ORIGINAL ARTICLE

The GPR88 agonist RTI-13951-33 reduces alcohol drinking and seeking in mice

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Abstract

GPR88 is an orphan G-protein-coupled receptor that is considered a potential target to treat neuropsychiatric disorders, including addiction. Most knowledge about GPR88 function stems from knockout mouse studies, and in vivo pharmacology is still scarce. Here we examine the effects of the novel brain-penetrant agonist RTI-13951-33 on several alcohol-related behaviours in the mouse. In the intermittent-access-two-bottle-choice paradigm, the compound reduced excessive voluntary alcohol drinking, while water drinking was intact. This was observed for C57BL/6 mice, as well as for control but not *Gpr88* knockout mice, demonstrating efficacy and specificity of the drug in vivo. In the drinking-in-the-dark paradigm, RTI-13951-33 also reduced binge-like drinking behaviour for control but not *Gpr88* knockout mice, confirming the alcohol consumption-reducing effect and in vivo specificity of the drug. When C57BL/6 mice were trained for alcohol self-administration, RTI-13951-33 decreased the number of nose-pokes over a 4-h session and reduced the number of licks and bursts of licks, suggesting reduced motivation to obtain alcohol. Finally, RTI-13951-33 did not induce any place preference or aversion but reduced the expression of conditioned place preference to alcohol, indicative of a reduction of alcohol-reward seeking. Altogether, data show that RTI-13951-33 limits alcohol intake under distinct conditions that require consummatory behaviour, operant response or association with contextual cues. RTI-13951-33 therefore is a promising lead compound to evaluate GPR88 as a therapeutic target for alcohol use disorders. More broadly, RTI-13951-33 represents a unique tool to better understand GPR88 function, disentangle receptor roles in development from those in the adult and perhaps address other neuropsychiatric disorders.

KEYWORDS
agonist, alcohol use disorder, GPR88, in vivo pharmacology, mouse behaviour

1 | INTRODUCTION

GPR88 is an orphan G-protein-coupled receptor expressed in the brains of rodents and humans and enriched in the striatum and cortex.[1] In humans, genetic studies found a positive association between Gpr88 and several psychiatric conditions including bipolar disorder, schizophrenia, childhood chorea, learning disabilities and speech retardation.[2,3] In mice, constitutive deletion of the *Gpr88* gene produced multiple behavioural phenotypes, including notably motor hyperactivity, motor coordination deficits, and risk-taking behaviours, as well as impaired cognitive flexibility, sensory processing and sensorimotor gating.[1,4–8] *Gpr88* knockout animals also showed increased voluntary alcohol drinking at both moderate and excessive levels and increased alcohol-seeking behaviour.[9] Magnetic resonance imaging of *Gpr88* knockout mice revealed broad modifications of functional connectivity throughout the brain. Predominant reshaping of sensorimotor networks was consistent with most behavioural phenotypes,[10] and remodelling of executive, reward and emotional networks involved in substance use disorders, including alcohol use disorder (AUD), was also observed.[9] At cellular level, constitutive *Gpr88* knockout mice showed that altered spine morphology and gene expression in striatum and amygdala,[6] altered excitability of GABAergic medium spiny neurons[5] and the conditional *Gpr88* gene deletion demonstrated distributed roles of the receptor expressed in either striatopallidal or striatonigral pathways for locomotor control.[11,12] Together therefore, human genetic studies and animal research suggest multiple GPR88 functions in the brain, and the receptor is considered a potential target to treat neuropsychiatric disorders, including schizophrenia, Parkinson's disease, and drug abuse.

Because current knowledge of GPR88 roles in the brain is mainly based on gene knockout studies in mice, which do not distinguish developmental roles from regulatory roles of the receptor in the adult, it is critical that pharmacological tools are developed to further investigate GPR88 function and therapeutic potential.[13] We have pioneered the design, synthesis and study of a family of GPR88 agonists, starting from 2-PCCA[14,15] to novel series of brain-penetrant drugs with improved potency and efficacy.[16–18] In particular, RTI-13951-33,[16] the second-generation lead GPR88 agonist, shows nanomolar potency ($EC_{50} = 25$ nM) at the recombinant receptor using an in vitro cAMP assay. Moreover, the compound efficiently stimulates GPR88-mediated G protein activity in striatal membranes prepared from control but not *Gpr88* knockout mice, indicating activity and specificity at the native receptor. Finally, the compound is highly water soluble and has favourable pharmacokinetic (PK) properties for animal behavioural studies.[16] In the rat PK study, RTI-13951-33 at 10 mg/kg dose, ip, had a brain C_{max} of 287 ng/ml and a half-life of approximately 1.5 h, which demonstrated a sufficient brain exposure for GPR88 modulation. When administered to rats intraperitoneally, RTI-13951-33 significantly reduced alcohol self-administration and alcohol intake in a dose-dependent manner without effects on locomotion and sucrose self-administration. Taken together, this first study suggested that RTI-13951-33 is active in vivo and influences alcohol consumption.

In this report, we further investigate the effects of RTI-13951-33 in mouse models of alcohol drinking, seeking and preference and also include *Gpr88* knockout mice in the study. Our results demonstrate that RTI-13951-33 is a promising lead compound in the evaluation of GPR88 as a therapeutic target for AUD.

2 | MATERIALS AND METHODS

2.1 | Animals

Male C57BL/6J mice were obtained from Jackson Laboratories (USA) for all experiments except for alcohol conditioned place preference (CPP) and morphine locomotion (Charles River, France). Total Gpr88$^{-/-}$ knockout mice were created and produced as previously described.[6] Briefly, to generate *Gpr88*-deficient mice, *Gpr88*-floxed mice, in which exon 2 is flanked by a loxP site (upstream) and a Lox-FRT neomycin-resistance cassette (downstream), were crossed with CMV-Cre mice expressing Cre recombinase under the cytomegalovirus promoter. This led to germ-line deletion of Gpr88 exon 2 under a mixed background (13.96% C57B1/6; 60.94% C57B1/6J; 0.05% FVB/N; 25% 129/SvPas; 0.05% SJL/J). Mice were bred at the Douglas Research Center for all experiments except for alcohol CPP and morphine locomotion (Charles River, France). Animals were group-housed (3–5 animals per cage) for all the experiments (except during the two-bottle-choice procedures, see below) under a 12-h light/dark cycle. Mice were 3–5 months old and weighted 25–35 g at the time of the experiments. Temperature and humidity were controlled, and food and water were available ad libitum. All procedures in this report were conducted in accordance with the guidelines set forth by the Canadian Council of Animal Care and by the Animal Care Committees of McGill University/Douglas Mental Health University Institute for all experiments, except for alcohol CPP and morphine locomotion experiments that were approved by the Regional Committee of Ethic in Animals Experiment of Strasbourg (CREMEAS, APAFIS n°27402-2020093017453593).

2.2 | Drugs and treatments

Alcohol solution was prepared from ethyl alcohol absolute anhydrous (190 proof) diluted to 20% alcohol (v/v). Morphine (10 mg/kg; injected s. c. at a volume of 10 ml/kg) was provided by the NIDA Drug Supply Program (NIH) and dissolved in sterile 0.9% saline solution. RTI-13951-33 (20, 30 and 60 mg/kg) was dissolved in sterile 0.9% saline to be administered ip at 10 ml/kg. In the previous behavioural studies in rats, RTI-13951-33 at 10 and 20 mg/kg doses, but not 5 mg/kg dose, reduced alcohol self-administration. Given that mouse has a faster metabolic rate than rat, we chose 20 mg/kg as the lowest dose for behavioural studies in mice.

2.3 | Locomotor activity

Mice were initially placed in the centre of the locomotor chamber and spontaneous locomotor activity was recorded for 30-min habituation. For spontaneous locomotion, mice were then briefly removed from the chamber, injected with saline or RTI-13951-33 (30 or 60 mg/kg, ip) and placed back in the chamber for 60 min. For morphine-induced locomotion, mice were briefly removed from the chamber, co-injected with saline or RTI-13951-33 (30 or 60 mg/kg, ip) and morphine (10 mg/kg, subcutaneously), and placed back in the chamber for 120 min.

2.4 | Intermittent access—two-bottle choice (IA20%2BC)

Animals were single-housed. The IA20%2BC drinking procedure was conducted for 8 weeks as previously described.[9] Briefly, after 1 week of acclimation, animals were given 24 h of concurrent access to one bottle of 20% alcohol (v/v) in tap water and one bottle of water starting at 12 a.m. on Monday, Wednesday and Friday with 24 or 48 h of alcohol-deprivation periods between the alcohol-drinking sessions. The water and alcohol bottles were weighed after 24 h access. The position (left or right) of each solution was alternated between sessions as a control for side preference and the possible loss of solutions due to the handling of the bottles was controlled by weighing bottles in empty cages. Body weights were recorded weekly throughout the study. Animals were injected with saline or RTI-13951-33 (30 mg/kg, ip) 30 min before being tested in the 24-h drinking session.

2.5 | Drinking in the dark

Animals were single-housed under a 12 h reversed light/dark cycle. Oral alcohol intake was determined using the drinking-in-the-dark (DID) paradigm during 2 weeks. The procedure was adapted from Thiele et al.[19] Briefly, for each week, drinking sessions were conducted 2 h every day during three consecutive days and 4 h on the fourth and final day, with one bottle containing tap water, while the other contained alcohol diluted to 20% alcohol (v/v) in tap water. The bottles were weighed every day, and the mice were weighed at the beginning of the experiment. Animals were injected with saline or RTI-13951-33 (30 mg/kg, ip) 30 min before being tested in the 4-h drinking session.

2.6 | Operant alcohol self-administration

Operant self-administration of alcohol was adapted from Laguesse et al.[20] Prior to the operant training, animals were first subjected to the IA20%2BC for 6 weeks as described above. Only animals that consume more than 8 g/kg/24 h during the last week of IA20%2BC were selected for the operant self-administration training phase. All animal training and testing sessions were performed during the dark phase of their light/dark cycle. All training occurred in mouse operant chambers (Imetronic, France), housed in sound- and light-attenuated boxes that were equipped with fans to provide ventilation and white noise. C57BL/6J mice were trained for 8 weeks with five daily self-administration sessions per week (Monday to Friday). Animals were trained to self-administer 20% (v/v) alcohol in daily 4-h sessions using a fixed-ratio 1 (FR 1) schedule. When the alcohol session began, the light in the chamber was turned on. An active nose-poke caused a 10 μl of alcohol to be dispensed into the liquid cup and a cue light above the nose-poke hole to illuminate. Reward delivery was paired with a 1-s tone (85 db) and the illumination of the cue-light above the magazine. Inactive nose-poke produced no alcohol delivery. The number and timing of the active and inactive nose-pokes, magazine visits, and alcohol deliveries were recorded during each session. Animals were injected with saline or RTI-13951-33 (30 mg/kg, ip) 30 min before being tested in the 4-h self-administration session. This study was performed with a 'within-subjects' design in which mice received both treatments in counterbalanced order. RTI-13951-33 injections were separated by 1 week of standard self-administration, allowing the nose-poke responding for alcohol to return to baseline.

2.7 | CPP to RTI-13951-33

The test was conducted in an apparatus (PanLab) composed of two compartments that only differ by their colour of walls and floor texture. A central compartment separates the two others. On preconditioning (Day 1), mice were given access to the entire apparatus for 30 min. For the conditioning phase, we used an unbiased CPP design. Compartments were randomly assigned, and mice were conditioned for six consecutive days with one session per day. On Days 2, 4 and 6, mice were given an ip injection of saline or RTI-13951-33 (30 mg/kg) and immediately placed into the drug-paired compartment for 30 min. On Days 3, 5 and 7, animals were injected with saline only and immediately placed in the saline-paired compartment for 30 min. On the post-conditioning (Day 8), animals were allowed to explore the entire apparatus for 30 min. The CPP score was calculated as the percentage of time spent in the drug-paired compartment on the test day minus the percentage of time spent in the same compartment on the preconditioning day.

2.8 | CPP to alcohol

Alcohol CPP was conducted as previously described.[9] Briefly, the test was conducted in the same apparatus (Imetronic, France). On Day 1, mice were given access to the entire apparatus for 20 min (preconditioning). For the conditioning phase, we used a biased CPP design, in which alcohol treatment was associated with the less preferred compartment during the preconditioning test. Animals that spent more than 70% of their time in either of the compartments during the preconditioning test were excluded from the experiment. On Day

2, conditioning training started with one conditioning trial per day for 6 days as follows: Mice were administered (ip) a saline solution and confined immediately to one of the compartments for 5 min (unpaired compartment). On the next day, mice were administered a saline solution (saline group) or alcohol (1.8 g/kg, 20% v/v, alcohol group) and were confined to the other compartment (drug-paired compartment). This schedule was repeated twice more until Day 6. On Day 8, animals were injected with saline or RTI-13951-33 (30 mg/kg, ip) and 30 min later were allowed to explore the entire apparatus for 20 min (post-conditioning test). CPP score was calculated as the percentage of time spent in the drug-paired compartment on the test day minus the percentage of time spent in the same compartment on the preconditioning day.

2.9 | Statistical analysis

For behavioural experiments, data were analysed with unpaired t-test and two or three-way ANOVA with or without repeated measures (RM-ANOVA) (GraphPad Prism or Statistica). Significant main effects and interactions of the ANOVAs were further investigated with the Bonferroni post hoc tests or method of contrast analysis. Statistical significance was set at $p < 0.05$.

3 | RESULTS

3.1 | RTI-13951-33 decreases locomotor activity

Because Gpr88 knockout mice show hyperactivity,[5,6] we anticipated that the GPR88 agonist may alter spontaneous locomotor activity in C57BL/6 mice. In a first experiment, we found that intraperitoneal (ip) administration of 20 mg/kg RTI-13951-33 had no effect on locomotor activity (data not shown), as was the case in the rat previously.[16] In a second experiment, we tested the doses of 30 and 60 mg/kg (Figure 1A, left panels). Before drug administration, there was no difference between saline and RTI-13951-33 groups for the distance travelled during the 30-min habituation (one-way ANOVA test, $F[2,41] = 0,159$, $p = 0.85$) and following saline injection (one-way ANOVA test, $F[2,41] = 0,0.88$, $p = 0.42$). RTI-13951-33 administration significantly decreased locomotor activity, at the two doses (one-way ANOVA test, $F[2,41] = 6.49$, $p = 0.0035$; saline vs. RTI-13951-33 at 30 mg/kg, $p < 0.05$; saline vs. RTI-13951-33 at 60 mg/kg, $p < 0.01$), and in a dose-dependent manner ($p < 0.01$). Further analysis of locomotor activity throughout the 60 min (Figure 1A, right panel) confirmed that RTI-13951-33 administration reduces spontaneous activity (two-way ANOVA with repeated measures; main effect of treatment, $F[2,41] = 5.6$, $p = 0.006$; main effect of time, $F[14,565] = 18.79$, $p < 0.001$; and significant treatment–time interaction, $F[28,565] = 2.04$, $p = 0.001$). A Bonferroni post hoc analysis revealed that the RTI-13951-33 effect was significant during the first 5 min for the 30 mg/kg dose and during the first 15 min for the 60 mg/kg dose (Table S1).

Because the level of spontaneous activity is low and a reduction is not easily detectable, we further tested the effect of RTI-13951-33 upon co-administration of morphine (10 mg/kg, ip), known to enhance locomotor activity in mice. Analysis by 20-min sections (Figure 1B, left panels) showed that the two doses of RTI-13951-33 (30 and 60 mg/kg) significantly decreased the distanced travelled during the first 20 min (one-way ANOVA $F[2,30] = 2.35$, $p = 0.0012$; Bonferroni post hoc: saline vs. RTI-13951-33 at 30 mg/kg, $p < 0.05$; saline vs. RTI-13951-33 at 60 mg/kg, $p < 0.001$). From 20 to 40 min, only the dose of 60 mg/kg reduced morphine-induced hyperlocomotion (one-way ANOVA, $F[2,30] = 1.55$, $p = 0.003$; Bonferroni post hoc: saline vs. RTI-13951-33 at 30 mg/kg, $p > 0.05$; saline vs. RTI-13951-33 at 60 mg/kg, $p < 0.01$). Finally, RTI-13951-33 had no effect on locomotion in the 40- to 60-min time period (one-way ANOVA, $F[2,30] = 1.04$, $p = 0.36$). Analysis throughout the entire 120-min time period (Figure 1B, right panel) showed that morphine increases locomotor activity with the expected 2-h time course and confirmed that RTI-13951-33 reduces morphine-induced hyperlocomotion (two-way ANOVA with repeated measures; main effect of treatment, $F[2,30] = 2.57$, $p = 0.09$; main effect of time, $F[23,690] = 21.24$, $p < 0.001$; significant treatment-time interaction, $F[46,690] = 2.14$, $p < 0.001$). Bonferroni post hoc test revealed that the RTI-13951-33 effect on locomotion was significant from 35 to 40 min for the lower dose, and from 10 to 65 min for the higher dose (Table S1).

Altogether, we show that RTI-13951-33 reduces locomotor activity in mice at the two 30 and 60 mg/kg doses, an effect consistent with hyperactivity observed in Gpr88 knockout mice. To avoid confounding effects of the compound in alcohol-related behaviours in subsequent experiments, we selected the minimally active dose of 30 mg/kg and tested mouse behaviour 30 min after RTI-13951-33 administration.

3.2 | RTI-13951-33 reduces voluntary alcohol drinking

We previously showed that Gpr88 knockout mice drink more alcohol in continuous (moderate consumption) or intermittent (excessive consumption) in two-bottle-choice paradigms.[9] Here we first assessed whether RTI-13951-33 would reduce excessive voluntary alcohol consumption in C57BL/6 mice using the IA20%2BC paradigm. After 8 weeks of training, animals were pre-injected with saline or RTI-13951-33 (30 mg/kg, ip) 30 min prior to the presentation of alcohol or water for a 24-h drinking session. As shown in Figure 2A (left panel), RTI-13951-33 decreased the level of total alcohol consumption (one-tailed t-test, $t[17] = 1.954$, $p = 0.03$) and no difference in water intake was detected ($t[17] = 0.41$, $p = 0.68$). Analysis of alcohol intake over the first 6 h of the session (Figure 2A, right panels) revealed a significant reduction of alcohol intake in the RTI-13951-33-treated group compared with control group (two-way ANOVA with repeated measures; main effect of treatment, $F[1,16] = 5.86$, $p = 0.027$; main effect of time, $F[2,31] = 6.74$, $p = 0.005$; no significant treatment-time interaction, $F[2,31] = 0.09$, $p = 0.9$).
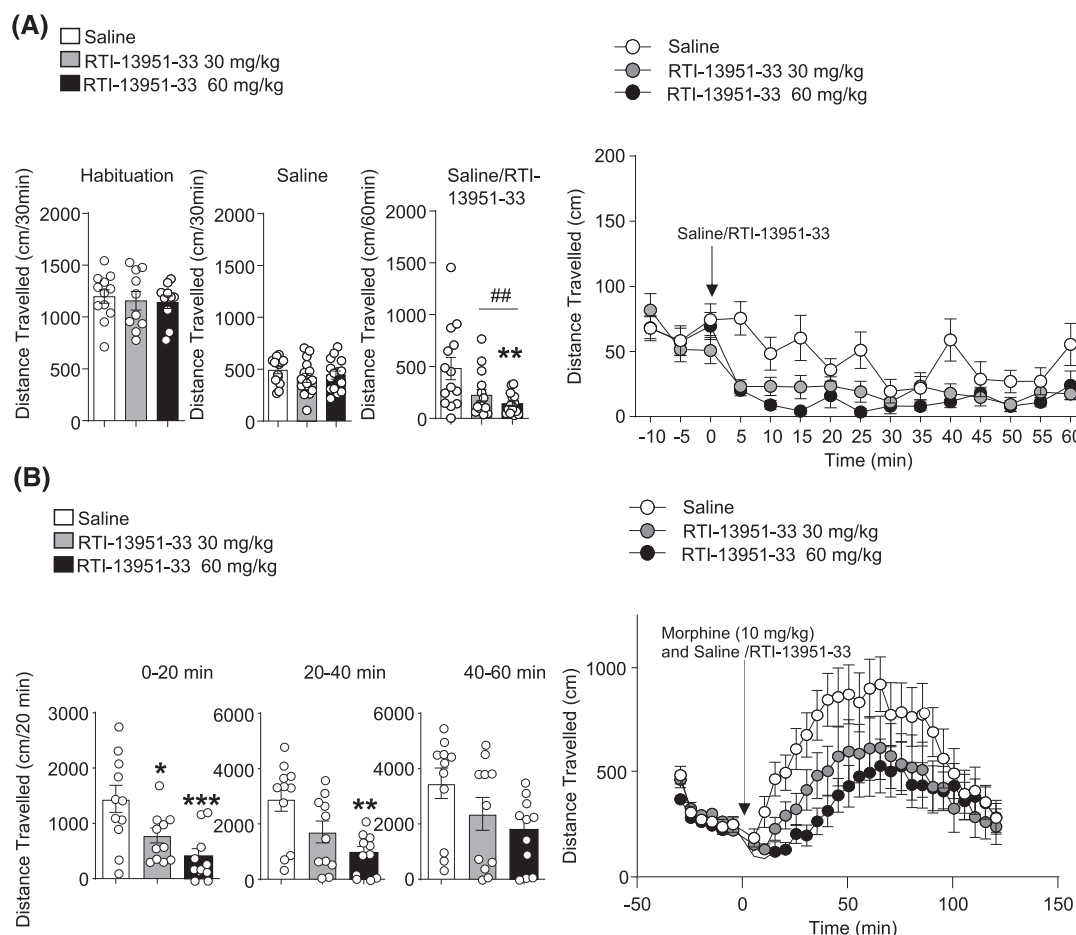
FIGURE 1 RTI-13951-33 reduces locomotor activity. (A) RTI-13951-33 dose-dependently reduced spontaneous locomotor activity in C57BL/6J mice. Left panels. Total distance travelled during the 30-min habituation, then 30 min after saline administration (ip), and finally 60 min after saline or RTI-13951-33 (30 or 60 mg/kg, ip) administration. Versus saline: **$p < 0.01$; versus 30 mg/kg: ##$p < 0.01$; $n = 14$–15/group. Right. Time course of locomotor activity after saline or RTI-13951-33 (30 and 60 mg/kg, ip) administration (see Table S1 for statistical analysis). Saline or RTI-13951-33 were given at the time indicated by the arrow. Data show average distance travelled in 5 min. Error bars represent standard error of means ($n = 14$–15/group). (B) RTI-13951-33 dose-dependently reduced morphine-induced hyperlocomotion in C57BL/6J mice. After 30 min of habituation, morphine (10 mg/kg) was injected simultaneously with saline or RTI-13951-33 (30 or 60 mg/kg, ip), and activity measured for 120 min. Left panels. Total distance travelled during the first 0–20 min, 20–40 min and 40–60 min time periods of the test. Versus saline: *$p < 0.05$; **$p < 0.01$. $n = 11$/group. Right. Time course of locomotor activity after morphine/saline or RTI-13951-33 (30 and 60 mg/kg, ip) co-administration (see Table S1 for statistical analysis). Saline or RTI-13951-33 was given at the time indicated by the arrow. Data show average distance travelled in 5 min. Error bars represent standard error of means ($n = 11$/group).

Subsequent analyses using the method of contrasts showed that the main effect of RTI-13951-33 treatment was observed during the second 2-h period of the session ($p = 0.02$).

In order to determine the in vivo specificity of RTI-13951-33, we measured the effect of RTI-13951-33 on alcohol intake in the IA20% 2BC paradigm using *Gpr88* knockout mice and their controls. As before, animals were exposed to alcohol for 8 weeks and, on the test day, were injected with saline of RTI-13951-33 (30 mg/kg, ip) 30 min prior to the presentation of alcohol or water for a 24-h drinking session. Two-way ANOVA showed a significant effect of treatment ($F[1,56] = 6.38$, $p = 0.01$) and a significant effect of genotype ($F[1,56] = 30.3$, $p < 0.0001$) with no treatment–genotype interaction ($F[1,56] = 2.27$, $p = 0.13$). Subsequent analysis (Figure 2B, left panel) using method of contrast showed that, consistent with our previous study,

alcohol intake was significantly higher in Gpr88$^{-/-}$ mice compared with Gpr88$^{+/+}$ controls in the saline-treated group ($+48\%$, $p = 0.036$). In addition, RTI-13951-33 reduced alcohol intake in Gpr88$^{+/+}$ ($p = 0.026$) but not Gpr88$^{-/-}$ mice ($p = 0.89$). This result shows that the GPR88 agonist also efficiently reduces alcohol-drinking behaviour in mouse strain with a different genetic background (75% C57BL/6-25% SvPas, see Section 2) at the dose of 30 mg/kg and demonstrates that GPR88 is required for the RTI-13951-33 effect. Similar to our finding in C57BL/6J mice, no difference in water consumption was found across genotypes for both treatments (Figure 2B, right panel), as two-way ANOVA showed no effect of treatment ($F[1,56] = 0.1$, $p = 0.71$), no effect of genotype ($F[1,56] = 2.01$, $p = 0.16$) and no treatment–genotype interaction ($F[1,56] = 0.09$, $p = 0.76$).
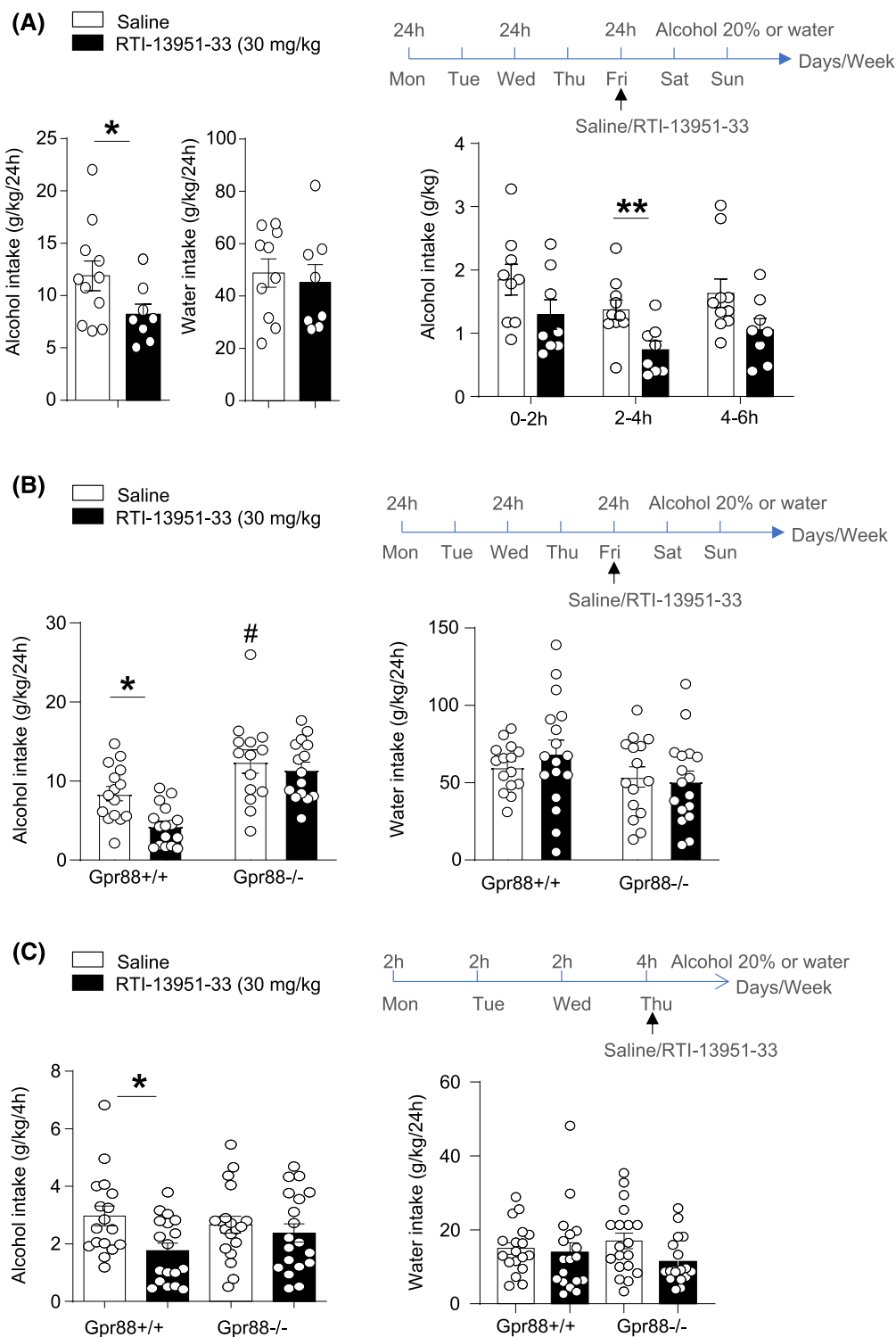
FIGURE 2 RTI-13951-33 reduces alcohol drinking in the two-bottle choice. (A) C57BL/6J mice were subjected to the intermittent access drinking procedure (IA20%-2BC) for 8 weeks. On the test day, saline or RTI-13951-33 (30 mg/kg, ip) was administered 30 min before the start of the 24-h session ($n = 8$–11/group). Total alcohol or water intake is shown. (B) Gpr88 knockout mice (Gpr88$^{-/-}$) and their controls (Gpr88$^{+/+}$) went through the same IA20%-2BC procedure, and data are shown as in A ($n = 14$–16/group). (C) Gpr88 knockout mice (Gpr88$^{-/-}$) and their controls (Gpr88$^{+/+}$) were subjected to drinking-in-the-dark (DID) procedure for 2 weeks. On the test day, saline or RTI-13951-33 (30 mg/kg ip) was administered 30 min before the start of the 4-h session ($n = 17$–19/group). Data are presented as mean ± s.e.m. treatment effect: *$p < 0.05$; **$p < 0.05$, genotype effect #$p < 0.05$.

Next, we evaluated the effects of the GPR88 agonist on alcohol binge-like consumption. To this aim, we used the DID assay[19] and tested *Gpr88* knockout mice and their controls. After 2 weeks of DID, RTI-13951-33 (30 mg/kg, ip) was injected 30 min prior to the presentation of alcohol or water for the 4-h drinking session (Friday, second week). Two-way ANOVA showed significant effect of treatment (F[1,69] = 6.03, $p = 0.01$), no effect of genotype (F[1,69] = 0.25, $p = 0.6$) and no treatment–genotype interaction (F[1,69] = 2.29,

$p = 0.13$). Subsequent analysis using showed that binge-like drinking does not differ between *Gpr88* knockout mice and their controls ($p = 0.89$) and that RTI-13951-33 significantly decreased the volume of alcohol intake only in Gpr88$^{+/+}$ mice ($p = 0.03$, Figure 2C, left panel). No effect on water intake was detected (Figure 2C, right panel) as two-way ANOVA showed no effect of treatment (F[1,69] = 2.73, $p = 0.1$), no effect of genotype (F[1,69] = 0.01, $p = 0.9$) and no treatment–genotype interaction (F[1,69] = 1.26, $p = 0.26$). This result

shows that RTI-13951-33 decreases binge-like alcohol consumption, an effect that is not observed in mice lacking GPR88.

Altogether these data show that RTI-13951-33 at the dose of 30 mg/kg efficiently reduces voluntary alcohol drinking in a GPR88-dependent manner. This result is concordant with increased voluntary alcohol drinking in *Gpr88* knockout mice from our previous study. The absence of RTI-13951-33 on water intake in all the tests suggests that a potential locomotor activity-reducing effect of RTI-13951-33, although unlikely at this dose, does not impair the drinking behaviour.

3.3 | RTI-13951-33 reduces alcohol self-administration

We previously observed that *Gpr88* knockout mice show increased operant response and motivation to obtain alcohol.[9] Here we assessed whether the GPR88 agonist also alters alcohol self-administration. C57BL/6J mice that underwent 6 weeks of IA20%2 BC were trained to self-administer 20% alcohol in operant chambers. After reaching a stable baseline of responding, mice received saline or RTI-13951-33 (30 mg/kg, ip) 30 min before the 4-h alcohol self-administration session, using a within-subjects design (Weeks 14 and 15, Figure 3A). Total locomotor activity in the operant chamber was identical for saline- and RTI-13951-33-treated mice, suggesting that the GPR88 agonist did not impair mouse activity under our conditions ($t[18] = 0.92$, $p = 0.36$) (Figure 3B).

The number of active and inactive nose-pokes are shown in Figure 3C for each 1-h period of the total 4-h session time. RTI-13951-33 did not significantly modify the number of active nose-pokes across the session (two-way ANOVA; no effect of treatment, $F[1,14] = 2.3$, $p = 0.14$; no effect of time, $F[3,42] = 2.3$, $p = 0.11$; no treatment–time interaction, $F[3,42] = 1.44$, $p = 0.24$). However, the total number of active nose-pokes over the 4-h session was significantly reduced (paired t-test, $t[7] = 2.17$, $p = 0.05$). The number of inactive nose-pokes was low and was not modified by drug treatment, either across the four 1-h periods (two-way ANOVA; no effect of treatment, $F[1,14] = 0.96$, $p = 0.34$; significant effect of time, $F[3,42] = 3.55$, $p = 0.047$; and no treatment–time interaction, $F[3,42] = 0.33$, $p = 0.8$). Also, the total number of inactive nose-pokes over the 4-h session was not changed between the two groups (paired t-test, $t[7] = 1.05$, $p = 0.33$).

We quantified the licking behaviour for each 60-min period of the total 4-h session (Figure 3D). As expected, there was a significant increase of lick numbers for the saline group over time, but this was not observed for the RTI-13951-33-treated group (two-way ANOVA; significant effect of treatment, $F[1,14] = 5.65$, $p = 0.03$; significant effect of time, $F[3,42] = 5.6$, $p = 0.019$; significant treatment–time interaction, $F[3,42] = 5.13$, $p = 0.004$). Bonferroni post hoc tests showed a significant difference between the two groups in the last 2 h- of the session (180 min, $p = 0.048$; 240 min $p = 0.041$). The total number of licks over the 4-h session was significantly reduced in the RTI-13951-33-treated group (paired t-test, $t[7] = 2.43$, $p = 0.045$).

No drug effect was observed for the alcohol contact duration ($t[7] = 0.62$, $p = 0.54$) and interlick intervals (ILI) (paired t-test, $t[7] = 0.25$, $p = 0.52$) over the 4-h session. We also quantified the number of burst of licks, defined as three or more licks with an ILI of less than 1 s (Figure 3E). As for the licks, there was a significant increase of burst number for the saline group in the last 2 h, which was not observed for the RTI-13951-33 group (two-way ANOVA; significant effect of treatment $F[1,14] = 7.17$, $p = 0.01$; significant effect of time, $F[3,42] = 7.01$, $p = 0.0035$; significant treatment–time interaction, $F[3,42] = 5.38$, $p = 0.003$); Bonferroni post hoc tests revealed a significant difference between the two groups in the last 2 h of the session (180 min, $p = 0.05$; 240 min $p = 0.014$). The total number of bursts over the 4-h session was significantly reduced in the RTI-13951-33-treated group (paired t-test, $t[7] = 2.92$, $p = 0.022$). There was no significant change in burst duration (paired t-test, $t[7] = 0.45$, $p = 0.32$) and number of licks per burst (paired t-test, $t[7] = 0.22$, $p = 0.62$). The microstructural pattern of licks and bursts for each mouse is shown in Figure S1, the pattern of bursts over session and the cumulative number of bursts over session are shown in Figure 3F. These representations highlight individual variability and in addition also clearly shows that RTI-13951-33 reduces the number of licks and bursts particularly in the last 2 h of the session.

Altogether, these results demonstrate that RTI-13951-33 reduces alcohol self-administration and suggest that the drug decreases both motivation to obtain alcohol (nose-pokes) and alcohol intake (licks and bursts). These data are also consistent with our previous finding of increased alcohol self-administration in *Gpr88* knockout mice.[9]

3.4 | RTI-13951-33 reduces conditioned place preference to alcohol

Finally, we assessed whether RTI-13951-33 alters alcohol seeking in the alcohol CPP paradigm.[21] We first tested whether RTI-13951-33 alone induces place aversion or preference. Mice were conditioned with RTI-13951-33 (30 mg/kg) or saline for six consecutive days (one session per day). As shown in Figure 4A, the two groups of mice displayed a similar CPP score with no aversion or preference for the drug-paired compartment (paired t-test, $t[21] = 0.39$, $p = 0.86$). We next tested whether RTI-13951-33 modifies the expression of alcohol-induced CPP, or alcohol seeking, after CPP has been established. Mice were conditioned for alcohol (1.8 g/kg) or saline for six consecutive days (one session per day). On the post-conditioning day, mice were pre-injected with saline or RTI-13951-33 (30 mg/kg) and 30 min later tested for alcohol-induced CPP. As shown in Figure 4B, one-way ANOVA revealed a significant effect of treatment ($F[2,43] = 12.22$, $p < 0.0001$). Bonferroni post hoc tests revealed that mice treated with alcohol showed increased preference for the drug-paired compartment compared with saline-treated group ($p < 0.001$) and that GPR88 agonist reduced alcohol CPP ($p = 0.036$).

These data suggest that the GPR88 agonist reduces alcohol-reward seeking, a response that is likely independent from alcohol reward (see Section 4).
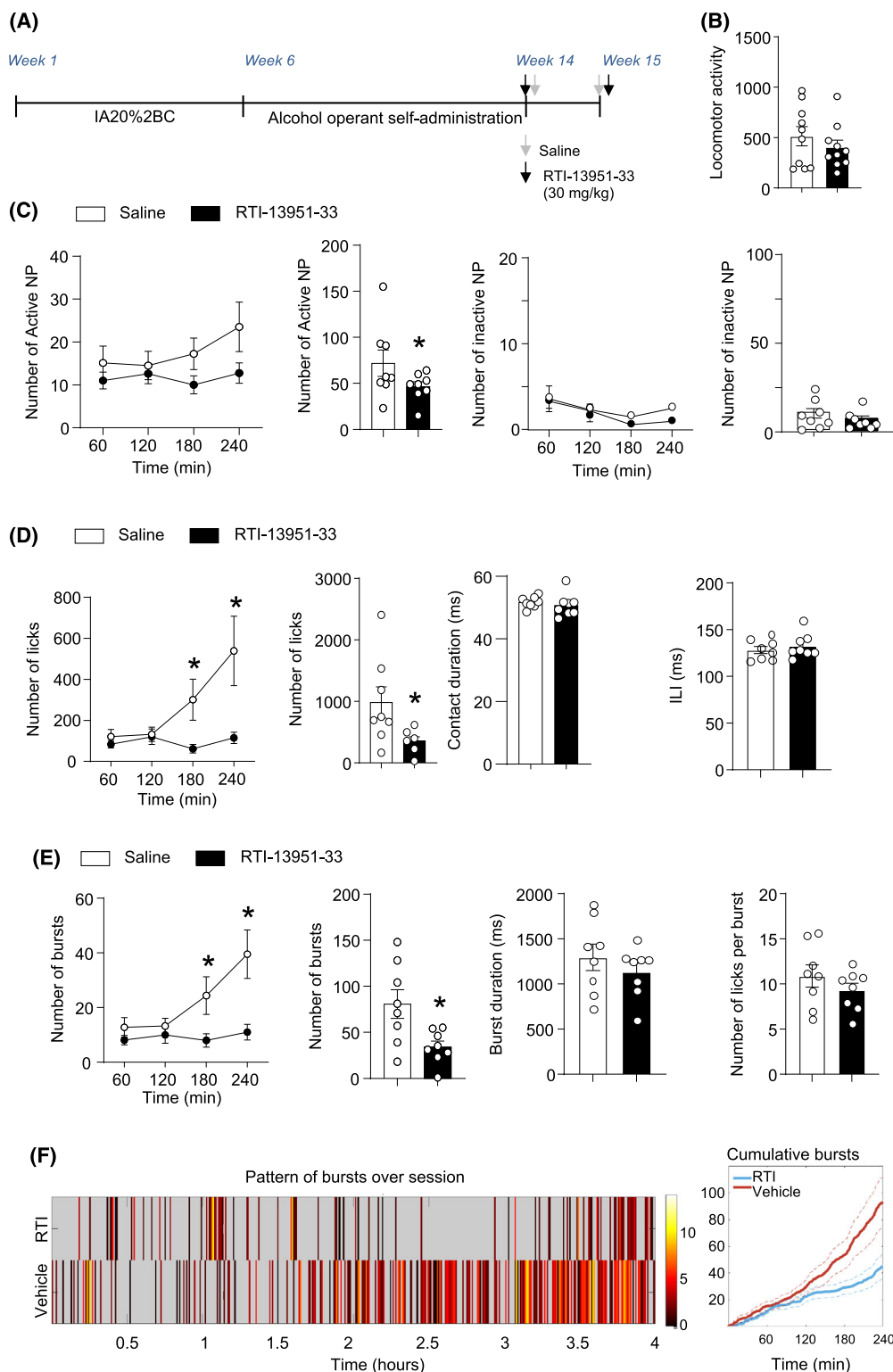
FIGURE 3 RTI-13951-33 reduces alcohol operant self-administration. (A) Time line of the self-administration procedure in C57BL/6J mice. After 6-weeks IA20%2BC and 8-weeks self-administration (SA) training, saline or RTI-13951-33 (30 mg/kg ip) was administered 30 min before the 4-h session ($n = 8$/group). (B) Total locomotor activity for saline or RTI-13951-33 groups during the session. (C) Operant behaviour. Number of active (left) and inactive (right) nose-pokes per hour and in total is shown. (D) Licking behaviour. Number of licks per hour, total number of licks, alcohol contact duration, and interlick intervals (ILIs) are shown. (E) Bursts. Number of bursts per hour, total number of bursts, burst duration and number of licks per burst are shown. (F) Microstructural pattern of bursts throughout the entire 4-h session. Left: pattern of bursts and cumulative bursts over 4-h session are shown for RTI-13951-33- and saline-treated animals (minimal precision 1 s). Right: mean cumulative number of bursts over time for each group (bold line) with their respective standard error of the mean (dashed lines). Individual patterns are shown in Figure S1
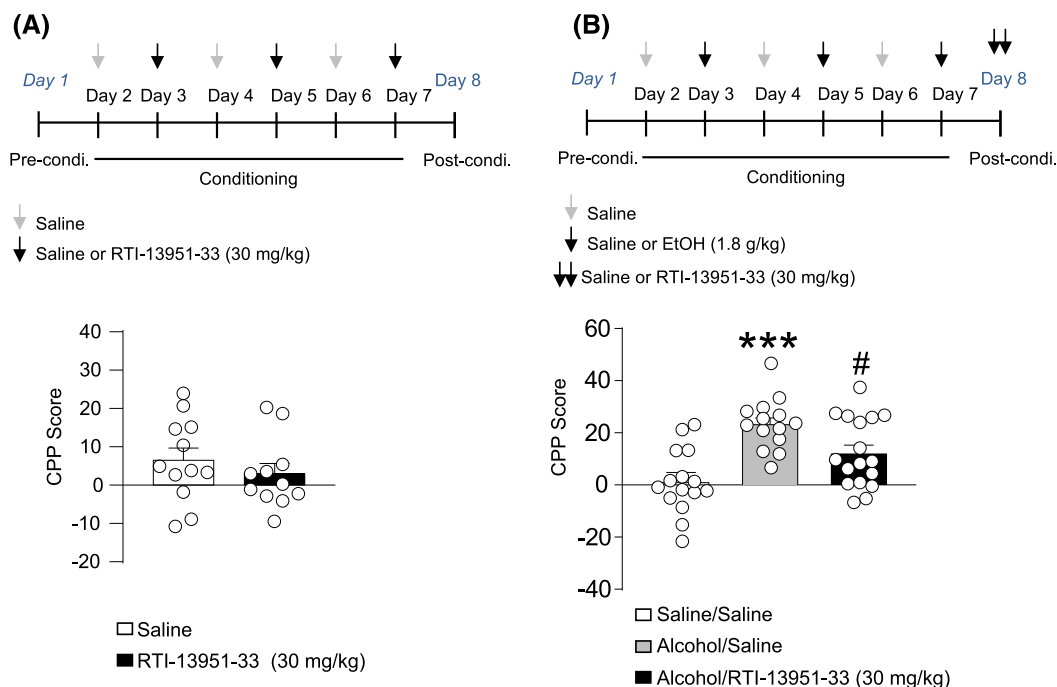
FIGURE 4 RTI-13951-33 reduces conditioned place preference to alcohol. (A) RTI-13951-33 does not in conditioned place preference (CPP) in C57BL/6J mice. Top: timeline of the experiment. Following pretest (Day 1), conditioning sessions were conducted daily for 6 days, with saline or RTI-13951-33 (30 mg/kg) injections, and the test conducted on day 8 (free 20-min exploration of the apparatus). Bottom: CPP scores (% time spent in the drug compartment minus percentage of time spent in the same compartment during pretest) are shown ($n = 11$–12/group). (B) RTI-13951-33 reduces CPP to alcohol. Top: time line of the experiment. Following pretest (Day 1), mice were conditioned to saline or alcohol (1.8 g/kg) for 6 days. On the test day (Day 8), animals received saline or RTI-13951-33 (30 mg/kg) and were placed into the CPP chamber 30 min later for a 20-min exploration time. Bottom: CPP scores (% time spent on the saline-paired side minus percentage of time spent on the alcohol-paired side ($n = 11$–17/group). Saline versus alcohol/saline, ***$p < 0.001$; alcohol/saline versus alcohol/RTI-13951-33, #$p < 0.05$.

4 | DISCUSSION

Our data first demonstrate that the GPR88 agonist RTI-13951-33 reduces locomotor activity, suggesting that the drug efficiently crosses the blood brain barrier in mice and acts on the brain. Second, we show that RTI-13951-33 diminishes excessive alcohol drinking in the IA20%2BC and that this effect is GPR88-dependent, demonstrating in vivo specificity of the compound in a classical rodent model of excessive alcohol drinking.[22,23] Third, we found that RTI-13951-33 also reduces other alcohol-related behaviours in mice, and these include (i) binge-like voluntary alcohol drinking, (ii) operant alcohol self-administration and (iii) alcohol seeking in an alcohol place conditioning paradigm. Overall, RTI-13951-33 attenuated alcohol intake in all the tested behavioural conditions, whether these conditions require a consummatory behaviour, operant response or association with contextual cues. The pharmacological activation of GPR88 therefore represents a promising approach for AUD research, as all these rodent behaviours[23] model different aspects of a multifaceted pathology.[24] Of note, we have performed all these studies in males only, as a proof-of-principle, and studying the effects of RTI-13951-33, or improved compounds, in females also will be important in the future.

An important observation is the fact that GPR88 activation by RTI-13951-33 produces effects that oppose the effects of the *Gpr88*

gene knockout. Thus, in the present study, RTI-13951-33 reduced locomotion, alcohol drinking (IA20%2 BC and DID) and alcohol self-administration, while our previous studies showed the exact opposite for the mutant mice.[6,9] These findings partly address the long-standing question of a potential developmental role of the receptor. In fact, most studies addressing GPR88 function have used constitutive *Gpr88* knockout animals,[4–6,8,10] in which the receptor was absent since the very beginning of development. Behavioural phenotypes of these knockout animals, therefore, may result either from a developmental role of the receptor in shaping neuronal activity and brain connectivity, or from a tonic receptor activity in the adult brain. Our other studies using conditional knockout approaches[11,12] showed that a number of behavioural phenotypes could be distributed over GPR88 expression either in the striatopallidal pathway or in the striatonigral pathway. In this case, the gene knockout had occurred later in development but still prenatally, and mutant mouse deficits could also stem from developmental adaptations within striatopallidal/striatonigral pathways. Finally, there is evidence for a nonclassical nuclear localization of the receptor, which was proposed to contribute to cortical development.[25] Our finding that the GPR88 agonist RTI-13951-33 efficiently reduces alcohol drinking demonstrates that knockout mice phenotypes are not necessarily developmental but indeed reflect a receptor activity in the adult, at least for behavioural responses in the present report.

The effect of RTI-13951-33 on alcohol-induced CPP seemed different, as both the *Gpr88* gene knockout[9] and RTI-13951-33 administration reduced alcohol CPP. These results however are not in opposition, as the constitutive gene knockout does not allow dissociating the acquisition from the expression of alcohol CPP. Further, the knockout mice study showed reduced alcohol-induced dopamine release in the nucleus accumbens; therefore, the lower alcohol CPP was likely due to reduced alcohol reward limiting the drug–context association.[9] Here, in the pharmacological experiment, alcohol place conditioning took place normally and RTI-13951-33 was administered post-conditioning, as the animal freely explored the two compartments. The observed reduced CPP, in this case, is considered a hallmark of reduced alcohol-reward seeking behaviour.[21,26] Thus, this particular experiment does not assess RTI-13951-33 effect on alcohol reward but suggests that activating GPR88 may efficiently reduce alcohol-reward seeking.

It is interesting to note that the RTI-13951-33 effect seems to operate early in the locomotor study (first hour) and later in alcohol experiments (2–4 h in both IA20%2BC and self-administration experiments). In the pharmacokinetic (PK) study,[16] we have observed that RTI-13951-33 (10 mg/kg, ip) reached the peak brain concentration at 1 h and maintained significant brain exposure across a 4-h testing period, which is therefore compatible with the 2–4 h efficacy observed in voluntary drinking and operant experiments. The apparent distinct time course of RTI-13951-33 effects in locomotion and alcohol drinking experiments may indicate that several distinct cellular or network mechanisms are engaged by RTI-13951-33 to modulate these different behavioural responses.

The mechanisms of action of RTI-13951-33 is necessarily complex, as multiple biological processes and brain networks underlie behaviours that were examined in this study. At cellular level, GPR88 acts as a Gi/o coupled receptor in both transfected cells[15] and striatal tissues[6]; therefore, we may speculate that RTI-13951-33 inhibits neuronal activity in alcohol-responding pathways. RTI-13951-33's activation of GPR88 may also influence the activity of other GPCRs, as there is evidence that GPR88 acts as a brake on GPCR signalling. In vitro data showed that increasing GPR88 expression in recombinant cells reduces signalling and trafficking of multiple GPCRs in close proximity, and alters β-arrestin recruitment.[27] Ex vivo data showed that G protein coupling of delta and mu opioid receptors is enhanced in the striatum of *Gpr88* knockout mice.[6] Finally, in vivo experiments showed that behavioural phenotypes of *Gpr88* knockout mice are attenuated by delta opioid receptor blockade[6] and that morphine effects are modified in these mice.[27] RTI-13951-33 may therefore modify neuronal activities in multiple ways, and further signalling and electrophysiological experiments will better characterize the drug effect on neurons.

At circuit level, RTI-13951-33 may alter the activity of networks relevant to alcohol reward processing, motivation to seek alcohol and alcohol-related context learning. These include notably the basal ganglia (reward and incentive salience), the extended amygdala (negative emotional states) and prefrontal/insular cortices (executive functions),[28] where GPR88 is heavily expressed. It will be important in the future to assess in vivo pharmacological properties of RTI-13951-33 more broadly, and in particular using paradigms assessing appetitive properties of and motivational drive for other drugs of abuse and natural rewards, impulsive/compulsive behaviours, learning and memory as well as emotional responses. Extensive characterization of in vivo RTI-13951-33 effects may reveal unexpected properties of potential therapeutic interest.

In conclusion, RTI-13951-33 represents a useful pharmacological tool for studying GPR88 functions in the brain and a promising lead compound for drug discovery targeting GPR88 to treat alcohol addiction, and possibly other neuropsychiatric disorders. Optimization of RTI-13951-33 to further improve potency and PK properties including assessment of oral bioavailability with improved compounds is currently under investigation.

CONFLICT OF INTEREST

The authors report no conflict of interest.

AUTHOR CONTRIBUTIONS

SBH and BLK designed and led the project, analysed and interpreted the data and wrote the manuscript. ED contributed to the project design and edited the manuscript. SBH and ED performed the locomotion experiments. SBH and MC performed the alcohol-related behavioural experiments. MS contributed to analysis of self-administration experiments. CJ developed RTI-13951-33 and contributed to the project design. MTR and AMD synthesized and characterized RTI-13951-33 for behavioural experiments. All authors gave final approval of the version submitted.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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