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Opioid receptor types involved in the development of nicotine physical dependence in an invertebrate (*Planaria*) model

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ABSTRACT

Recent data suggest that opioid receptors are involved in the development of nicotine physical dependence in mammals. Evidence in support of a similar involvement in an invertebrate (*Planaria*) is presented using the selective opioid receptor antagonist naloxone, and the more receptor subtype-selective antagonists CTAP (D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH_2) (μ, MOR), naltrindole (δ, DOR), and *nor*-BNI (norbinaltorphimine) (κ, KOR). Induction of physical dependence was achieved by 60-min pre-exposure of planarians to nicotine and was quantified by abstinence-induced withdrawal (reduction in spontaneous locomotor activity). Known MOR and DOR subtype-selective opioid receptor antagonists attenuated the withdrawal, as did the non-selective antagonist naloxone, but a KOR subtype-selective antagonist did not. An involvement of MOR and DOR, but not KOR, in the development of nicotine physical dependence or in abstinence-induced withdrawal was thus demonstrated in a sensitive and facile invertebrate model.

1. Introduction

The abuse of nicotine (continued use despite negative health consequences) is a multifaceted phenomenon involving several neurotransmitter systems. Proximal mediation of nicotine-induced effects occurs *via* the activation of ligand-gated nAChRs (nicotinic acetylcholine receptors) –– primarily homomeric α_7 and heteromeric $\alpha_4\beta_2$ subtypes (Millar and Gotti, 2009). Distal (behavioral) effects are mediated *via* downstream receptors for several neurotransmitters, such as catecholamines (*e.g.*, norepinephrine and dopamine), 5-HT (5-hydroxytryptamine, serotonin), GABA (γ-aminobutyric acid), glutamate, cannabinoids, hypothalamic hypocretin peptides, and endogenous opioids (Clarke and Reuben, 1996; De Vries and Schoffelmeer, 2005; Di Matteo et al., 1999; Fu et al., 2000; Hollander et al., 2008; Isola et al., 2009; Liechti and Markou, 2008; Maldonado et al., 2006; Marty et al., 1985; McGehee et al., 1995; Pontieri et al., 1996; Scherma et al., 2008; Wilkie et al., 1993; Yang et al., 1996).

A recent review of neurobiological mechanisms underlying nicotine dependence places particular emphasis on the endogenous opioid system (Berrendero et al., 2010). Evidence that this system has an important role in nicotine abuse includes: nicotine stimulates the release of endogenous opioid peptides, alters the expression of endogenous opioid peptides, and induces the dopamine release in nucleus accumbens, which is attenuated by opioid antagonists and in β-endorphin or enkephalin knockout mice (Berrendero et al., 2005; Britt and McGehee, 2008; Dhatt et al., 1995; Goktalay et al., 2006; Maisonneuve and Glick, 1999; Tanda and Di Chiara, 1998; Trigo et al., 2009). Of the opioid-receptor subtypes μ (MOR), δ (DOR), and κ (KOR), the μ-subtype has been most closely associated with nicotine-induced effects (reviewed in (Berrendero et al., 2010)). The involvement of κ- and δ-subtypes is less clear (Berrendero et al., 2010; Hahn et al., 2000; Heidbreder et al., 1996). Nicotine activates μ-opioid receptors in human anterior cingulate cortex (Scott et al., 2007), and smoking initiation, reward, and dependence have been linked to μ-opioid receptor polymorphisms (Perkins et al., 2008; Zhang et al., 2006). Studies in vertebrates (*e.g.*, (Balerio et al., 2004; Biala et al., 2005; Goktalay et al., 2006; Ise et al., 2000; Malin et al., 1993)), including knockout animals (Berrendero et al., 2002, 2005; Galeote et al., 2009; Trigo et al., 2009), suggest that abstinence-induced withdrawal from nicotine involves opioid receptors and that changes in locomotor activity are a manifestation of nicotine-induced modulation of opioid and other neurotransmitter systems related to nicotine physical dependence (Decker et al., 1995).

Although models of nicotine physical dependence and withdrawal in vertebrates are available (as reviewed in (Berrendero et al., 2010)), they are relatively effort- and time-intensive and they often require antagonists to precipitate quantifiable withdrawal. A simpler *in vivo* model would be advantageous. Following several pioneering contributions that paved the way to the recognition of planarians as a suitable animal in different experimental conditions (*e.g.*, (Carolei et al., 1975; Venturini et al., 1981, 1983)), we have shown that planarians

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offer a simple model to study neurotransmitter processes related to drug use and abuse, including development of physical dependence and abstinence-induced or antagonist-induced withdrawal (reviewed in monograph (Raffa and Rawls, 2008)). We recently published the use of this model to study nicotine pharmacology (Rawls et al., 2011), including abstinence-induced nicotine withdrawal. Planarians have been established as a model to study opioid behavior pharmacology (Raffa and Rawls, 2008). We now describe the use of this model to study the involvement of opioid-receptor subtypes in the development of nicotine physical dependence and abstinence-induced withdrawal.

2. Methods

2.1. Subjects and compounds

Planarians (*Dugesia dorotocephala*) were purchased from Carolina Biological Supply (Burlington, NC, USA) and were tested on the same day or the day following receipt. Nicotine, mecamylamine, scopolamine, naloxone, CTAP, naltrindole, and *nor*-BNI were purchased from Sigma-Aldrich Corp. (St. Louis, MO). Solutions were prepared daily in water (1 ml Amquel® per 1 gal water). All of the experiments were conducted using plastic Petri dishes that contained water or test compound(s) under standard laboratory conditions. Each of the experiments used independent groups of planarians and each planarian was used only once.

2.2. Behavioral experiments

Individual planarians (n = 5–13 per group) were pretreated in nicotine (100 μM), nicotine (100 μM) plus antagonist (scopolamine, 10 μM; mecamylamine, 50 μM; naloxone, 10 μM; CTAP, 10 μM; naltrindole, 10 μM; *nor*-BNI, 10 μM), previously shown to antagonize an agonist in this preparation, or water for 60 min. They were then placed individually into a Petri dish containing nicotine (100 μM), antagonist (the same concentration as used in pretreatment phase) or water for 5 min, and spontaneous locomotor velocity (*p*LMV) was quantified as the number of gridlines (0.5 cm apart) crossed or re-crossed over the five-minute observation interval (Raffa and Valdez, 2001).

2.3. Data analysis

Comparisons of group means ± the standard error of the mean (S.E.) were first evaluated by two-way ANOVA and then, if appropriate, by *post-hoc* analysis. Slopes were calculated using linear regression analysis [with 95% confidence limits]. In all cases, a value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Abstinence-induced nicotine withdrawal

Consistent with several previous reports (for example, (Raffa et al., 2001, 2003; Raffa and Valdez, 2001)), drug-naïve planarians displayed nearly constant (linear) *p*LMV of about 13–16 gridlines per minute when they were tested in water and nicotine-exposed planarians displayed an abstinence-induced withdrawal (*i.e.*, a significant reduction in *p*LMV when they were tested in water (slope = 16.69 [16.34–17.03]), but not when they were tested in the same concentration of nicotine (100 μM) (slope = 12.09 [10.62–13.55]) ($P < 0.05$) (Fig. 1)).

3.2. Nicotinic vs muscarinic AChRs

Co-incubation of planarians for 60 min with nicotine (100 μM) with the nicotinic AChR antagonist mecamylamine (50 μM) (slope = 9.50 [9.09–9.91]), which had no effect of its own, significantly ($P < 0.05$) reduced nicotine abstinence-induced withdrawal (slope = 15.13 [13.76–16.49]) (Fig. 2). Importantly, these planarians still displayed a linear *p*LMV over the five-min observation period. They resume normal *p*LMV after a short recovery period (Raffa and Rawls, 2008). Possible confounding interpretations, such as change in pH, osmolarity, *etc.*, were eliminated in previous studies (Raffa and Valdez, 2001).

In contrast to the nicotinic AChR antagonist, co-incubation with the muscarinic AChR antagonist scopolamine (10 μM) had no effect ($P > 0.05$) on subsequent abstinence-induced nicotine withdrawal (Fig. 3).

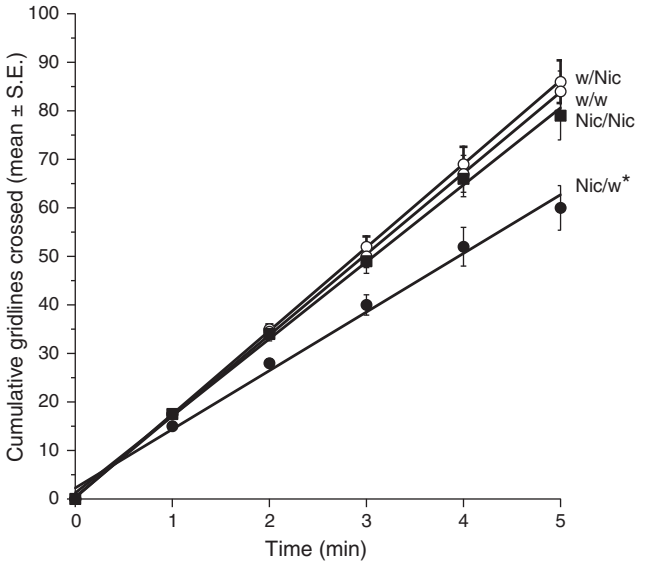

Fig. 1. Spontaneous planarian locomotor velocity (*p*LMV) measured as the mean number of gridlines crossed (± S.E.) during a 5-min observation period. Planarians were pretreated either in water (w) or nicotine (Nic, 100 μM) then tested either in water or nicotine at the same concentration used during the pretreatment. Nicotine-naïve animals displayed no difference in *p*LMV when tested in water or nicotine. Nicotine-pretreated animals displayed no difference *p*LMV from nicotine-naïve animals when tested in nicotine. However, planarians pretreated in nicotine for 60 min and then tested in water displayed significantly reduced *p*LMV. *$P < 0.05$ compared to w/w.

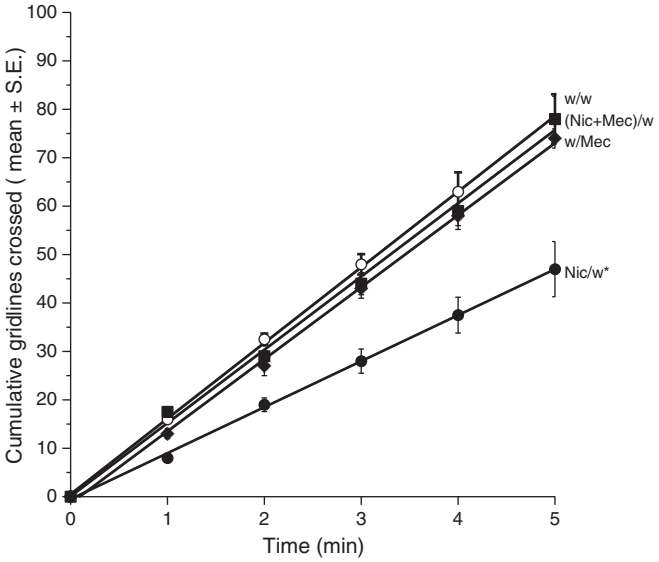

Fig. 2. Abstinence-induced nicotine (Nic) withdrawal in water (w) was attenuated when the planarians were coincubated with 50 μM mecamylamine (Nic + Mec/w) for 60 min and then tested in water. *$P < 0.05$ compared to w/w.

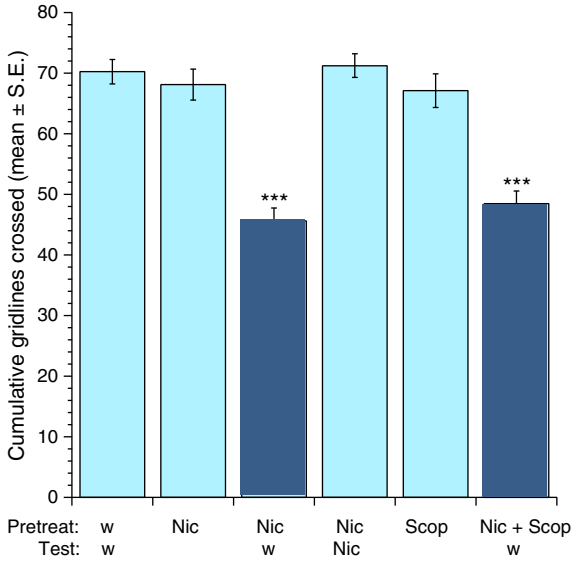

Fig. 3. Planarians pretreated in water (w), nicotine (Nic, 100 μM), scopolamine (Scop, 10 μM), or nicotine (100 μM) plus scopolamine (10 μM) (Nic + Scop) were tested either in water or nicotine at the concentration during pretreatment. Abstinence-induced nicotine withdrawal (Nic/w *vs* Nic/Nic) was unaffected by the muscarinic AChR antagonist scopolamine. $^{***}P < 0.001$ compared to w/w.

3.3. Opioid receptors

The *p*LMV of planarians co-administered nicotine and naloxone (at a dose that had no effect of its own on *p*LMV) (slope = 15.53 [14.91–16.14]) significantly ($P < 0.05$) reduced nicotine withdrawal (slope = 11.11 [10.82–11.35]); not significantly different ($P > 0.05$) than the *p*LMV of planarians pretreated and tested in water (Fig. 4a).

3.4. Opioid receptor subtypes

Co-incubation of planarians for 60 min with nicotine (100 μM) and the selective MOR antagonist CTAP (10 μM) (slope = 16.16 [15.36–16.96]) reduced significantly ($P < 0.05$) nicotine abstinence-induced withdrawal (slope = 13.89 [13.30–14.47]). Likewise, co-incubation of planarians for 60 min with nicotine (100 μM) together with the selective DOR antagonist naltrindole (10 μM) (slope = 13.86 [13.16–14.56]) significantly reduced ($P < 0.05$) nicotine abstinence-induced withdrawal (slope = 10.64 [9.73–11.56]). In contrast, *p*LMV of planarians that were co-administered nicotine (100 μM) and the selective KOR antagonist *nor*-BNI (slope = 12.31 [11.78–12.85]) was not significantly different ($P > 0.05$) than the *p*LMV of planarians pretreated with nicotine (100 μM) and tested in water (slope = 11.87 [11.34–12.40]) (Fig. 4b,c,d). The antagonists had no effect of their own on *p*LMV (Table 1).

4. Discussion

The endogenous opioid pathway is involved in nicotine rewarding effects and the development of nicotine physical dependence, as summarized in a recent review (Berrendero et al., 2010). The reinforcement properties (which are inferred from self-administration) of nicotine in rats are mediated at least in part through opioid receptor subtypes (Liu and Jernigan, 2011). Anatomical and physiological interactions between the nAChR that mediate nicotine's *in vivo* effects and the endogenous opioid systems (opioid receptors) are well established (*e.g.*, (Berrendero et al., 2005; Britt and McGehee, 2008; Maisonneuve and Glick, 1999; Tanda and Di Chiara, 1998)) and positron emission tomography imaging (PET scan) in humans has shown that nicotine activates MOR located in anterior cingulate cortex (Scott et al., 2007). Although evidence from animal models and clinical trials has been equivocal regarding efficacy of the opioid antagonist naloxone or the longer-acting naltrexone (Byars et al., 2005; Corrigall and Coen, 1991; Covey et al., 1999; DeNoble and Mele, 2006; Epstein and King, 2004; Gorelick et al., 1988; Hutchison et al., 1999; Ismayilova and Shoaib, 2010; Karras and Kane, 1980; King et al., 2006; King and Meyer, 2000; Krishnan-Sarin et al., 1999, 2003; Liu et al., 2009; Nemeth-Coslett and Griffiths, 1986; Ray et al., 2006, 2007; Rohsenow et al., 2007; Rukstalis et al., 2005; Sutherland et al., 1995; Wewers et al., 1998; Wong et al., 1999), antagonist therapy might be effective in sub-populations of smokers, particularly those who have higher rates of depressive symptoms (Walsh et al., 2008).

A recent study reported the contribution of opioid-receptor subtypes to nicotine behavioral effects in rats (Liu and Jernigan, 2011). The authors propose that such information can help guide drug-discovery efforts related to amelioration or treatment of nicotine abuse. Since studies in rodents involve methodological complexities and are time- and resource-intensive, a simpler, more rapid, and mammalian-sparing model would be of complementary value. We have shown that planarians provide a convenient model for a wide variety of phenomena related to drug use and drug-abuse, including physical dependence and withdrawal (Raffa and Rawls, 2008).

Prior work by others (Pagán et al., 2009) has shown that planarians display abstinence-induced withdrawal subsequent to the discontinuation of nicotine exposure. In Pagán et al. (2009), abstinence-induced withdrawal was assessed by observing withdrawal signs (Raffa and Desai, 2005). In Rawls et al. (2011), withdrawal was assessed using decreased spontaneous motility, an endpoint used to quantify planarian withdrawal from cocaine, amphetamines, benzodiazepines, and opioids (*e.g.*, (Raffa and Valdez, 2001; Rawls et al., 2007, 2009)). We used this methodology in the present study, since decreased locomotor activity is a withdrawal sign in planarians (Raffa and Desai, 2005). Abstinence-induced withdrawal signs can be difficult to quantify in mammals, although a well-documented withdrawal syndrome comprised of somatic effects (*e.g.*, forelimb tremor, head twitches, jumping, and piloerection) and affective signs (*e.g.*, anhedonia) is precipitated by administration of cholinergic antagonists to nicotine-dependent rats (Kenny and Markou, 2001; Malin, 2001). An attractive feature of the planarian model is that the physical dependence development is rapid (under 60 min), robust, and easily quantified. An as yet unexplored possibility is that the reduced locomotor activity reflects a state similar to the immobility displayed by nicotine-withdrawn rats in the forced swim test (Chae et al., 2008).

Nicotine physical dependence involves overlapping stages of development, expression, and maintenance that may be sensitive to changes in opioid-mediated transmission (Hadjiconstantinou and Neff, 2011). Our study was aimed at the development stage, as planarians were concurrently exposed to nicotine and opioid receptor antagonists and then withdrawn and placed into drug-free environment for behavioral analysis. We found that antagonism of MOR and DOR during the development of nicotine physical dependence attenuates subsequent abstinence-induced withdrawal in planarians. Limited knowledge about the pharmacological effects of nicotine in planarians (Pagan et al., 2013; Rawls et al., 2011), especially as related to its interaction with endogenous opioids and opioid receptors, precludes extensive speculation about specific mechanisms; however, a link between the nicotine abstinence syndrome and endogenous opioid system has already been demonstrated for rats and mice, (Biala et al., 2005; Malin et al., 2006). The evidence that morphine reverses withdrawal signs in rats in abstinence-induced nicotine withdrawal and nicotine reduces naloxone-precipitated morphine withdrawal signs suggests that common neurobiological mechanisms underlie nicotine and opioid withdrawal (Malin et al., 1993; Zarrindast and Farzin, 1996). Further, the abstinence syndrome in nicotine-dependent rats is more severe following naloxone-precipitated withdrawal than after abstinence-induced withdrawal (Malin et al., 1993, 2006). Although exacerbated abstinence syndrome induced by

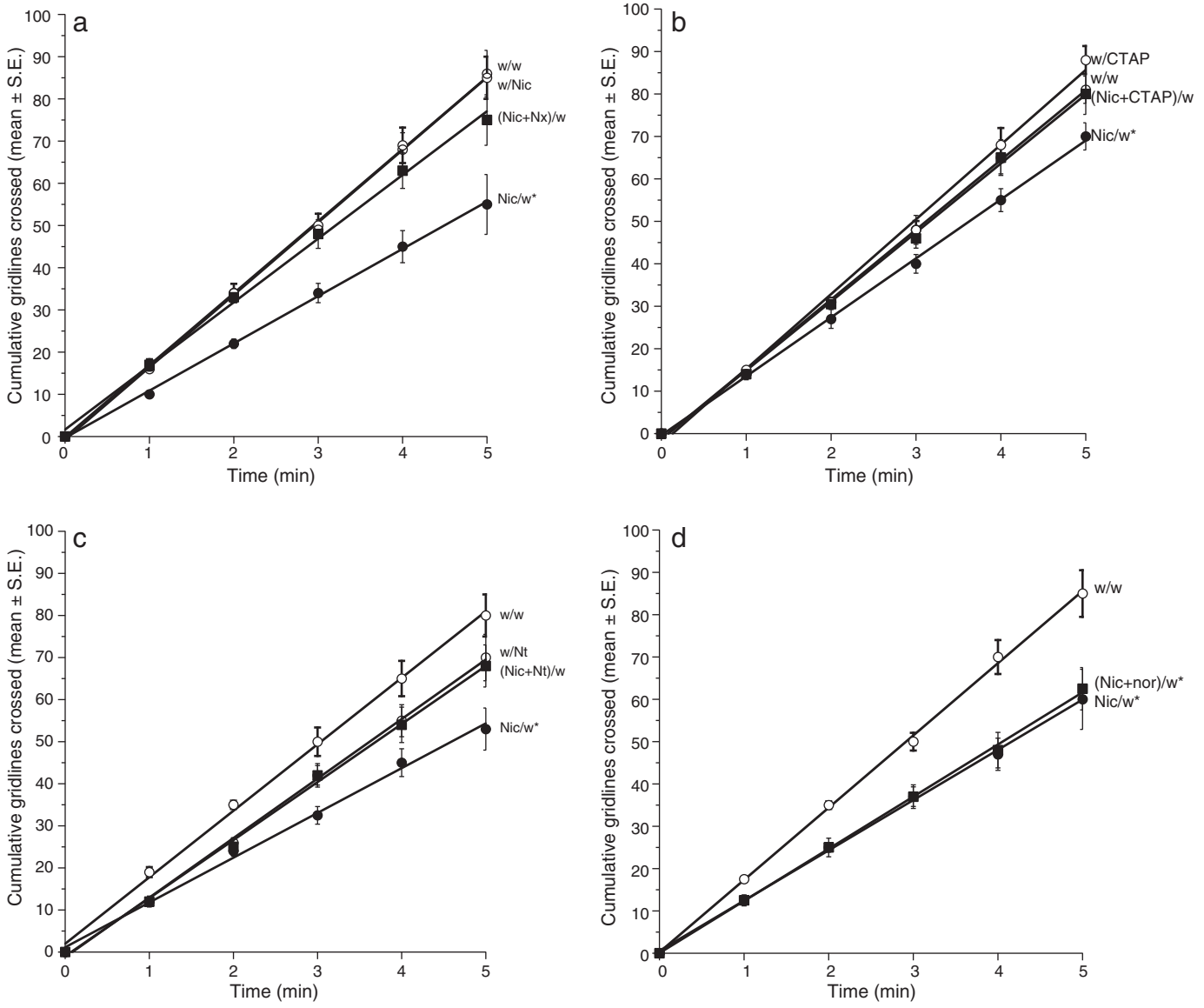

Fig. 4. Abstinence-induced nicotine (Nic) withdrawal in water (w) was attenuated by the opioid receptor antagonist naloxone (Nx) (a) and by the subtype-selective μ- (b) and δ- (c) opioid receptor antagonists CTAP and naltrindole (Nt), respectively, but not by the κ-opioid receptor antagonist *nor*-BNI (nor) (d). *$P < 0.05$ compared to w/w.

opioid receptor antagonism in nicotine-dependent rats was not observed in planarians, the difference is likely due to the timing of naloxone exposure. Naloxone was administered after physical dependence had already developed in rats, but was administered simultaneously with nicotine during the development of physical dependence (Malin et al., 1993) in our study.

Taken together, the most parsimonious explanation for our results is that nicotine produces an enhancement in the activity of the MOR and DOR systems that contribute to the normal development of physical dependence, conceivably by increasing the synthesis of opioid peptides such as β-endorphin and Met-enkephalin (Conte-Devolx et al., 1981; Hadjiconstantinou and Neff, 2011; Hexum and Russett, 1987; Marty et al., 1985; Pierzchala et al., 1987; Pomerleau, 1998). When an opioid antagonist is administered during nicotine exposure, the conjectured increase in opioid peptide synthesis would still occur, but ensuing downstream activation of opioid receptor subtypes would be prevented, thus leading to a subsequent reduction in withdrawal response on discontinuation of nicotine exposure. Another possibility is that its inhibition of nicotine physical dependence in planarians is related to antagonism of nicotine acetylcholine receptors (nAChRs), since naloxone and naltrexone have been shown to bind to nAChRs (Almeida et al., 2000; Tomé et al., 2001).

It is interesting to note that the KOR antagonist did not affect the development of nicotine physical dependence in planarians, indicating that the effect was subtype-selective for MOR and DOR. This is consistent with recent data showing that chronic nicotine exposure of rats upregulates five enkephalin opioid peptides in the striatum without producing any change in dynorphin synthesis (Petruzziello et al., 2013). Enkephalins and dynorphins exert opposing actions on dopamine neurons (increasing and decreasing dopamine release respectively) and are components of circuits promoting positive or negative motivational and affective states (Smith et al., 2012).

Table 1
Control experiments. The antagonists had no effect of their own on *p*LMV (5-min cumulative mean ± S.E.). N = 7–13 planarians per group.

Pretreat	Test	*p*LMV	*P*
Water	Water	73 ± 3	>0.05
Naloxone	Water	85 ± 3	>0.05
Naltrindole	Water	79 ± 2	>0.05
nor-BNI	Water	87 ± 3	>0.05
CTAP	Water	83 ± 4	>0.05

The present results do not address the mechanism of dependence development or withdrawal. In rodents, nicotine increases synthesis and release of endogenous opioid peptides in nucleus accumbens by activation of dopamine and glutamate receptor-mediated pathways. After acute administration of nicotine, the peptides influence the release of dopamine and modulate locomotor activity, which is increased by enkephalins and endorphins and decreased by dynorphins. During nicotine withdrawal in rodents, spontaneous locomotor activity is decreased and somatic signs of physical dependence are increased. The decrease in locomotor activity is thought to result from suppressed dopamine and enhanced dynorphin release in nucleus accumbens. Such information is not yet known for planarians and was beyond the scope of the present study. Nonetheless, when opioid receptor block was produced in planarians that were withdrawn from nicotine and already physically dependent, an enhancement of the withdrawal response occurred, similar to that observed in rats and mice (Biala et al., 2005; Malin et al., 1993), perhaps due to suppression of compensatory MOR or DOR systems that are normally activated to offset aversive effects associated with nicotine abstinence. Future studies using planarian models will investigate the effects of subtype-selective opioid receptor antagonists on the expression and maintenance of nicotine physical dependence and the rewarding properties of nicotine using conditioned place preference (Ramoz et al., 2012; Rawls et al., 2011).

Previous findings in rats (Liu and Jernigan, 2011) evaluated the effects of opioid receptor antagonists in the reinforcing effects of nicotine using a model of nicotine self-administration. The present study suggests the involvement of MOR- and DOR-, but not KOR-opioid receptor subtypes in the development of nicotine physical dependence or abstinence-induced withdrawal in an invertebrate model.

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