substance, levamisole, is showing up with increasing frequency in illicit cocaine powder and crack cocaine and can lead to a severe reduction in the number of white blood cells, a problem that is called agranulocytosis". The Drug Enforcement Agency (DEA) estimates that about 80% of the cocaine seized in the US is laced with levamisole (Wolford et al., 2012). DEA data from 2009 also noted an average concentration of approximately 10% levamisole detected in cocaine, and Buchanan et al. (2010) demonstrated the presence of levamisole (as high as 10%) in a patient's crack cocaine pipe, thus confirming levamisole as a cocaine adulterant. Speculation about the addition of LVM to cocaine centers on two hypotheses. One is that LVM increases the amount of 'product' which increases profits. LVM is cheap, has similar physicochemical properties to cocaine, and is easily accessible as a veterinary pharmaceutical in regions in which the laced cocaine originates.

A second hypothesis is that levamisole is added to cocaine to modify the pharmacological properties of cocaine. To probe the latter possibility, we used an established planarian assay to determine if levamisole affects cocaine's action *in vivo*. Planarians are aquatic flatworms with a centralized nervous system often

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considered to be the simplest 'brain' (Raffa and Rawls, 2008; Buttarelli et al., 2008). Planarians contain neurotransmitter systems, including glutamate, dopamine, serotonin, acetylcholine, and GABA (Eriksson and Panula, 1994; Vyas et al., 2011; Nishimura et al., 2010), and to a limited extent display mammalian-equivalent behavioral responses (stereotypical activity, abstinence-related withdrawal, behavioral sensitization, cross-sensitization, and place conditioning) following exposure to addictive substances (Palladini et al., 1996; Pagán et al., 2008, 2009, 2013; Rowlands and Pagán, 2008; Rawls et al., 2010, 2011; Ramoz et al., 2012). The present experiments characterized levamisole and cocaine interactions using two behavioral endpoints. One was stereotypical activity, defined as the number of C-shape movements across a defined time interval (Passarelli et al., 1999; Rawls et al., 2011). The other was conditioned place preference (CPP), an assay in which planarians exposed to a distinct environment in the presence of a positively reinforcing substance will later show preference for that same environment when given a choice (Zhang et al., 2013; Ramoz et al., 2012). Drug combination analysis employing isobolographic theory was used to quantify levamisole–cocaine interactions (Tallarida, 2011, 2012). The isobolographic method is derived from the principle of dose equivalence and is the standard pharmacological approach to analyze observed combination dose effects for comparison with expected, or additive, effects. Its aim is to assess synergistic and/or additive interactions between compounds administered simultaneously (Tallarida, 2011, 2012).

2. Experimental procedures

2.1. Subjects and drugs

Planarians (*Dugesia dorotocephala*) were purchased from Carolina Biological Supply (Burlington, NC, USA). Upon arrival in the laboratory, planarians were maintained in the aqueous solution provided by Carolina Biological Supply, acclimated to room temperature (21 °C), and tested within 3 days of receipt. (−)-Cocaine hydrochloride was generously provided by the National Institute on Drug Abuse (Bethesda, MD, USA). Levamisole hydrochloride was purchased from Sigma–Aldrich (St. Louis, MO, USA). Stock solutions of each drug were prepared daily in a vehicle of tap water containing AmQuel® water conditioner. Treatment solutions were diluted with tap water containing AmQuel® water conditioner. Concentrations of cocaine were based on prior behavioral outcomes in planarians (Owaisat et al., 2012; Pagán et al., 2013; Rawls et al., 2010), and levamisole concentrations were determined empirically using previously reported Ki values as a guide (Anagnostou et al., 1996).

2.2. Behavioral experiments

2.2.1. Stereotypical activity

Individual planarians were placed randomly into a transparent petri dish (5.5 cm diameter) containing a solution of cocaine (0, 0.1, 1, 3, 5 mM) or levamisole (0, 0.1, 0.3, 0.75, 1 mM) for 5 min and stereotyped movements were quantified (Ramoz et al., 2012). The concentration-effect data for each individual drug was used to determine the constant–potency ratio of the two drugs at a specified effect level (*i.e.* equi-effective doses of each drug). From this value the isobole, which indicates additivity for the predicted effect of the combination, was constructed and used to determine if the combination was additive, sub-additive or synergistic (super-additive) (Tallarida, 2011, 2012). Combination doses used in actual experiments were determined based on individual drug potencies.

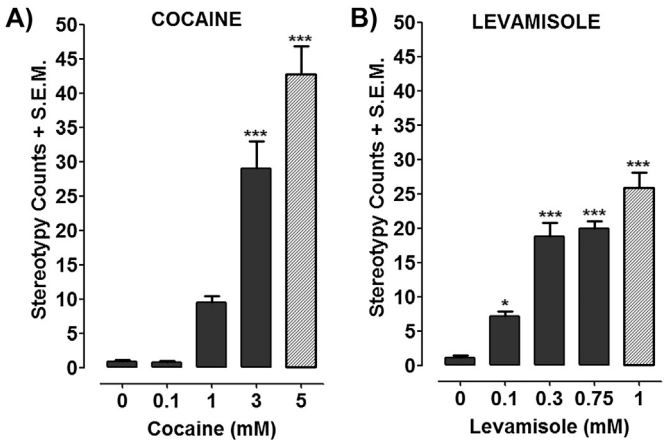

Fig. 1. Cocaine and levamisole produce stereotyped movements in planarians. Planarians were exposed to different concentrations of cocaine (1A) or levamisole (1B) for 5 min. The number of C-shape movements over the 5-min exposure interval were determined and presented as mean stereotypy counts ±S.E.M. $N = 8$ planarians/group. $^{***}p < 0.001$ or $^{*}p < 0.05$ compared to the respective water control in cocaine (1A) or levamisole (1B) experiments.

2.2.2. Conditioned place preference (CPP)

CPP) experiments were divided into 3 different phases: 1) pre-conditioning (pre-test); 2) conditioning; and 3) post-conditioning (post-test). Because planarians display a natural preference for a dark environment (Raffa et al., 2003), we used a biased, counterbalanced conditioning design to assess cocaine and levamisole preference (Ramoz et al., 2012). In a biased design, the preference of each individual animal for a particular environment is determined prior to conditioning by placing the animal in the apparatus, and then by assessing the amount of time the animal spends in each compartment. The least-preferred compartment for each animal is then assigned to be the drug-paired compartment. For the pre-conditioning phase, dark and "ambient" light environments were created by covering half (both the top and bottom) of a petri dish containing water with black construction paper. An individual planarian was then placed at the midline of the dish and given free access to roam both the light and dark environments of the dish. The time spent in the least-preferred setting over a 5-min interval was then determined. This value is called the pre-test time. The least-preferred environment, as determined during pre-conditioning, is designated as the environment in which drug conditioning occurs and is therefore called the 'drug-paired' environment. For conditioning, planarians were exposed to either cocaine (0, 0.001, 0.01, 0.1, 1, 100 μM) or levamisole (0, 0.01, 0.1, 1 μM) for 30 min in the least-preferred (drug-paired) environment. For the situation in which the 'drug-paired' environment is ambient light, the petri dish is uncovered during the conditioning phase to allow exposure to the light. For the opposite situation in which the drug-paired environment is the dark, the entire petri dish is covered with black construction paper to enable exposure to a dark environment. Immediately following conditioning, the post-conditioning phase was performed in a manner identical to that described for pre-conditioning. Planarians were placed at the midline of a petri dish containing water and allowed free access to the light and dark sides of the dish for 5 min. Time spent in the drug-paired side (the original least-preferred environment) was determined (post-test), and a preference score was calculated as the difference between the post-test and pre-test times. A similar protocol has been used by our laboratory to demonstrate that planarians display CPP to different addictive substances including designer cathinones, nicotine, and sugar (Rawls et al., 2011; Ramoz

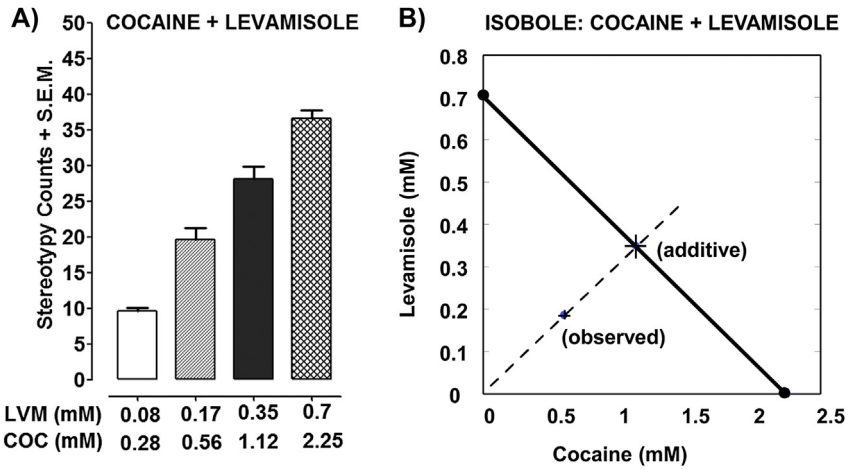

Fig. 2. Cocaine and levamisole interact synergistically to produce stereotyped movements in planarians. Cocaine and levamisole were determined to have a constant–potency ratio (cocaine:levamisole) of 3.20, with potencies of 2.25 mM for cocaine and 0.703 for levamisole, from the data in Fig. 1. (2A) Four combinations maintaining the constant–potency ratio of 3.20 (*i.e.* cocaine concentration (mM)/levamisole concentration (mM): 2.25/0.703; 1.12/0.35; 0.56/0.17; 0.28/0.08) were administered to planarians for 5 min. The number of C-shape movements were determined over the 5 min exposure interval and presented as mean stereotypy counts ± S.E.M. $N = 8$ planarians/group. (2B) Isobole indicating additivity for the combination was determined from the constant–potency ratio and presented as the diagonal line with intercepts 2.25 +/0.097 (horizontal) and 0.703 +/0.125 (vertical). The observed dose-combination point (0.598 mM for cocaine, 0.186 mM for levamisole) determined from Fig. 2A data lies significantly below the isobole, thereby indicating synergism for the combination.

et al., 2012; Zhang et al., 2013). Cocaine produced significant CPP whereas levamisole did not (see Results). Thus, for combined administration, inactive concentrations (0.01, 1 μM) of levamisole were tested in combination with graded concentrations (0.001, 0.1, 100 μM) of cocaine.

2.3. Data analysis

Comparisons of group means (±S.E.M.) for experiments involving individual drugs were evaluated by one-way ANOVA, and in cases of significance, followed by Dunnett's test. For combined administration, isobolographic analysis, plus two-way ANOVA followed by Bonferroni's test, was used. Values of $p < 0.05$ were considered statistically significant.

3. Results

3.1. Combination of cocaine and levamisole elicits synergistic stereotypical activity

Effects of acute exposure to cocaine or levamisole on stereotyped movements are presented in Fig. 1. For cocaine (Fig. 1A), one-way ANOVA revealed a significant main effect [$F(4, 35) = 58.12$, $p < 0.0001$]. Post-hoc analysis indicated that 3 and 5 mM cocaine produced significantly greater stereotypical activity than water-exposed controls ($p < 0.001$). The maximal number of stereotyped movements (42.75 ± 4.11) was produced by 5 mM cocaine. For levamisole (Fig. 1B), one-way ANOVA revealed a significant main effect [$F(4, 35) = 47.78$, $p < 0.0001$]. Post-hoc analysis indicated that concentrations of 0.3, 0.75 and 1 mM levamisole produced significantly greater stereotypical activity compared to water-exposed controls ($p < 0.001$). The maximal number of stereotyped movements (25.88 ± 2.18) was produced by 1 mM levamisole.

The individual concentration-effect curve of each individual drug (determined from the data in Fig. 1) allows a determination of equally effective doses (*e.g.* effect 20% is inserted into the equation of each curve and the dose that produces this effect is determined). Specifically, cocaine gives a concentration of 2.25 mM whereas levamisole gives a concentration of 0.703 mM. These ED_{20} values for cocaine and levamisole reveal that the agents have a constant-potency ratio (cocaine:levamisole) of 3.20 with potencies of 2.25 mM for cocaine and 0.703 for levamisole. In other words, these values are the equi-effective doses of cocaine and levamisole that produce the same effect level (an effect of 20 stereotyped movements). From those values the isobole, which indicates additivity for the combination of cocaine and levamisole, was determined and shown in Fig. 2B as the diagonal line with intercepts 2.25 +/0.097 (horizontal) and 0.703 +/0.125 (vertical). Four combinations of cocaine and levamisole, all of which maintained the constant–potency ratio of 3.20 (*i.e.* cocaine concentration (mM)/levamisole concentration (mM): 2.25/0.703; 1.12/0.35; 0.56/0.17; 0.28/0.08), were tested in actual experiments and their effects on stereotyped movements are shown in Fig. 2A. As was the case with the individual agents (Fig. 1), the constant-potency combinations of cocaine and levamisole produced concentration-dependent

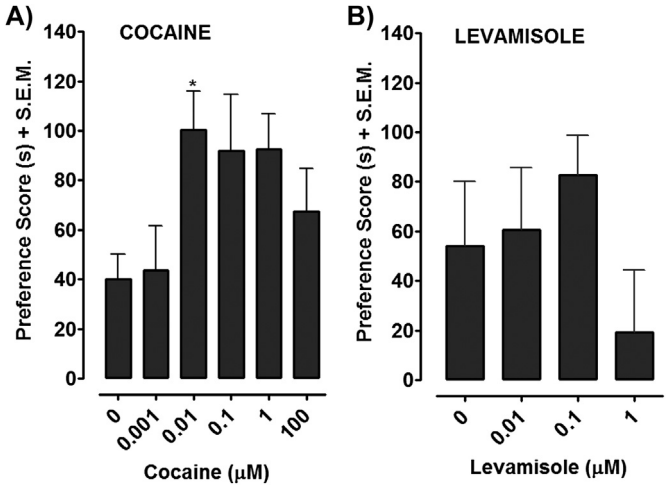

Fig. 3. Cocaine, but not levamisole, produces CPP in planarians. Data are presented as the mean preference score (s) + S.E.M. (difference between post-conditioning and pre-conditioning times) from planarians in which cocaine (3A) or levamisole (3B) was paired with ambient light during the conditioning phase. $N = 8–12$ planarians/group. $^{*}p < 0.05$ compared to water control.

increases in stereotyped movements (Fig. 2A). Fig 2B shows the isobole. This is a plot of all dose combinations (cocaine and levamisole) that are expected to give the specified effect when there is no interaction. The observed dose combination point (0.598 mM for cocaine, 0.186 mM for levamisole) that achieved an effect level of 20 stereotyped movements, however, is below the isobole, thereby indicating synergism, *i.e.* the effect is achieved with this lower dose combination (Tallarida, 2012).

3.2. Levamisole enhances cocaine-induced conditioned place preference (CPP)

The place conditioning effects of cocaine and levamisole are presented in Fig. 3. For cocaine (Fig. 3A), one-way ANOVA indicated a significant main effect on CPP [$F(5, 46) = 2.832$, $p < 0.05$]. Conditioning with a concentration of 0.01 μM cocaine produced a significantly greater preference shift (100 ± 15 s) than water controls (40 ± 10 s) ($p < 0.05$). For levamisole (Fig. 3B), a significant main effect was not indicated by one-way ANOVA [$F(3, 28) = 1.271$, $p > 0.05$].

Using the CPP data obtained for the individual drugs as a guide (Fig. 3), we combined inactive concentrations of levamisole (0.01, 1 μM) with submaximal (0.001 μM), as well as higher and more efficacious concentrations (0.1, 100 μM), of cocaine. In the case in which an inactive concentration of levamisole is administered with an active concentration of cocaine, the expected effect becomes one in which the preference shift produced by the combination is simply equal to the preference shift produced by cocaine alone. The analysis is then one in which the effect of the active drug, cocaine, is statistically compared before and after the addition of levamisole. For actual experiments (Fig. 4), two-way ANOVA revealed a significant effect of levamisole [$F(2, 60) = 3.51$, $p < 0.05$]. Bonferroni analysis indicated that the preference shift produced by a submaximal concentration of cocaine (0.001 μM) was enhanced in the presence of levamisole ($p < 0.05$) (Fig. 4). The shift produced by cocaine (0.001 μM) alone was 43 ± 18 s; in the presence of 0.01 and 1 μM the preference shift was 123 ± 23 s and 119 ± 13 s, respectively. Levamisole did not significantly affect the preference shift elicited by higher concentrations of cocaine (0.1, 100 μM) ($p > 0.05$).

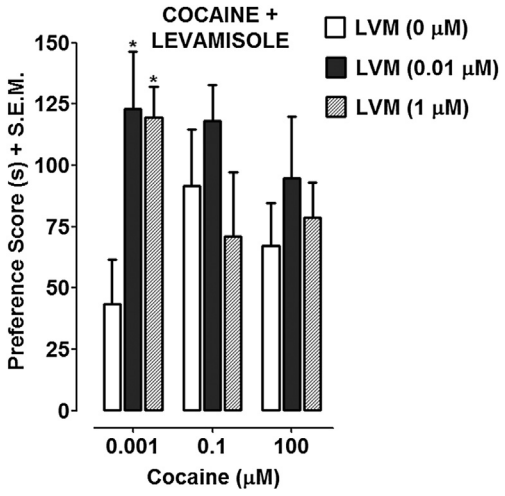

Fig. 4. Levamisole enhances cocaine-induced CPP. Data are presented as the mean preference score (s) + S.E.M. (difference between post-conditioning and pre-conditioning times) from planarians in which increasing concentrations of cocaine (the active agent) were co-administered with water or levamisole (LVM) (the inactive agent) in the ambient light (non-preferred environment) during the conditioning phase. $N = 8$ planarians/group. *$p < 0.05$ compared to water (LVM [0 μM]) + cocaine group.

4. Discussion

The aim of our study was to provide experimental information about effects of levamisole on cocaine's action *in vivo* and that effort revealed synergism for the cocaine and levamisole interaction. We demonstrated that two neuropharmacological effects of cocaine, stereotyped movements and CPP, were enhanced in select cases in which cocaine was administered in combination with levamisole. As is often the case when synergism is first detected, we do not yet know the precise mechanism, but the very detection of synergism is an important first step in exploring mechanism and impact. Understanding the pharmacology of a prevalent combination such as cocaine and levamisole is critical because poly-drug use (*e.g.* cocaine + alcohol, heroin + cocaine) can increase dangers that each drug poses by itself and make it more difficult to identify efficacious medications to manage dependence, craving, and relapse. An example of the challenge posed by poly-drug addiction is the impact of alcohol dependence on the efficacy of modafinil in treating cocaine dependence (Anderson et al., 2009; Vocci and Elkashef, 2005). In this case, modafinil treatment reduces cocaine use in subjects that are not dependent on alcohol but, in cocaine users who are alcohol dependent, modafinil loses its efficacy.

Effects of cocaine on the endpoints studied here (stereotypy and CPP) are established across different species, including planarians (Pagán et al., 2013; Rawls et al., 2010). Although effects of levamisole on motor and reward systems are not well defined, some of its biological actions are consistent with an agent that enhances effects of cocaine through direct or indirect augmentation of monoamine activity. For example, levamisole inhibits monoamine oxidase (MAO) and activates nicotinic receptors (*e.g.* α3β4, α3β2), and both MAO inhibitors and nicotinic agonists enhance cocaine effects and increase dopamine transmission (Hernando et al., 2012; Levandoski et al., 2003; Agarwal et al., 1990). Levamisole also elevates levels of endogenous opioids associated with drug highs and attenuates the opiate withdrawal syndrome in rats (Spector et al., 1998). Furthermore, levamisole, when used as adjuvant therapy for colon cancer, has been reported to cause mood-elevating effects (Goldin et al., 1982). On the basis of those combined data, one explanation for the levamisole synergism with cocaine in activity and CPP assays is enhanced dopamine activity, perhaps through the simultaneous block of dopamine catabolism, or activation of nicotinic acetylcholine receptors, by levamisole and blockade of dopamine uptake by cocaine. Pharmacodynamic mechanisms are not the only potential explanations for the synergy; pharmacokinetic interactions, such as levamisole inhibiting catabolism of cocaine into inactive metabolites, or a chemical interaction between the two drugs that leads to enhancement of the pharmacological properties of cocaine, may have played a role.

When administered by themselves, cocaine and levamisole increased stereotyped movements. Although drugs possessing abuse liability often increase stereotypical activity in laboratory animals (Koob, 1992), the relationship, if any, between stereotyped movements in planarians, rats and mice is uncertain. Part of the reason is that stereotypy is broadly defined as a repetitive, ritualistic movement and is not uniformly quantified across different species. For rodents a scoring system is frequently used to quantify individual stereotypical movements, such as rearing, grooming, sniffing, and head bobbing (Tanda et al., 2007), and photocell beam interruptions are used to quantify cumulative stereotyped movements (Rasmussen et al., 2011; Hummel and Unterwald, 2003). For planarians we quantify stereotypical activity as C-shape movements, which are detected following exposure to different addictive substances including cocaine, synthetic cathinones, nicotine, and caffeine (Zhang et al., 2013; Ramoz et al., 2012; Rawls et al., 2010; Pagán et al., 2008). C-shape movements in planarians have also

been described as "seizure-like activity" based on evidence that proconvulsants induce C-shape movements that are prevented by concurrent exposure to antiepileptic agents (Ramakrishnan and Desaer, 2011; Raffa et al., 2010). Furthermore, planarians exposed to cholinergic agonists exhibit muscle contractions resembling C-shape movements (Nishimura et al., 2010; Rawls et al., 2011; Owaisat et al., 2012). Given that seizure-like activity has been reported in mice following administration of higher doses of levamisole (Rehni and Singh, 2010), and that levamisole activates nicotinic acetylcholine receptors involved in seizures (Levandoski et al., 2003), it cannot be discounted that a proconvulsant action of levamisole was partly responsible for producing the C-shape movements in planarians.

The effectiveness of cocaine in planarian motor and CPP assays varied with concentration; lower concentrations produced the greatest preference shifts whereas 1000-fold higher concentrations were required to elicit stereotyped movements. Similar dose-related trends have been observed in corresponding rat assays and suggest that the rewarding and aversive effects of cocaine are separable by dose. For example, in rat CPP studies, cocaine displays an inverted U-shaped dose response in which the highest doses (*e.g.* 30 mg/kg) produced diminished responses due to their aversive effects (Bardo et al., 1995; Tzschentke, 2007; Zakharova et al., 2009). Doses of cocaine at the high end of the inverted U-shaped curve also produce more robust stereotypical activity (Estevez et al., 1979; Bhattacharyya and Pradhan, 1979) and, at even higher doses, eventually seizure (Zagnoni and Albano, 2002). Our results with planarians suggest a similar phenomenon for cocaine in which its rewarding effects are more robust at lower concentrations that do not elicit significant increases in stereotypical activity.

Levamisole enhanced CPP produced by submaximal concentrations of cocaine but did not alter preference shifts produced by higher, and more effective, concentrations of cocaine. Levamisole's preferential effect on submaximal concentrations of cocaine may be related to the magnitude of the preference shift produced by cocaine itself. For instance, the more effective concentrations of cocaine are likely to be associated with a 'ceiling effect', a phenomenon in which a drug produces a maximum effect so that increasing the drug dosage or, as in the present case, adding another type of drug does not increase overall efficacy (Lutfy and Cowan, 2004; Tallarida, 2012). Indeed, when cocaine was administered to planarians by itself, the maximal preference shift was about 100 s. For combinations of cocaine and levamisole, the maximal preference shift detected was about 125 s. The relative similarity in magnitude of those preference shifts, coupled with the inverted U-shaped dose response curve of cocaine by itself, supports the interpretation that levamisole synergism with cocaine is more likely to be detected at submaximal concentrations of cocaine.

A comment is perhaps warranted on a liability of the planarian assay, which is the challenge of quantifying changes in motility following acute exposure to stimulant drugs. Planarians display slight to modest increases in motility immediately following exposure to concentrations of amphetamine and nicotine in the lower portion of their concentration curves (Raffa and Martley, 2005). However, acute exposure to modest to higher concentrations decreases motility (Pagán et al., 2009; Rawls et al., 2011).

In conclusion, we provide the first evidence that levamisole enhances cocaine's action *in vivo*. Most important is the identification of levamisole synergism with cocaine, which was detected in two different assays, including CPP, in which the environmental preference for cocaine was augmented by the presence of levamisole. The synergism between cocaine and levamisole now requires further, and more detailed, investigation in mammalian models of CPP and self-administration to determine if levamisole affects the positive reinforcing and drug seeking properties of cocaine.

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Conflict of interest

All authors declare that they have no conflicts of interest.

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