Nicotine increases alcohol self-administration in male rats via a μ-opioid mechanism within the mesolimbic pathway

Esi Domi1 | Li Xu1,2 | Marvin Pätz3 | Anton Nordeman1 | Gaëlle Augier1 | Lovisa Holm1 | Sanne Toivainen1 | Eric Augier1 | Anita C. Hansson3 | Markus Heilig1

1Center for Social and Affective Neuroscience, BKV, Linköping University, Linköping, S-581 85, Sweden
2Psychosomatic Medicine Center, Sichuan Academy of Medical Sciences, Sichuan Provincial People's Hospital, Chengdu, China
3Institute of Psychopharmacology, Central Institute of Mental Health, Medical Faculty Mannheim, Heidelberg University, Mannheim, Germany

Correspondence
Markus Heilig, Center for Social and Affective Neuroscience, BKV, Linköping University, Linköping, S-581 85, Sweden.
Email: markus.heilig@liu.se

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Background and Purpose: Alcohol and nicotine use disorders are commonly comorbid. Both alcohol and nicotine can activate opioid systems in reward-related brain regions, leading to adaptive changes in opioid signalling upon chronic exposure. The potential role of these adaptations for comorbidity is presently unknown. Here, we examined the contribution of μ and κ-opioid receptors to nicotine-induced escalation of alcohol self-administration in rats.

Experimental Approach: Chronic nicotine was tested on alcohol self-administration and motivation to obtain alcohol. We then tested the effect of the κ antagonist CERC-501 and the preferential μ receptor antagonist naltrexone on basal and nicotine-escalated alcohol self-administration. To probe μ or κ receptor adaptations, receptor binding and G-protein coupling assays were performed in reward-related brain regions. Finally, dopaminergic activity in response to alcohol was examined, using phosphorylation of DARPP-32 in nucleus accumbens as a biomarker.

Key Results: Nicotine robustly induced escalation of alcohol self-administration and motivation to obtain alcohol. This was blocked by naltrexone but not by CERC-501. Escalation of alcohol self-administration was associated with decreased DAMGO-stimulated μ receptor signalling in the ventral tegmental area (VTA) and decreased pDARPP-32 in the nucleus accumbens shell in response to alcohol.

Conclusions and Implications: Collectively, these results suggest that nicotine contributes to escalate alcohol self-administration through a dysregulation of μ receptor activity in the VTA. These data imply that targeting μ rather than κ receptors may be the preferred pharmacotherapeutic approach for the treatment of alcohol use disorder when nicotine use contributes to alcohol consumption.

KEYWORDS
alcohol, comorbidity, nicotine, κ receptors, μ

Abbreviations: Acb, nucleus accumbens; AcbC, nucleus accumbens core; AcbS, nucleus accumbens shell; AMG, amygdala; AUD, alcohol use disorder; BLA, basolateral amygdala; CeA, central amygdala; Cg, cingulate cortex; CPu, caudate putamen; dmPFC, dorsomedial PFC; IL, infralimbic cortex; MeA, medial amygdala; OFC, orbitofrontal cortex; PFC, prefrontal cortex; PrL, prelimbic cortex; vmPFC, ventromedial PFC; VTA, ventral tegmental area.

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1 | INTRODUCTION

Alcohol and nicotine use disorders are two of the leading preventable causes of death worldwide. These two conditions are commonly comorbid, with a large majority of people with alcohol use disorder (AUD) also being diagnosed with a comorbid addiction to nicotine (Sussman, Lisha, & Griffiths, 2011). Cigarette smoking is associated with drinking severity among people with alcohol use disorder (Grant, Hasin, Chou, Stinson, & Dawson, 2004) and increases alcohol drinking in normal subjects (Barrett, Tichauer, Leyton, & Pihl, 2006). Nicotine use also predicts higher addiction rates, higher severity and also poorer pharmacological treatment outcomes in alcohol use disorder (Fucito et al., 2012).

Consistent with the human research, preclinical studies show that exposure to nicotine increases alcohol self-administration in non-dependent (Olausson, Ericson, Lof, Engel, & Soderpalm, 2001), alcohol-dependent (Leao et al., 2015) and also alcohol-prefering rats (Hauser et al., 2012). Possible biological mechanisms proposed as a basis for this interaction mainly include the stress hormone system and adaptations in the mesolimbic dopamine system (Doyon et al., 2013). Nicotine-alcohol interaction has also been shown to activate interconnected brain regions involved in stress and reward processes such as nucleus accumbens core (AcbC) and nucleus accumbens shell (AcbS), ventral tegmental area (VTA), prefrontal cortex (PFC) and amygdala (AMG) (Leao et al., 2015). However, despite these advances, it remains unclear how nicotine facilitates the transition to excessive drinking.

Endogenous opioid systems play a pivotal role in the reinforcement and motivational aspects of alcohol and nicotine addiction (Trigo, Martin-Garcia, Berrendero, Robledo, & Maldonado, 2010). Chronic exposure to nicotine or alcohol induces profound and specific changes in the expression and release of β-endorphin, dynorphin and their corresponding receptors, depending on the various stages of the addiction process (for review, see Drews & Zimmer, 2010). Opioid receptors and their peptide ligands are directly or indirectly affected by drugs of abuse (Kieffer & Evans, 2009). Conversely, µ opioid receptor antagonism reduces alcohol drinking and relapse-like behaviours in rodents (Dhaher et al., 2012; Stromberg, Casale, Volpicelli, Volpicelli, & O’Brien, 1998), as well as relapse to heavy drinking and alcohol craving in humans (Jonas et al., 2014; King et al., 2012; O’Malley, Krishnan-Sarin, Farren, Sinha, & Kreek, 2002). Pharmacological studies also provide evidence for µ receptor blockade to attenuate nicotine reinforcement. For instance, the µ receptor antagonist, glycyglutamate, blocks conditioned place preference to nicotine and mecamylamine-induced nicotine withdrawal in rats (Goktalay, Cavun, Levendusky, Hamilton, & Millington, 2006).

In contrast, interventions targeting κ opioid receptors influence alcohol taking without influencing the reinforcing properties of nicotine. κ receptor agonists can reduce ethanol reinforcement in non-dependent animals, but this is mediated through their aversive effects (Noble, Lenoir, & Marie, 2015), which also render them unsuitable as clinical therapeutics (Bals-Kubik, Ableitner, Herz, & Shippenberg, 1993). More importantly, κ antagonists attenuate alcohol reward but do not reduce the rewarding properties of nicotine in preclinical models. In an additional and potentially important distinction, κ antagonism specifically attenuates escalated alcohol self-administration but does not affect basal consumption (Kissler et al., 2014; Walker, Valdez, McLaughlin, & Bakalkin, 2012). This suggests that κ antagonists may have a potential as therapeutics in alcohol use disorder, where consumption typically has escalated beyond initial levels.

First-generation κ antagonists were unsuitable candidates for clinical development, because of a slow onset of action and long-lasting effects (Bruchas et al., 2007). Recently, selective, short-acting κ antagonists with acceptable human safety and tolerability have become available, with CERC-501 as an example of this class (Lowe et al., 2014). Using CERC-501, we found that κ receptor antagonism suppressed escalated drinking induced by intermittent two-bottle choice procedure while leaving baseline consumption unaffected (Domi et al., 2018).

Escalation of alcohol drinking is a key clinical feature in alcohol use disorders and extensive work has been devoted by the alcohol field to modelling this phenomenon in rodents (Becker & Ron, 2014). Along this line, our aim here was to investigate the role of CERC-501 and the preferential µ receptor antagonist, naltrexone, in a model of nicotine-induced escalation of alcohol self-administration and motivation to obtain alcohol (Le, Wang, Harding, Juztsch, & Shaham, 2003).

Behavioural pharmacology experiments were combined with an analysis of receptor binding and G-protein coupling in brain regions involved in drug addiction: AcbC, AcbS, dorsomedial and ventromedial PFC (dmPFC and vmPFC), orbitofrontal cortex (OFC), basolateral AMG (BLA), central AMG (CeA), medial AMG (MeA) and VTA.
Moreover, we measured phosphorylation of DARPP-32, which is reliably triggered by dopamine release and D1 receptor activation, as a biomarker to assess whether nicotine-induced escalation of drinking involves modulation of dopaminergic activity in the nucleus accumbens (Acb) in response to alcohol. Control behaviours, including saccharin self-administration and locomotor activity, were evaluated to determine the specificity of the results of nicotine-induced escalation of alcohol self-administration.

2 | METHODS

2.1 | Animals

Male Wistar rats were used to enable comparison of the current results with prior literature on models of escalated alcohol self-administration induced by nicotine (Le et al., 2003).

A total of 175 male Wistar rats (Charles River), weighing 250–300 g (7–9 weeks) at the beginning of the experiments, were pair-housed with free access to tap water and food pellets. Rats were maintained in a temperature- and humidity-controlled vivarium on a 12-h light/dark cycle (lights off at 7:00 a.m.). Behavioural experiments were conducted during the dark phase. Rats were handled three times before each experiment. All animal care and experiments were conducted in accordance with the Swedish National Committee for animal research, and the protocol was approved by the Local Ethics Committee for Animal Care and Use at Linkoping University. Animal studies are reported in compliance with the ARRIVE guidelines (Kilkenny, Browne, Cuthill, Emerson, & Altman, 2010) and the editorial on reporting animal studies (McGrath & Lilley, 2015) with the recommendations made by the British Journal of Pharmacology.

2.2 | Alcohol self-administration

Operant training and testing were performed in the operant chambers (Med Associates Inc., St Albans, VT, USA; 30.5 × 29.2 × 24.1 cm) housed in sound-attenuating cubicles. Each operant chamber was equipped with two retractable levers positioned laterally to a liquid cup receptacle. Animals were trained to self-administer 20% (v/v) alcohol without sucrose/saccharin fading as described (Augier et al., 2014; Augier, Dulman, Singley, & Heilig, 2017; Simms, Bitto-Onon, Chatterjee, & Bartlett, 2010). Rats were initially trained to lever press on a fixed ratio 1 (FR1) 5-s time-out (TO) schedule to self-administer 20% alcohol during 30-min sessions. Two levers were extended to mark the onset of the session and to signal alcohol availability. Pressing once on the lever associated with alcohol (active) was reinforced by the delivery of 100 μl of 20% alcohol in water in the adjacent drinking well and initiated a concomitant 5-s time-out period signalled by the illumination of the cue light above the lever. Responses on the inactive lever and during the time-out period were recorded but had no programmed consequences. Sessions were conducted 5 days a week until performance stabilized (defined as a minimum of 15 sessions and no change greater than 15% in the total number of reinforcers earned during the last three sessions). Once a stable self-administration baseline was reached, the sessions were conducted under a fixed ratio 2 (FR2) until a stable baseline of lever pressing was achieved (defined as for fixed ratio1). After establishing a baseline of fixed ratio 2, rats were treated with either saline or nicotine 0.8 mg kg⁻¹ solution s.c. 20 min prior to the session, for 14 consecutive days as previously described (Le et al., 2003; Leao et al., 2015). Animals that did not acquire the self-administration procedure or earned less than 10 reinforcers during the 30 min session at the baseline were excluded from the study. Motivation of the animals to obtain alcohol was measured using a progressive ratio schedule (Hodos, 1961) under the same experimental conditions as those used in the fixed ratio schedule, except that the response requirement or cost was increased within session after each reinforcer earned, according to the following formula: 1, 2, 3, 4, 6, 8, 10, 12, 16, 20, 24, 28, 32 ... The progressive ratio session terminated after 30 min had elapsed without a reward. The break point was defined as the last completed response requirement during the progressive ratio session.

2.3 | Saccharin self-administration

Saccharin self-administration was performed under the same conditions as for alcohol self-administration. Briefly, rats were trained to self-administer 0.2% saccharin in 30-min sessions under an fixed ratio2 5-s time-out schedule of reinforcement. Once a stable self-administration baseline was reached (a total of 15 fixed ratio2 sessions), rats were assigned to nicotine or saline treatments based on their baseline response and further tested for 14 additional sessions.

2.4 | Locomotor activity

The distance travelled (cm) was determined in the operant chambers using MED-PC IV (Med Associates, St. Albans, VT, USA). During the self-administration session, each time a rat broke the infrared beams localized at each side of the operant box represented an event recorded automatically by the program. For every movement from one beam to another, one move of 18.1 cm (the distance between the two infrared beams) was counted. Every event occurring at the same times (two at the same second, either from the same infrared or from two different) was considered duplicate and removed from the total count. The effect of naltrexone on locomotor activity was examined for 30 min (to match the duration of the alcohol self-administration sessions) in sound-attenuated chambers equipped with an open field (43 × 43 cm) containing infrared beam detectors MED-PC IV (Med Associates). The automated system analysed total distance (horizontal locomotor activity) in the arena as a locomotion index across every 5-min bin. After each trial, the field was cleaned with water and dried using paper towels.
2.5 | Blood alcohol concentration

Blood samples (100 μl) taken from the rat tail vein were spun down in a centrifuge (15 min, 2,000 g), and the plasma samples were stored at −20°C until determination of blood alcohol concentrations (BACs). Alcohol content was then assayed in duplicates, from 5 μl plasma aliquots using an oxygenate alcohol analyser (Analog Instruments, Street Stourbridge, England). Data are expressed as mg dl−1.

2.6 | Tissue preparation

Rats (N = 32) after alcohol (n = 16) or saccharin self-administration (n = 16) received chronic treatment with saline (n = 8) or nicotine (n = 8) for 14 days as described above and were used for the receptor autoradiography and GTPγS assays at the end of the behavioural procedure. Rats were anesthetized by isoflurane 20 min after the last nicotine injection (i.e. at the time the escalated self-administration session would have started, no alcohol was self-administered that day, to avoid confounding short-term effects of differential alcohol self-administration) and killed by decapitation. Brains were quickly removed, flash frozen in isopentane at −40°C, and stored at −80°C until further usage. Coronal brain sections were taken from the PFC (cingulate cortex [Cg]; prelimbic cortex [PrL]; infralimbic cortex [IL]; OFC, bregma levels: +2.7/+3.7); caudate putamen (CPu), AcbC, AcbS, bregma levels: +1.2/+2.2, BLA, CeA, MeA, bregma levels: −2.8/−3.3; and VTA bregma levels: −5.2/−5.6 according to the rat brain atlas (Paxinos & Watson, 2005). The sections were obtained with a cryostat (Leica CM1950) with a thickness of 12 μm and mounted onto gelatin-coated glass slides.

μ and κ receptor autoradiographies and DAMGO-stimulated μ receptor GTPγS assay were performed as previously described (Bjork et al., 2013; Hermann et al., 2017). For details, see Supporting Information.

2.7 | U50,488H-stimulated κ receptor G-protein coupling (GTPγS assay)

Slides were rinsed in preincubation buffer (50 mM Tris-HCl; 3 mM MgCl2; 0.2 mM EGTA; 100 mM NaCl; pH = 7.4) for 10 min at room temperature. To stop the reaction, slides were dipped in ice-cold washing buffer (50 mM Tris-HCl; 3 mM MgCl2; 0.2 mM EGTA; 100 mM NaCl; pH = 7.4) for 10 min at room temperature. After completing receptor autoradiographies or [35S]-GTPγS assays, Fujifilm BAS imaging plates (Fujifilm, Tokyo, Japan) were exposed to the sections. Imaging plates were scanned with a phosphoimager (Typhoon FLA 700, GE Healthcare, Germany). The mean grey values were measured using the MCID software (MCID Image Analysis Software Solutions for Life Sciences). For receptor-binding studies, non-specific binding (residual binding in the presence of saturating concentrations of cold antagonist) was subtracted from total binding to obtain specific binding. For the activity assays, mean grey values were measured of the [35S]-GTPγS binding under basal and stimulated conditions. The percentage of stimulation was calculated for every region and animal. Autoradiography and GTPγS data were plotted to a [3H]- and [14C]-standard, respectively. Density values of the receptor autoradiographs in (fmol mg−1) was calculated based on the specific activity of the radioligand and the saturation binding equation (B = Bmax*[R]/(Kd + [R]), solving for Bmax, Bmax = maximal bound receptor, Kd = receptor affinity, nM). Data were expressed as fmol mg−1 protein (mean ± SEM) (Bjork et al., 2013; Hermann et al., 2017; Hirth et al., 2016; Sommer, Costa, & Hansson, 2014). Density values of the [35S]-GTPγS assays were presented in nCi g−1 (mean ± SEM) (Bjork et al., 2013). Measurements could only be taken when regions of interest were clearly visualized; when the group size was lower than n = 5, the data are considered as preliminary and were not analysed. Because of the individual differences, stimulated GTPγS values are presented as % stimulation and not as raw stimulated values in Tables S2 and S4. Raw values and statistical analysis of μ receptor, κ receptor binding, basal levels and DAMGO-stimulated μ receptor, or U50,488H-stimulated κ receptor GTPγS binding in CG, OFC, PrL, IL, CPu, AcbC, AcbS, BLA, MeA, CeA, and VTA are provided in Table S4.

2.8 | Image analysis

The immuno-related procedures used comply with the recommendations made by the British Journal of Pharmacology (Alexander et al., 2018). Rats (N = 18) were deeply anesthetized with isoflurane and transcardially perfused with 0.9% saline followed by 4% paraformaldehyde (PFA). Brains were removed and postfixed in 4% PFA for 2 h and then transferred into 30% sucrose solution till sinking. Coronal brain sections (20 μm) were processed in a cryostat microtome and stored in cryoprotectant.

Floating brain sections were washed in PBS 3 × 10 min and then blocked in a solution of 4% BSA and 0.2% Triton X-100 dissolved in PBS for 1 h at room temperature. For labelling p DARPP-32 and
DAPI-32, a monoclonal rabbit anti-pDARPP-32 (Cell Signaling Technology Cat# 12438, RRID:AB_2797914, 1:200 dilution) and a polyclonal rabbit anti-DARPP-32 (Cell Signaling Technology Cat# 2306, RRID:AB_823479, 1:200 dilution) were used as the primary antibodies. Sections were incubated in primary antibodies separately for 2 h at room temperature. Sections were rinsed in PBS three times, mounted on slides and coverslipped with prolonged diamond antifade mountant with DAPI (P36962, Invitrogen). All images were acquired through a Zeiss LSM 780 upright confocal microscope (20x magnification). Quantification of pDARPP-32 and DARPP-32 was carried out with ImageJ 1.48 software (ImageJ, RRID:SCR_003070). Images of AcbS and AcbC (1,024 × 1,024 μm) were taken unilaterally from a minimum of three sections across two slides from each animal at levels corresponding to 1.2–1.70 mm anterior to bregma (Paxinos & Watson, 2005). Cell counts were averaged across sections for each animal. Individual cells were identified based on DAPI staining of the nuclei. The percentage of cells expressing pDARPP-32 or DARPP-32 was determined by dividing the number of positive cells by the total number of DAPI-labelled nuclei in the same image.

2.10 | Materials

Nicotine hydrochloride tartrate salt (Sigma-Aldrich, Stockholm, Sweden) was dissolved in saline, and the dose 0.8 mg kg\(^{-1}\) (expressed as the free base) was injected s.c. 20 min prior to self-administration sessions. Alcohol solution (20%) was prepared volume/volume (v/v) in tap water from 95% alcohol. Naltrexone, \(K_d = 3.1 \pm 0.32\) [nM] 1997, (Sigma-Aldrich) was dissolved in a sterile saline solution and administered s.c. at the volume of 1 ml kg\(^{-1}\) at the doses 0, 0.3, and 1 mg kg\(^{-1}\). CERC-501, \(K_d = 0.8 \pm 0.2\) [nM] (Rorick-Kehn et al., 2014) (CERECOR; Baltimore, USA), was dissolved in distilled water with the addition of 85% lactic acid and administered p.o. at the volume of 1 ml kg\(^{-1}\) at the doses 0.3, and 10 mg kg\(^{-1}\). CERC-501 and naltrexone doses were selected based on prior work (Rorick-Kehn et al., 2014; Williams & Broadbridge, 2009). Drugs were prepared freshly on the experimental day, and rats were habituated to the route of the administration before the test.

For the analysis of \(\mu\) and \(\kappa\) receptor binding and coupling assays, we used the \(\mu\) receptor agonist DAMGO ([\(\text{Tyrosyl-3,5-3H}\)] , \(K_d = 0.7 \pm 0.1\) [nM]; Sharif & Hughes, 1989; Tocris, Bristol, UK), \([3H]-\text{DAMGO}\) (specific activity 50–51 Ci mmol\(^{-1}\); PerkinElmer, Massachusetts, USA) and the \(\kappa\) agonist U50,488H (\(K_d = 3.8 \pm 0.2\) [nM]; Kim, Eun, Soh, Eun, & Cho, 1996; Tocris), \([3H]-\text{U50,488H}\) (specific activity 44.6 Ci mmol\(^{-1}\); PerkinElmer). To determine non-specific bindings, unlabelled selective \(\mu\) receptor antagonist CTOP (\(K_d = 0.96\) nM) and \(\kappa\) antagonist nor-BNI (\(K_d = 0.15 \pm 0.1\) [nM]) were used (Tocris). \([35S]-\text{GTP}\gamma\text{S}\) and GDP were obtained from PerkinElmer and Sigma-Aldrich Chemie GmbH Germany, respectively.

To label pDARPP-32 and DARPP-32, we used a monoclonal rabbit anti-pDARPP-32 (Thr 34) and a polyclonal rabbit anti-DARPP-32. As a secondary antibody, we used Alexa Fluor 555-labelled donkey anti-rabbit.

2.11 | Blinding and randomization

The laboratory animals were assigned to the experimental groups randomly. The laboratory animals responses, the treatments were assessed blindly. All samples were analysed in a blinded manner.

2.12 | Data and statistical analysis

The data and statistical analysis comply with the recommendations of the British Journal of Pharmacology on experimental design and analysis in pharmacology (Curtis et al., 2018). Statistical analysis was carried only for groups with a group size of \(n \geq 5\), and the experimental groups were designed accordingly, using randomization. The sample sizes and animal numbers were determined by power analysis of pre-existing data. Data were analysed to confirm normal distribution (Shapiro–Wilk test), and the homogeneity of variance was confirmed by Levene’s or Bartlett’s test. The declared group size represents the number of independent values, on which the statistical analysis was performed.

The effects of nicotine on alcohol, saccharin self-administration, and locomotor activity were analysed using repeated measures ANOVA with time (sessions) as a within-subject factor and pretreatment (nicotine vs. saline treatment) as a between-subject factor. Drinking latency, front-loading response, blood alcohol concentrations, DARPP-32 phosphorylation, and total DARPP in AcbC and AcbS were analysed using unpaired Student’s t-test. The effects of naltrexone and CERC-501 on escalated alcohol self-administration and motivation to self-administer alcohol were evaluated using factorial ANOVA with pretreatment (CERC-501 vs. saline or naltrexone vs. saline) and treatment (nicotine vs. saline) as between-subject factors. The autoradiographic image analysis was carried out using factorial ANOVA with treatment (nicotine vs. saline) and self-administration condition (alcohol vs. saccharin) as between-subject factors. All post hoc analyses were conducted using a Newman–Keuls test only when the F value attained \(P < 0.05\), and there was no significant inhomogeneity of variances. For determining whether groups differ, the level of probability (\(P\)) was set at \(P < 0.05\) for constituting the threshold for statistical significance. The data are presented as the mean ± SEM. Data were analysed using STATISTICA, Stat Soft 13.0 (RRID:SCR_014213). Statistical details including the degrees of freedom and \(F\) and \(P\) values are reported in the Tables S5 and S6.

2.13 | Data transformation

Agonist-stimulated GTP\gamma\text{S} binding was calculated as per cent of baseline value in the same region and animal. Values were expressed
as % stimulation and statistically compared region-wise. Additional analysis of the effects of naltrexone are presented in the Supporting Information relatively (as % variation) to the corresponding controls. No data were excluded.

2.14 Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Harding et al., 2018), and are permanently archived in the Concise Guide to PHARMACOLOGY 2019/20 (Alexander et al., 2019).

3 RESULTS

3.1 Effects of the κ receptor antagonist CERC-501 on nicotine-induced escalation of alcohol self-administration and motivation to obtain alcohol

In the absence of other treatment, nicotine ($n = 30$) induced a transient decrease in alcohol self-administration compared to saline ($n = 29$), followed by a robust escalation that lasted throughout the experiment. There was a decrease in the number of reinforcers earned on the first nicotine treatment day, followed by a robust escalation of alcohol self-administration from day 7 that persisted until the end of the treatment (Figure 1a).

After stable escalation was obtained in the nicotine group, rats receiving saline and nicotine, respectively, were further divided into three subgroups each and were treated p.o. with vehicle or CERC-501 (3 or 10 mg kg$^{-1}$) 45 min prior to nicotine ($n = 10$ per group), or saline injections ($n = 10, 9$ and $10$ for 0, 3 and 10 mg kg$^{-1}$, respectively). CERC-501 had no significant effect on basal or nicotine-induced escalated alcohol self-administration. Although a trend for a suppression was present, this was primarily driven by a decrease in non-escalated self-administration (Figure 1b). Inactive lever pressing was not significantly affected by CERC-501 or nicotine or their interaction (Figure 1c).

Finally, nicotine markedly enhanced motivation to obtain alcohol, measured using a progressive ratio schedule, but this was unaffected by CERC-501 (Figure 1d).

3.2 Effects of the μ receptor-preferring antagonist naltrexone on nicotine-induced escalation of alcohol self-administration and motivation to obtain alcohol

After re-establishing a baseline of alcohol self-administration, rats treated with nicotine ($n = 39$) were divided into three subgroups ($n = 13$) and treated with naltrexone (0, 0.3, or 1.0 mg kg$^{-1}$) s.c. 30 min prior to nicotine or saline injections ($n = 38; n = 13, 12$, and $13$ for...
each dose, respectively). The number of alcohol rewards was significantly higher in the nicotine-treated group compared to saline. Naltrexone (0.3 and 1 mg kg\(^{-1}\)) suppressed both basal and nicotine-induced escalated alcohol self-administration (Figure 2a). There was no significant effect of neither nicotine nor naltrexone treatment on the inactive lever (Figure 2b). The effect of naltrexone was also analysed as % decrease compared to the saline or nicotine vehicle groups. Nicotine significantly increased the % number of reinforcers. Naltrexone (0.3 and 1 mg kg\(^{-1}\)) reduced % decrease from control in both basal and nicotine-induced escalated alcohol self-administration Figure S1a.

Naltrexone also reduced basal and nicotine-induced escalated break points for alcohol (Figure 2c). The data expressed as % decrease of progressive ratio responding to vehicle groups are shown in Figure S1b.

To examine the behavioural specificity of naltrexone on alcohol self-administration, we assessed its effect on general locomotor behaviour (n = 18). Repeated measures ANOVA showed no significant effect of naltrexone on locomotor activity (Figure S2).

### 3.3 Effects of chronic nicotine on operant responding for saccharin reinforcement and locomotor activity

In a separate cohort of rats (n = 32 per group), nicotine induced an escalation of alcohol self-administration similar to that reported above; a transient decrease in the number of reinforcers the first day and an escalation that became significant on day 6 and lasted throughout the experiment (Figure 3a). We further tested whether nicotine-induced escalation was specific to alcohol by assessing the effects of nicotine treatment on 0.2% saccharin self-administration. In contrast to the effects on alcohol self-administration, chronic nicotine only induced a transient decrease of saccharin self-administration that was not followed by an escalation. Saccharin responding decreased the first 3 days of nicotine treatment and then returned to a level indistinguishable from that of saline-pretreated rats (Figure 3b).

To exclude the possibility of a ceiling effect in the saccharin group and ensure that nicotine-induced escalation was behaviourally specific to alcohol, we also tested the effect of nicotine in a separate batch of rats (n = 9 per group), using a lower concentration of saccharin (0.02%) chosen to produce similar rate of responses to the ones produced with alcohol self-administration (Pucilowski, Overstreet, Rezvani, & Janowsky, 1993). Using this solution as reinforcer, rats reached a stable baseline of 52.6 ± 6.2 reinforcers on the fixed ratio 2 schedule of reinforcement and then underwent nicotine treatment for 14 days. Chronic nicotine induced a transient decrease of saccharin self-administration; in contrast to the effects on alcohol self-administration, this was not followed by an escalation (Figure S3a). Nicotine also left motivation for saccharin responding unaffected (Figure S3b).

Drinking latency was defined as the average time to the first alcohol response on the last 4 days of escalated alcohol self-administration. Nicotine significantly reduced this measure in alcohol self-administration without affecting saccharin (Figure S4a,b).

We also analysed the patterns of self-administration for both alcohol and saccharin sessions (depicted as mean number of reinforcers per 5-min bins, earned during the last four stabilized sessions before and after escalation, n = 6 per group). Nicotine increased the front-loading response specifically during alcohol self-administration when compared to saline controls. During alcohol, but not saccharin...
sessions, the number of reinforcers earned during the first 5 min was significantly higher in the nicotine-treated rats (Figure 3c,d). Representative patterns for alcohol and saccharin self-administration are shown in Figure 3e,f.

A separate cohort of rats (n = 9 per group) was used to measure blood alcohol concentrations after nicotine-induced escalation of alcohol self-administration. Nicotine-treated rats had higher blood alcohol concentrations compared to saline control. Moreover, blood alcohol concentrations showed a positive and significant correlation with the number of alcohol reinforcers obtained during the 30-min self-administration session (Figure S4c,d).

Chronic nicotine did not significantly affect locomotor activity during alcohol self-administration (Figure 3g). Nicotine only transiently affected locomotor activity during saccharin self-administration, decreasing it during the first 2 days, while no significant differences were present throughout the remainder of the procedure (Figure 3h).
3.4 | Effects of alcohol and nicotine on µ receptor binding and signalling in the VTA

Rats exposed to alcohol (n = 16) or saccharin (n = 16) and treated with nicotine (n = 8) or saline (n = 8) were sacrificed for the autoradiographic assay after the last nicotine injection (see schematic representation in Figure S5).

µ binding in the VTA was differentially affected by alcohol and nicotine. Alcohol self-administration decreased binding, while nicotine increased it (Figure 4a).

DAMGO-stimulated µ receptor signalling in the VTA was decreased in both saccharin and alcohol self-administering nicotine-treated rats. Alcohol self-administration alone induced a similar decrease of µ receptor signalling when compared to the saccharin control group. In the saccharin control, stimulated binding was decreased (34%) by nicotine treatment. The combination of alcohol and nicotine induced a profound decrease in DAMGO-stimulated µ receptor-GTPγS binding (50%), when compared to nicotine or alcohol alone (Figure 4b).

3.5 | Effects of alcohol and nicotine on µ receptor binding and signalling in the Acb and CPu

In the AcbS, alcohol self-administration, compared to saccharin, resulted in decreased µ receptor binding irrespective of nicotine, while nicotine produced a modest reduction in µ receptor binding in the saccharin group only. Alcohol self-administration significantly decreased µ receptor binding compared to saccharin self-administration, both in the saline and the nicotine group, while nicotine only decreased µ receptor binding in saccharin self-administering animals (Figure 5a).

DAMGO-stimulated µ receptor signalling in the AcbS was increased by alcohol self-administration but was unaffected by nicotine (Figure 5b).

Alcohol self-administration decreased µ receptor binding in the AcbC, while nicotine did not affect it (Figure 5c).

However, DAMGO-stimulated µ receptor signalling in the AcbC was not influenced neither by alcohol self-administration nor nicotine (Figure 5d). In the CPu, alcohol self-administration or nicotine pre-treatment did not affect µ receptor binding or DAMGO-stimulated µ receptor signalling (Figure 5e,f).

Results of κ receptor binding and U50,488H-stimulated κ receptor GTPγS in the VTA and AcbS are provided in Figure S6.

3.6 | Effects of chronic nicotine on alcohol-induced phosphorylation of DARPP-32 in the AcbS

Analysis of DARPP-32 phosphorylation requires rapid tissue processing to avoid dephosphorylations, which put practical limitations on the size of DARPP-32 phosphorylation experiments. We therefore first carried out preliminary experiments (n = 5 per group), in which we assessed the ability of our alcohol challenge dose alone (alcohol 20% [v/v], 0.5 g kg⁻¹) to induce DARPP-32 compared to a saline challenge control (Figure S7). The dose 0.5 g kg⁻¹ was chosen in order to match the amount of alcohol (g kg⁻¹) self-administered during the 30-min session in the non-escalated rats calculated based on the number of alcohol reinforcers earned and their body weight. In
a separate cohort of rats \( n = 9 \) per group, we compared pDARPP-32 following alcohol challenge between nicotine-escalated and non-escalated rats. In AcbS, nicotine-escalated rats showed a decreased pDARPP-32 response to alcohol (Figure 6a), and there was no effect of escalation in AcbC (Figure 6b).

Total DARPP-32 was not affected by the escalation neither in AcbS nor in AcbC (Figure S8a,b).

4 | DISCUSSION

We report that chronic non-contingent nicotine administration elicited a robust escalation of alcohol self-administration and increased the motivation to obtain alcohol in male rats. This effect was behaviourally specific for alcohol. Nicotine did not affect locomotion or saccharin reinforcement, essentially excluding the possibility.
that non-specific hyperactivity or globally altered hedonic behaviour account for the observations. Escalation of alcohol self-administration was reduced by blockade of \( \mu \) but not \( \kappa \) receptors. The combination of nicotine and alcohol was accompanied by \( \mu \) receptor desensitization in the VTA and a blunted dopamine-dependent response to alcohol in AcbS. This suggests that nicotine may attenuate the reinforcing value of alcohol and promote escalation of alcohol self-administration in order to restore its reinforcing value.

We found increased alcohol self-administration induced by nicotine, similar to what has been reported previously in preclinical (Le, Funk, Lo, & Coen, 2014) and clinical research (Barrett et al., 2006). Consistent with previous studies, increased alcohol self-administration emerged after approximately 1 week of nicotine treatment (Le et al., 2003). Developing tolerance to the chronic effects of nicotine can lead to an escalation of alcohol self-administration (Perkins, 2002). However, repeated nicotine administration did not result in reduced locomotor activity during escalated drinking, showing a possible cross-sensitization, rather than a cross-tolerance between the drugs. The latency to initiate alcohol lever pressing was significantly reduced by nicotine, potentially corresponding to an increased urge to drink observed in people with alcohol use disorder who are also smokers (Verplaetse & McKee, 2017).

Neuroadaptations within the opioid system that are induced by chronic alcohol or nicotine, together with effects of opioid agonists and antagonists on the rewarding properties of both drugs, have previously identified the opioid system as a potential treatment target in alcohol and nicotine addiction (Drews & Zimmer, 2010). Antagonism of \( \mu \) and \( \kappa \) receptors has shown efficacy in reducing alcohol intake in rodents (Walker & Koob, 2008). For \( \mu \) antagonism, this is also the case in people with alcohol use disorder (Jonas et al., 2014). The \( \mu \) receptor-preferring antagonist naltrexone is approved for clinical treatment of alcohol addiction, while \( \kappa \) blockade prevents escalated drinking induced by prolonged alcohol exposure or stress, without affecting basal alcohol consumption (Walker & Koob, 2008).

Similarly, we recently reported that the selective \( \kappa \) antagonist, CERC-501, reduced escalated alcohol self-administration induced by intermittent access to alcohol 20%, while showing less of an effect on non-escalated drinking (Domi et al., 2018). In contrast, our present study did not find a preferential activity of CERC-501 on escalated alcohol self-administration when escalation resulted from nicotine treatment. Of importance, a recent study in a human laboratory model of smoking behaviour did not support a role for CERC-501 in the treatment of nicotine use disorder (Jones et al., 2019). Consistent with our previous data, CERC-501 did not alter basal levels of drinking. Moreover, a recent study reported that the irreversible \( \kappa \) antagonist, nor-BNI, further increased drinking in a model of concurrent i.v. alcohol and nicotine self-administration (Larraga, Belluzzi, & Leslie, 2017). These findings prompt caution when considering \( \kappa \) antagonists...
as a treatment option for people with alcohol use disorder who smoke, who represent approximately 80% of the population afflicted with alcohol use disorders (John et al., 2003).

In contrast to CERC-501, the μ receptor-antagonist naltrexone dose-dependently reduced baseline alcohol self-administration as well as nicotine-induced escalation of alcohol self-administration. Multiple lines of evidence support a role of μ receptors in the reinforcing properties of alcohol and nicotine (for review, see Berrendero, Robledo, Trigo, Martín-Garcia, & Maldonado, 2010; Hansson et al., 2018; Nutt, 2014). μ antagonists or genetic deletion of the receptor has been shown to abolish nicotine-induced conditioned place preference (Walters, Cleck, Kuo, & Blendy, 2005). For naltrexone, approved for treatment of alcohol use disorder, mixed results have been reported with regard to nicotine use disorder, both in preclinical and clinical research (David, Lancaster, Stead, & Evins, 2006; King & Meyer, 2000). However, naltrexone may preferentially improve smoking quit rates in heavy drinkers and is more effective in treating alcohol use disorder in nicotine users (King, Cao, Vanier, & Wilcox, 2009). Recent clinical findings provided evidence for a greater naltrexone efficacy in reducing heavy drinking in nicotine users (Anton et al., 2018). Accordingly, our results support the notion that people with alcohol use disorder who are also nicotine users would be particularly likely to benefit from naltrexone treatment. Notably, alcohol and nicotine interactions may cause sexually dimorphic effects on the endogenous opioid system that could result in differential effects of naltrexone in male and female subjects; therefore, findings from this study might not apply to females.

Several studies have investigated the effects of chronic nicotine on the densities, affinities, and functional activities of μ and κ receptors (Berrendero et al., 2010). For instance, it has been shown that 14 days of nicotine treatment induces an upregulation of μ receptors in the striatum of female rats (Wewers, Dhatt, Snively, & Tejwani, 1999). Moreover, an uncoupling and desensitization of κ receptors in the striatum and Acb has been observed during nicotine withdrawal without showing altered densities of the receptors (McCarthy, Zhang, Neff, & Hadjiconstantinou, 2011). However, no data have, to our knowledge, been available on possible adaptations of μ and κ receptor binding and signalling during concomitant use of alcohol and nicotine.

In the present study, the main changes in alcohol-escalated rats occurred within the mesolimbic system, where the drug combination affected the binding and signalling of both μ and κ receptors. Opioid receptors are present in areas of the brain involved in reward processing, such as VTA, Acb, PFC and extended AMG (Mansour, Fox, Burke, Akl, & Watson, 1995). These brain areas have also shown increased activity in a model of transition to compulsive alcohol drinking mediated by chronic nicotine exposure (Leao et al., 2015). Our data suggest that following chronic nicotine treatment during alcohol self-administration, μ receptors in the VTA may undergo desensitization through decreased G-protein coupling. Chronic nicotine and alcohol both reduced DAMGO-stimulated μ receptor [35S]-GTPyS binding and this reduction was additive. Our observation that decreased signalling was accompanied by increased μ receptor binding sites suggests that receptor binding sites may be up-regulated to compensate for the possible desensitization but that this compensation is only partial, as the net effect is nevertheless down-regulated signalling. A possible desensitization or constitutive signalling of the receptor may occur through uncoupling of μ receptors from G proteins. Chronic nicotine may also cause a down-regulation of the G-protein-coupled receptor kinase 2 and thereby significantly impede the ability of DAMGO to promote μ receptor endocytosis (He & Whistler, 2011). The lack of correlation between changes in the signalling and density of μ receptors supports the generally accepted notion that in different brain areas, the kinetics of desensitization might not overlap with binding, and this might be due to receptor uncoupling (Luttrell & Lefkowitz, 2002).

We found that alcohol alone, but not nicotine, increased μ receptor coupling and decreased binding specifically in AcbS; there were no receptor changes in the core. Contrary to μ, κ receptor coupling in the shell was reduced by alcohol alone. Furthermore, alcohol alone increased U50,488H-stimulated κ receptor G-protein coupling in the AcbC, which was attenuated by chronic nicotine. It is, however, in our view unlikely that this adaptation is involved in escalation of alcohol self-administration, since the κ antagonist CERC-501 did not affect nicotine-induced escalation of drinking. There were no changes in neither κ receptor binding nor signalling within the VTA and there were no changes of and κ receptors in the CPUs.

The neuroadaptations of μ and κ receptor systems in alcohol dependence remain debated. An influential PET study using the selective μ receptor ligand [11C]-carfentanil reported stable elevations of μ receptors in the striatum of people with alcohol use disorder following initiation of abstinence and these elevations correlated with self-reports of alcohol craving (Heinz et al., 2005). It has since been argued that measures of [11C]-carfentanil binding to assess receptor densities in PET studies may in part also be influenced by changes in endogenous opioid release. In contrast to the PET data, decreased rather than increased μ receptor binding was found in striatal post-mortem brain tissue in both the ventral striatum and caudate of alcohol-dependent patients, while κ receptors were not affected by disease state in that analysis (Hermann et al., 2017).

Binding capacity remains an important metric, but the functional consequences of neuroadaptations encompassing opioid receptors are ultimately determined by the magnitude of intracellular signals transduced in response to ligand-mediated receptor activation. In the present study, we used [35S]-GTPyS binding, a classical measure of G-protein coupling and signalling (Harrison & Traynor, 2003). A limitation of our analysis is that it was only carried out under saturated conditions, thus precluding us from detecting any potential effects that might result from changes in ligand binding affinity. At these saturated conditions, however, we found that a profound down-regulation of DAMGO-stimulated μ receptor [35S]-GTPyS binding was induced by alcohol and nicotine in the VTA of escalated rats. This suggests that maximal μ receptor responses to both nicotine and alcohol could be desensitized in the VTA, potentially contributing to a tolerance for the rewarding potential of both drugs. This decreased signalling can in turn be hypothesized to promote alcohol use as an attempt to maintain hedonic state. Interestingly, a recent PET study shows a blunted
Moreover, an elevated vulnerability to develop alcohol use disorder (Turton et al., 2018). A classical mechanism through which opioids modulate mesolimbic dopamine transmission and reward. We therefore predicted that a reduction of μ receptor signalling in the VTA would reduce opioid inhibition of GABAergic neurons in this structure, ultimately increasing their inhibitory tone onto the dopamine neurons and resulting in decreased dopamine release in the Acb. To examine this hypothesis, we exposed rats to the combination of nicotine and alcohol, challenged them with a standard dose of alcohol, and assessed DARPP-32 phosphorylation, a D1-dependent intracellular signalling response to dopamine (Svenningsson, Naim, & Greengard, 2005). In the AcbS, this biomarker of dopamine activity was attenuated by prior exposure to nicotine and alcohol, indicating a downregulated dopamine response to alcohol.

Increased dopamine transmission has been suggested to increase the incentive salience of drug-associated stimuli and results in an increased risk of developing addiction. The role of dopamine transmission seems, however, to change over time as addiction develops. For instance, alcohol increases dopamine release in the Acb of healthy subjects, presumably promoting its reinforcing properties and initiation of an addictive process (Volkow, Fowler, Wang, & Swanson, 2004). In contrast, once an alcohol use disorder develops, there is a blunted DA release (Diana, 2011). Although nicotine alone has been shown to acutely increase DA release in AcbS, its chronic administration attenuates dopamine release both in the core and shell subdivisions (Nisell, Marcus, Nomikos, & Svensson, 1997). A blunted dopamine transmission has been associated with increased susceptibility to drug and alcohol abuse (Martinez et al., 2005). Data in agreement with this notion and with our present results have been provided by Doyon et al. (2013). Using a similar escalation model, they found that nicotine pretreatment decreased alcohol-induced dopamine transmission (Doyon et al., 2013).

In the present study, we show that nicotine-induced changes in AcbS dopamine responses to alcohol might depend on the functional state of the endogenous opioid system within the mesolimbic pathway. Future dynamic measurements of dopamine in the Acb at baseline and stimulated conditions might provide better insights for understanding the dopamine changes in nicotine-induced escalation of alcohol drinking. Of clinical relevance, targeting μ receptors, rather than κ receptors, may be the preferred pharmacotherapeutic approach for the treatment of alcohol use disorder patients who are also smokers.

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AUTHOR CONTRIBUTIONS
E.D. designed the project, performed the behavioural tests and data analysis, and wrote the manuscript. L.X., L.H., and A.N. performed the immunohistochemical staining. A.C.H and M.P. performed the autoradiographic assays and data analysis. G.A., S.T., and E.A. performed the behavioural tests and data analysis. M.H. designed the project, supervised the experiments, and contributed to writing the manuscript. All authors reviewed the final text version.

CONFLICT OF INTEREST
The authors declare that they have no conflict of interest.

DECLARATION OF TRANSPARENCY AND SCIENTIFIC RIGOUR
This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the BJP guidelines for Design & Analysis, Immunoblotting and Immunochemistry, and Animal Experimentation, and as recommended by funding agencies, publishers and other organizations engaged with supporting research.

ORCID
Esi Domi https://orcid.org/0000-0001-5726-4814

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