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A novel and selective nociceptin receptor (NOP) agonist (1-(1-((cis)-4-isopropylcyclohexyl)piperidin-4-yl)-1H-indol-2-yl)methanol (AT-312) decreases acquisition of ethanol-induced conditioned place preference in mice

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Abstract:	Background: Nociceptin/Orphanin FQ (N/OFQ), the endogenous peptide agonist for the opioid receptor-like (ORL1) receptor (also known as NOP or the nociceptin receptor), has been shown to block the acquisition and expression of ethanol-induced conditioned place preference (CPP). We report the characterization of a novel small-molecule NOP ligand AT-312 (1-(1-((cis)-4-isopropylcyclohexyl)piperidin-4-yl)-1H-indol-2-yl)methanol) in receptor binding and GTPgS functional assays in vitro. We then investigated the effect of AT-312 on the rewarding action of ethanol in mice using the CPP paradigm. Further, using mice lacking the NOP receptor and their wild-type controls, we also examined the involvement of NOP in the effect of AT-312. Motivational effects of AT-312 alone were also assessed in the CPP paradigm. Methods: Female mice lacking NOP and/or their wild-type controls received conditioning in the presence or absence of the NOP agonist [AT-312 (1, 3 and 10 mg/kg) or the control NOP agonist SCH221510 (10 mg/kg)] followed by saline/ethanol for 3 consecutive days (twice daily) and tested for CPP in a drug-free state on the next day. Results: Our in vitro data showed that AT-312 is a high affinity, selective NOP full agonist with 17-fold selectivity over the mu opioid receptor and

	>200-fold selectivity over the kappa opioid receptor. The results of our in vivo studies showed that AT-312 reduced ethanol CPP at the lowest dose (1 mg/kg) tested but completely abolished ethanol CPP at higher doses (3 or 10 mg/kg) compared to their vehicle-treated control group. AT-312 (3 mg/kg) did not alter ethanol-induced CPP in mice lacking NOP, confirming that AT-312 reduced ethanol CPP through its action at the NOP receptor. AT-312 (3 mg/kg) did not induce reward or aversion when administered alone, showing that the novel small molecule NOP agonist shows efficacy in blocking ethanol-induced CPP via the NOP receptor. Conclusions: Together, these data suggest that small molecule NOP agonists have the potential to reduce alcohol reward and may be promising as medications to treat alcohol addiction.

TITLE PAGE

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ABSTRACT

Background: Nociceptin/Orphanin FQ (N/OFQ), the endogenous peptide agonist for the opioid receptor-like (ORL1) receptor (also known as NOP or the nociceptin receptor), has been shown to block the acquisition and expression of ethanol-induced conditioned place preference (CPP). Here, we report the characterization of a novel small-molecule NOP ligand AT-312 (1-(1-((cis)-4-isopropylcyclohexyl)piperidin-4-yl)-1H-indol-2-yl)methanol) in receptor binding and GTPγS functional assays in vitro. We then investigated the effect of AT-312 on the rewarding action of ethanol in mice using the CPP paradigm. Further, using mice lacking the NOP receptor and their wild-type controls, we also examined the involvement of NOP in the effect of AT-312. Motivational effects of AT-312 alone were also assessed in the CPP paradigm. **Methods:** Female mice lacking NOP and/or their wild-type controls received conditioning in the presence or absence of the NOP agonist [AT-312 (1, 3 and 10 mg/kg) or the control NOP agonist SCH221510 (10 mg/kg)] followed by saline/ethanol for 3 consecutive days (twice daily) and tested for CPP in a drug-free state on the next day. **Results:** Our *in vitro* data showed that AT-312 is a high affinity, selective NOP full agonist with 17-fold selectivity over the mu opioid receptor and >200-fold selectivity over the kappa opioid receptor. The results of our *in vivo* studies showed that AT-312 reduced ethanol CPP at the lowest dose (1 mg/kg) tested but completely abolished ethanol CPP at higher doses (3 or 10 mg/kg) compared to their vehicle-treated control group. AT-312 (3 mg/kg) did not alter ethanol-induced CPP in mice lacking NOP, confirming that AT-312 reduced ethanol CPP through its action at the NOP receptor. AT-312 (3 mg/kg) did not induce reward or aversion when administered alone, showing that the novel small molecule NOP agonist shows efficacy in blocking ethanol-induced CPP via the NOP receptor.

Conclusions: Together, these data suggest that small molecule NOP agonists have the potential to reduce alcohol reward and may be promising as medications to treat alcohol addiction.

Key Words: NOP agonist, AT-312, Alcohol reward, NOP knockout mouse, Ethanol-induced conditioned place preference

Introduction

Alcoholism and alcohol-related disorders are major public health issues and place an enormous burden on society and economy (Esser et al., 2017; Esser et al., 2014). When alcohol-related accidents are factored in, alcohol is among the top three causes of death in the US (Mokdad et al., 2004). Of the estimated 18 million alcohol-dependent individuals in the population, only about 1 million actually receive/seek adequate treatment, which mainly involves psychosocial support in conjunction with limited pharmacotherapy. While no single medication or strategy has been shown to be very effective, it is generally accepted that having pharmacotherapy as an adjunct to behavioral interventions is the best approach for treating alcohol dependence and maintaining abstinence. For this however, the current repertoire of pharmacotherapeutic options needs to be significantly expanded. Only three pharmacotherapeutic agents are currently approved for the treatment of alcohol dependence in the US, oral and intramuscular naltrexone (NTX), acamprosate, and disulfiram. Of these, NTX, an opioid receptor antagonist, has shown limited efficacy in reducing craving after stopping alcohol drinking; acamprosate, whose mechanism of action is unclear, improves abstinence rates, whereas disulfiram, produces an aversive reaction to alcohol. These are still not widely adopted by physicians who treat alcohol-dependent patients, mostly due to lack of confidence about their efficacy and a range of unpleasant side effects that limit patient compliance. There still remains a need for new approaches and treatments for alcohol dependence.

Unlike other drugs of abuse, alcohol does not act at one receptor target, but dysregulates many neurotransmitter systems, ion channels, and neurocircuitry in several brain areas, particularly the ventral tegmental area, nucleus accumbens, central amygdala and bed nucleus of stria terminalis (Gilpin and Koob, 2008; Koob and Volkow, 2010).

Among these, the endogenous opioid system is well known to play a key role in the rewarding and reinforcing effects of alcohol (Altshuler et al., 1980; Froehlich et al., 1990; Gianoulakis, 2004; Hubbell et al., 1986; Marfaing-Jallat et al., 1983; Weiss et al., 1990). Indeed, as stated above, NTX, approved for use in the US as an anti-alcohol pharmacotherapy, decreases alcohol consumption and craving in humans, and decreases the rewarding properties of ethanol in animal models (Altshuler et al., 1980; Benjamin et al., 1993; Farren and O'Malley, 1997; Froehlich et al., 1990; Gianoulakis et al., 1996; Hubbell et al., 1991; Ji et al., 2008; Kornet et al., 1991; Marfaing-Jallat et al., 1983; Myers et al., 1986; O'Malley et al., 2002; Oslin et al., 1997; Samson and Doyle, 1985; Volpicelli et al., 1992; Volpicelli et al., 1986; Weiss et al., 1990). The mu, delta and kappa opioid receptors and their respective endogenous ligands β-endorphins, enkephalins and dynorphin have all been shown to be involved in various stages of alcohol addiction cycle (Hall et al., 2001; Oswald and Wand, 2004; Roberts et al., 2000).

A growing body of evidence suggests that the fourth member of the opioid receptor-ligand family, the nociceptin opioid receptor NOP (previously called the opioid receptor-like (ORL1) receptor) and its endogenous neuropeptide ligand, nociceptin/orphanin FQ (N/OFQ) are involved in alcohol reward and reinforcement (Ciccocioppo et al., 1999; Kuzmin et al., 2007; Kuzmin et al., 2003; Ciccocioppo et al., 2002). Similarly to other members of the opioid receptor family, the NOP receptor is widely distributed in areas of the brain implicated in motivational behaviors as well as negative affect, such as the ventral tegmental area, nucleus accumbens,

lateral hypothalamus and the central amygdala (Neal et al., 1999a; Neal et al., 1999b). The endogenous ligand of the NOP, N/OFQ, acts to alter neurotransmitter release, particularly dopamine, GABA, and glutamate, all of which are also implicated in alcohol reward (Di Giannuario et al., 1999; Kallupi et al., 2014; Lutfy et al., 2001; Murphy et al., 1996; Murphy and Maidment, 1999; Murphy et al., 2004; Sakoori and Murphy, 2004). N/OFQ is also considered to have an 'anti-opioid' action in the brain [for a review, see (Mogil and Pasternak, 2001)]. Exogenous administration of N/OFQ has been shown to suppress basal and drug-stimulated dopamine release in the NAc (Di Giannuario et al., 1999; Lutfy et al., 2001; Murphy et al., 1996; Murphy and Maidment, 1999; Murphy et al., 2004; Sakoori and Murphy, 2004), and the rewarding properties of several common drugs of abuse [reviewed in (Lutfy and Zaveri, 2016)]. In particular, intracerebroventricular (i.c.v) administration of N/OFQ has been shown to block acquisition of conditioned place preference (CPP) induced by morphine (Ciccocioppo et al., 2000; Murphy et al., 1999), cocaine (Sakoori and Murphy, 2004), amphetamines (Kotlinska et al., 2003), and alcohol (Ciccocioppo et al., 1999; Kuzmin et al., 2007; Kuzmin et al., 2003). A small molecule NOP agonist Ro 64-6198, given systemically, was also shown to block both the acquisition and expression of alcohol CPP in mice (Kuzmin et al., 2003) and alcohol self-administration in rats (Kuzmin et al., 2007). Another potent NOP agonist MT-7716 was shown to decrease alcohol intake in alcohol-preferring Marchigian Sardinian (msP) rats and attenuate alcohol withdrawal symptoms in alcohol-dependent Wistar rats (Ciccocioppo et al., 2014b). Recently, SR-8993, a selective NOP agonist was reported to reduce anxiety associated with alcohol withdrawal as well as home cage and limited access alcohol drinking in Wistar rats (Aziz et al., 2016). Interestingly the level of N/OFQ is altered following restraint stress in the amygdala

(Ciccocioppo et al., 2014a). These studies suggest that NOP agonists may be potentially promising treatment agents for alcoholism and alcohol use disorders.

In the present study, we characterized a novel small-molecule NOP ligand AT-312 (1-(1-((cis)-4-isopropylcyclohexyl)piperidin-4-yl)-1H-indol-2-yl)methanol), for its selectivity and affinity toward the NOP and classical opioid receptors using radioligand binding assays and determined its efficacy in the GTP(γ)S functional assay conducted in Chinese hamster ovary (CHO) cells transfected with the human opioid receptors. We also determined its bioavailability and brain penetration, which showed appreciable plasma exposure and a brain-to-plasma ratio greater than 1 after systemic (subcutaneous, s.c.) administration (Table 2). Using this route of administration, we further determined its efficacy in reducing the rewarding action of ethanol in the CPP paradigm, a widely used animal model of drug reward (Bardo and Bevins, 2000). To demonstrate that the effect of AT-312 in reducing alcohol CPP in mice is due to its activity at the NOP receptor, we compared its efficacy in reducing CPP induced by ethanol in mice lacking the NOP receptor and their wild-type littermates/controls. We also investigated the effect of a known NOP agonist SCH 221510 on alcohol reward in this same paradigm as a control.

MATERIALS AND METHODS

Cells

Human NOP, mu, delta, and kappa opioid receptors were individually expressed in Chinese hamster ovary cells stably transfected with the human receptor cDNA, as we have described previously (Zaveri et al., 2001; Toll et al., 2009). The HORL, HDOR, HKOR-FLAG19 cells were grown in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum, in the presence of 0.4 mg/ml G418 and 0.5% penicillin/streptomycin, in 150-mm tissue culture dishes. The HKOR-CN cells were grown in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum, in the presence of 0.5% penicillin/streptomycin and no G418. The HMOR cells in 50% F12/DMEM with 10% fetal bovine serum, in the presence of 0.4 mg/ml G418 and 0.5% penicillin/streptomycin. Kappa-CN cells were used for KOP radioligand binding assays, while Kappa-FLG19 cells were used in KOP [^{35}S]GTPγS functional assays.

Animals

Female mice lacking NOP (Nishi et al., 1997) and their wild-type littermates/controls (2-6 months old), fully backcrossed on C57BL/6J mouse strain, bred in house, were used throughout. We used female mice because they exhibit a robust CPP response compared to male mice using the current 3-day conditioning paradigm (Nguyen et al., 2012; Tseng et al., 2013). Mice were housed 2-4 per cage with free access to laboratory chow and tap water and kept under a 12 h light/12 h dark cycle in a temperature- and humidity-controlled room. The light was on 6 AM and off at 6 PM. All experiments were conducted during the light cycle between the hours of 10:00 AM to 5:00 PM and were according to the National Institute of Health for the proper use of animals in research and approved by the Institutional Animal Care and Use Committee at Western University of Health Sciences (Pomona, California, USA).

153 **Drugs**

154 AT-312 (1-(1-((cis)-4-isopropylcyclohexyl)piperidin-4-yl)-1H-indol-2-yl)methanol)
155 (Figure 1) was synthesized at Astraea Therapeutics, and was of >99% chemical purity as fully
156 characterized by nuclear magnetic resonance spectroscopy, LC-MS and elemental analysis.
157 SCH221510 (Figure 1) was purchased from Tocris. These test compounds were dissolved in 1-
158 2% DMSO and then diluted to the desired concentration with 0.5% aqueous
159 hydroxypropylcellulose (HPC) and injected subcutaneously (s.c.) in a volume of 0.1 ml/10g of
160 body weight. Controls received 0.1 ml/10g of body weight of the appropriate vehicle (1-2%
161 DMSO in 0.5% of HPC).

162 **In vitro Characterization**

163 **Membrane preparation.** The cell lines are grown to confluency, then harvested for
164 membrane preparation. The membranes are prepared in 50 mM Tris buffer (pH 7.4). Cells are
165 scraped and centrifuged at 500 × g for 12 mins. The cell pellet is homogenized in 50 mM Tris
166 with a Fisher Scientific PowerGen 125 rotor-stator type homogenizer, centrifuged at 20,000 × g
167 for 25 mins, washed and recentrifuged once more at 20,000 × g for 25 mins, and aliquoted at a
168 concentration of 3 mg/ml protein per vial and stored in a -80 ˚C freezer till further use.

169 **Receptor Binding.** Compounds were dissolved at 10 mM stock in 100% DMSO. The
170 assay was performed in a 96-well polystyrene plate with triplicates of six concentrations of each
171 test compound (1µM – 0.01 nM), adding 100 µl of compound and 100 µl of tritiated ligands
172 [^{3}H]DAMGO (51.0 Ci/mmole, K_d 0.59 nM for MOP), [^{3}H]DPDPE (42.0 Ci/mmole, K_d 1.11 nM
173 for DOP), [^{3}H]U69593 (41.7 Ci/mmole, K_d 1.05 nM for KOP), and [^{3}H]N/OFQ (130 Ci/mmole,
174 K_d 0.12 nM for NOP). Nonspecific binding was determined using 1.0 µM of the unlabeled
175 nociceptin for NOP, 10 µM unlabeled DAMGO for MOP, 1.0 µM unlabeled DPDPE for DOP,

and 10 μM unlabeled U69,593 for KOP. Assays were initiated by addition of 800 μl of membrane per well. Samples were incubated for 60 min at 25˚C in a total volume of 1.0 ml. In NOP receptor experiments, 1 mg/ml BSA was added to the compound dilution buffer. The incubation was terminated by rapid filtration through 0.5% PEI-soaked glass fiber filter mats (GF/C Filtermat A, Perkin-Elmer) on a Tomtec Mach III cell harvester and washed 5 times with 0.5 ml of ice-cold 50 nM Tris-HCl, pH 7.4 buffer. The filters were dried overnight and soaked with scintillation cocktail before counting on a Wallac Beta plate 1205 liquid scintillation counter. Radioactivity was determined as counts per minutes (cpm). IC_{50} values were determined using at least six concentrations of test compound, and calculated using Graphpad/Prism (ISI, San Diego, CA). K_i values were determined by the method of Cheng and Prusoff (Cheng and Prusoff, 1973).

[^{35}S]GTPγS binding Assay. [^{35}S]GTPγS binding was conducted as we have described previously (Toll et al., 2009; Traynor and Nahorski, 1995; Zaveri et al., 2001). Cells were scraped from tissue culture dishes into 20 mM Hepes, 1 mM EDTA, then centrifuged at 500 × g for 10 min. Cells were re-suspended in this buffer and homogenized using a Polytron Homogenizer. The homogenate was centrifuged at 27,000 × g for 15 min, and the pellet resuspended in Buffer A, containing: 20 mM Hepes, 10 mM MgCl2, 100 mM NaCl, pH 7.4. The suspension was recentrifuged at 27,000 × g and suspended once more in Buffer A. For the binding assay, membranes (8-15 μg protein) were incubated with [^{35}S]GTPγS (50 pM), GDP (10 μM), and the appropriate compound, in a total volume of 1.0 ml, for 60 min at 25˚C. Samples were filtered over glass fiber filters and counted as described for the binding assays. Statistical analysis was conducted using Prism.

In vivo Pharmacology

Experiment 1: To determine the effect of NOP agonists on ethanol-induced CPP. We used an unbiased CPP paradigm, widely used as an animal model of drug reward (Bardo and Bevins, 2000), to determine the effect of AT-312 on the rewarding action of ethanol. The details of the CPP apparatus and paradigm have been provided elsewhere (Nguyen et al., 2012; Tseng et al., 2013). Briefly, mice were tested for preconditioning place preference on day 1. On this day, mice were placed in the central neutral chamber and allowed to freely explore the conditioning chambers through this smaller central chamber. The amount of time that mice spent in each CPP chamber was recorded. On days 2-4, mice were conditioned with ethanol in the presence and absence of the NOP agonist. In the morning on each day, mice were treated with vehicle or one of the doses of AT-312 (1, 3 or 10 mg/kg, s.c.; n = 6-9 mice per group) followed, 15 min later, by ethanol (2 g/kg, i.p.) and then immediately confined to the drug-paired chamber (DPCh) for 15 min. In the afternoon, mice received vehicle followed by saline and were conditioned in the vehicle-paired chamber (VPCh). The order of conditioning were reversed for some mice to counterbalance the treatment and chamber assignment as well as the use of wild-type versus knockout mice for the morning and afternoon conditioning. Mice were then tested under a drug-free state for postconditioning place preference on day 5, as described for day 1. SCH221510 has been previously reported by Varty and colleagues (Varty et al., 2008) as a NOP agonist. Thus, we used this compound as the control NOP agonist and determined its effect on the rewarding action of alcohol. To this end, mice were tested for preconditioning place preference on day 1, conditioned with ethanol in the presence or absence of SCH221210 (10 mg/kg) on days 2-4 and then tested for CPP on day 5, as described above.

Experiment 2: To characterize the role of the NOP receptor in the inhibitory action of AT-312 on alcohol CPP: Mice lacking NOP and their wild-type controls were tested for preconditioning place preference, received conditioning with ethanol (2 g/kg, i.p.) in the presence or absence of AT-312 (3 mg/kg, s.c.; n = 7 mice per treatment per each genotype) on days 2-4 and then were tested for postconditioning place preference on day 5. On each test day, the amount of time that mice spent in the CPP chambers was recorded, as described above.

Experiment 3: To assess the motivational effect of AT-312 in the place conditioning paradigm: Mice were tested for baseline place preference on day 1, received conditioning on days 2-4 and were tested for postconditioning place preference on day 5. On each conditioning day, mice were treated with vehicle or AT-312 (3 mg/kg, s.c.; n = 5 mice per treatment) followed by saline and placed in the vehicle-paired (VPCh) or drug-paired chamber (DPCh). In the afternoon, mice were treated with the alternate treatment and conditioned to the opposite chamber. The amount of time that mice spent in the CPP chamber was recorded on each test day (days 1 and 5), as described above.

Experiment 4: To determine the effect of pentobarbital on ethanol-induced CPP: Mice were tested for preconditioning place preference on day 1, treated with saline or pentobarbital (25 mg/kg, s.c.; n = 6 mice per treatment) 15 min before ethanol (2 g/kg, i.p.) on each conditioning day and were confined to the drug paired chamber (DPCh). Animals were treated with saline 15 min before saline and confined to the vehicle-paired chambers (VPCh). These treatments were given either in the morning or afternoon in a counterbalanced manner. Each conditioning session lasted for 15 min and was conducted on days 2-4. Mice were then tested for CPP on day 5, as described above.

Data Analysis. Data are presented as mean (±S.E.M.) of the amount of time that mice spent in the drug-paired chamber (DPCh) or DPCh vs. vehicle-paired chamber (VPCh) on preconditioning test day (day 1, D1) and postconditioning test day (day 5, D5) and were analyzed using repeated measures two- or three-way analysis of variance (ANOVA). The Bonferroni's post-hoc test was used to reveal significant changes between different groups. $P<0.05$ was considered significant.

RESULTS

In vitro NOP Receptor Binding affinity and Opioid Receptor Selectivity of AT-312

The chemical structure of AT-312 is shown in Fig. 1. The receptor binding affinity of AT-312 was determined using radioligand displacement assays conducted in membranes from CHO cells stably expressing the human NOP, MOP, DOP and KOP receptors. As shown in Table 1, AT-312 showed high binding affinity for the NOP receptor, yielding a subnanomolar Ki value of 0.34 ± 0.13 nM in competition with [^{3}H]N/OFQ as the radioligand. In similar experiments using [^{3}H]DAMGO, [^{3}H]U69593 and [^{3}H]DPDPE at the MOP, KOP and DOP receptors respectively, AT-312 showed binding selectivity of 17-fold versus MOP, 216-fold versus KOP and 378-fold versus DOP receptors. The NOP agonist SCH221510 tested in the same assays showed NOP binding Ki of 13.7 nM, about 40-fold lower affinity at NOP than AT-312. Also, the NOP binding affinity of SCH221510 was only 5-fold selective versus MOP, 3.6-fold versus KOP and 29-fold versus DOP receptors. AT-312 therefore, exhibits significantly higher binding affinity and selectivity for NOP compared to the positive control SCH221510. The high affinity of AT-312 for the NOP receptor is similar to that observed for other reported NOP agonists Ro 64-6198 and MT-7716 (Zaveri, 2016).

As observed for other piperidinyl NOP ligands from our own compound library as well as those reported in the literature, AT-312 did not show appreciable affinity for the DOP receptor. Affinity profiling in a panel of 68 receptors and ion channels showed that, at a concentration of 100 nM, AT-312 did not bind to any non-opioid off-target receptors, whereas at 10 μM, it inhibited the specific binding of radioligands at the α1 adrenergic receptor by 60%, dopamine D4 receptor (86%), dopamine D3 receptor (100%), muscarinic M1 and M2 receptors (85%), NK2 receptor (92%), Ca^{+2} channel (L-type) (73%) and Na+ channel (site 2) (94%) and

norepinephrine transporter (65%). Overall, AT-312 appears to be a selective NOP receptor ligand.

In vitro Functional Efficacy of AT-312

The intrinsic efficacy of AT-312 at the NOP and traditional opioid receptors was determined using the GTPγS binding assay conducted in membranes of CHO cells stably transfected with the NOP and classical opioid receptors. Table 1 shows the in vitro functional efficacy profile of AT-312 and SCH221510. AT-312 is a full agonist at the NOP receptor, showing potency (EC_{50}) of 30 nM and 100% agonist stimulation compared to the endogenous NOP agonist N/OFQ. In contrast, it showed only a partial agonist efficacy of 25% at the MOP receptor and significantly lower potency compared to the MOP opioid full agonist DAMGO (Table 1). AT-312 had no agonist efficacy at the KOP receptor. In these experiments, SCH221510 was also found to be a full agonist at the NOP receptor, with comparable potency as that of AT-312 (Table 1). However, it also showed significant agonist stimulation at the KOP receptor, in contrast to AT-312.

AT-312, a novel NOP agonist, dose-dependently blocked the development of ethanol-induced CPP. The novel NOP agonist AT-312 dose-dependently reduced the rewarding action of ethanol (Fig. 2). Repeated measures ANOVA of the amount of time that mice spent in the drug-paired chamber (DPCh) on pre- and postconditioning days revealed a significant effect of treatment ($F_{3,26} = 21.35$; $P < 0.01$) but no effect of time ($F_{1,26} = 4.48$; $P = 0.08$) and no significant interaction between the two factors ($F_{3,26} = 10.38$; $P = 0.08$). The *post-hoc* test showed that the amount of time that mice spent in the DPCh was significantly ($P<0.05$) increased following ethanol conditioning in the vehicle-treated control group (Fig. 2, compare D5 vs. D1 for the mice treated with vehicle before ethanol on the conditioning days and this response was

reduced by AT-312 in a dose-dependent manner (Fig. 2). In particular, the two higher doses of AT-312 (3 and 10 mg/kg) blocked ethanol-induced CPP [compare the amount of time between the vehicle-treated group on day 5 vs. the AT-312 (3 mg/kg) group ($P<0.01$) as well as against AT-312 (10 mg/kg) on this day ($P<0.001$)]. Together, these results suggest that AT-312 dose-dependently abolished the rewarding action of alcohol.

AT-312 reduced the rewarding action of ethanol in wild-type but not in NOP knockout mice. The amount of time that mice lacking NOP and their wild-type littermates/controls spent in the ethanol-paired chamber on the preconditioning (D1) and postconditioning (D5) test days is shown in Figure 3. Three-way ANOVA revealed a significant effect of time ($F_{1,1}=26$; $P<0.0001$), a significant effect of context ($F_{1,1} = 8.27$; $P<0.01$) but no effect of genotype ($F_{1,1} = 1.58$; $P>0.05$). However, there was a significant interaction between time, context and genotype ($F_{1,1} = 6.39$; $P<0.02$). The post-hoc test showed that conditioning with ethanol induced a significant ($P<0.05$) CPP in both wild-type and knockout mice pretreated with vehicle prior to ethanol on the conditioning days, as evidenced by a significant increase in the amount of time that vehicle-treated control mice of either genotype spent in the ethanol-paired on day 5 compared to day 1 (Fig. 3, left panel; compare each bar on D5 vs. D1 for each genotype). The CPP response was significantly ($P<0.001$) reduced by AT-312 (10 mg/kg) in wild-type mice (Fig. 3, compare wild-type mice (NOP+/+) treated with AT-312 vs. vehicle on D5). On the other hand, mice lacking NOP spent the same amount of time in the DPCh on day 5 regardless of whether they were injected with the NOP agonist or vehicle (Fig. 3, compare NOP-/- treated with AT-312 group vs. vehicle-treated NOP-/- as well as against AT-312-treated NOP+/+ on D5). This result suggests that AT-312 exerts its inhibitory effect on the rewarding

action of ethanol via the NOP receptor. AT-312 reduced locomotor activity in wild-type mice but this response was absent in mice lacking NOP (data not shown).

AT-312 given alone did not have any motivational effect in the place conditioning paradigm. Figure 4 shows the amount of time that mice, treated with vehicle in both conditioning chambers (Vehicle) and those that received vehicle in one chamber and AT-312 (3 mg/kg) in the other chamber, spent in the drug-paired chamber (DPCh). Two-way ANOVA revealed no significant effect of treatment ($F_{1,8} = 0.75$; $P>0.05$), no significant effect of time ($F_{1,8} = 0.45$; $P>0.05$) and no significant interaction between the two factors ($F_{1,8} = 1.69$; $P>0.05$), showing that AT-312 at this dose (3 mg/kg) did not possess motivational effects of its own.

Ethanol-induced CPP was reduced in mice treated with SCH221510, a NOP agonist. We also determined the effect of a known NOP agonist on ethanol-induced CPP. Considering that this compound was less selective toward the NOP compared to AT-312, we determined the effect of a relatively higher dose (10 mg/kg) of this compound on the rewarding action of ethanol. Figure 5 shows the amount of time that mice treated with vehicle or the NOP agonist spent in the ethanol-paired chamber (DPCh). Two-way repeated measures ANOVA revealed a significant effect of time that mice spent in the ethanol-paired chamber on day 5 vs. day 1 ($F_{1,14} = 20.44$; $P<0.02$) but no significant effect of treatment ($F_{1,14} = 4.53$; $P>0.05$) and no significant interaction between the two factors ($F_{1,14} = 3.47$; $P>0.05$). The Bonferroni post-hoc test showed that the amount of time that mice spent in the ethanol-paired chamber was increased in vehicle-pretreated mice, suggesting that ethanol induced a significant CPP in the control group (Fig. 5, compare the amount of time that vehicle-pretreated mice spent in the DPCh on D5 vs. D1). However, this response was reduced in mice treated with SCH221510.

Pentobarbital reduced motor activity but failed to alter the rewarding action of ethanol. Figure 6 illustrates the amount of time that mice spent in the DPCh on the pre- and postconditioning test days. Three-way repeated measure ANOVA revealed a significant effect of context (DPCh vs. VPCh; $F_{1,1} = 11.61$; $P<0.002$) but no significant effect of time ($F_{1,1} = 0.21$; $P>0.05$) and no significant effect of treatment ($F_{1,1} = 0.50$; $P>0.05$). Although there was a significant context × time interaction ($F_{1,1} = 13.40$; $P<0.0001$, there was no treatment × context ($F_{1,1} = 0.001$; $P>0.05$) or time × context × treatment ($F_{1,1} = 0.001$; $P>0.05$) interaction. The post hoc test showed that ethanol induced a comparable CPP response in both groups, showing that pentobarbital did not alter the rewarding action of ethanol. Interestingly, pentobarbital induced a robust motor sedative effect and potentiated the sedative effect of alcohol on each conditioning day (data not shown).

DISCUSSION

The main findings of the present study are that the novel NOP agonist AT-312 reduced the acquisition of CPP induced by ethanol, and that this effect was absent in mice lacking the NOP receptor. Similar, albeit less potent effects were also observed on alcohol reward in mice treated with the control NOP agonist SCH221510. The current results also demonstrate that AT-312 did not have motivational effects of its own at a dose (3 mg/kg) that completely abolished ethanol CPP in wild-type mice. Together, these results are consistent with previous studies with NOP agonists N/OFQ and Ro 64-6198, and confirm that NOP agonists can reduce acquisition of ethanol CPP in mice via selective action at the NOP receptor.

AT-312 is a selective and high affinity NOP full agonist, belonging to a novel class of NOP ligands structurally unrelated to NOP agonists Ro 64-6198, MT-7716, SR-8993 and others that have shown efficacy in reducing the rewarding effects of alcohol in various animal models and paradigms (Zaveri, 2016). A growing body of evidence suggests that NOP may be a potential target to reduce the rewarding and reinforcing actions of alcohol and other addictive drugs [(see recent reviews (Lutfy and Zaveri, 2016; Witkin et al., 2014; Zaveri, 2011; Zaveri, 2016)]. Consistent with existing literature, this novel NOP agonist AT-312 dose-dependently reduced the rewarding action of alcohol and appeared to be more potent than the known NOP agonist, SCH221510. Although further studies are needed to define the mechanism for the greater effect of AT-312 compared to SCH221510, we speculate that it may be due to its higher affinity toward NOP. Our in vitro studies show that AT-312 exhibits at least 20-fold binding selectivity toward the NOP versus the MOP receptor and is a full agonist at NOP but a weak partial agonist at the MOP receptor, with no appreciable agonist efficacy at the KOP receptor. In comparison, SCH221510 has only a four-fold binding selectivity versus the KOP receptor and has significant agonist efficacy at both the MOP and KOP receptors in the same assays (Table 1).

However, further studies are needed to assess the contribution of each receptor in the inhibitory effects of the two NOP agonists.

Considering that AT-312 displayed higher affinity for and acted as a partial agonist at the mu opioid receptor, one may argue that the inhibitory action of the drug may be due to its interaction with the MOP receptor or both receptor systems rather than NOP only. In order to address this issue, we used mice lacking NOP and their wild-type controls and tested if the inhibitory effect of AT-312 is mediated via the NOP receptor. We rationalized that if AT-312 inhibits the rewarding action of alcohol via the NOP receptor, the drug would fail to alter the rewarding action of alcohol in mice lacking the NOP receptor. Consistent with this hypothesis, we observed that while the novel NOP agonist significantly reduced the rewarding action of alcohol in wild-type mice, the drug failed to alter ethanol-induced CPP in mice lacking NOP. This result suggests that AT-312 reduces the rewarding action of ethanol via the NOP receptor. However, further research is needed to assess the contribution of MOP receptor partial agonist activity in this response. Nevertheless, it is noteworthy to state that buprenorphine, a MOP partial agonist, was found to reduce alcohol consumption via its interaction with NOP (Ciccocioppo et al., 2007) and also found to reduce cocaine self-administration due to its agonist activity at the NOP and the MOP receptors (Kallupi et al., 2017).

Given that ethanol induced aversion in mice pretreated with the highest dose of AT-312 (Fig. 2), one may argue that the NOP agonist may have reduced ethanol-induced CPP by inducing aversion. However, the lower dose of AT-312 (3 mg/kg), which also completely blocked ethanol induced CPP in wild-type but not knockout mice (Fig. 3), failed to induce aversion when given with alcohol (Fig. 3) or alone (Fig. 4). This is in accord with an earlier report showing that NOP agonists may be devoid of any motivational effects (Devine et al.,

1996). Such a property could be useful to treat drug reward since the NOP agonist would not alter basal hedonic homeostasis, which may be advantageous for patient compliance and other normal daily functions.

NOP agonists are known to reduce motor activity (Devine et al., 1996). Consistent with this, we found that AT-312 suppressed motor activity in wild-type mice and this response was absent in mice lacking NOP receptor, showing that this effect of AT-312 was also mediated via the NOP receptor. It is generally accepted that drugs that reduce locomotor activity can confound behavioral responding. This may affect the outcome of the CPP response if one tests animals in the presence of a sedative drug. However, we tested the animals for CPP under a drug-free state on the postconditioning test days and found no significant differences in locomotor activity between the vehicle-conditioned and drug-conditioned groups. Additionally, we believe that not all drugs that reduce motor activity during conditioning block the CPP response. Interestingly, alcohol is sedative in mice; yet, it induces a robust CPP response. We further demonstrated this by conducting an experiment using pentobarbital, which is a known sedative hypnotic and examined its effect on ethanol-induced CPP. While pentobarbital significantly reduced locomotor activity during conditioning, it failed to alter the CPP response on the test day (Fig. 6), suggesting that the ability of AT-312 to reduce the rewarding action of ethanol may not be simply due to its sedative effect during conditioning.

The rewarding action of alcohol and other drugs of abuse has been linked to their ability to increase extracellular dopamine in the nucleus accumbens (Di Chiara and Imperato, 1988). Although the mechanism of inhibitory action of AT-312 is not clear at this time, we speculate that the NOP agonist reduces the ability of alcohol to elevate extracellular dopamine levels in the nucleus accumbens. Indeed, previous studies have shown that intracerebroventricular

administration of N/OFQ reduced elevation of accumbal dopamine induced by morphine (Di Giannuario et al., 1999) and cocaine (Lutfy et al., 2001; Sakoori and Murphy, 2004). The NOP agonist also attenuated the rewarding action of morphine (Ciccocioppo et al., 2000; Murphy et al., 1999), cocaine (Sakoori and Murphy, 2004) and ethanol (Kuzmin et al., 2003). Thus, we propose that the NOP agonist reduces dopaminergic neurotransmission by acting in the ventral tegmental area and/or nucleus accumbens to reduce the rewarding action of alcohol. However, further studies are needed to identify the neuroanatomical sites of action of the NOP agonist in this regard.

A recent report shows that NOP receptor knockout rats exhibit reduced alcohol consumption compared to their wild-type controls although saccharine intake was not different between the rats of the two genotypes (Kallupi et al., 2017). A similar reduction in ethanol self-administration was observed in rats treated with a novel orally bioavailable NOP antagonist (Rorick-Kehn et al., 2016). Interestingly, we did not observe reduced ethanol-induced CPP in mice lacking NOP although these authors found decreased ethanol self-administration in NOP knockout rats (Kallupi et al., 2017) or in wild-type rats treated with the NOP antagonist (Rorick-Kehn et al., 2016). A parsimonious explanation of such discordant effects is that the two studies measured two different responses. Notably, Kuzmin and colleagues also found that male mice lacking N/OFQ tended to show a stronger response to ethanol (Kuzmin et al., 2003). Alternately, the NOP system has been implicated in feeding, and N/OFQ has hyperphagic effects [for a review, see (Witkin et al., 2014)]. Thus, it is possible that food and drink consumption could be reduced in animals lacking the NOP receptor or its endogenous agonist. However, saccharin consumption was not altered in rats lacking NOP receptors (Kallupi et al., 2017). Nevertheless, it is possible that NOP system is involved in consumption of food and drinks with caloric values

and thus one would expect a difference in outcomes of the two studies. It is of interest to note that the novel bioavailable NOP antagonist reduced consumption of highly palatable food to the regular chow level (Statnick et al., 2016).

The other explanation for the discrepant results could be species differences in the current and earlier studies. Interestingly, we found enhanced cocaine-induced CPP in mice lacking NOP (Marquez et al., 2008), whereas these authors reported reduced cocaine-induced CPP in NOP knockout rats (Kallupi et al., 2017). The sex of animals may have contributed to this discrepant data since we used female mice in this study. Our earlier studies have shown that female mice exhibit greater ethanol-induced CPP than male mice, hence were used here (Nguyen et al., 2012). On the other hand, these other studies used male rats to study the role of NOP receptors in alcohol self-administration (Kallupi et al., 2017; Rorick-Kehn et al., 2016).

In summary, we found that a novel NOP agonist, AT-312, reduced the rewarding effects of ethanol in the CPP paradigm. The inhibitory effect of the NOP agonist was absent in NOP knockout mice, showing that the action of AT-312 was via the NOP receptor. AT-312 did not possess any motivational effects of its own at a dose that robustly reduced ethanol-induced CPP. Thus, the NOP receptor may be a potential target for the development of pharmacotherapy to treat alcohol use disorders.

Conflict of Interest

The authors declare no conflicts of interest. NTZ, WEP and MEM are employees of Astraea Therapeutics.

Author Contributions

PM, WEP and MEM conducted experiments, AH conducted genotyping and took care of the mouse breeding colony, NZ developed the compound, supervised its *in vitro* characterization and wrote the manuscript, and KL designed the CPP experiments, analyzed the behavioral data and wrote the manuscript.

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660

661 **FIGURE LEGENDS**

662 **Figure 1. Structures of NOP agonists AT-312 and SCH221510**

663 **Figure 2. The effect of AT-312, a novel NOP agonist, on ethanol-induced CPP in C57BL/6J**
664 **mice:** Data are mean (±S.E.M.) of the amount of time that mice spent in the drug-paired chamber
665 (DPCh) before (D1) (preconditioning test day) and after (D5) conditioning (post-conditioning
666 test day). Mice treated with vehicle or AT-312 (1, 3 or 10 mg/kg, s.c.) 15 min before ethanol on
667 the conditioning days. *P<0.05, indicates a significant increase in the amount of time in the
668 DPCh on D5 vs. D1; ++P<0.01, +++P<0.001, significantly different from the control group on
669 D5.

670 **Figure 3. The action of AT-312, a novel NOP agonist, on ethanol-induced CPP in mice**
671 **lacking NOP and their wild-type littermates/controls:** Data are mean (±S.E.M.) of the amount
672 of time that mice spent in the drug-paired chamber (DPCh) before (D1) (preconditioning test
673 day) and after (D5) conditioning (post-conditioning test day). Mice lacking NOP **[NOP (-/-)]** and
674 their wild-type littermates **[NOP (+/+)]** were treated with vehicle (left panel) or AT-312 (10
675 mg/kg, right panel) 15 min before ethanol on conditioning days. ***P<0.001; *P<0.05 DPCh vs.
676 VPCh

677 **Figure 4. Motivational effect of AT-312, a novel NOP agonist, in the place conditioning**
678 **paradigm:** Data are mean (±S.E.M.) of the amount of time that mice spent in the drug-paired
679 chamber (DPCh) on test days before (D1) and after (D5) conditioning. Mice received vehicle or
680 AT-312 (3 mg/kg, right panel) 15 min before saline on the conditioning days.

681 **Figure 5. Effects of SCH221510 (SCH; 10 mg/kg) on ethanol CPP in C57BL/6J mice:** Data
682 are mean (±S.E.M.) of the amount of time that mice spent in the drug-paired chamber (DPCh) on

test days before (D1) and after (D5) conditioning. Mice were treated with vehicle or SCH221510 15 min before ethanol on the conditioning days. *P<0.05, significant difference in the amount of time between D5 vs D1 for the vehicle-treated group

Figure 6. Effects of pentobarbital (Pento; 25 mg/kg) on ethanol CPP in C57BL/6J mice: Data are mean (±S.E.M.) of the amount of time that mice spent in the drug-paired (DPCh) and vehicle-paired chamber (VPCh) on test days before (D1) and after (D5) conditioning. Mice were treated with vehicle or pentobarbital 15 min before ethanol on the conditioning days. *P<0.05, significant difference in the amount of time that mice spent in the DPCh vs. VPCh on D5.

Table 1: In vitro pharmacological profile of NOP agonists in binding and functional assays at the opioid receptors*

	Receptor Binding Ki (nM)				[^{35}S] GTPγS NOP		[^{35}S] GTPγS MOP		[^{35}S] GTPγS KOP	
	NOP	MOP	KOP	DOP	EC_{50} (nM)	% Stim	EC_{50} (nM)	% Stim	EC_{50} (nM)	% Stim
N/OFQ	0.08 ± 0.03	133 ± 30	247 ± 3.4	ND	4.0 ± 0.1	100	>10,000		>10,000	
DAMGO		2.96 ± 0.54					32.6 ± 4.06	100		
DPDPE				1.11 ± 0.07						
U69,593			1.05 ± 0.02						60.14 ± 7.45	100
AT-312	0.34 ± 0.13	5.99 ± 0.97	73.5 ± 28.3	128.7± 57.4	29.9 ± 1.4	102.3 ± 0.75	81.5 ± 15.9	24.6 ± 2.4	>10,000	-
SCH221510	13.7 ± 2.30	65.4 ± 11.3	49.7 ± 11.3	403.7 ± 109.7	18.9 ± 5.9	95.1 ± 7.8	139.3 ± 4.6	76.8 ± 13.1	142.0 ± 15.6	82.72 ± 0.22

** GTP(γ)S functional assays only carried out if binding affinity Ki<100 nM. The functional efficacy at the delta opioid receptor was therefore not determined for AT-312 and SCH221510. Values are the Mean ± SEM of three independent experiments run in triplicate. Functional activity was determined by stimulation of [^{35}S]GTPγS binding to cell membranes, % stimulation was obtained as a percentage of stimulation of the standard agonists N/OFQ (for NOP), DAMGO (for MOP) and U69,593 (for KOP) taken as 100%.*

Table 2: In vivo pharmacokinetic profile of AT-312 after subcutaneous administration in mice

PK parameters	Dose, route (10 mg/kg, s.c.)
Plasma C_{max}	1263 nM
Plasma t_{max}	1 h
Brain C_{max} *	5465 nM
Brain t_{max}	1 h
Brain-to-plasma ratio at C_{max} *	4.33
Brain-to-plasma ratio AUC*	4.68

* Total brain concentrations; AUC (Area under the curve)

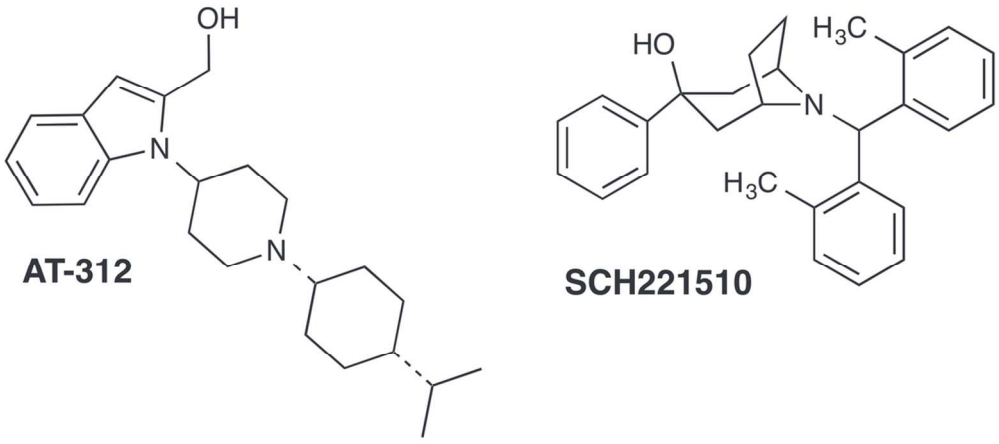

Figure 1. Chemical structures of NOP agonists AT-312 and SCH221510

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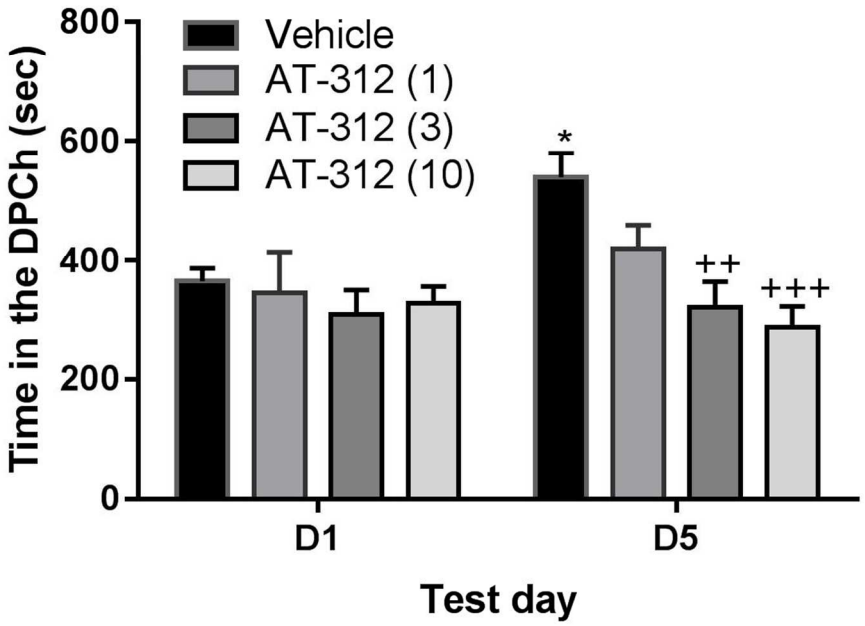

Figure 2. The effect of AT-312, a novel NOP agonist, on ethanol-induced CPP in C57BL/6J mice: Data are mean (±S.E.M.) of the amount of time that mice spent in the drug-paired chamber (DPCh) before (D1) (preconditioning test day) and after (D5) conditioning (post-conditioning test day). Mice treated with vehicle or AT-312 (1, 3 or 10 mg/kg, s.c.) 15 min before ethanol on the conditioning days. *P<0.05, indicates a significant increase in the amount of time in the DPCh on D5 vs. D1; ++P<0.01, +++P<0.001, significantly different from the control group on D5.

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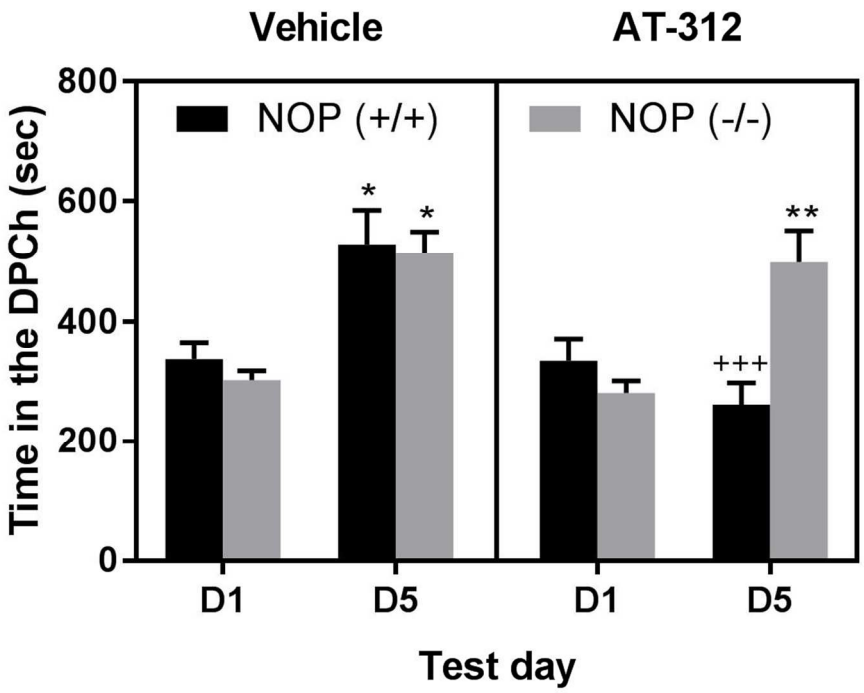

Figure 3. The action of AT-312, a novel NOP agonist, on ethanol-induced CPP in mice lacking NOP and their wild-type littermates/controls: Data are mean (±S.E.M.) of the amount of time that mice spent in the drug-paired chamber (DPCh) before (D1) (preconditioning test day) and after (D5) conditioning (post-conditioning test day). Mice lacking NOP [NOP (-/-)] and their wild-type littermates [NOP (+/+)] were treated with vehicle (left panel) or AT-312 (10 mg/kg, right panel) 15 min before ethanol on conditioning days. ***P<0.001; *P<0.05 DPCh vs. VPCh

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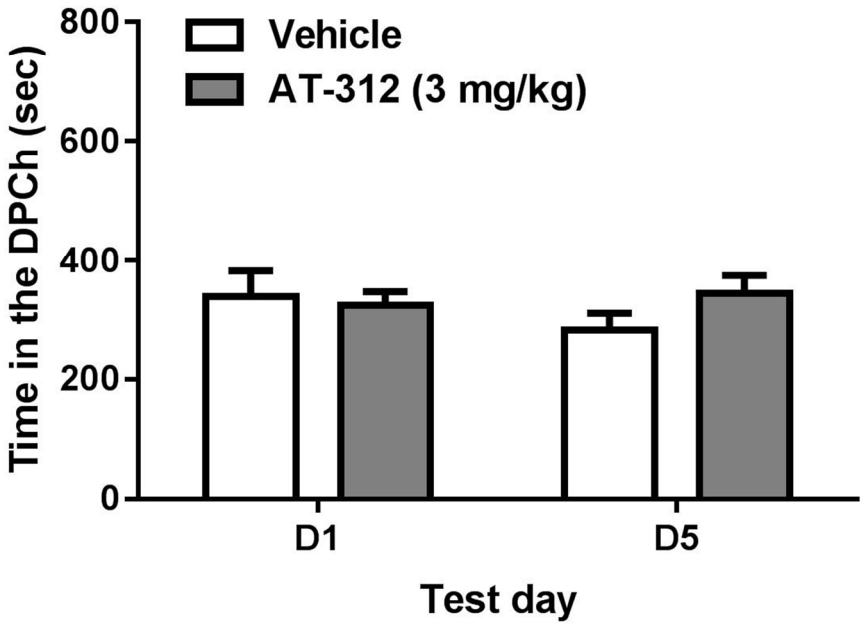

Figure 4. Motivational effect of AT-312, a novel NOP agonist, in the place conditioning paradigm: Data are mean (±S.E.M.) of the amount of time that mice spent in the drug-paired chamber (DPCh) on test days before (D1) and after (D5) conditioning. Mice received vehicle or AT-312 (3 mg/kg, right panel) 15 min before saline on the conditioning days.

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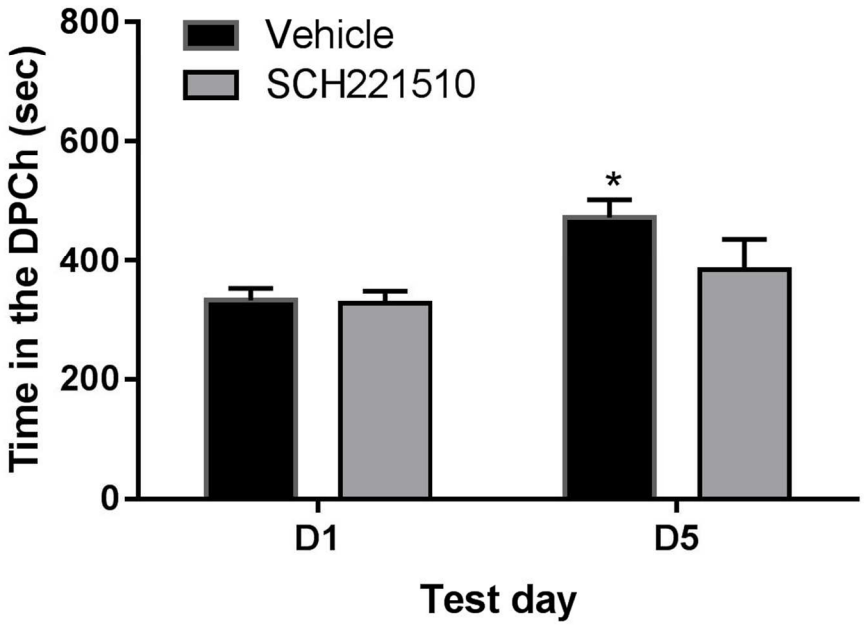

Figure 5. Effects of SCH221510 (SCH; 10 mg/kg) on ethanol CPP in C57BL/6J mice: Data are mean (±S.E.M.) of the amount of time that mice spent in the drug-paired chamber (DPCh) on test days before (D1) and after (D5) conditioning. Mice were treated with vehicle or SCH221510 15 min before ethanol on the conditioning days. *P<0.05, significant difference in the amount of time between D5 vs D1 for the vehicle-treated group

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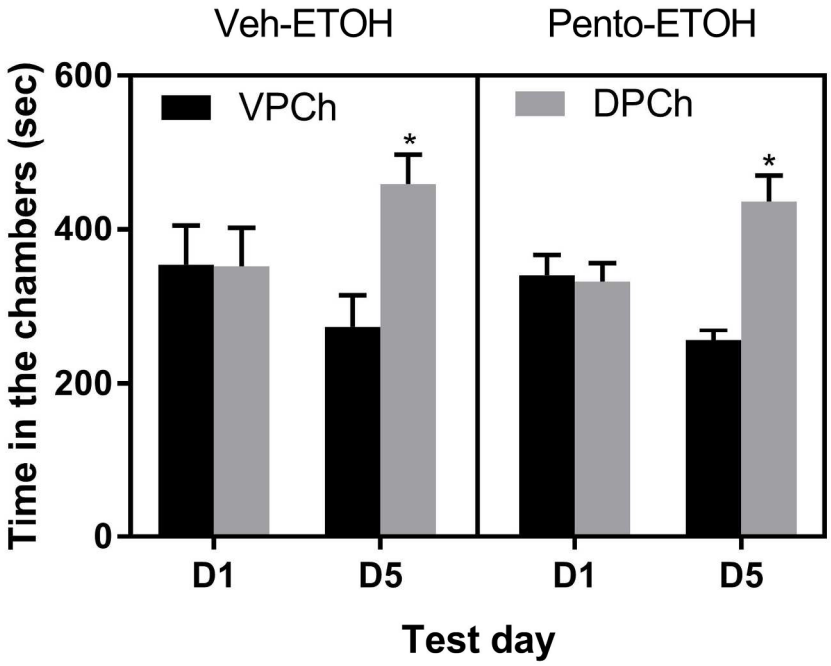

Figure 6. Effects of pentobarbital (Pento; 25 mg/kg) on ethanol CPP in C57BL/6J mice: Data are mean (±S.E.M.) of the amount of time that mice spent in the drug-paired (DPCh) and vehicle-paired chamber (VPCh) on test days before (D1) and after (D5) conditioning. Mice were treated with vehicle or pentobarbital 15 min before ethanol on the conditioning days. *P<0.05, significant difference in the amount of time that mice spent in the DPCh vs. VPCh on D5.

195x149mm (300 x 300 DPI)

Response to Critique

Manuscript ID ACER-17-3119

Title: A novel and selective nociceptin receptor (NOP) agonist (1-(1-((cis)-4-isopropylcyclohexyl)piperidin-4-yl)-1H-indol-2-yl)methanol (AT-312) decreases acquisition of ethanol-induced conditioned place preference in mice

Dear Dr. Phillips,

We would like to thank you and the reviewers for the insightful reviews and positive comments. We have incorporated all the changes requested by the reviewers and added new experiments (Figure 6) to address their questions. We believe these changes have enhanced the quality of our manuscript. Below please find our point-by-point response letter. Changes in the manuscript are highlighted in grey.

Reviewer: 1

• *Drug-induced changes in locomotion can confound behavioral responding. Does AT-312 impact general locomotor behavior?*

Response: The reviewers raised a crucial point regarding motor activity, which, as is generally accepted, can impact the outcome of the CPP response. In our experiments, we'd like to bring attention to the following: First of all, the animals were tested under a *drug-free state* and there was no difference in locomotor activity between different groups on the postconditioning 'test day'. However, if the reviewers are concerned about the locomotor activity during the conditioning sessions, yes, AT-312 caused motor suppression on each conditioning day, particularly at the higher 10 mg/kg dose. It also potentiated the motor sedative effect of ethanol when the two drugs were given together in wild-type mice. This response was absent in mice lacking NOP, showing that the motor sedative effect of the drug is also mediated via the NOP receptor. However, we discovered that not all drugs that reduce motor activity during the conditioning would block the CPP response. To illustrate this point, we conducted an experiment using pentobarbital (25 mg/kg) to assess if a drug that causes motor suppression would also block ethanol-induced CPP. Our results show that pentobarbital induces a robust *decrease in locomotor activity* but *failed* to alter ethanol-induced CPP. The result of this experiment and the related discussion are now included in our Revised manuscript. Given that alcohol itself causes a significant motor suppression and still induces a robust CPP, and that pentobarbital caused motor sedation but failed to alter CPP, we do not believe that motor suppression during conditioning was the sole reason for the ability of AT-312 to reduce ethanol-induced CPP.

• *Can the authors provide an explanation for the observation that AT-312 has a 50-fold higher affinity than SCH221510, but a 2-fold lower potency for GTPyS. Does it have a short residence time at the receptor (Rosethorne et al. Mol Pharmacol, 2016, 89(4))? If this is the case it may also have differences in beta-arrestin recruitment.*

Response: The 2-fold difference in potency (EC50) in the GTPgS assay is within the experimental variability in the GTPgS assays, done at different times and is not a significant difference. However, both are full agonists (nearly 100% stimulation) at NOP.

What we think the reviewer is asking is the explanation for the lower potency (higher EC50) of AT-312 relative to its high binding affinity (Ki), compared to SCH221510. This difference between the Ki and the EC50 is also a common occurrence with the NOP receptor ligands (see Adapa and Toll, Neuropeptides, 1997 for a discussion on this phenomenon for N/OFQ itself). Taking all this into account, AT-312 is still a full agonist at NOP and of a higher affinity than SCH221510.

We have no information regarding the residence time at the receptor. All radioligand binding affinity assays and the GTPgS functional assays are conducted for a 1-h time, a standard protocol for these assays.

We are not aware of any correlations of GTPgS EC50 to beta-arrestin recruitment, to the best of our knowledge.

• *When the authors discuss the Kallupi NOP KO rats consuming less alcohol, they can also add that the LY2940094 NOP antagonist reducing alcohol SA and seeking also in rats (Rorick-Kehn et al, ACER, 2016, 40(5)).*

Response: This information has been added to and discussed in the Discussion section of the revised manuscript. We also discussed their earlier paper regarding the effect of the NOP antagonist on palatability of food in this section.

• *Given some of these controversies between the agonist, antagonist, knockout animals and alcohol CPP and consumption, it would be a great addition to the manuscript if the authors could determine if a dose of 3 mg/kg AT-312 reduces voluntary alcohol intake in mice.*

Response: This is an excellent suggestion and we are conducting these experiments. It may take couple more months to confirm our findings. We will report these results in due course.

• *Please provide a reference for AT-312 synthesis and its purity before testing.*

Response: The synthesis of AT-312 is not communicated in a journal publication yet. However, we have added the full chemical name and information about the chemical purity to the Methods section. The structure is also given, in Figure 1.

Reviewer: 2

Comments to the Author

My major concern is that NOP agonists usually produce locomotor impairment. Here no control experiments were carried out to evaluate this behavioral effect that can severely impact CPP results. I would recommend to include these data and in case AT-312, like other NOP agonists, produces sedation or locomotor impairment in the discussion this limitation should be addressed in depth.

Response: As stated above, AT-312 caused motor suppression which was absent in mice lacking NOP. However, as stated above, the mice were tested for preference (on the test day) under a drug-free state. Also, we conducted a control study with pentobarbital and showed that pentobarbital reduced motor activity but failed to alter the CPP response. Thus, we believe that the inhibitory

effect of the NOP agonist to reduce ethanol CPP is not due to its motor impairment. The inhibition of ethanol CPP by AT-312 is absent in mice lacking NOP.

Additional comments:

In the Abstract please indicate that female mice were used. Please report the binding affinity of AT-312 also against DOP.

Response: The abstract is modified to indicate female mice. The DOP affinity of AT-312 is in Table 1 (Ki = 128.71 ± 57.44)

In the main text the number of mice used for each experiment should be given

Response: This has been incorporated in our revised manuscript (please see the Method section of the revised manuscript).

Experiment 2 "AT-312 reduced the rewarding…" statistical analysis is missing (F value, DF etc) . This should be a 3 way anova with lines, treatment and time as factors. Figure 3 should be modified accordingly by reporting al the data in one single panel

Response: The figure has been modified and a 3-way ANOVA was used to analyze the data. This information has been included in the Results section of our revised manuscript.

Experiment 3 "AT-312 given alone…" were data analyzed by one or two-way anova? Here a two-way with time and treatment should be used.

Response: We analyzed the data using a two way ANOVA. This information has been highlighted in the Result section of our revised manuscript.

Experiment 4 "Ethanol-induced CPP was reduced…" here the use of a two-way anova should be indicated. In Figure 4 the dose of SCH should be reported.

Response: This information is incorporated and highlighted in the Result section of the revised manuscript.

I have some problems with the statistics in general because I cannot understand the Degree of Freedom. When treatment x time (interaction) is reported the DF cannot be the same than for simple treatment or time. Not having information about the N of mice used I cannot fully understand where the problem is.

Response: We have included the sample size under each experiment in the Method section. We also included stats for all the experiments and what type of ANOVA we used. In our studies, we basically have two times with two treatments or two genotypes, or two genotypes and two treatments. Therefore the degree of freedom will be (2-1 = 1 for each condition) and (1 x 1 for the interaction). This is what it shows on the Prism GraphPad analyses and this is our understanding of the degrees of freedom. However, if the reviewer is aware of something else that the software and we are missing, please let us know and we will be happy to incorporate it in our manuscript.

The first paragraph of the Introduction section is lacking of references. Please add 2 or 3 appropriate citations to support your epidemiological information.

Response: This has been addressed, as recommended.

Introduction lines (85-88) here the first work showing the effect of NOP agonist on alcohol related behaviors should be reported (PMID: 9952048)

Response: This paper (Ciccocioppo et al. Psychopharmacology (Berl). 1999 Jan;141(2):220-4, has been cited in our revised manuscript.

Introduction line 94 and line 112, relevant citations from Roberto's lab on NOP modulation of GABA , glutamate system in the CeA and anxiety are missing (PMID: 24403138; PMID: 24169802)

Response: We have included these references in our revised manuscript.

Materials and methods, Drug section, please indicate the rout of drug administration; experiment 1, line 192, indicate the route of alcohol administration.

Response: The route of drug and alcohol administration has been included in the Methods section of our revised manuscript.

Discussion: Authors attempted to reconcile their data (and other published work) with recent observation on NOP KO rats showing reduced motivation for alcohol and other drugs of abuse. Their arguments are robust but they also neglected the fact that recent publications are showing that, consistent with data on NOP KO rats, NOP antagonists reduce alcohol abuse related behaviors (PMID: 27084498; PMID: 27435979). These studies should be considered and a bit more and deeper discussion would help to improve the quality of the study.

Response: We have discussed all the possibilities that we think of based on the results of the current study and previous reports. The information about the recent study with the NOP antagonist and ethanol self-administration has been added to the Discussion. We also included the information about the effect of the antagonist on palatable food. We robustly discussed the current data while we carefully discussed previous studies as well.

TITLE PAGE

Title: A novel and selective nociceptin receptor (NOP) agonist (1-(1-((cis)-4-isopropylcyclohexyl)piperidin-4-yl)-1H-indol-2-yl)methanol (AT-312) decreases acquisition of ethanol-induced conditioned place preference in mice

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Running Title: AT-312, a novel NOP agonist blocks alcohol reward

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ABSTRACT

Background: Nociceptin/Orphanin FQ (N/OFQ), the endogenous peptide agonist for the opioid receptor-like (ORL1) receptor (also known as NOP or the nociceptin receptor), has been shown to block the acquisition and expression of ethanol-induced conditioned place preference (CPP). Here, we report the characterization of a novel small-molecule NOP ligand AT-312 (1-(1-((cis)-4-isopropylcyclohexyl)piperidin-4-yl)-1H-indol-2-yl)methanol) in receptor binding and GTPγS functional assays in vitro. We then investigated the effect of AT-312 on the rewarding action of ethanol in mice using the CPP paradigm. Further, using mice lacking the NOP receptor and their wild-type controls, we also examined the involvement of NOP in the effect of AT-312. Motivational effects of AT-312 alone were also assessed in the CPP paradigm. **Methods:** Female mice lacking NOP and/or their wild-type controls received conditioning in the presence or absence of the NOP agonist [AT-312 (1, 3 and 10 mg/kg) or the control NOP agonist SCH221510 (10 mg/kg)] followed by saline/ethanol for 3 consecutive days (twice daily) and tested for CPP in a drug-free state on the next day. **Results:** Our *in vitro* data showed that AT-312 is a high affinity, selective NOP full agonist with 17-fold selectivity over the mu opioid receptor and >200-fold selectivity over the kappa opioid receptor. The results of our *in vivo* studies showed that AT-312 reduced ethanol CPP at the lowest dose (1 mg/kg) tested but completely abolished ethanol CPP at higher doses (3 or 10 mg/kg) compared to their vehicle-treated control group. AT-312 (3 mg/kg) did not alter ethanol-induced CPP in mice lacking NOP, confirming that AT-312 reduced ethanol CPP through its action at the NOP receptor. AT-312 (3 mg/kg) did not induce reward or aversion when administered alone, showing that the novel small molecule NOP agonist shows efficacy in blocking ethanol-induced CPP via the NOP receptor.

Conclusions: Together, these data suggest that small molecule NOP agonists have the potential to reduce alcohol reward and may be promising as medications to treat alcohol addiction.

Key Words: NOP agonist, AT-312, Alcohol reward, NOP knockout mouse, Ethanol-induced conditioned place preference

Introduction

Alcoholism and alcohol-related disorders are major public health issues and place an enormous burden on society and economy (Esser et al., 2017; Esser et al., 2014). When alcohol-related accidents are factored in, alcohol is among the top three causes of death in the US (Mokdad et al., 2004). Of the estimated 18 million alcohol-dependent individuals in the population, only about 1 million actually receive/seek adequate treatment, which mainly involves psychosocial support in conjunction with limited pharmacotherapy. While no single medication or strategy has been shown to be very effective, it is generally accepted that having pharmacotherapy as an adjunct to behavioral interventions is the best approach for treating alcohol dependence and maintaining abstinence. For this however, the current repertoire of pharmacotherapeutic options needs to be significantly expanded. Only three pharmacotherapeutic agents are currently approved for the treatment of alcohol dependence in the US, oral and intramuscular naltrexone (NTX), acamprosate, and disulfiram. Of these, NTX, an opioid receptor antagonist, has shown limited efficacy in reducing craving after stopping alcohol drinking; acamprosate, whose mechanism of action is unclear, improves abstinence rates, whereas disulfiram, produces an aversive reaction to alcohol. These are still not widely adopted by physicians who treat alcohol-dependent patients, mostly due to lack of confidence about their efficacy and a range of unpleasant side effects that limit patient compliance. There still remains a need for new approaches and treatments for alcohol dependence.

Unlike other drugs of abuse, alcohol does not act at one receptor target, but dysregulates many neurotransmitter systems, ion channels, and neurocircuitry in several brain areas, particularly the ventral tegmental area, nucleus accumbens, central amygdala and bed nucleus of stria terminalis (Gilpin and Koob, 2008; Koob and Volkow, 2010).

Among these, the endogenous opioid system is well known to play a key role in the rewarding and reinforcing effects of alcohol (Altshuler et al., 1980; Froehlich et al., 1990; Gianoulakis, 2004; Hubbell et al., 1986; Marfaing-Jallat et al., 1983; Weiss et al., 1990). Indeed, as stated above, NTX, approved for use in the US as an anti-alcohol pharmacotherapy, decreases alcohol consumption and craving in humans, and decreases the rewarding properties of ethanol in animal models (Altshuler et al., 1980; Benjamin et al., 1993; Farren and O'Malley, 1997; Froehlich et al., 1990; Gianoulakis et al., 1996; Hubbell et al., 1991; Ji et al., 2008; Kornet et al., 1991; Marfaing-Jallat et al., 1983; Myers et al., 1986; O'Malley et al., 2002; Oslin et al., 1997; Samson and Doyle, 1985; Volpicelli et al., 1992; Volpicelli et al., 1986; Weiss et al., 1990). The mu, delta and kappa opioid receptors and their respective endogenous ligands β-endorphins, enkephalins and dynorphin have all been shown to be involved in various stages of alcohol addiction cycle (Hall et al., 2001; Oswald and Wand, 2004; Roberts et al., 2000).

A growing body of evidence suggests that the fourth member of the opioid receptor-ligand family, the nociceptin opioid receptor NOP (previously called the opioid receptor-like (ORL1) receptor) and its endogenous neuropeptide ligand, nociceptin/orphanin FQ (N/OFQ) are involved in alcohol reward and reinforcement (Ciccocioppo et al., 1999; Kuzmin et al., 2007; Kuzmin et al., 2003; Ciccocioppo et al., 2002). Similarly to other members of the opioid receptor family, the NOP receptor is widely distributed in areas of the brain implicated in motivational behaviors as well as negative affect, such as the ventral tegmental area, nucleus accumbens,

lateral hypothalamus and the central amygdala (Neal et al., 1999a; Neal et al., 1999b). The endogenous ligand of the NOP, N/OFQ, acts to alter neurotransmitter release, particularly dopamine, GABA, and glutamate, all of which are also implicated in alcohol reward (Di Giannuario et al., 1999; Kallupi et al., 2014; Lutfy et al., 2001; Murphy et al., 1996; Murphy and Maidment, 1999; Murphy et al., 2004; Sakoori and Murphy, 2004). N/OFQ is also considered to have an 'anti-opioid' action in the brain [for a review, see (Mogil and Pasternak, 2001)]. Exogenous administration of N/OFQ has been shown to suppress basal and drug-stimulated dopamine release in the NAc (Di Giannuario et al., 1999; Lutfy et al., 2001; Murphy et al., 1996; Murphy and Maidment, 1999; Murphy et al., 2004; Sakoori and Murphy, 2004), and the rewarding properties of several common drugs of abuse [reviewed in (Lutfy and Zaveri, 2016)]. In particular, intracerebroventricular (i.c.v) administration of N/OFQ has been shown to block acquisition of conditioned place preference (CPP) induced by morphine (Ciccocioppo et al., 2000; Murphy et al., 1999), cocaine (Sakoori and Murphy, 2004), amphetamines (Kotlinska et al., 2003), and alcohol (Ciccocioppo et al., 1999; Kuzmin et al., 2007; Kuzmin et al., 2003). A small molecule NOP agonist Ro 64-6198, given systemically, was also shown to block both the acquisition and expression of alcohol CPP in mice (Kuzmin et al., 2003) and alcohol self-administration in rats (Kuzmin et al., 2007). Another potent NOP agonist MT-7716 was shown to decrease alcohol intake in alcohol-preferring Marchigian Sardinian (msP) rats and attenuate alcohol withdrawal symptoms in alcohol-dependent Wistar rats (Ciccocioppo et al., 2014b). Recently, SR-8993, a selective NOP agonist was reported to reduce anxiety associated with alcohol withdrawal as well as home cage and limited access alcohol drinking in Wistar rats (Aziz et al., 2016). Interestingly the level of N/OFQ is altered following restraint stress in the amygdala

(Ciccocioppo et al., 2014a). These studies suggest that NOP agonists may be potentially promising treatment agents for alcoholism and alcohol use disorders.

In the present study, we characterized a novel small-molecule NOP ligand AT-312 (1-(1-((cis)-4-isopropylcyclohexyl)piperidin-4-yl)-1H-indol-2-yl)methanol), for its selectivity and affinity toward the NOP and classical opioid receptors using radioligand binding assays and determined its efficacy in the GTP(γ)S functional assay conducted in Chinese hamster ovary (CHO) cells transfected with the human opioid receptors. We also determined its bioavailability and brain penetration, which showed appreciable plasma exposure and a brain-to-plasma ratio greater than 1 after systemic (subcutaneous, s.c.) administration (Table 2). Using this route of administration, we further determined its efficacy in reducing the rewarding action of ethanol in the CPP paradigm, a widely used animal model of drug reward (Bardo and Bevins, 2000). To demonstrate that the effect of AT-312 in reducing alcohol CPP in mice is due to its activity at the NOP receptor, we compared its efficacy in reducing CPP induced by ethanol in mice lacking the NOP receptor and their wild-type littermates/controls. We also investigated the effect of a known NOP agonist SCH 221510 on alcohol reward in this same paradigm as a control.

MATERIALS AND METHODS

Cells

Human NOP, mu, delta, and kappa opioid receptors were individually expressed in Chinese hamster ovary cells stably transfected with the human receptor cDNA, as we have described previously (Zaveri et al., 2001; Toll et al., 2009). The HORL, HDOR, HKOR-FLAG19 cells were grown in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum, in the presence of 0.4 mg/ml G418 and 0.5% penicillin/streptomycin, in 150-mm tissue culture dishes. The HKOR-CN cells were grown in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum, in the presence of 0.5% penicillin/streptomycin and no G418. The HMOR cells in 50% F12/DMEM with 10% fetal bovine serum, in the presence of 0.4 mg/ml G418 and 0.5% penicillin/streptomycin. Kappa-CN cells were used for KOP radioligand binding assays, while Kappa-FLG19 cells were used in KOP [^{35}S]GTPγS functional assays.

Animals

Female mice lacking NOP (Nishi et al., 1997) and their wild-type littermates/controls (2-6 months old), fully backcrossed on C57BL/6J mouse strain, bred in house, were used throughout. We used female mice because they exhibit a robust CPP response compared to male mice using the current 3-day conditioning paradigm (Nguyen et al., 2012; Tseng et al., 2013). Mice were housed 2-4 per cage with free access to laboratory chow and tap water and kept under a 12 h light/12 h dark cycle in a temperature- and humidity-controlled room. The light was on 6 AM and off at 6 PM. All experiments were conducted during the light cycle between the hours of 10:00 AM to 5:00 PM and were according to the National Institute of Health for the proper use of animals in research and approved by the Institutional Animal Care and Use Committee at Western University of Health Sciences (Pomona, California, USA).

Drugs

AT-312 (1-(1-((cis)-4-isopropylcyclohexyl)piperidin-4-yl)-1H-indol-2-yl)methanol) (Figure 1) was synthesized at Astraea Therapeutics, and was of >99% chemical purity as fully characterized by nuclear magnetic resonance spectroscopy, LC-MS and elemental analysis. SCH221510 (Figure 1) was purchased from Tocris. These test compounds were dissolved in 1-2% DMSO and then diluted to the desired concentration with 0.5% aqueous hydroxypropylcellulose (HPC) and injected subcutaneously (s.c.) in a volume of 0.1 ml/10g of body weight. Controls received 0.1 ml/10g of body weight of the appropriate vehicle (1-2% DMSO in 0.5% of HPC).

In vitro Characterization

Membrane preparation. The cell lines are grown to confluency, then harvested for membrane preparation. The membranes are prepared in 50 mM Tris buffer (pH 7.4). Cells are scraped and centrifuged at 500 × g for 12 mins. The cell pellet is homogenized in 50 mM Tris with a Fisher Scientific PowerGen 125 rotor-stator type homogenizer, centrifuged at 20,000 × g for 25 mins, washed and recentrifuged once more at 20,000 × g for 25 mins, and aliquoted at a concentration of 3 mg/ml protein per vial and stored in a -80 ˚C freezer till further use.

Receptor Binding. Compounds were dissolved at 10 mM stock in 100% DMSO. The assay was performed in a 96-well polystyrene plate with triplicates of six concentrations of each test compound (1µM – 0.01 nM), adding 100 µl of compound and 100 µl of tritiated ligands [^{3}H]DAMGO (51.0 Ci/mmole, K_d 0.59 nM for MOP), [^{3}H]DPDPE (42.0 Ci/mmole, K_d 1.11 nM for DOP), [^{3}H]U69593 (41.7 Ci/mmole, K_d 1.05 nM for KOP), and [^{3}H]N/OFQ (130 Ci/mmole, K_d 0.12 nM for NOP). Nonspecific binding was determined using 1.0 µM of the unlabeled nociceptin for NOP, 10 µM unlabeled DAMGO for MOP, 1.0 µM unlabeled DPDPE for DOP,

and 10 μM unlabeled U69,593 for KOP. Assays were initiated by addition of 800 μl of membrane per well. Samples were incubated for 60 min at 25˚C in a total volume of 1.0 ml. In NOP receptor experiments, 1 mg/ml BSA was added to the compound dilution buffer. The incubation was terminated by rapid filtration through 0.5% PEI-soaked glass fiber filter mats (GF/C Filtermat A, Perkin-Elmer) on a Tomtec Mach III cell harvester and washed 5 times with 0.5 ml of ice-cold 50 nM Tris-HCl, pH 7.4 buffer. The filters were dried overnight and soaked with scintillation cocktail before counting on a Wallac Beta plate 1205 liquid scintillation counter. Radioactivity was determined as counts per minutes (cpm). IC_{50} values were determined using at least six concentrations of test compound, and calculated using Graphpad/Prism (ISI, San Diego, CA). K_i values were determined by the method of Cheng and Prusoff (Cheng and Prusoff, 1973).

[^{35}S]GTPγS binding Assay. [^{35}S]GTPγS binding was conducted as we have described previously (Toll et al., 2009; Traynor and Nahorski, 1995; Zaveri et al., 2001). Cells were scraped from tissue culture dishes into 20 mM Hepes, 1 mM EDTA, then centrifuged at 500 × g for 10 min. Cells were re-suspended in this buffer and homogenized using a Polytron Homogenizer. The homogenate was centrifuged at 27,000 × g for 15 min, and the pellet resuspended in Buffer A, containing: 20 mM Hepes, 10 mM MgCl2, 100 mM NaCl, pH 7.4. The suspension was recentrifuged at 27,000 × g and suspended once more in Buffer A. For the binding assay, membranes (8-15 μg protein) were incubated with [^{35}S]GTPγS (50 pM), GDP (10 μM), and the appropriate compound, in a total volume of 1.0 ml, for 60 min at 25˚C. Samples were filtered over glass fiber filters and counted as described for the binding assays. Statistical analysis was conducted using Prism.

In vivo Pharmacology

Experiment 1: To determine the effect of NOP agonists on ethanol-induced CPP. We used an unbiased CPP paradigm, widely used as an animal model of drug reward (Bardo and Bevins, 2000), to determine the effect of AT-312 on the rewarding action of ethanol. The details of the CPP apparatus and paradigm have been provided elsewhere (Nguyen et al., 2012; Tseng et al., 2013). Briefly, mice were tested for preconditioning place preference on day 1. On this day, mice were placed in the central neutral chamber and allowed to freely explore the conditioning chambers through this smaller central chamber. The amount of time that mice spent in each CPP chamber was recorded. On days 2-4, mice were conditioned with ethanol in the presence and absence of the NOP agonist. In the morning on each day, mice were treated with vehicle or one of the doses of AT-312 (1, 3 or 10 mg/kg, s.c.; n = 6-9 mice per group) followed, 15 min later, by ethanol (2 g/kg, i.p.) and then immediately confined to the drug-paired chamber (DPCh) for 15 min. In the afternoon, mice received vehicle followed by saline and were conditioned in the vehicle-paired chamber (VPCh). The order of conditioning were reversed for some mice to counterbalance the treatment and chamber assignment as well as the use of wild-type versus knockout mice for the morning and afternoon conditioning. Mice were then tested under a drug-free state for postconditioning place preference on day 5, as described for day 1. SCH221510 has been previously reported by Varty and colleagues (Varty et al., 2008) as a NOP agonist. Thus, we used this compound as the control NOP agonist and determined its effect on the rewarding action of alcohol. To this end, mice were tested for preconditioning place preference on day 1, conditioned with ethanol in the presence or absence of SCH221210 (10 mg/kg) on days 2-4 and then tested for CPP on day 5, as described above.

Experiment 2: To characterize the role of the NOP receptor in the inhibitory action of AT-312 on alcohol CPP: Mice lacking NOP and their wild-type controls were tested for preconditioning place preference, received conditioning with ethanol (2 g/kg, i.p.) in the presence or absence of AT-312 (3 mg/kg, s.c.; n = 7 mice per treatment per each genotype) on days 2-4 and then were tested for postconditioning place preference on day 5. On each test day, the amount of time that mice spent in the CPP chambers was recorded, as described above.

Experiment 3: To assess the motivational effect of AT-312 in the place conditioning paradigm: Mice were tested for baseline place preference on day 1, received conditioning on days 2-4 and were tested for postconditioning place preference on day 5. On each conditioning day, mice were treated with vehicle or AT-312 (3 mg/kg, s.c.; n = 5 mice per treatment) followed by saline and placed in the vehicle-paired (VPCh) or drug-paired chamber (DPCh). In the afternoon, mice were treated with the alternate treatment and conditioned to the opposite chamber. The amount of time that mice spent in the CPP chamber was recorded on each test day (days 1 and 5), as described above.

Experiment 4: To determine the effect of pentobarbital on ethanol-induced CPP: Mice were tested for preconditioning place preference on day 1, treated with saline or pentobarbital (25 mg/kg, s.c.; n = 6 mice per treatment) 15 min before ethanol (2 g/kg, i.p.) on each conditioning day and were confined to the drug paired chamber (DPCh). Animals were treated with saline 15 min before saline and confined to the vehicle-paired chambers (VPCh). These treatments were given either in the morning or afternoon in a counterbalanced manner. Each conditioning session lasted for 15 min and was conducted on days 2-4. Mice were then tested for CPP on day 5, as described above.

243 *Data Analysis.* Data are presented as mean (±S.E.M.) of the amount of time that mice
244 spent in the drug-paired chamber (DPCh) or DPCh vs. vehicle-paired chamber (VPCh) on
245 preconditioning test day (day 1, D1) and postconditioning test day (day 5, D5) and were
246 analyzed using repeated measures two- or three-way analysis of variance (ANOVA). The
247 Bonferroni's post-hoc test was used to reveal significant changes between different groups.
248 $P<0.05$ was considered significant.
249

RESULTS

In vitro NOP Receptor Binding affinity and Opioid Receptor Selectivity of AT-312

The chemical structure of AT-312 is shown in Fig. 1. The receptor binding affinity of AT-312 was determined using radioligand displacement assays conducted in membranes from CHO cells stably expressing the human NOP, MOP, DOP and KOP receptors. As shown in Table 1, AT-312 showed high binding affinity for the NOP receptor, yielding a subnanomolar Ki value of 0.34 ± 0.13 nM in competition with [^{3}H]N/OFQ as the radioligand. In similar experiments using [^{3}H]DAMGO, [^{3}H]U69593 and [^{3}H]DPDPE at the MOP, KOP and DOP receptors respectively, AT-312 showed binding selectivity of 17-fold versus MOP, 216-fold versus KOP and 378-fold versus DOP receptors. The NOP agonist SCH221510 tested in the same assays showed NOP binding Ki of 13.7 nM, about 40-fold lower affinity at NOP than AT-312. Also, the NOP binding affinity of SCH221510 was only 5-fold selective versus MOP, 3.6-fold versus KOP and 29-fold versus DOP receptors. AT-312 therefore, exhibits significantly higher binding affinity and selectivity for NOP compared to the positive control SCH221510. The high affinity of AT-312 for the NOP receptor is similar to that observed for other reported NOP agonists Ro 64-6198 and MT-7716 (Zaveri, 2016).

As observed for other piperidinyl NOP ligands from our own compound library as well as those reported in the literature, AT-312 did not show appreciable affinity for the DOP receptor. Affinity profiling in a panel of 68 receptors and ion channels showed that, at a concentration of 100 nM, AT-312 did not bind to any non-opioid off-target receptors, whereas at 10 μM, it inhibited the specific binding of radioligands at the α1 adrenergic receptor by 60%, dopamine D4 receptor (86%), dopamine D3 receptor (100%), muscarinic M1 and M2 receptors (85%), NK2 receptor (92%), Ca^{+2} channel (L-type) (73%) and Na+ channel (site 2) (94%) and

norepinephrine transporter (65%). Overall, AT-312 appears to be a selective NOP receptor ligand.

In vitro Functional Efficacy of AT-312

The intrinsic efficacy of AT-312 at the NOP and traditional opioid receptors was determined using the GTPγS binding assay conducted in membranes of CHO cells stably transfected with the NOP and classical opioid receptors. Table 1 shows the in vitro functional efficacy profile of AT-312 and SCH221510. AT-312 is a full agonist at the NOP receptor, showing potency (EC_{50}) of 30 nM and 100% agonist stimulation compared to the endogenous NOP agonist N/OFQ. In contrast, it showed only a partial agonist efficacy of 25% at the MOP receptor and significantly lower potency compared to the MOP opioid full agonist DAMGO (Table 1). AT-312 had no agonist efficacy at the KOP receptor. In these experiments, SCH221510 was also found to be a full agonist at the NOP receptor, with comparable potency as that of AT-312 (Table 1). However, it also showed significant agonist stimulation at the KOP receptor, in contrast to AT-312.

AT-312, a novel NOP agonist, dose-dependently blocked the development of ethanol-induced CPP. The novel NOP agonist AT-312 dose-dependently reduced the rewarding action of ethanol (Fig. 2). Repeated measures ANOVA of the amount of time that mice spent in the drug-paired chamber (DPCh) on pre- and postconditioning days revealed a significant effect of treatment ($F_{3,26} = 21.35$; $P < 0.01$) but no effect of time ($F_{1,26} = 4.48$; $P = 0.08$) and no significant interaction between the two factors ($F_{3,26} = 10.38$; $P = 0.08$). The *post-hoc* test showed that the amount of time that mice spent in the DPCh was significantly ($P<0.05$) increased following ethanol conditioning in the vehicle-treated control group (Fig. 2, compare D5 vs. D1 for the mice treated with vehicle before ethanol on the conditioning days and this response was

reduced by AT-312 in a dose-dependent manner (Fig. 2). In particular, the two higher doses of AT-312 (3 and 10 mg/kg) blocked ethanol-induced CPP [compare the amount of time between the vehicle-treated group on day 5 vs. the AT-312 (3 mg/kg) group ($P<0.01$) as well as against AT-312 (10 mg/kg) on this day ($P<0.001$)]. Together, these results suggest that AT-312 dose-dependently abolished the rewarding action of alcohol.

AT-312 reduced the rewarding action of ethanol in wild-type but not in NOP knockout mice. The amount of time that mice lacking NOP and their wild-type littermates/controls spent in the ethanol-paired chamber on the preconditioning (D1) and postconditioning (D5) test days is shown in Figure 3. Three-way ANOVA revealed a significant effect of time ($F_{1,1}=26$; $P<0.0001$), a significant effect of context ($F_{1,1} = 8.27$; $P<0.01$) but no effect of genotype ($F_{1,1} = 1.58$; $P>0.05$). However, there was a significant interaction between time, context and genotype ($F_{1,1} = 6.39$; $P<0.02$). The post-hoc test showed that conditioning with ethanol induced a significant ($P<0.05$) CPP in both wild-type and knockout mice pretreated with vehicle prior to ethanol on the conditioning days, as evidenced by a significant increase in the amount of time that vehicle-treated control mice of either genotype spent in the ethanol-paired on day 5 compared to day 1 (Fig. 3, left panel; compare each bar on D5 vs. D1 for each genotype). The CPP response was significantly ($P<0.001$) reduced by AT-312 (10 mg/kg) in wild-type mice (Fig. 3, compare wild-type mice (NOP+/+) treated with AT-312 vs. vehicle on D5). On the other hand, mice lacking NOP spent the same amount of time in the DPCh on day 5 regardless of whether they were injected with the NOP agonist or vehicle (Fig. 3, compare NOP-/- treated with AT-312 group vs. vehicle-treated NOP-/- as well as against AT-312-treated NOP+/+ on D5). This result suggests that AT-312 exerts its inhibitory effect on the rewarding

action of ethanol via the NOP receptor. AT-312 reduced locomotor activity in wild-type mice but this response was absent in mice lacking NOP (data not shown).

AT-312 given alone did not have any motivational effect in the place conditioning paradigm. Figure 4 shows the amount of time that mice, treated with vehicle in both conditioning chambers (Vehicle) and those that received vehicle in one chamber and AT-312 (3 mg/kg) in the other chamber, spent in the drug-paired chamber (DPCh). Two-way ANOVA revealed no significant effect of treatment ($F_{1,8} = 0.75$; $P>0.05$), no significant effect of time ($F_{1,8} = 0.45$; $P>0.05$) and no significant interaction between the two factors ($F_{1,8} = 1.69$; $P>0.05$), showing that AT-312 at this dose (3 mg/kg) did not possess motivational effects of its own.

Ethanol-induced CPP was reduced in mice treated with SCH221510, a NOP agonist. We also determined the effect of a known NOP agonist on ethanol-induced CPP. Considering that this compound was less selective toward the NOP compared to AT-312, we determined the effect of a relatively higher dose (10 mg/kg) of this compound on the rewarding action of ethanol. Figure 5 shows the amount of time that mice treated with vehicle or the NOP agonist spent in the ethanol-paired chamber (DPCh). Two-way repeated measures ANOVA revealed a significant effect of time that mice spent in the ethanol-paired chamber on day 5 vs. day 1 ($F_{1,14} = 20.44$; $P<0.02$) but no significant effect of treatment ($F_{1,14} = 4.53$; $P>0.05$) and no significant interaction between the two factors ($F_{1,14} = 3.47$; $P>0.05$). The Bonferroni post-hoc test showed that the amount of time that mice spent in the ethanol-paired chamber was increased in vehicle-pretreated mice, suggesting that ethanol induced a significant CPP in the control group (Fig. 5, compare the amount of time that vehicle-pretreated mice spent in the DPCh on D5 vs. D1). However, this response was reduced in mice treated with SCH221510.

Pentobarbital reduced motor activity but failed to alter the rewarding action of ethanol. Figure 6 illustrates the amount of time that mice spent in the DPCh on the pre- and postconditioning test days. Three-way repeated measure ANOVA revealed a significant effect of context (DPCh vs. VPCh; $F_{1,1} = 11.61$; $P<0.002$) but no significant effect of time ($F_{1,1} = 0.21$; $P>0.05$) and no significant effect of treatment ($F_{1,1} = 0.50$; $P>0.05$). Although there was a significant context × time interaction ($F_{1,1} = 13.40$; $P<0.0001$, there was no treatment × context ($F_{1,1} = 0.001$; $P>0.05$) or time × context × treatment ($F_{1,1} = 0.001$; $P>0.05$) interaction. The post hoc test showed that ethanol induced a comparable CPP response in both groups, showing that pentobarbital did not alter the rewarding action of ethanol. Interestingly, pentobarbital induced a robust motor sedative effect and potentiated the sedative effect of alcohol on each conditioning day (data not shown).

DISCUSSION

The main findings of the present study are that the novel NOP agonist AT-312 reduced the acquisition of CPP induced by ethanol, and that this effect was absent in mice lacking the NOP receptor. Similar, albeit less potent effects were also observed on alcohol reward in mice treated with the control NOP agonist SCH221510. The current results also demonstrate that AT-312 did not have motivational effects of its own at a dose (3 mg/kg) that completely abolished ethanol CPP in wild-type mice. Together, these results are consistent with previous studies with NOP agonists N/OFQ and Ro 64-6198, and confirm that NOP agonists can reduce acquisition of ethanol CPP in mice via selective action at the NOP receptor.

AT-312 is a selective and high affinity NOP full agonist, belonging to a novel class of NOP ligands structurally unrelated to NOP agonists Ro 64-6198, MT-7716, SR-8993 and others that have shown efficacy in reducing the rewarding effects of alcohol in various animal models and paradigms (Zaveri, 2016). A growing body of evidence suggests that NOP may be a potential target to reduce the rewarding and reinforcing actions of alcohol and other addictive drugs [(see recent reviews (Lutfy and Zaveri, 2016; Witkin et al., 2014; Zaveri, 2011; Zaveri, 2016)]. Consistent with existing literature, this novel NOP agonist AT-312 dose-dependently reduced the rewarding action of alcohol and appeared to be more potent than the known NOP agonist, SCH221510. Although further studies are needed to define the mechanism for the greater effect of AT-312 compared to SCH221510, we speculate that it may be due to its higher affinity toward NOP. Our in vitro studies show that AT-312 exhibits at least 20-fold binding selectivity toward the NOP versus the MOP receptor and is a full agonist at NOP but a weak partial agonist at the MOP receptor, with no appreciable agonist efficacy at the KOP receptor. In comparison, SCH221510 has only a four-fold binding selectivity versus the KOP receptor and has significant agonist efficacy at both the MOP and KOP receptors in the same assays (Table 1).

However, further studies are needed to assess the contribution of each receptor in the inhibitory effects of the two NOP agonists.

Considering that AT-312 displayed higher affinity for and acted as a partial agonist at the mu opioid receptor, one may argue that the inhibitory action of the drug may be due to its interaction with the MOP receptor or both receptor systems rather than NOP only. In order to address this issue, we used mice lacking NOP and their wild-type controls and tested if the inhibitory effect of AT-312 is mediated via the NOP receptor. We rationalized that if AT-312 inhibits the rewarding action of alcohol via the NOP receptor, the drug would fail to alter the rewarding action of alcohol in mice lacking the NOP receptor. Consistent with this hypothesis, we observed that while the novel NOP agonist significantly reduced the rewarding action of alcohol in wild-type mice, the drug failed to alter ethanol-induced CPP in mice lacking NOP. This result suggests that AT-312 reduces the rewarding action of ethanol via the NOP receptor. However, further research is needed to assess the contribution of MOP receptor partial agonist activity in this response. Nevertheless, it is noteworthy to state that buprenorphine, a MOP partial agonist, was found to reduce alcohol consumption via its interaction with NOP (Ciccocioppo et al., 2007) and also found to reduce cocaine self-administration due to its agonist activity at the NOP and the MOP receptors (Kallupi et al., 2017).

Given that ethanol induced aversion in mice pretreated with the highest dose of AT-312 (Fig. 2), one may argue that the NOP agonist may have reduced ethanol-induced CPP by inducing aversion. However, the lower dose of AT-312 (3 mg/kg), which also completely blocked ethanol induced CPP in wild-type but not knockout mice (Fig. 3), failed to induce aversion when given with alcohol (Fig. 3) or alone (Fig. 4). This is in accord with an earlier report showing that NOP agonists may be devoid of any motivational effects (Devine et al.,

1996). Such a property could be useful to treat drug reward since the NOP agonist would not alter basal hedonic homeostasis, which may be advantageous for patient compliance and other normal daily functions.

NOP agonists are known to reduce motor activity (Devine et al., 1996). Consistent with this, we found that AT-312 suppressed motor activity in wild-type mice and this response was absent in mice lacking NOP receptor, showing that this effect of AT-312 was also mediated via the NOP receptor. It is generally accepted that drugs that reduce locomotor activity can confound behavioral responding. This may affect the outcome of the CPP response if one tests animals in the presence of a sedative drug. However, we tested the animals for CPP under a drug-free state on the postconditioning test days and found no significant differences in locomotor activity between the vehicle-conditioned and drug-conditioned groups. Additionally, we believe that not all drugs that reduce motor activity during conditioning block the CPP response. Interestingly, alcohol is sedative in mice; yet, it induces a robust CPP response. We further demonstrated this by conducting an experiment using pentobarbital, which is a known sedative hypnotic and examined its effect on ethanol-induced CPP. While pentobarbital significantly reduced locomotor activity during conditioning, it failed to alter the CPP response on the test day (Fig. 6), suggesting that the ability of AT-312 to reduce the rewarding action of ethanol may not be simply due to its sedative effect during conditioning.

The rewarding action of alcohol and other drugs of abuse has been linked to their ability to increase extracellular dopamine in the nucleus accumbens (Di Chiara and Imperato, 1988). Although the mechanism of inhibitory action of AT-312 is not clear at this time, we speculate that the NOP agonist reduces the ability of alcohol to elevate extracellular dopamine levels in the nucleus accumbens. Indeed, previous studies have shown that intracerebroventricular

administration of N/OFQ reduced elevation of accumbal dopamine induced by morphine (Di Giannuario et al., 1999) and cocaine (Lutfy et al., 2001; Sakoori and Murphy, 2004). The NOP agonist also attenuated the rewarding action of morphine (Ciccocioppo et al., 2000; Murphy et al., 1999), cocaine (Sakoori and Murphy, 2004) and ethanol (Kuzmin et al., 2003). Thus, we propose that the NOP agonist reduces dopaminergic neurotransmission by acting in the ventral tegmental area and/or nucleus accumbens to reduce the rewarding action of alcohol. However, further studies are needed to identify the neuroanatomical sites of action of the NOP agonist in this regard.

A recent report shows that NOP receptor knockout rats exhibit reduced alcohol consumption compared to their wild-type controls although saccharine intake was not different between the rats of the two genotypes (Kallupi et al., 2017). A similar reduction in ethanol self-administration was observed in rats treated with a novel orally bioavailable NOP antagonist (Rorick-Kehn et al., 2016). Interestingly, we did not observe reduced ethanol-induced CPP in mice lacking NOP although these authors found decreased ethanol self-administration in NOP knockout rats (Kallupi et al., 2017) or in wild-type rats treated with the NOP antagonist (Rorick-Kehn et al., 2016). A parsimonious explanation of such discordant effects is that the two studies measured two different responses. Notably, Kuzmin and colleagues also found that male mice lacking N/OFQ tended to show a stronger response to ethanol (Kuzmin et al., 2003). Alternately, the NOP system has been implicated in feeding, and N/OFQ has hyperphagic effects [for a review, see (Witkin et al., 2014)]. Thus, it is possible that food and drink consumption could be reduced in animals lacking the NOP receptor or its endogenous agonist. However, saccharin consumption was not altered in rats lacking NOP receptors (Kallupi et al., 2017). Nevertheless, it is possible that NOP system is involved in consumption of food and drinks with caloric values

and thus one would expect a difference in outcomes of the two studies. It is of interest to note that the novel bioavailable NOP antagonist reduced consumption of highly palatable food to the regular chow level (Statnick et al., 2016).

The other explanation for the discrepant results could be species differences in the current and earlier studies. Interestingly, we found enhanced cocaine-induced CPP in mice lacking NOP (Marquez et al., 2008), whereas these authors reported reduced cocaine-induced CPP in NOP knockout rats (Kallupi et al., 2017). The sex of animals may have contributed to this discrepant data since we used female mice in this study. Our earlier studies have shown that female mice exhibit greater ethanol-induced CPP than male mice, hence were used here (Nguyen et al., 2012). On the other hand, these other studies used male rats to study the role of NOP receptors in alcohol self-administration (Kallupi et al., 2017; Rorick-Kehn et al., 2016).

In summary, we found that a novel NOP agonist, AT-312, reduced the rewarding effects of ethanol in the CPP paradigm. The inhibitory effect of the NOP agonist was absent in NOP knockout mice, showing that the action of AT-312 was via the NOP receptor. AT-312 did not possess any motivational effects of its own at a dose that robustly reduced ethanol-induced CPP. Thus, the NOP receptor may be a potential target for the development of pharmacotherapy to treat alcohol use disorders.

Conflict of Interest

The authors declare no conflicts of interest. NTZ, WEP and MEM are employees of Astraea Therapeutics.

Author Contributions

PM, WEP and MEM conducted experiments, AH conducted genotyping and took care of the mouse breeding colony, NZ developed the compound, supervised its *in vitro* characterization and wrote the manuscript, and KL designed the CPP experiments, analyzed the behavioral data and wrote the manuscript.

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660

661 **FIGURE LEGENDS**

662 **Figure 1. Structures of NOP agonists AT-312 and SCH221510**

663 **Figure 2. The effect of AT-312, a novel NOP agonist, on ethanol-induced CPP in C57BL/6J**
664 **mice:** Data are mean (±S.E.M.) of the amount of time that mice spent in the drug-paired chamber
665 (DPCh) before (D1) (preconditioning test day) and after (D5) conditioning (post-conditioning
666 test day). Mice treated with vehicle or AT-312 (1, 3 or 10 mg/kg, s.c.) 15 min before ethanol on
667 the conditioning days. *P<0.05, indicates a significant increase in the amount of time in the
668 DPCh on D5 vs. D1; ++P<0.01, +++P<0.001, significantly different from the control group on
669 D5.

670 **Figure 3. The action of AT-312, a novel NOP agonist, on ethanol-induced CPP in mice**
671 **lacking NOP and their wild-type littermates/controls:** Data are mean (±S.E.M.) of the amount
672 of time that mice spent in the drug-paired chamber (DPCh) before (D1) (preconditioning test
673 day) and after (D5) conditioning (post-conditioning test day). Mice lacking NOP **[NOP (-/-)]** and
674 their wild-type littermates **[NOP (+/+)]** were treated with vehicle (left panel) or AT-312 (10
675 mg/kg, right panel) 15 min before ethanol on conditioning days. ***P<0.001; *P<0.05 DPCh vs.
676 VPCh

677 **Figure 4. Motivational effect of AT-312, a novel NOP agonist, in the place conditioning**
678 **paradigm:** Data are mean (±S.E.M.) of the amount of time that mice spent in the drug-paired
679 chamber (DPCh) on test days before (D1) and after (D5) conditioning. Mice received vehicle or
680 AT-312 (3 mg/kg, right panel) 15 min before saline on the conditioning days.

681 **Figure 5. Effects of SCH221510 (SCH; 10 mg/kg) on ethanol CPP in C57BL/6J mice:** Data
682 are mean (±S.E.M.) of the amount of time that mice spent in the drug-paired chamber (DPCh) on

test days before (D1) and after (D5) conditioning. Mice were treated with vehicle or SCH221510 15 min before ethanol on the conditioning days. *P<0.05, significant difference in the amount of time between D5 vs D1 for the vehicle-treated group

Figure 6. Effects of pentobarbital (Pento; 25 mg/kg) on ethanol CPP in C57BL/6J mice: Data are mean (±S.E.M.) of the amount of time that mice spent in the drug-paired (DPCh) and vehicle-paired chamber (VPCh) on test days before (D1) and after (D5) conditioning. Mice were treated with vehicle or pentobarbital 15 min before ethanol on the conditioning days. *P<0.05, significant difference in the amount of time that mice spent in the DPCh vs. VPCh on D5.

Table 1: In vitro pharmacological profile of NOP agonists in binding and functional assays at the opioid receptors*

	Receptor Binding Ki (nM)				[^{35}S] GTPγS NOP		[^{35}S] GTPγS MOP		[^{35}S] GTPγS KOP	
	NOP	MOP	KOP	DOP	EC_{50} (nM)	% Stim	EC_{50} (nM)	% Stim	EC_{50} (nM)	% Stim
N/OFQ	0.08 ± 0.03	133 ± 30	247 ± 3.4	ND	4.0 ± 0.1	100	>10,000		>10,000	
DAMGO		2.96 ± 0.54					32.6 ± 4.06	100		
DPDPE				1.11 ± 0.07						
U69,593			1.05 ± 0.02						60.14 ± 7.45	100
AT-312	0.34 ± 0.13	5.99 ± 0.97	73.5 ± 28.3	128.7± 57.4	29.9 ± 1.4	102.3 ± 0.75	81.5 ± 15.9	24.6 ± 2.4	>10,000	-
SCH221510	13.7 ± 2.30	65.4 ± 11.3	49.7 ± 11.3	403.7 ± 109.7	18.9 ± 5.9	95.1 ± 7.8	139.3 ± 4.6	76.8 ± 13.1	142.0 ± 15.6	82.72 ± 0.22

** GTP(γ)S functional assays only carried out if binding affinity Ki<100 nM. The functional efficacy at the delta opioid receptor was therefore not determined for AT-312 and SCH221510. Values are the Mean ± SEM of three independent experiments run in triplicate. Functional activity was determined by stimulation of [^{35}S]GTPγS binding to cell membranes, % stimulation was obtained as a percentage of stimulation of the standard agonists N/OFQ (for NOP), DAMGO (for MOP) and U69,593 (for KOP) taken as 100%.*

Table 2: In vivo pharmacokinetic profile of AT-312 after subcutaneous administration in mice

PK parameters	Dose, route (10 mg/kg, s.c.)
Plasma C_{max}	1263 nM
Plasma t_{max}	1 h
Brain C_{max} *	5465 nM
Brain t_{max}	1 h
Brain-to-plasma ratio at C_{max} *	4.33
Brain-to-plasma ratio AUC*	4.68

* Total brain concentrations; AUC (Area under the curve)