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The κ -opioid receptor antagonist *nor*-BNI inhibits cocaine and amphetamine, but not cannabinoid (WIN 52212-2), abstinence-induced withdrawal in planarians: an instance of ‘pharmacologic congruence’

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Abstract

The broad applicability of receptor theory to diverse species, from invertebrates to mammals, provides evidence for the evolution in complexity of pharmacologic receptor diversification and of receptor-effector signal transduction mechanisms. However, pre-mammalian species have less receptor subtype differentiation and thus might share signal transduction pathways to a greater extent than do mammals, a phenomenon that we term ‘pharmacologic congruence’. We have demonstrated previously that the lowest species considered to have a centralized nervous system, planarians, display both abstinence-induced and antagonist-precipitated withdrawal signs, indicative of the development of physical dependence. We report here: (1) amphetamine abstinence-induced withdrawal, and (2) the attenuation of cocaine and amphetamine, but not cannabinoid agonist (WIN 52212-2), abstinence-induced withdrawal by the opioid receptor antagonist naloxone and by the selective κ -opioid receptor subtype antagonist *nor*-BNI (*nor*-Binaltorphimine), but not by the selective μ -opioid or the δ -opioid receptor subtype antagonists CTAP (D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH₂) and naltrindole. These results provide evidence that the withdrawal from cocaine and amphetamine, but not cannabinoids, in planarians is mediated through a common *nor*-BNI-sensitive (κ -opioid receptor-like) pathway.

1. Introduction

Withdrawal from abused drugs is a complex phenomenon. However, we previously reported a simple withdrawal in *Planaria*, a type of freshwater flatworm (Raffa et al., 2001). Planarians have a centralized cluster of cephalic nerve cell bodies and a ladder-shaped spinal process. They also contain mammalian-relevant neurotransmitters such as dopamine, norepinephrine, 5-HT (5-hydroxytryptamine, serotonin), amino acids, and opioids (e.g., Algeri et al., 1983; Welsh and Williams, 1970). Planarians respond with characteristic behaviors or changes in their locomotor activity (motility) following exposure to dopaminergic agonists, antagonists, or neuronal reuptake inhibitors, such as cocaine (e.g., Algeri et al., 1983; Venturini et al., 1983, 1989; Palladini et al., 1996). They also display withdrawal signs during abstinence-induced or antagonist-precipitated withdrawal. These behaviors include (Raffa and Desai,

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2005): ‘HeadBop’ (a ‘nodding’ movement of head accompanying forward movement); ‘Squirming’ (uncoordinated, ‘jerky’ movements); ‘Clinging’ (intertwining with another planarian if present); ‘HeadSwing’ (axial rotation of the head about the long axis while tail is anchored); ‘TailTwist’ (tip of body twisted), and ‘Corkscrew’ (a spiral motion around the long axis). The occurrence of the behaviors is reversible – most pronounced during the first 5-min observation period and decline during the subsequent observation periods. We also previously reported that planarians undergo both abstinence-induced or antagonist-precipitated withdrawal from cocaine and cannabinoid receptor agonists (Raffa and Valdez, 2001; Raffa et al., 2005; Rawls et al., 2006, 2007). The withdrawal is dose-related to the exposure concentration and is not due to extraneous factors such as the solution pH, osmolarity, or temperature.

During the course of these studies, we noted (e.g., Rawls et al., 2006) that the nitric oxide synthase inhibitor L-NAME (L-Nitro-arginine methyl ester) attenuates abstinence-induced withdrawal from the diverse compounds cocaine and WIN 55212-2 ((*R*)-(+)-[2,3-Dihydro-5-methyl-3-[(4-morpholino)methyl]pyrrolo-[1,2,3-*de*]-1,4-benzoxazin-6-yl](1-naphthyl) methanone) (Compton et al., 1992). These findings implied some congruence of transduction pathways. The purpose of the present set of experiments was to investigate whether pathways involved in opioid withdrawal in planarians (Raffa et al., 2003) are congruent with those for withdrawal from cocaine (Raffa and Valdez, 2001), amphetamine (reported herein), or cannabinoid (Rawls et al., 2006).

2. Results

2.1 Amphetamine

Consistent with our previous findings (e.g., Raffa and Valdez, 2001; Raffa et al., 2001; Raffa et al., 2003), drug-naïve planarians displayed a nearly constant *p*LMV of about 14 gridlines per minute when tested in water. This resulted in an essentially linear relationship between *p*LMV and observation time throughout the 5-min observation period. In previous studies we have shown that this near linear behavior persists for at least 10 minutes (Raffa et al., 2001). When incubated in amphetamine (10 μ M) for 1 h, then tested in the same concentration of amphetamine, planarians displayed *p*LMV over the 5-min observation period that was not significantly different from drug-naïve animals. In contrast, planarians that were incubated in 10 μ M amphetamine for 1 h, then tested in water, demonstrated a large decrease ($F = 37.642$; $df = 35$; $P < 0.0001$) in *p*LMV during the 5-min observation period (Fig. 1). This result is consistent with abstinence-induced withdrawal (e.g., Raffa and Valdez, 2001; Raffa et al., 2003; Rawls et al., 2006). Other possible confounding factors, such as pH, osmotic effect, etc., have been eliminated in previous studies (Raffa et al., 2001).

We next tested the effect of co-incubation of planarians in amphetamine (10 μ M) and naloxone (10 μ M) for 1 h on subsequent *p*LMV during the 5-min observation period. This dose of naloxone had been previously shown to attenuate opioid withdrawal in planarians (Raffa et al., 2003). The results are summarized in graphical form in Fig. 2A. Co-incubation of planarians with naloxone along with amphetamine significantly attenuated the subsequent abstinence-induced amphetamine withdrawal ($P < 0.05$). Importantly, the planarians still displayed a linear *p*LMV over the 5-min observation period. Pretreatment or testing in naloxone alone had no effect on the *p*LMV (data not shown).

Co-incubation of planarians with either the selective μ -opioid receptor subtype antagonist CTAP (10 μ M) (Fig. 2B) or the selective δ -opioid receptor subtype antagonist naltrindole (10 μ M) (Fig. 2C) along with amphetamine did not significantly alter the subsequent abstinence-induced amphetamine withdrawal. However, co-incubation of planarians with the selective κ -opioid receptor subtype antagonist *nor*-BNI (10 μ M) along with amphetamine significantly

attenuated the subsequent abstinence-induced amphetamine withdrawal ($P < 0.05$) (Fig. 2D). The planarians still displayed a linear $pLMV$ over the 5-min observation period. Pretreatment or testing in *nor*-BNI alone had no effect on the $pLMV$ (data not shown).

2.2. Cocaine

Consistent with previous findings (e.g., Raffa and Valdez, 2001; Raffa et al., 2001; Raffa et al., 2003), cocaine-naïve planarians displayed a nearly constant (linear) $pLMV$ when tested in water, whereas cocaine-exposed planarians displayed abstinence-induced withdrawal when tested in water ($F = 9.159$; $df = 29$; $P < 0.01$). The withdrawal was not induced when cocaine-exposed planarians were tested in the same concentration (10 μM) of cocaine.

Co-incubation of planarians for 1 h with naloxone together with cocaine (10 μM) significantly attenuated ($P < 0.05$) the subsequent abstinence-induced cocaine withdrawal when the planarians were tested in water. The planarians still displayed a linear $pLMV$ over the 5-min observation period. Pretreatment or testing in naloxone alone had no effect on $pLMV$ ($P > 0.05$) (data not shown). The attenuation by naloxone of cocaine withdrawal was dose-dependent on the naloxone concentration (10, 20, and 40 μM). The results are summarized in Fig. 3A.

Co-incubation of planarians with either the selective μ -opioid receptor subtype antagonist CTAP (10 μM) or the selective δ -opioid receptor antagonist naltrindole (10 μM) along with cocaine (10 μM) did not significantly alter the subsequent abstinence-induced cocaine withdrawal (data not shown). However, co-incubation of planarians with the selective κ -opioid receptor subtype antagonist *nor*-BNI (10, 20, and 40 μM) along with cocaine dose-relatedly attenuated the subsequent abstinence-induced cocaine withdrawal (Fig. 3B). The planarians still displayed a linear $pLMV$ over the 5-min observation period. Pretreatment or testing in *nor*-BNI alone had no effect on the $pLMV$ (data not shown).

2.3. WIN 52212-2

WIN 52212-2-naïve planarians displayed a nearly constant $pLMV$ when tested in water and WIN 52212-2-exposed planarians displayed abstinence-induced withdrawal when they were tested in water ($F = 8.397$; $df = 55$). The withdrawal was not induced when WIN 52212-2-exposed (1-h) planarians were tested in the same concentration (10 μM) of WIN 52212-2. These results are consistent with our previous report (Rawls et al., 2006).

Co-incubation of planarians for 1 h with naloxone together with WIN 52212-2 (10 μM) did not significantly alter ($P < 0.05$) the subsequent abstinence-induced WIN 52212 withdrawal when the planarians were tested in water. The results are summarized in Table 1. These results are not inconsistent with the naloxone-sensitive stimulation of planarian motor activity (Buttarelli et al., 2002), as discussed below.

3. Discussion

The present findings are part of a larger investigation of planarian withdrawal from abused drugs (e.g., Raffa and Valdez, 2001; Raffa et al., 2003; Umeda et al., 2004; Raffa and Desai, 2005; Rawls et al., 2006) including combinations of drugs (Raffa et al., 2006, 2007). Planarians are useful for such studies because of the simplicity of the model and the existing literature on the behavioral responses to a variety of neurotransmitters and selective ligands (e.g., Needleman, 1967; Carolei et al., 1975; Algeri et al., 1983; Venturini et al., 1989; Palladini et al., 1996; Passarelli et al., 1999).

The results of this study confirm planarian abstinence-induced withdrawal from cocaine (Raffa and Valdez, 2001) and the synthetic cannabinoid agonist WIN 52212-2 (Rawls et al., 2006)

and demonstrate for the first time to our knowledge an abstinence-induced withdrawal from amphetamine. Evidence that the metric and protocol used in this study measures withdrawal has been presented in the previous cited publications. In this regard, it is important to note that no alteration in *p*LMV occurred when planarians were exposed to drug-containing solution and then observed in the same concentration of drug. A change in the *p*LMV was observed only when drug-pretreated planarians were tested in water, i.e., the *removal* of the drug. Hence, the change in *p*LMV was not drug-induced, but rather was abstinence-induced.

We previously demonstrated an abstinence-induced withdrawal from the κ -opioid agonist U-50,488H (*trans*-($\pm$)-3,4-Dichloro-*N*-methyl-*N*-(2-[1-pyrrolidinyl]cyclohexyl)-benzeneacetamide) in planarians (Raffa et al., 2003). The magnitude of the withdrawal was correlated with the concentration of U-50,488H and significantly less withdrawal ($P < 0.05$) was observed for the less active enantiomer [*1R,2R*]U-50,488. Furthermore, the withdrawal was significantly attenuated ($P < 0.05$) by the nonselective opioid antagonist naloxone (10 μ M) and by the selective κ -opioid receptor subtype antagonist *nor*-BNI (20 μ M). Thus, planarian abstinence-induced withdrawal from a κ -opioid receptor agonist is mediated through a κ -opioid receptor pathway. In the present study, we report that the previously-described abstinence-induced withdrawal from cocaine (Raffa and Valdez, 2001) is also mediated through a κ -opioid receptor pathway – i.e., it was attenuated by a non-selective opioid receptor antagonist (naloxone) and a selective κ -opioid receptor subtype antagonist (*nor*-BNI), but not by a selective μ -opioid receptor subtype antagonist (CTAP) or by a selective δ -opioid receptor subtype antagonist (naltrindole). We also report that planarians undergo abstinence-induced withdrawal from amphetamine. Pretreatment with naloxone or *nor*-BNI, but not CTAP or naltrindole, attenuated the abstinence-induced withdrawal from amphetamine. By definition, the presence of a withdrawal syndrome following cessation of drug exposure indicates the presence of physical dependence. Therefore, our results provide pharmacological evidence that a common κ -opioid receptor pathway mediates cocaine and amphetamine physical dependence in a planarian model. It should be noted, however, that not all planarian withdrawal is mediated through the same pathway, as evidenced by the *lack* of effect of naloxone on the abstinence-induced withdrawal from WIN 52212-2, a cannabinoid agonist.

A previous report (Butarelli *et al.*, 2002) examined the direct effect of several doses of WIN 52212-2 on planarian locomotor activity using methodology similar to ours and reported an increase in locomotor activity at concentrations of WIN 52212-2 at or above 50 μ g; no increase was observed at 20 μ g (12.54 μ M). Since we used 10 μ M in our experiments, there is no discrepancy between the studies. Furthermore, the previous study found that the direct effect was antagonized by naloxone, suggesting a congruent pathway with cannabinoids not detected in the present study.

The combined findings that physical dependence (manifested as an abstinence-induced withdrawal) from an opioid, amphetamine, and cocaine is attenuated by the same antagonists is surprising and intriguing. Our results suggest that abstinence-induced withdrawal from several otherwise diverse substances in this primitive model is mediated through the same common, κ -opioid receptor-sensitive, pathway. Although the design of the study does not allow elucidate of any details about this pathway, the data suggest some convergence of transduction pathways. The convergence must be downstream to the proximal κ -opioid receptor, since cocaine and amphetamine do not have affinity for κ -opioid receptors. The lack of attenuation of WIN 52212-2 abstinence-induced dependence/withdrawal suggests that the cannabinoid pathway is independent of the shared opioid/cocaine/amphetamine pathway. A caveat is that before the concept of congruence can be accepted completely, it will be necessary to more fully characterize the receptor selectivity of these ligands in planarians. This is currently underway.

In summary, this is the first demonstration of abstinence-induced withdrawal from amphetamine in planarians and, further, is the first demonstration that abstinence-induced withdrawal (physical-dependence) from a κ -opioid, amphetamine, and cocaine – but not cannabinoid agonist (WIN 52212-2) – is attenuated by the opioid receptor antagonist naloxone and the selective κ -opioid receptor antagonist *nor*-BNI. These results suggest some degree of congruence in the mechanisms involved in the development of physical dependence and expression of withdrawal in planarians and prompt the question: is a similar, or analogous, congruence extant in mammals?

4. Experimental Procedure

4.1. Animals and chemicals

Planarians (*Dugesia dorotocephala*) were purchased from the Carolina Biological Supply Co. (Burlington, NC), were acclimated to laboratory conditions, and were used within 72 h. Each planarian was used only once ($N = 4 - 16$ per group). All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). Amphetamine, cocaine, CTAP (D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH₂), naloxone, naltrindole, and *nor*-BNI were dissolved in water. A stock solution of 1 mM WIN 52212-2 was prepared in 10% cremophor/water and serial dilutions were made using water (Rawls et al., 2002).

4.2. Procedure

Following pretreatment (described below) for 1 h (Raffa and Valdez, 2001), planarians were placed singly into a clear plastic Petri dish (14 cm diameter) containing room-temperature water (pH = 7.0) placed over grid paper (gridlines spaced 0.5 cm apart). The spontaneous locomotor velocity (*p*LMV) of each planarian was measured by counting the number of gridlines each planarian crossed per minute over a 5-minute observation period. Five groups were examined: (i) planarians pretreated in water, then tested in water — as control for exposure to vehicle; (ii) planarians pretreated in test substance then tested in the same concentration of the test substance — to demonstrate that no decrease in *p*LMV occurs during continued exposure to drug; (iii) planarians pretreated in test substance, then tested in water — the experimental group; (iv) planarians pretreated in antagonist, then tested in water — to demonstrate lack of effect of antagonist alone; and (v) planarians pretreated in test substance plus antagonist, then tested in water — to assess the ability of the antagonist to inhibit withdrawal.

4.3. Statistics

The group means were compared using one-way ANOVA followed by Tukey-Kramer multiple comparisons *post hoc* test. The criterion for significance was $P < 0.05$.

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Fig. 1.
*p*LMV (expressed as the mean $\pm$ s.e.m. of the cumulative number of gridlines crossed per minute) of planarians pretreated in water (w) or 10 μ M amphetamine (A) and tested in water or the same concentration of amphetamine.

Naloxone (NLX) attenuates amphetamine (AMPH) withdrawal

CTAP does not attenuate amphetamine (AMPH) withdrawal

NTI does not attenuate amphetamine (AMPH) withdrawal

nor-BNI attenuates amphetamine (AMPH) withdrawal

Fig. 2.

*p*LMV (expressed as the mean $\pm$ s.e.m. of the cumulative number of gridlines crossed per minute) of planarians tested in water after pretreatment with amphetamine (10 μ M) plus (**A**) naloxone (NLX; 10 μ M); (**B**) CTAP (10 μ M); (**C**) naltrindole (NTI; 10 μ M); or (**D**) *nor*-BNI (10 μ M). N = 10–16 planarians per group.

Fig. 3. pLMV (expressed as the mean $\pm$ s.e.m. of the cumulative number of gridlines crossed per minute) of planarians pretreated in water (w) or cocaine (C; 10 μ M) and tested in water or tested in water after pretreatment with cocaine (10 μ M) plus (A) naloxone (Nx; either 10, 20, or 40 μ M) or (B) *nor*-BNI (nB; 10, 20, or 40 μ M). N = 10 per group.

Table 1

Lack of naloxone antagonism of abstinence-induced withdrawal from WIN 52212-2 (10 μ M). Pretreatment = 1 h; test = 5 min. Naloxone concentration (μ M) shown in parentheses. P_1 : compared to water/water group; P_2 : compared to WIN 52212-2/water group.

Pretreatment	Test	$p\text{LMV} \pm \text{s.e.m.}$	P_1	P_2
water	water	73 ± 5	—	
WIN 52212-2	WIN 52212-2	58 ± 3	n.s.	
WIN 52212-2	water	36 ± 6	< 0.001	—
Naloxone (40)	water	73 ± 9		n.s.
WIN 52212-2 + Naloxone (20)	water	51 ± 9		n.s.
WIN 52212-2 + Naloxone (40)	water	28 ± 6		n.s.
WIN 52212-2 + Naloxone (80)	water	35 ± 7		n.s.