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Buprenorphine requires concomitant activation of NOP and MOP receptors to reduce cocaine consumption

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Abstract

Buprenorphine's clinical use is approved for the treatment of heroin addiction; however, evidence supporting its efficacy in cocaine abuse also exists. While for heroin it has been demonstrated that the effect of buprenorphine is mediated by its ability to activate μ-opioid peptide receptor (MOP) receptors, the mechanism through which it attenuates cocaine intake remains elusive. We explored this mechanism using operant models where rodents were trained to chronically self-administer cocaine for 2 hours daily. Buprenorphine (0.3, 1.0 and 3.0 mg/kg) given intraperitoneally 90 minutes before access to cocaine significantly and dose dependently reduced its intake. Pre-treatment with naltrexone or with the selective nociceptin/orphanin FQ peptide (NOP) antagonist SB-612111 did not prevent buprenorphine-induced reduction of cocaine intake. However, when naltrexone and SB-612111 were combined, the effect of buprenorphine on cocaine was completely prevented. To confirm that co-activation of MOP and NOP receptors is the underlying mechanism through which buprenorphine reduces cocaine intake, three compounds, namely, AT-034, AT-201 and AT-202, with a range of affinity and intrinsic activity profiles for MOP and NOP receptors, but weak ability for kappa-opioid peptide receptor (KOP) transmission, were tested. Consistent with our hypothesis based on buprenorphine's effects, results demonstrated that AT-034 and AT-201, which co-activate MOP and NOP receptors, reduced cocaine self-administration like buprenorphine. AT-202, which selectively stimulates NOP receptors, was not effective. Together, these data demonstrate that for buprenorphine, co-activation of MOP and NOP receptors is essential to reduce cocaine consumption. These results open new vistas on the treatment of cocaine addiction by developing compounds with mixed MOP/NOP agonist properties.

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Disclosure/Conflict of Interest

The authors report no biomedical financial interests or potential conflicts of interest. M.K. conducted the experiments and wrote the manuscript. N.Z. provided the compounds AT-034, AT-201 and AT-202 and helped with the interpretation of the data. G.d.G. performed the experiments and analyzed the data. Q.S., D.Y. and V.B.J. helped with the experiments and data collection. R.C. designed, supervised the study and contributed to prepare and write the manuscript.

Keywords

addiction; buprenorphine; cocaine; MOP and NOP receptors; self-administration

INTRODUCTION

Buprenorphine is a long-acting partial agonist at MOP and antagonist at DOP and KOP receptors and, together with methadone, represents a first line treatment for heroin addiction (Kosten 1990; Kleber 2003; Kakko *et al.* 2007). However, a growing body of evidence indicates that buprenorphine may also be effective in counteracting several effects of cocaine and in reducing its consumption. Studies in rats showed that buprenorphine attenuates the expression of cocaine sensitization and other cocaine-related behaviors by increasing basal levels of glutamate in the nucleus accumbens, which serve to decrease the effectiveness of cocaine or cocaine-associated cues (Placenza, Rajabi, & Stewart 2008). Several research groups reported the reduction of cocaine self-administration in rats following buprenorphine treatment (Sorge & Stewart 2006a, 2006b). On the other hand, clinical studies show mixed results. In some human laboratory studies, buprenorphine was able to reduce self-reported cocaine craving and cocaine self-administration (Kosten, Kleber, & Morgan 1989a; Schottenfeld *et al.* 1993; Montoya *et al.* 2004). In other studies, these findings were not confirmed (Mendelson *et al.* 1992; Strain *et al.* 1994; Foltin & Fischman 1996; Kouri, Lukas, & Mendelson 1996). Inconsistent results were also obtained in clinical trials in opiate-dependent subjects co-abusing cocaine, where in some cases, buprenorphine treatment was associated also with a reduction in cocaine use (Kosten, Kleber, & Morgan 1989b), whereas other trials did not confirm this effect (Compton *et al.* 1995; Schottenfeld *et al.* 1997). In one study, worse office-based buprenorphine treatment outcomes were observed in opioid-dependent cocaine users compared with cocaine-free patients (Sullivan *et al.* 2010). Conversely, in another work, the beneficial effects of office-based buprenorphine treatment in opioid-dependent cocaine users and non-users were comparable (Cunningham *et al.* 2013). A possible explanation for these variable effects of buprenorphine on cocaine consumption could be the different dose regimen used across the studies, patient population and treatment protocols. Intriguingly, in a study in which sublingual buprenorphine was used, it was found that in heroin addicts who also abused cocaine, buprenorphine reduced consumption of both drugs. However, the effect on cocaine occurred only at very high doses and appeared to be independent from that on heroin (Montoya *et al.* 2004). This finding was replicated in another cohort of heroin-addicted patients, in which buprenorphine was also given at high doses (Gerra, Fantoma, & Zaimovic 2006). In this latter report, it was also found that the combination of buprenorphine and the non-selective MOP, DOP and KOP antagonist naltrexone resulted in an even more pronounced effect (Gerra *et al.* 2006). Given that buprenorphine has a high ligand affinity for these receptors and that its effect on cocaine is not blocked but rather enhanced by naltrexone, it is tempting to speculate that this effect is not entirely dependent on activation of the classical opioid receptors.

This possibility is further supported by data showing that, when buprenorphine was tested in combination with low doses of naltrexone to block its MOP partial agonist effects, the

inhibitory effect on cocaine self-administration and cocaine-primed reinstatement was preserved (Witkin *et al.* 1991; Mello *et al.* 1993; Wee *et al.* 2012; Cordery *et al.* 2014).

Previous work showed that in addition to its activity at classical MOP, DOP and KOP receptors, buprenorphine also acts as an agonist/partial agonist at the nociceptin/orphanin FQ peptide (NOP) receptor (Bloms-Funke *et al.* 2000; Wnendt *et al.* 1999; Huang *et al.* 2001; Lutfy *et al.* 2003), an effect that occurs only at high doses of the drug (Lutfy *et al.* 2003). Consistent with this mechanism, in previous studies in alcohol-preferring rats, we have shown that at high doses, buprenorphine reduced alcohol intake through activation of NOP receptors, whereas at low doses, it enhanced drinking by activating MOP receptors (Ciccocioppo *et al.* 2007). Activation of the NOP receptor by the endogenous ligand nociceptin/orphanin FQ (N/OFQ) has been shown to reduce the expression of CPP engendered by either cocaine or methamphetamine (Kotlinska *et al.* 2002; Zhao *et al.* 2003), and microdialysis experiments revealed that intracranial N/OFQ injection prevented cocaine from stimulating mesoaccumbal dopamine (DA) efflux (Lutfy, Do, & Maidment 2001). Moreover, activation of NOP receptors by N/OFQ resulted in blockade of cocaine sensitization (Lutfy *et al.* 2002; Kotlinska *et al.* 2003; Bebawy *et al.* 2010), an effect that was absent in NOP receptor KO mice (Bebawy *et al.* 2010). Finally, it has been reported that mice lacking the NOP receptor show greater conditioned place preference for cocaine compared with wild-type littermates (Marquez *et al.* 2008).

Taken together, these data led us to hypothesize that NOP agonism may represent a component of the mechanism through which buprenorphine attenuates cocaine consumption. To explore this hypothesis, we conducted a series of pharmacological experiments in which we investigated the effect of buprenorphine on cocaine self-administration after pre-treatment with the classical opioid receptor antagonist naltrexone, with the selective NOP antagonist SB-612111 or a combination of both. We then used three new small-molecules NOP agonist/MOP partial agonists, namely, AT-034 (Journigan *et al.* 2014), AT-201 (Khroyan *et al.* 2007; Khroyan *et al.* 2009) and a selective NOP agonist AT-202 (Toll *et al.* 2009), (Table 1) representing a range of affinity/efficacy profiles for MOP and NOP receptors but weak ability to influence KOP activity, to evaluate their ability to mimic buprenorphine's effects on cocaine self-administration. To demonstrate the specificity of action of all the compounds on cocaine intake, their effect on sucrose self-administration was also investigated.

MATERIALS AND METHODS

Animals

Male Wistar rats ($N = 54$) (Charles River, Calco, Italy) were employed for this study. At the beginning of the experiments, animals' body weight ranged between 250 and 300 g. They were housed in groups of two in a room with artificial 12:12 hours light/dark cycle (lights off at 9:00 AM), constant temperature (20–22 °C) and humidity (45–55 percent). All animals were handled once daily for 5 minutes for 1 week before the beginning of the experiments. During the entire period of the experimental phase, rats were offered free access to tap water and food pellets (4RF18, Mucedola, Settimo Milanese, Italy). Experiments were conducted during the dark phase of the light/dark cycle. All procedures

were performed during the dark phase and conducted in adherence to the European Community Council Directive for Care and Use of Laboratory Animals and the National Institutes of Health Guidelines for Care and Use of Laboratory Animals.

Intravenous surgery

Incisions were made to expose the right jugular vein and the back between the shoulders; a catheter made from silicon tubing (ID = 0.020 in, OD = 0.037 in) was subcutaneously positioned between these two points. For 1 week after surgery, rats were treated daily with 0.2 ml of the antibiotic sodium cefotaxime (262 mg/ml). For the duration of the experiments, catheters were daily flushed with 0.2–0.3 ml of heparinized saline solution. Patency of the catheter was assumed if there was an immediate loss of reflexes. Experiments began 1 week after surgery.

Drugs

Cocaine hydrochloride (Johnson Matthey, Edinburgh, UK) was dissolved in sterile physiological saline at a concentration of 0.25 mg/0.1 ml and given intravenously (Knoll *et al.* 2011). Naltrexone (Sigma-Aldrich) was dissolved in distilled water and was administered intraperitoneally (i.p.) at the doses of 0.25, 1.0, 2.5 and 5.0 mg/kg/ml 60 minutes prior to the test phase. Buprenorphine hydrochloride, available as temgesic 0.3 mg/ml, was purchased from commercial sources (pharmacy), in injectable formulation 1 ml per ampule. It was administered i.p. at the doses of 0.3, 1.0 and 3.0 mg/kg/ml 90 minutes prior to the test phase. SB-612111 (Eli Lilly & Co., USA) was dissolved in a vehicle containing H_3PO_4 and distilled water in 3:3 parts. It administered per os via gavage in 10.0 and 30.0 mg/kg/ml 60 minutes prior to the test phase. AT-034, AT-201 and AT-202 (NOP/MOP agonist compounds, Table 1) were synthesized by Dr. Zaveri (Astraea Therapeutics, USA). These compounds were dissolved in 2 percent DMSO and 98 percent hydroxymethylcellulose. They were administered subcutaneously 60 minutes prior to the test phase.

SELF-ADMINISTRATION APPARATUS

The self-administration stations consisted of operant conditioning chambers (Med Associate Inc.) enclosed in sound-attenuating, ventilated environmental cubicles. Each chamber was equipped with two retractable levers located in the front panel of the chamber. A plastic tube that was connected to the catheter before the beginning of the session delivered cocaine. An infusion pump was activated by responses on the right (active) lever, while responses on the left (inactive) lever were recorded but did not result in any programmed consequences. Activation of the pump resulted in a delivery of 0.1 ml of fluid. An IBM compatible computer controlled the delivery of cocaine solution and recording of the behavioral data. Following 1 week of recovery from surgery rats ($N = 54$) were trained to self-administer cocaine under a fixed ratio 5 (FR-5) schedule of reinforcement; every five active lever presses resulted in the delivery of one cocaine dose (0.25 mg/0.1 ml, intravenously). Following each cocaine infusion, we presented a 20-second time-out period during which responses at the active lever had no programmed consequences.

COCAINE SELF-ADMINISTRATION PARADIGM

After intravenous catheter implantation, animals were left for 1 week in their home cages in order to recover from surgery. Animals were trained to self-administer cocaine under an FR-1 schedule of reinforcement, for five consecutive days. Then they were moved to an FR-5 (with 20-second time-out) for the entire duration of the experiment. Once a stable baseline of cocaine infusion was achieved (for at least 15 consecutive days), drug treatment in a Latin square design began. Drugs were injected systemically: orally, i.p. or subcutaneously, 60–90 minutes before the test began. Experiments were conducted every 3 days. Between drug tests, cocaine self-administration baseline was re-established.

STATISTICAL ANALYSIS

For data evaluation, the analysis of variance (ANOVA) was used followed by Newman–Keuls *post hoc* tests. In details, drug effects were analyzed by one factor (treatment) within subject ANOVA. A between-subjects one-way ANOVA was used to analyze the data of experiment depicted in Fig. 4. Experiment depicted in Fig. 1c was instead analyzed by means of a paired Student's *t*-test. The Newman–Keuls test was used for *post hoc* comparisons when appropriate. Statistical significance was set at $P < 0.05$.

RESULTS

Buprenorphine reduces cocaine but not saccharin self-administration in a dose-dependent manner

Rats ($n = 8$) were trained to lever press for cocaine (0.25 mg/0.1 ml) under an FR-1 schedule of reinforcement. After learning acquisition, they were moved to a (FR-5) schedule. Training continued until rats exhibited a stable level of responding under this contingency. Responses on the inactive lever were registered but resulted in no scheduled consequences. Rats ($n = 8$) in a within-subject-counterbalanced Latin square design were then treated with buprenorphine (0.0, 0.3, 1.0 and 3.0 mg/kg) given i.p. 90 minutes prior to the test phase. One-way ANOVA revealed a significant inhibition of cocaine intake following buprenorphine [$F(3, 7) = 5.72$; $P < 0.01$]. *Post hoc* Newman–Keuls analyses (Fig. 1a) confirmed that buprenorphine decreased cocaine self-administration in a dose-dependent manner with the effect being significant at 0.3 mg/kg ($P < 0.05$) and also at 1.0 and 3.0 mg/kg ($P < 0.01$). To control for the selectivity of the effect of buprenorphine on cocaine, another group of rats ($n = 6$) was trained to self-administration saccharin, and the effect of the intermediate dose (1.0 mg/kg) of buprenorphine was tested in a within-subject-counterbalanced design. Paired Student's *t*-test analysis did not show any statistically significant [$t = 1.44$, $df = 5$, P = NS] effect of buprenorphine on saccharin (Fig. 1c). In both experiments, inactive lever responses were not affected by buprenorphine [$F(3, 7) = 1.25$; P = NS] (Fig. 1b) and [$t = 1$, $df = 5$, P = NS] (Fig. 1d).

The non-selective opioid antagonist naltrexone and the NOP antagonist SB-612111 do not reduce cocaine self-administration

In another group of rats ($n = 7$), we investigated the effect of a wide range of doses of naltrexone (0.25, 1.0, 2.5 and 5.0 mg/kg) on cocaine intake. Results revealed no effect of the

opioid antagonist on cocaine-related operant responding [$F(4, 6) = 1.416$; P = NS] (Fig. 2a). Inactive lever presses were not affected by the treatment with naltrexone [$F(4, 6) = 0.68$; P = NS] (Fig. 2b). A second group of rats ($n = 9$) was also trained to self-administration cocaine, and the effect of the selective NOP antagonist SB-612111 (0.0, 10.0 and 30.0 mg/kg) was explored. Same as previously, results demonstrated that rats rapidly acquired a stable baseline of cocaine responding, while one-way ANOVA showed no effect [$F(2, 8) = 0.13$; P = NS] of SB-612111 on cocaine intake (Fig. 2c). Inactive lever was not affected by the treatment [$F(2, 8) = 0.58$; P = NS] (Fig. 2d).

Pre-treatment with naltrexone or SB-612111 does not block the effect of buprenorphine on cocaine self-administration

To evaluate the role of MOP and NOP receptors in mediating buprenorphine-induced reduction of cocaine self-administration, we tested the effect of naltrexone (0.25, 1.0 and 2.5 mg/kg), of SB-612111 (10.0 and 30.0 mg/kg) and of their combination against buprenorphine. Naltrexone was administered 30 minutes prior to buprenorphine (1 mg/kg) and 90 minutes later; rats ($n = 10$) were tested for cocaine self-administration. The experiment was carried out in a within-subject Latin square-counterbalanced design. One-way ANOVA revealed a statistically significant overall effect of treatment [$F(4, 9) = 11.94$; $P < 0.0001$] on cocaine self-administration. *Post hoc* Newman–Keuls comparison indicated that buprenorphine (1 mg/kg) alone significantly decreased cocaine self-administration compared with vehicle-treated rats ($P < 0.001$). Treatment with naltrexone did not alter cocaine self-administration *per se* nor did it counteract the inhibitory effect of buprenorphine on cocaine self-administration (Fig. 3a). Responses on the inactive lever were not influenced [$F(4, 9) = 1.55$; P = NS] by treatments (Fig. 3b). The same effect was observed when the NOP receptors were selectively blocked by SB-612111, 30 minutes prior to buprenorphine administration. Overall ANOVA yields a statistically significant effect of the treatment [$F(3, 8) = 50.04$; $P < 0.0001$]. *Post hoc* Newman–Keuls analyses indicate that buprenorphine alone significantly decreased cocaine self-administration compared with the control group (buprenorphine 1.0 mg/kg versus vehicle $P < 0.001$), confirming the results of the previous experiment. On the other hand, SB-612111 alone did not alter cocaine self-administration compared with the control group nor affected buprenorphine-induced reduction of cocaine self-administration ($P > 0.05$). The responses on the left lever were not influenced by the treatment [$F(3, 8) = 0.37$; P = NS] (Fig. 3d).

The combination of naltrexone and SB-612111 blocks the effect of buprenorphine on cocaine self-administration

Finally, NOP and MOP receptors were simultaneously blocked by concomitant administration of SB-612111 (30 mg/kg) and naltrexone (2.5 mg/kg), given prior to buprenorphine (1 mg/kg). One-way ANOVA revealed a significant overall effect of treatment [$F(4, 45) = 34.30$; $P < 0.0001$]. *Post hoc* Newman–Keuls comparisons indicated that animals were treated directly with buprenorphine significantly ($P < 0.001$) reduced the intake of cocaine. The same effect was observed in the animals treated with SB-612111 or naltrexone alone prior to buprenorphine ($P < 0.001$). Interestingly however, the buprenorphine-induced reduction of cocaine self-administration was significantly inhibited by simultaneous treatment with naltrexone and SB-612111 ($P < 0.001$) suggesting that

concomitant inhibition of both receptors is required to reverse the effect of buprenorphine on cocaine self-administration (Fig. 4a). Treatment with SB-612111 plus naltrexone and buprenorphine was also significantly different ($P < 0.001$) from groups treated with buprenorphine plus SB-612111 or plus naltrexone. No drug effects were observed at the inactive control lever [$F(4, 45) = 0.61$; P = NS] (Fig. 4b).

Buprenorphine-like AT-034 and AT-201 but not nociceptin/orphanin FQ-like compound AT-202 selectively reduce cocaine self-administration

Separate groups of rats ($n = 7–9$) were trained to self-administer cocaine and saccharin solutions in operant chambers. AT-034, AT-201 and AT-202 (1.0, 3.0 and 10 mg/kg) were administered 60 minutes prior to the test phase. Experiments were carried out in a within-subject Latin square-counterbalanced design. One-way ANOVA revealed a statistically significant overall effect of AT-034 on cocaine self-administration [$F(3, 6) = 4.47$; $P < 0.01$]. *Post hoc* Newman–Keuls comparisons indicated that AT-034 at 3.0 and 10.0 mg/kg significantly reduced cocaine self-administration compared with controls ($P < 0.05$) (Fig. 5a). ANOVA also revealed a statistically significant effect of AT-034 on saccharin self-administration [$F(3, 8) = 9.13$; $P < 0.001$]. *Post hoc* comparisons revealed a significant ($P < 0.001$) inhibition of saccharin at 10 mg/kg suggesting lack of specificity at this dose (Fig. 6a). Treatment did not affect inactive lever responses either for cocaine [$F(3, 6) = 0.73$; P = NS] (Fig. 5b) or saccharin [$F(3, 8) = 2.8$; P = NS] (Fig. 6b). For AT-201, one-way ANOVA revealed an overall statistically significant effect of treatment [$F(3, 6) = 4.57$; $P < 0.05$]. *Post hoc* Newman–Keuls comparisons indicated that AT-201 significantly reduced cocaine self-administration at 3.0 mg/kg ($P < 0.05$) and 10.0 mg/kg ($P < 0.05$) compared with the vehicles (Fig. 5c). ANOVA also revealed a statistically significant effect of AT-201 on saccharin self-administration [$F(3, 7) = 14.01$; $P < 0.0001$] (Fig. 6c). *Post hoc* Newman–Keuls test revealed that compared with controls, AT-201 significantly reduced saccharin self-administration at the highest dose tested, 10.0 mg/kg ($P < 0.001$). The treatment with AT-201 did not affect inactive lever responses for cocaine [$F(3, 6) = 0.13$; P = NS] (Fig. 5d) or saccharin [$F(3, 7) = 0.5$; P = NS] (Fig. 6d). For AT-202, one-way ANOVA failed to reveal an overall significant effect of treatment on cocaine self-administration [$F(3, 8) = 2.96$; P = NS] (Fig. 5e) as well as on saccharin self-administration [$F(3, 8) = 0.37$; P = NS] (Fig. 6e). The treatment with AT-202 did not affect inactive lever responses for cocaine [$F(3, 8) = 2.5$; P = NS] (Fig. 5f) or saccharin [$F(3, 8) = 1.57$; P = NS].

DISCUSSION

In the past decade, pre-clinical and clinical literature has focused significant attention on the use of buprenorphine in cocaine abuse. This opioid agent has unique features because it is a high-affinity partial agonist at MOP receptors, an antagonist at KOP and DOP receptors and a low-affinity partial agonist at NOP receptors (Huang *et al.* 2001). This unique pharmacological profile of buprenorphine may be responsible for the promising effects observed on cocaine addiction in laboratory animals and in humans (McCann 2008). However, its exact mechanism of action still remains elusive. To shed light on this mechanism, we conducted an extensive pharmacological investigation using a classical opioid receptor antagonist (i.e. naltrexone), NOP receptor antagonists and their combination,

as well as new tool compounds mimicking some but not all pharmacological properties of buprenorphine. Consistent with the current literature, our results demonstrate that administration of buprenorphine dose dependently reduced operant responding for cocaine. The effect was selective, because over the same dose range, buprenorphine did not affect saccharin self-administration. We then tested the effect of the non-selective classical opioid antagonist naltrexone and the selective NOP blocker SB-612111. Results revealed that neither of the two compounds modified cocaine self-administration, although a trend to a reduction of lever pressing for cocaine was observed following naltrexone. These findings are consistent with results of previous studies showing that naltrexone does not modify the pattern of cocaine self-administration under continuous reinforcement schedules at doses that are instead able to modify heroin-related responding behavior (Ettenberg *et al.* 1982; Giuliano *et al.* 2013). However, this contrasts with few other studies in which an effect of opioid antagonist on cocaine intake was suggested (Gerra *et al.* 2006; Bidlack 2014). These discrepant results may depend on several methodological factors, including opioid antagonist dosages, cocaine unit dose employed and reinforcement schedules used in the self-administration studies (De Vry, Donselaar, & Van Ree 1989; Corrigall & Coen 1991). Importantly, here, we show that blockade of classical MOP, DOP and KOP by naltrexone or selective inhibition of NOP receptors by SB-612111 is not sufficient to prevent the inhibitory action of buprenorphine on cocaine self-administration. This finding contrasts with a recent report which showed that naltrexone is able to prevent the inhibitory effect of buprenorphine on cocaine intake (Wee *et al.* 2012). However, in the same study, it was also shown that this effect of naltrexone is variable depending on the reinforcement schedule used and occurs at relatively high doses. Based on these findings, we argued that the inhibitory effect of buprenorphine on cocaine intake might have been due to its ability to simultaneously activate MOP and NOP receptors. To demonstrate this hypothesis, we co-administered naltrexone and SB-612111 prior to buprenorphine. As predicted, the drug combination completely blocked the inhibitory effect of buprenorphine on cocaine self-administration. To further strengthen our hypothesis of a concomitant activation of MOP and NOP receptor as a mechanism responsible of buprenorphine effects on cocaine, we used a series of tool compounds with a range of affinities and selectivity for MOP, KOP and NOP receptors. Specifically, we tested AT-201 (previously called SR16435), which is a high-affinity partial agonist at NOP and MOP receptors, AT-034, which is a moderate-affinity partial agonist at NOP but is a high-affinity agonist at MOP, and AT-202 (previously called SR16835), which is a high-affinity and potent NOP agonist with moderate-affinity and partial agonist activity at MOP (Khroyan *et al.* 2007; Toll *et al.* 2009; Khroyan *et al.* 2011; Sukhtankar *et al.* 2013; Journigan *et al.* 2014) (Table 1). Results showed that AT-034 and AT-201 attenuate cocaine self-administration but at the highest dose (10 mg/kg), also reduced saccharin self-administration, whereas at the lower dose (3 mg/kg), their effect was selective for cocaine. The NOP full agonist AT-202 given at pharmacologically effective doses (Toll *et al.* 2009) did not show effects on cocaine and saccharin intake. Notably, in a previous study, it was found that AT-202 dose dependently increased nicotine taking in nicotine-dependent and non-dependent rats, while it did not affect alcohol intake in a co-administration paradigm (Cippitelli *et al.* 2016). Here, neither cocaine nor saccharin consumption was modified by AT-202, suggesting that intake facilitation was specific for nicotine. Together, these findings provide two important pieces of information. First, at low

doses, the effect of these agents is specific for cocaine, indicating that concomitant activation of MOP and NOP may likely attenuate psychostimulant reinforcement. Second, the efficacy in preventing cocaine intake is linked to a specific balance between MOP and NOP affinity and efficacy with highest effects achieved following partial agonism at both receptors. In fact, AT-202 that acts as a full agonist at NOP appeared to have no effect on cocaine self-administration. Clearly, these results should be interpreted with some caution because pharmacokinetic factors or activity on other receptor types might have also contributed to shape the effect of these still not-fully characterized molecules. In the recent years, several studies have shown that antagonists at KOP receptors may also attenuate cocaine self-administration in animal models. Because buprenorphine acts as a KOP partial agonist or antagonist (depending on the pharmacological test), it has been proposed that its efficacy to attenuate cocaine consumption is mediated by KOP rather than MOP receptors (Mello *et al.* 1993; Cordery *et al.* 2014). This hypothesis has attracted a significant clinical attention, and the possibility to combine buprenorphine with naltrexone has been envisioned. The rationale behind this combination being that naltrexone acting as a preferential antagonist at MOP receptors at low doses should be able to prevent the abuse liability of buprenorphine, by blocking its binding at MOP while leaving unaltered or even potentiating the antagonism at KOP (Gerra *et al.* 2006; Wee *et al.* 2012; Bidlack 2014). However, this hypothesis suffers from significant weaknesses. For example, naltrexone and buprenorphine block KOP at the same nanomolar concentration (Codd *et al.* 1995; Huang *et al.* 2001). Moreover, naltrexone, being a non-selective opioid antagonist, blocks MOP and KOP with similar potency demonstrating a KOP/MOP binding ratio of about 6 (Codd *et al.* 1995). Hence, at therapeutic doses, this antagonist should be able to fully block not only MOP but, like buprenorphine, also KOP receptors. It therefore remains obscure why naltrexone given alone should not reproduce the same beneficial effect of buprenorphine on cocaine. On contrary, the efficacy of this opioid antagonist in attenuating cocaine use is poorly supported at both pre-clinical (Ettenberg *et al.* 1982; Giuliano *et al.* 2013) and clinical levels in which lack of efficacy following high dosage (100 mg/d) has been documented (Schmitz *et al.* 2009). In addition, our experiments demonstrate that the efficacy of AT-034 and AT-201 in reducing cocaine intake was comparable to that of buprenorphine, whereas their binding at KOP receptor was about 20 and 60 times lower, respectively, which further discourages the hypothesis of an involvement of KOP receptors in mediating the effects observed here on cocaine self-administration. More importantly, buprenorphine has produced mixed results on cocaine-dependent individuals in clinical trials, with the greatest efficacy being demonstrated at very high doses (above 16 mg/day) and in individuals either co-abusing or co-dependent on opiates (Kosten *et al.* 1992; Schottenfeld *et al.* 1993; Montoya *et al.* 2004). Extrapolating from human positron emission tomography (PET) imaging studies with [^{11}C]-carfentanil, it is possible to assert that starting from 12 mg/day, buprenorphine reaches a full occupation of MOP opioid receptors (Greenwald, Schuh, & Stine 2003). But considering that the affinity of buprenorphine for KOP is higher than that at MOP, it is likely that at this dose, a full occupation of KOP receptors also occurs (Huang *et al.* 2001). Hence, the increased efficacy of buprenorphine on cocaine-addicted individuals that is observable at doses normally higher than 16 mg/day is unlikely attributable to buprenorphine's ability to bind neither to MOP nor to KOP receptors. As discussed previously, if at low/intermediate doses, buprenorphine specifically binds to the classical MOP, KOP and DOP opioid receptor,

then at higher doses, it may also occupy NOP receptors resulting in significant effects on pain and alcohol drinking (Lutfy *et al.* 2003; Ciccocioppo *et al.* 2007; Khroyan *et al.* 2011). Here, we provided evidence supporting a new intriguing hypothesis according to which buprenorphine blunts the motivation for cocaine by simultaneously activating MOP and NOP receptors. This hypothesis opens new vistas in the development of new pharmacotherapies for cocaine addiction. For instance, an attractive recent development is the clinical advancement of molecules like cebranopadol®. This compound has nanomolar affinity for NOP and MOP and lower affinity (about 30-fold) for KOP receptors, appears to have none or very limited abuse liability and is under current development for the treatment of pain (Lambert, Bird, & Rowbotham 2015). In addition to its use as analgesic, it would be extremely interesting to test this compound or analog molecules in cocaine abuse and dependence. This is a relatively close possibility because this medication already reached Phase 3 of clinical development and its safety profile in humans has been already documented.

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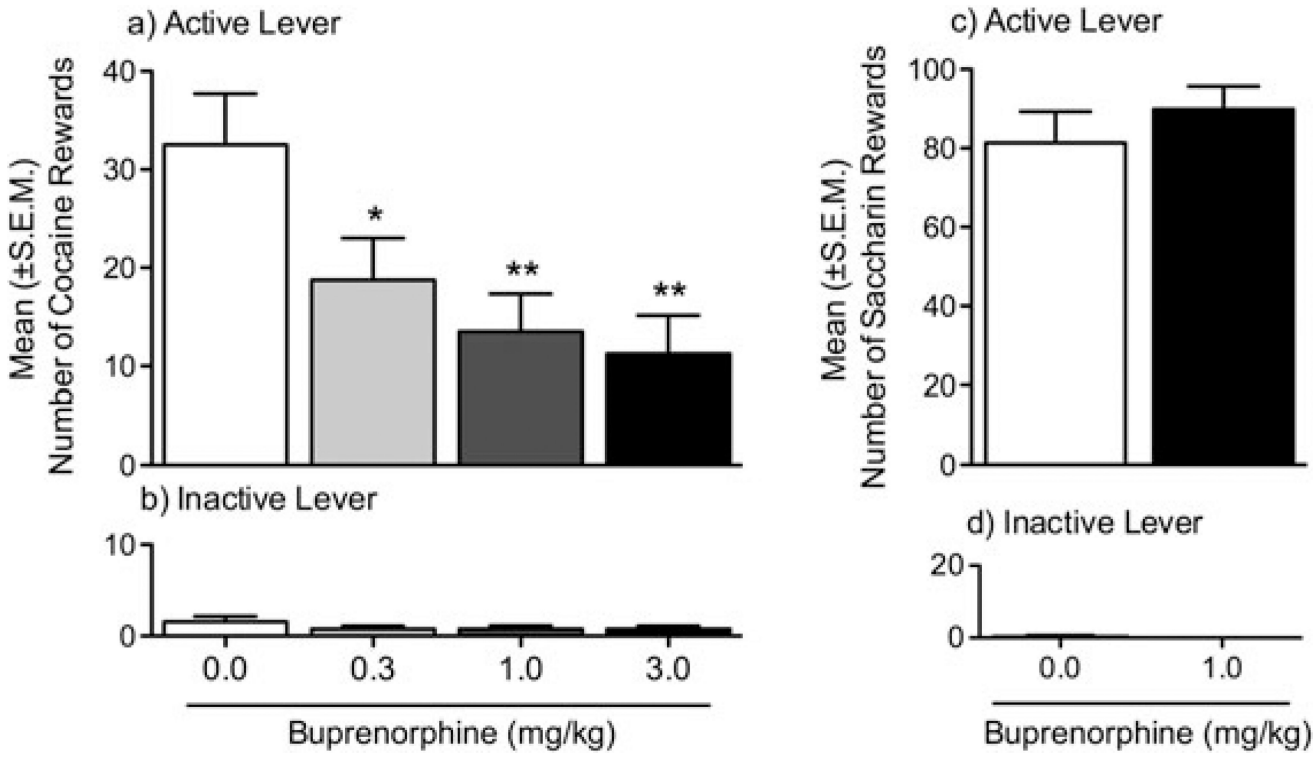

Figure 1.

Effect of buprenorphine on cocaine and saccharin self-administration: at all doses tested, buprenorphine significantly and in a dose-dependent manner reduced (a) the number of cocaine rewards (b), whereas inactive lever response was not affected by treatment. (c) At the middle, effective dose of 1.0 mg/kg buprenorphine neither affected saccharin self-administration (d) nor modified inactive lever responses. Difference from controls (0.0): $*P < 0.05$ or $**P < 0.01$

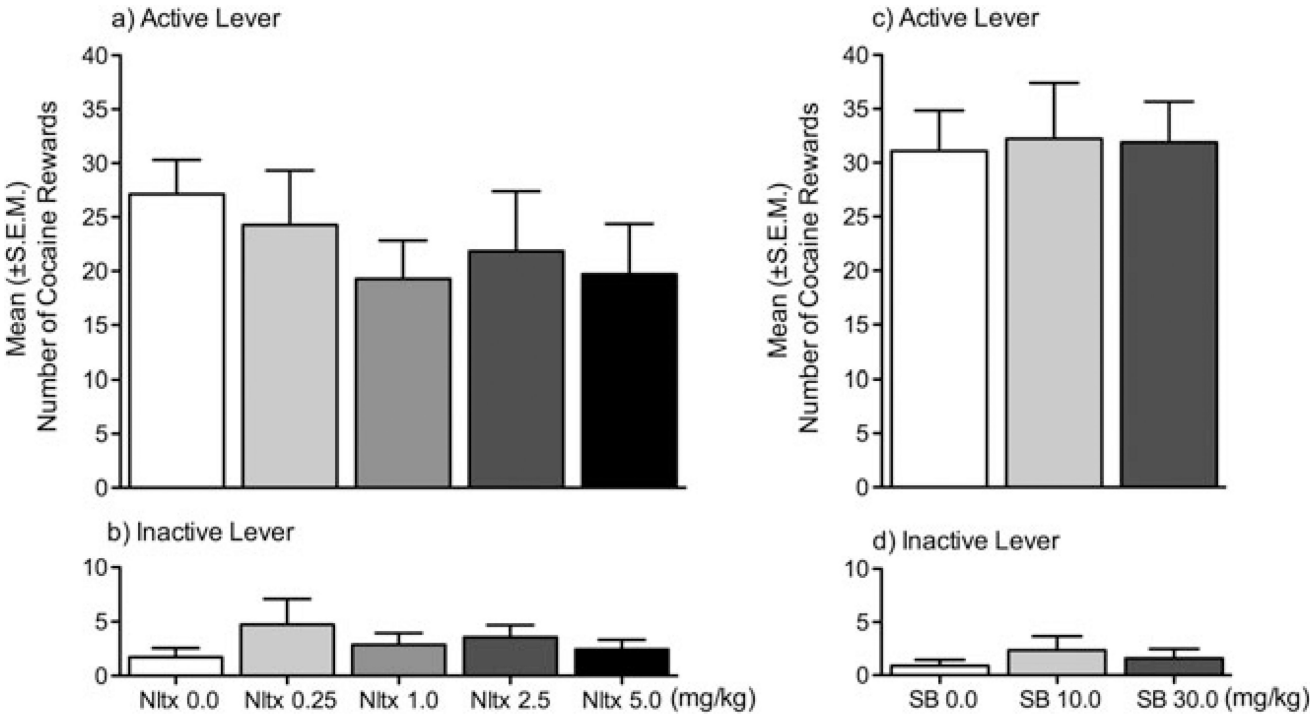

Figure 2.

Effect of naltrexone (Nltx) and of SB-612111 (SB) on cocaine self-administration: Nltx neither modified (a) cocaine self-administration (b) nor affected inactive control lever responding. Similarly, SB was unable to change cocaine-related operant responding at both (c) active and (d) inactive levers

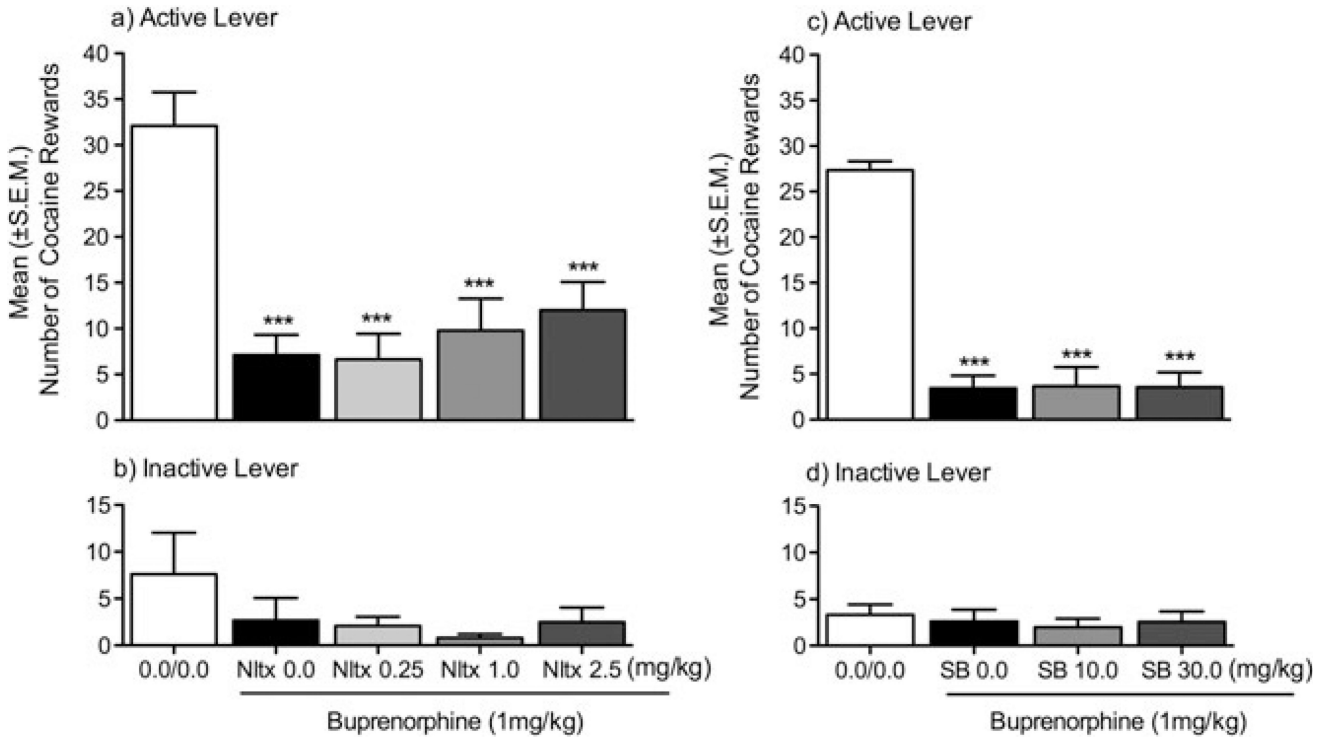

Figure 3.

Effect of naltrexone (Nltx) and of SB-612111 (SB) on buprenorphine-induced (1.0 mg/kg) reduction of cocaine self-administration: (a) Buprenorphine (1.0 mg/kg) significantly reduced cocaine self-administration. At all dose tested, Nltx (0.0, 0.25, 1.0 and 2.5 mg/kg) was unable to prevent this effect of buprenorphine. (b) Inactive lever responding was not modified by drug treatments. (c) Again, buprenorphine (1.0 mg/kg) significantly reduced cocaine self-administration. The effect was not modified by any of the SB doses (0.0, 10.0, and 30 mg/kg) employed. (d) Inactive lever responding was not modified by drug treatments. Difference from controls (0.0/0.0): ***$P < 0.001$

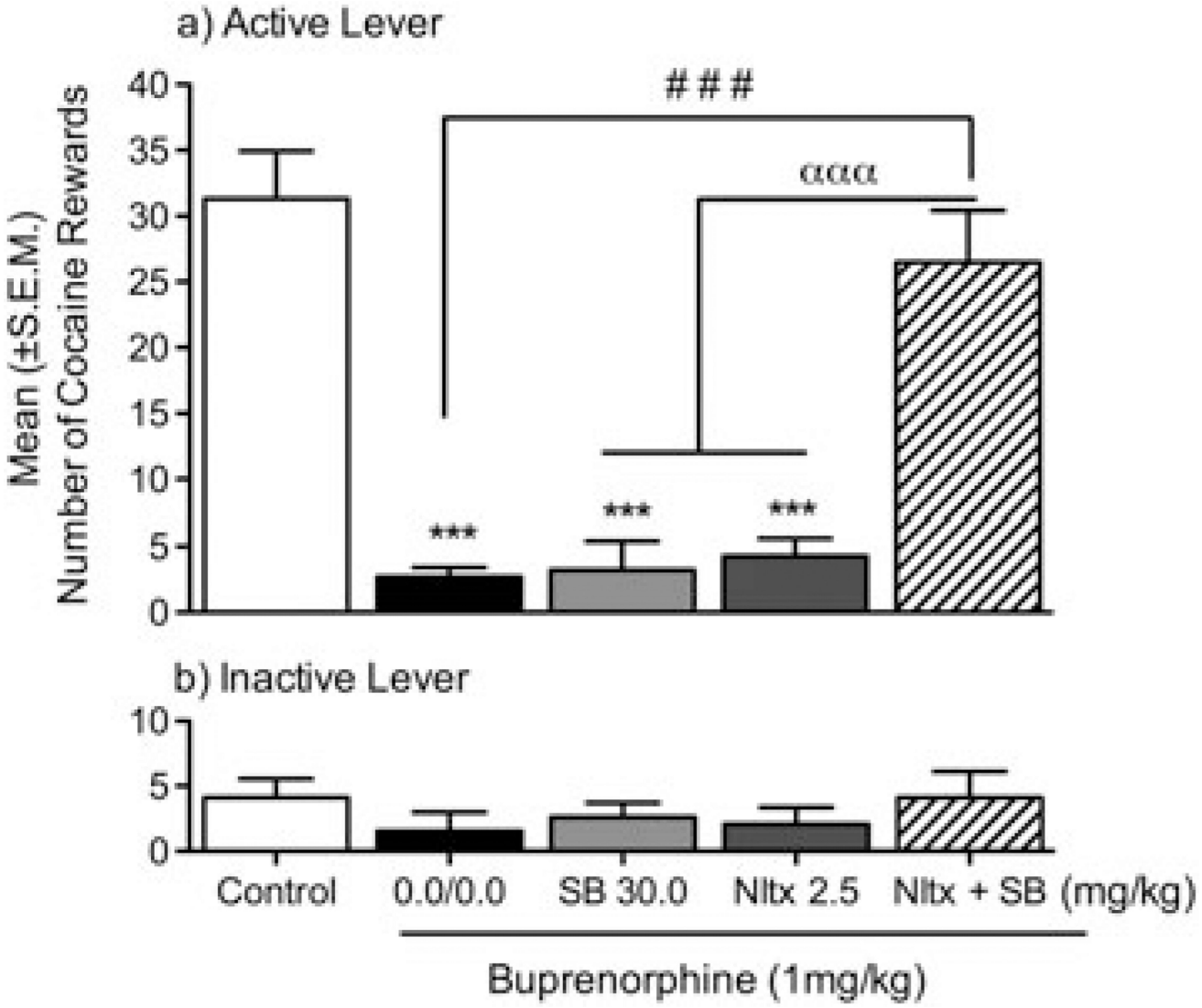

Figure 4.

Effect of naltrexone 2.5 mg/kg (Nltx 2.5) and of SB-612111 30.0 mg/kg (SB 30) or their combination (Nltx + SB) on buprenorphine-induced reduction of cocaine self-administration. (a) Buprenorphine (1 mg/kg) significantly reduced cocaine self-administration compared with vehicle-treated group (control). The effect was not modified by pre-treatment with Nltx 2.5 or SB 30.0 alone. However, when Nltx 2.5 and SB 30.0 were combined, they completely reversed the inhibitory effect of buprenorphine. (b) Inactive lever response was not affected by drug treatments. Differences from vehicle-treated animals (control): $^{***}P < 0.001$. Difference between buprenorphine plus vehicles (0.0/0.0) and the group treated with naltrexone plus SB-612111 (Nltx + SB): $^{\#\#\#}P < 0.0001$. Difference between naltrexone (Ntlx 2.5) or SB-612111 (SB 30) alone with naltrexone plus SB-612111 (Nltx + SB): $^{\alpha\alpha\alpha}P < 0.001$

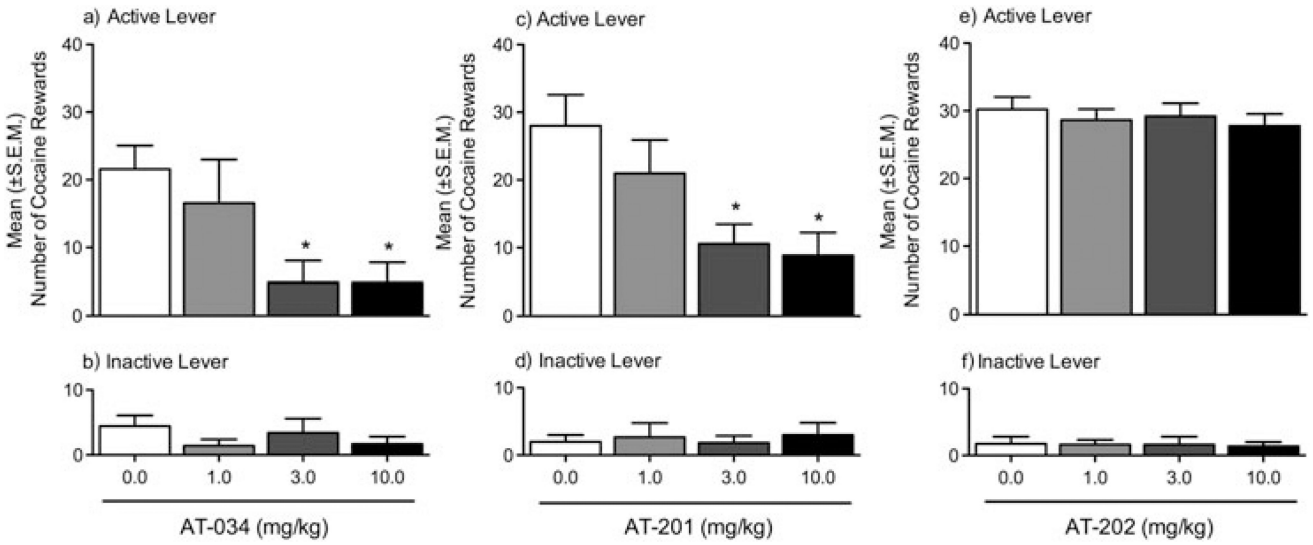

Figure 5.

Effect of AT-034 (0.0, 1.0, 3.0 or 10.0 mg/kg), AT-201 (0.0, 1.0, 3.0 or 10.0 mg/kg) or AT-202 (0.0, 1.0, 3.0 or 10.0 mg/kg) on cocaine self-administration: AT-034 significantly reduced (a) cocaine self-administration; (b) inactive lever responses was not affected by treatment. (c) Similarly, AT-0201 significantly reduced cocaine self-administration, while (d) inactive lever responses were not changed. AT-202 instead was ineffective and neither reduced (e) cocaine self-administration (f) nor inactive lever responding. Difference from controls (0.0): $*P < 0.05$

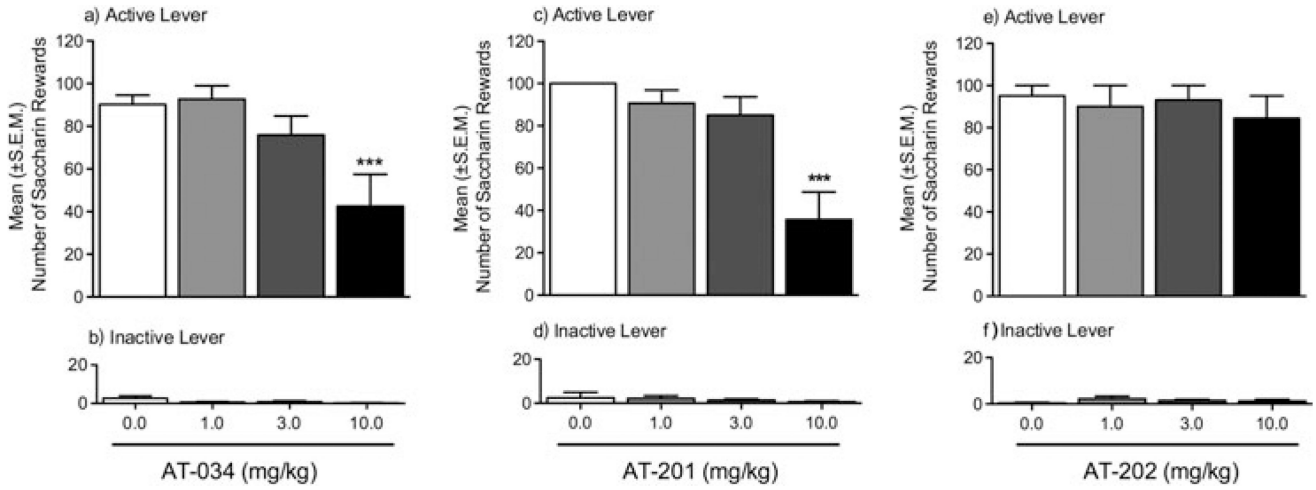

Figure 6.

Effect of AT-034 (0.0, 1.0, 3.0 or 10.0 mg/kg), AT-201 (0.0, 1.0, 3.0 or 10.0 mg/kg) or AT-202 (0.0, 1.0, 3.0 or 10.0 mg/kg) on saccharin self-administration: AT-034 significantly reduced (a) saccharin self-administration; (b) inactive lever responses were not affected by treatment. (c) Similarly, AT-0201 significantly reduced saccharin self-administration; (d) inactive lever responses were not changed. AT-202 instead neither modified (e) saccharin self-administration (f) nor inactive lever responding. Difference from controls (0.0): *** $P <$ 0.001

Table 1

Binding affinities and functional activities of compounds AT-034, AT-201 and AT-202 on NOP, MOP and KOP receptors

	Receptor binding K_i (nM)			[^{35}S]GTPγS NOP		[^{35}S]GTPγS MOP		[^{35}S]GTPγS KOP	
	NOP	MOP	KOP	EC_{50} nM	Percent Stim	EC_{50} nM	Percent Stim	EC_{50} nM	Percent Stim
AT-034	76.6 ± 3.6	1.97 ± 0.2	88.4 ± 14.2	124 ± 6.1	71 ± 1.4	39.5 ± 7.4	96.1 ± 6.6	>10K	–
AT-201	7.5 ± 0.8	2.70 ± 0.1	31.7 ± 4.8	28.7 ± 0.6	45.0 ± 5	29.5 ± 10	30 ± 0.5	>10K	–
AT-202	11.4 ± 0.9	79.8 ± 3.8	681 ± 61	46.1 ± 20.5	107 ± 7.4	129 ± 48	18 ± 1.6	>10K	–

Data for AT-034 originally reported as compound 7f in (Journigan *et al.* 2014). AT-201 was previously known as SR16435 and has been extensively characterized in Khroyan *et al.* (2007) and compared with buprenorphine in antinociception assays in Khroyan *et al.* (2009). AT-202 was previously known as SR16835, and its effect on morphine reward is characterized in Toll *et al.* (2009). NOP = nociceptin/orphanin FQ peptide.