Is R(+) Baclofen the best option for the future of Baclofen in alcohol dependence pharmacotherapy? Insights from the preclinical side

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Abstract
For several decades, studies conducted to evaluate the efficacy of RS(±)-Baclofen in the treatment of alcohol dependence yielded contrasting results. Human and animal studies recently questioned the use of the racemic drug in patients since a potential important role of the different enantiomers has been revealed with an efficacy thought to reside with the active R(+) enantiomer. Here we conducted experiments in the postdependent rat model of alcohol dependence to compare the efficacy of R(+)Baclofen or S(−)-Baclofen to that of RS(±)-Baclofen on ethanol intake, seeking, and relapse. R(+)Baclofen was more effective than RS(±)-Baclofen in reducing ethanol intake and seeking during acute withdrawal and during relapse after abstinence. We also used an original population approach in order to identify drug responders. We found a significant proportion of responders to S(−)-Baclofen and RS(±)-Baclofen, displaying an increase in ethanol intake, and this increasing effect on alcohol intake was not seen in the R(+)Baclofen group. At an intermediate dose of R(+)Baclofen, devoid of any motor side effects, we identified a very large proportion of responders (75%) with a large decrease in ethanol intake (90% decrease). Finally, the response to RS(±)-Baclofen on ethanol intake was correlated to plasma level of Baclofen. R(+)Baclofen and RS(±)-Baclofen were effective in reducing sucrose intake. Our study has important clinical implication since it suggests that the wide variability in the therapeutic responses of patients to RS(±)-Baclofen may come from the sensitivity to the R(+)Baclofen but also to the one of the S(−)-Baclofen that can promote an increase in ethanol intake.

Keywords
alcohol use disorder, GABAB receptor, operant oral alcohol self-administration, R(+)Baclofen, RS(±)-Baclofen, S(−)-Baclofen
Alcohol dependence (AD) is a chronic disease associated with high rates of mortality and morbidity. The 1-year prevalence of AD in the European Union was estimated at 3.4% among people 18 to 64 years of age (women 1.7%, men 5.2%), resulting in close to 11 million affected people.¹ In France, the prevalence is also estimated to 3.4% and a recent study demonstrated that alcohol is the first cause of hospitalization.² Despite the high prevalence of AD and its substantial impact on mortality and disease, it has the widest treatment gap at 92% in Europe.³ Several kinds of medication are currently available for the treatment of AD with different goals and specific mechanisms of action. Medications are used either to maintain abstinence (ie, disulfiram, acamprosate, naltrexone, GHB, and RS(±)-Baclofen) or to reduce drinking (ie, nalmefene and RS(±)-Baclofen). However several studies and meta-analyses of drug clinical trials for AD demonstrate consistent effectiveness with modest effect sizes for efficacy in reducing heavy drinking or maintaining abstinence. For example, the numbers needed to treat for benefit for acamprosate and naltrexone have been calculated around 11 to 20.⁴

Among the AD pharmacotherapies, RS(±)-Baclofen, a selective gamma-aminobutyric acid-B (GABA-B) receptor agonist, has a very special status and its use remains highly controversial. Open-label studies and clinical trials have shown that RS(±)-Baclofen can increase rates of abstinence and reduce alcohol craving and anxiety, with good tolerance and few side effect leading to its widespread off-label use in the treatment of AD, particularly in relapse prevention. A recent study in 30 patients with AD showed that RS(±)-Baclofen (30-75 mg) for at least 2 weeks attenuates alcohol cue reactivity in prefrontal brain regions associated with no changes in craving and decrease in the % of heavy drinking days.⁵ The promising results suggested by some studies and an impressive and intriguing lobbying by patient associations and clinicians insensitive to evidence-based medicine led in France to a “Temporary Recommendations for Use” in AD in 2014 and a Marketing Authorization Approval for doses up to 80 mg daily in 2018.⁶ Some recent publications failed to demonstrate a superior clinical outcome of RS(±)-Baclofen vs. placebo to maintain abstinence and reported that the current increasing use of RS(±)-Baclofen as a treatment for AD is premature.⁷,⁸ Some studies have reported concerns about adverse effects of RS(±)-Baclofen especially at high doses and particularly when taken with alcohol.⁹,¹⁰ The Cagliari Consensus statement suggested that RS(±)-Baclofen’s superioritiy over placebo is yet to be established, and further knowledge of dose-response relationships is required.¹¹ Controversial data about RS(±)-Baclofen efficacy in AD may come from numerous parameters such as the optimal dose, highly variable plasma levels of RS(±)-Baclofen, levels of comorbid anxiety, clinical endpoints (abstinence versus intake reduction), lack of identification of responder characteristics, and AD severity.¹²-¹⁵ Regarding highly variable plasma levels of RS(±)-Baclofen in AD patients, no study has been able to identify factors explaining inter-individual variability.¹⁶,¹⁷

Efficacy of racemic Baclofen (RS(±)-Baclofen) to reduce alcohol intake in rats has been demonstrated for more than three decades.¹⁸-²² However, it is striking to note that the doses used in the studies are very variable, generally ranging from 1 to 10 mg/kg for the reduction of alcohol intake or even 40 mg/kg for the reduction of withdrawal symptoms.²² Preclinical studies have also demonstrated mixed results using RS(±)-Baclofen since an increase in ethanol intake has also been shown in two-bottle choice and operant procedures.²⁴-²⁶ For example, RS(±)-Baclofen reduces ethanol and sucrose responding at 5mg/kg but increases ethanol and decreases sucrose responding at 1.25mg/kg.²⁶

Much less studies have explored the effects of the two enantiomers of RS(±)-Baclofen, the S(−)-Baclofen and the R(+)-Baclofen. The Baclofen that is used clinically is a racemic compound that breaks down into absolute configurations of R+ and S− and positive (+) and negative (−) molecular rotations. The biological action of the racemic drug Baclofen is known to reside with the active R(+)-Baclofen.²⁷ We have recently demonstrated that R(+)-Baclofen reduced alcohol intake, motivation to consume alcohol and alcohol relapse in a relevant animal model of binge drinking.²⁸-⁴⁰ Altogether these data demonstrated that R(+)-Baclofen is the most interesting enantiomer; however, the data have been obtained in inbred mice and selectively bred mice and rats and no study compared the effect of R(+)-Baclofen to those of RS(±)-Baclofen and no data are available regarding inter-individual responses.

In the present study, we tested the efficacy of the different Baclofen enantiomers on operant alcohol self-administration and relapse in outbred Long Evans rats. The use of outbred rats may be more appropriate to reveal interindividual variability to alcohol intake and response to Baclofen that is also analyzed in the present study. In addition we used here the postdependent state model of AD that is a more relevant model regarding the induction of a more severe AD phenotype and behavioral traits of the human disease with loss of control over ethanol intake and compulsive use.³¹,³² Because the identification of good responders is of high priority in AD patients, we looked at the individual response depending on the enantiomer and the dose. Identification of good responders is a critical issue, and this point has been raised for example in a recent study and a meta-analysis on Baclofen showing that higher daily alcohol intake could be associated with a greater effect of Baclofen.³³

The effect of RS(±)-Baclofen, the racemic Baclofen that is used in patients, is mediated by both R(+)-Baclofen and S(−)-Baclofen. Because S(−)-Baclofen may have opposite effects compared with those of R(+)-Baclofen, we explored individual responses to each enantiomer in order to better understand the potential reason of the wide variability observed in patients in their therapeutic responses to RS(±)-Baclofen.¹⁶ Finally, human pharmacokinetic studies have shown a wide interindividual variability.¹³ Thus, since the efficacy may be linked to pharmacokinetic factors, we quantified plasmatic Baclofen concentrations.
2 | MATERIALS AND METHODS

2.1 | Subjects

Twenty-four male Long-Evans rats (weighing 250-290 g at the beginning of the two-bottle choice procedure) were obtained from Charles River (L’Arbresle, France). Animals were individually housed under a 12-hour light/dark cycle (lights on at 7:00 am) in a temperature and humidity-controlled environment (22 ± 1°C), with food and water available ad libitum. Experiments were carried out in accordance to the guidelines for Care and Use of Laboratory Animals (National Institutes of Health) and the European community regulations for animal use in research (CEE no. 86/609) and were approved by the local research ethics committee (CREMEAP; no. APAFIS#2145-20151005157534). All efforts were made to minimize animal suffering and to reduce the number of animals used.

2.2 | Drug solutions

Ethanol solution for operant alcohol self-administration experiments was prepared by diluting ethanol 96% (VWR-Prolabo, Fontenay-sous-Bois, France) to 20% (v/v) in tap water. R(+) Baclofen, S(−)-Baclofen, and RS(±)-Baclofen were obtained from (Sigma Aldrich, Saint Quentin Fallavier, France). All drugs were dissolved in 0.9% sterile saline. Drug administration was given 30 minutes before the start of operant alcohol self-administration experiments via i.p. injections. Baclofen was administered at 0.5, 1, and 2 mg/kg, in a volume of 1 mL/kg of body weight. The dose of 1.5 mg/kg of R(+) Baclofen was also tested. All solutions were used at room temperature, and doses and routes of administration were chosen accordingly to the literature.

2.3 | Self-administration of high levels of ethanol in dependent rats

In order to facilitate high level of ethanol intake, rats were trained to consume 20% ethanol solution in a two-bottle-choice drinking procedure followed by several weeks of operant ethanol self-administration. For detailed description, see the Supporting Information section. After 5 weeks of stable levels of responding in operant ethanol self-administration (FR-3, 30 min), rats were collectively housed (two per cage) in pressured and ventilated chambers and exposed to chronic and intermittent ethanol vapor inhalation in order to induce dependence, as previously described. For detailed description, see the Supporting Information section. Animals were maintained under these conditions for 12 weeks until the start of any pharmacological treatment.

During the inhalation protocol, blood ethanol concentrations were regularly determined to adjust ethanol concentration in inhalation chambers. Blood ethanol samples were collected from sublingual vein, and BECs were determined using ANALOX AM1 Instrument analyzer (IMLAB, Lille, France).

2.4 | Drug injections

After operant responding stabilized in the procedures described above, rats received i.p. injections (volume: 1 mL/kg of body weight) of either the vehicle (saline) or the drug treatment, 30 minutes before the start of the operant session. Each rat received all the different treatments at the different doses tested in a Latin square counterbalanced design with, at least, a 1-day washout period between each dose.

2.4.1 | Experiment 1: Dose-response curves for RS(±)-Baclofen and its enantiomers

The doses tested for R(+)-Baclofen, S(−)-Baclofen, and RS(±)-Baclofen were 0.5, 1, and 2 mg/kg. All rats received drug treatments twice a week (every Tuesday and Thursday), taking 5 weeks to complete the dose-effect curves. On the other days of the week, operant ethanol self-administration sessions were conducted in the presence of saline injections.

2.4.2 | Experiment 2: 1.5mg/kg R(+)-Baclofen and homemade RS(±)-Baclofen

When Experiment 1 was finished, we decided to test, within the same group of rats, the 1.5 mg/kg dose of R(+)-Baclofen and also our homemade RS(±)-Baclofen (2 mg/kg) to be sure of the enantiomer ratio (1:1).

2.4.3 | Experiment 3: Effect of R(+)-Baclofen and RS(±)-Baclofen on ethanol seeking

Three weeks after, we evaluated the effect of 1.5 mg/kg of R (+)-Baclofen, 2 mg/kg of RS(±)-Baclofen, and vehicle (saline) on ethanol-seeking behavior. A single 30-minute session of extinction responding for ethanol was performed 24 hours after the last session of operant self-administration. During the extinction sessions, rats were exposed to the operant chamber and ethanol-associated cues (light above the lever and in the magazine), but lever-pressing did not result in any ethanol presentation. All three doses were tested in each rat (n=23), and extinction sessions were conducted once a week for 3 weeks. After each extinction session, ethanol self-administration rapidly recovered to baseline levels.

2.4.4 | Experiment 4: Effect of R(+)-Baclofen and RS(±)-Baclofen on relapse after abstinence

This paradigm consisted of introducing a period of forced abstinence. Here, the animals were not introduced into the operant chambers for 2 weeks, during which rats were housed in the home cage without...
ethanol vapor. The animals were divided into three groups (n=8) with equivalent baseline of ethanol self-administration. The baseline corresponded to the average of the last three operant sessions before abstinence. On test day (relapse), 1.5 mg/kg of R(+)-Baclofen, 2 mg/kg of RS(±)-Baclofen, or vehicle were injected 30 minutes before the start of the operant ethanol self-administration session, and ethanol was available during the 30-minute session.

2.4.5 | Experiment 5: Effect of R(+)-Baclofen and RS(±)-Baclofen on 1% sucrose operant self administration

Rats (n=8/group) were exposed to operant sucrose self-administration in 30-minute daily sessions under a fixed-ratio 3 schedule of reinforcement. Each response resulted in delivery of 0.1 mL of sucrose (1% w/v). Following a stable self-administration baseline, we tested the effect of R(+)-Baclofen (1 and 1.5 mg/kg), 2 mg/kg of RS(±)-Baclofen or vehicle, injected (i.p) 30 minutes before the operant session, on sucrose self-administration.

2.5 | Locomotor activity

Sedation is a common cited side-effect and limits tolerability in some patients; thus, we measured locomotor activity in our animals treated with either R(+)-Baclofen or RS(±)-Baclofen. The effect of high doses of R(+)-Baclofen (1.5 and 2mg/kg) and RS(±)-Baclofen (2mg/kg) were tested at the end of the study on rats that have already been tested for the three drugs. For a detailed description of the protocol, see the Supporting Information section.

2.6 | Baclofen quantification in plasma and correlation with its efficacy to reduce ethanol intake

Plasma samples collected from sublingual and retro-orbital sinus were used to determine the concentrations of baclofen by liquid chromatography-tandem mass spectrometry (LC-MS/MS) after protein precipitation, as previously described. Plasma samples were collected 30, 60, and 180 minutes after RS(±)-Baclofen (2mg/kg) injection in dependent rats. Correlation was tested between Baclofen efficacy and plasma Baclofen concentrations. Efficacy to reduce ethanol intake represents the percentage of change in ethanol intake before and after treatment. For a detailed description of the protocol, see the Supporting Information section.

2.7 | Statistical analyses

The SigmaStat 4.0 (Systat Software, Inc) and Prism 8 (GraphPad) softwares were used for all statistical analyses. Data were analyzed using one- or two-way analyses of variance (ANOVA), with or without repeated measures (RM) when normality and variance equality were confirmed; otherwise, a Kruskal-Wallis test was used. After confirming the significance of the primary findings using ANOVA or Kruskal-Wallis, a significance level of P<.05 was applied to all remaining post hoc analyses. The design was a within subject design for Experiments 1, 2, 3, and 5 and the dosage of plasmatic Baclofen. A between subject design was used for the Experiment 4 (Relapse). The repeated factors were the doses and the treatments for all the within subjects studies. The analysis of the distribution of the population in Experiment 1 was performed using a Chi square test.

For single comparisons, we used a Student’s t test (two-tailed); for multiple comparisons, we used a Tukey test after the ANOVA and the Dunn’s test after the Kruskal-Wallis test. Data were expressed as mean ± SEM (standard error of the mean) throughout the text and figures. A Pearson test was used for the correlation studies.

3 | RESULTS

3.1 | Effect of drugs on ethanol intake

Dependent rats were daily submitted to operant alcohol self-administration sessions for 30 minutes. We tested the effects of four different doses (0, 0.5, 1, and 2 mg/kg) of the two enantiomers of Baclofen (R(+)-Baclofen and S(−)-Baclofen) separately and the commercial racemic formula (RS(±)-Baclofen) on ethanol consumption (Figure 1). We found that S(−)-Baclofen did not reduce ethanol...
consumption at any doses. On the contrary, R(+)-Baclofen and RS(±)-Baclofen dose dependently reduced ethanol consumption with a maximum decrease from 0.55 to 0.059 g/kg of pure ethanol consumed for the R(+)-Baclofen at the dose of 2 mg/kg. The analysis of the data using a 2-way RM-ANOVA indicated a main effect of the factor treatment \((F_{2,138} = 38.8, P < .001)\), of the factor doses \((F_{2,138} = 48.2, P < .001)\), and revealed an interaction between both factors \((F_{6,138} = 13.4, P < .001)\). A Tukey test was performed for multiple comparisons between treatments and doses. This post hoc test did not show any difference between the doses within the S(−)-Baclofen treatment \((P > .05)\). Within the R(+)-Baclofen treatment, only the doses of 1 and 2 mg/kg were significantly different from the dose of 0 mg/kg \((P < .05 \text{ and } P < .001 \text{ respectively})\). Significant differences were also observed between the groups 0.5 and 1 mg/kg vs. the 2 mg/kg dose \((P < .001 \text{ for both})\). Concerning the RS(±)-Baclofen treatment, only the dose of 2 mg/kg differs significantly from the 0, 0.5 and 1 mg/kg doses \((P < .001)\). Within the dose of 0 mg/kg, no differences were observed between the treatments \((P > .9 \text{ for all comparisons})\). For the dose of 0.5, a significant difference between the treatments S(−)-Baclofen and R(+)-Baclofen \((P < .05)\) was observed, whereas for the dose of 1 mg/kg, a significant difference was obtained between the RS(±)-Baclofen and R(+)-Baclofen treatments \((P < .05)\). A marginal effect was nonetheless observed between the treatments S(−)-Baclofen and R(+)-Baclofen \((P = .059)\) for this latter dose. Within the dose of 2 mg/kg, all treatments differ from the others \((P < .001 \text{ for all comparisons})\). The effect of R(+-) and RS(±)-Baclofen is short lasting since the levels of ethanol self-administration are back to baseline the day after the injection (Figures S1A-S1C). For a detailed statistical analysis, see the Supporting information section.

The data analysis at the individual level revealed different profiles of response (Figure S2). Indeed, some animals did not respond to the drug, some others exhibited a decrease in ethanol consumption, but surprisingly, some individuals showed an increase in ethanol consumption after treatment. To estimate the proportion of responder vs. nonresponder rats, we tested two different thresholds of change as compared with the dose of 0 mg/kg, namely, 10 and 15% change. Data are presented only for the 10% change threshold because the one of 15% does not bring more information. As shown in Figure 2, we evaluated from the same set of data than in Figure 1, the proportion of each category of response for the doses of 1 mg/kg and 2 mg/kg (Figure 2B) of the three treatments and for the 10% change threshold. The striking finding in this analysis is the fact that for the dose of 1 mg/kg in both the S(−)-Baclofen and RS(±)-Baclofen treatments, 29 and 25% of the rats respectively showed an increase in ethanol consumption. The Chi square analysis revealed a significant difference \((P < .05)\) between the S(−) and R(+)-Baclofen (Figure 2A). For the dose of 2mg/kg, only the S(−)-Baclofen induced an increase in ethanol consumption in a large proportion of rats (45.8%). Only one of the rats receiving the R(+-)-Baclofen treatment showed an increase in ethanol intake (Figure 2B). The Chi square analysis revealed a significant difference in the proportions of responders vs. nonresponders between both the R(+-)-Baclofen and RS(±)-Baclofen vs. the S(−)-Baclofen treatment \((P < .001 \text{ and } P < .01 \text{ respectively})\). Interestingly, the proportion of rats showing an increase in consumption after the RS(±)-Baclofen treatment dropped to 4.2 after the dose of 2 mg/kg (Figure 2B). The Chi square analysis revealed a significant difference in the proportion of responders vs. nonresponders between both the R(+-)-Baclofen and RS(±)-Baclofen vs. the S(−)-Baclofen treatment \((P < .01 \text{ for both comparisons})\). Hence, in order to demonstrate if the doses of baclofen used here may induce locomotor side effects, we tested the effect of the doses of 1.5 and 2 mg/kg of R(+-)Baclofen vs. the effect 2 mg/kg of RS(±)-Baclofen on locomotor activity in an open field (Figure 3). We...
found that only the dose of 2 mg/kg of R(+)-Baclofen induced a decrease in the total distance travelled. The normality test failed; thus, a Kruskal-Wallis test was performed and indicated a main effect of the factor treatment (H = 19.1946, \( P < .001 \)). The Dunn's multiple comparison test revealed significant differences between the dose of 2 mg/kg of R(+)-Baclofen vs. the vehicle group (\( P < .001 \)) and vs. the treatment of RS(±)-Baclofen at 2 mg/kg. We thus tested this new dose of R(+)-Baclofen (1.5 mg/kg) on ethanol self-administration in comparison with the dose of 2 mg/kg of the homemade mixture 1:1 of R(+)-Baclofen/S(−)-Baclofen. We found that both treatments decreased ethanol consumption (Figure S3 and Supporting information) and that the effect is transient with a total recovery the day after the injection. A significant rebound in the amount of ethanol consumed was nevertheless noticed for the dose of 1.5 mg/kg of R(+)-Baclofen (Figure S4).

In Table 1, we depicted the percentage of rats in each category of response to the treatments determined from Experiment 1 and the corresponding variation in ethanol consumption. We found that the increase in ethanol consumption induced by the S(−)-Baclofen treatment (1 or 2 mg/kg) is of about 47%, whereas the decrease is of about 33%. The amplitudes of these increase and decrease are similar to the ones observed with the RS(±)-Baclofen treatment at 1 mg/kg (48% increase and 40% decrease) for the same order of proportions already observed (one third of the total population).

When the dose was increased to 2 mg/kg, only 1 rat continued to show an increased in ethanol consumption (+21.4%), whereas 87.5% of the rats exhibited a decrease of 56.1% in their ethanol consumption. In contrast, the dose of 1 mg/kg of R(+)-Baclofen induced an increase in ethanol consumption in only 8.3% of the rats for an increase of 34.5%. The majority of the rats (54.2%) exhibited a decrease of 41.8% in ethanol consumption. For the dose of 2 mg/kg, the effect is even larger with a decrease in ethanol consumption of 95.2% for 91.7% of the rats. Only one rat showed a limited increase of 16.7% in ethanol consumed. For the intermediate dose of R(−)-Baclofen (1.5 mg/kg, which has no locomotor effect), it has a similar global effect as for the RS(±)-Baclofen dose of 2 mg/kg, and it is noteworthy that 75% of the rats showed a decrease of more than

| TABLE 1 | Proportion of responders (increase, decrease, and no response) after treatment with each enantiomer or the racemic compound at different doses |
|---|---|---|---|---|
| 1 mg/kg | S(−)-Baclofen | % of Rats | % of Change | R(+)-Baclofen | % of Rats | % of Change | RS(±)-Baclofen | % of Rats | % of Change |
| Nonresponders | 41.7 | +0.1 | 35.7 | −3.9 | 37.5 | +1.6 |
| Intake increased | 29.2 | +47.9 | 8.3 | +34.5 | 25.0 | +48.6 |
| Intake decreased | 29.2 | −34.6 | 54.2 | −41.8 | 37.5 | −40.1 |
| 2 mg/kg | S(−)-Baclofen | % of Rats | % of Change | R(+)-Baclofen | % of Rats | % of Change | RS(±)-Baclofen | % of Rats | % of Change |
| Nonresponders | 25.0 | +6.5 | 4.2 | 0 | 8.3 | +6.1 |
| Intake increased | 33.3 | +46.9 | 4.2 | 16.7 | 4.2 | +21.4 |
| Intake decreased | 41.7 | −31.5 | 91.7 | −95.2 | 87.5 | −56.1 |
| 1.5 mg/kg | R(+)-Baclofen | % of Rats | % of Change |
| Nonresponders | 16.7 | −7.9 |
| Intake increased | 8.3 | +30.9 |
| Intake decreased | 75 | −89.3 |
89% of their ethanol consumption, whereas only 8.3% showed an increase of 30.9%.

### 3.2 Effect of drugs on ethanol seeking

The effect of the R(+)-Baclofen at 1.5 mg/kg and the RS(±)-Baclofen at 2 mg/kg on seeking was evaluated during a single session in which no delivery of ethanol occurred even after the three consecutive presses on the active lever and the appearance of the cues (light and sound). We found that both treatments decreased the seeking (number of the active lever presses) as compared with the vehicle administration (Figure 4A). The one-way RM-ANOVA revealed a main effect of the factor treatment ($F_{(2,44)} = 50.03, P < .001$). The post hoc test indicated a significant difference between the groups R(+)-Baclofen (1.5 mg/kg) and RS(±)-Baclofen (2 mg/kg) vs. the vehicle group ($P < .001$). The analysis also revealed a marginal difference between the R(+)-Baclofen (1.5 mg/kg) and RS(±)-Baclofen (2 mg/kg) treatment ($P = .056$).

### 3.3 Effect of drugs on relapse after abstinence

One major challenge for the treatment of AD is to prevent the relapse phenomenon after abstinence. After 10 days of abstinence, rats were submitted to new sessions of self-administration with regular access to the 20% ethanol solution (FR3 – 30 min) and treated with either the vehicle, R(+)-Baclofen (1.5 mg/kg) or RS(±)-Baclofen (2 mg/kg). We found that both treatments blocked the relapse as compared with the vehicle (Figure 4B). A two-way RM-ANOVA revealed a main effect of the factor time point ($F_{(1,21)} = 21.96, P < .001$) and of the factor treatment $F_{(2,21)} = 10.57, P < .001$ and an interaction between both factors $F_{(2,21)} = 7.94, P < .01$. The post hoc analysis indicated a significant difference between the Baseline and the relapse for both...
treatments ($P < .001$) but not for the vehicle group ($P > .05$). Within the relapse time point, the analysis revealed a significant difference between the vehicle group and both treatment groups ($P < .001$).

### 3.4 Effect of drugs on 1% sucrose intake

The latter treatments were tested on operant self-administration of a 1% sucrose solution. We found that both treatments decreased sucrose self-administration (Figure 4C). The one-way ANOVA performed revealed a significant main effect of the factor treatment ($F_{(2,22)} = 7.2$, $P < .01$). The post hoc analysis indicated a significant difference between the vehicle group and the R(+)−Baclofen group (1.5 mg/kg) ($P < .01$) and a marginal effect between the vehicle group and the RS(±)-Baclofen (2 mg/kg) ($P = .053$).

### 3.5 Correlation between Baclofen plasma concentrations and its efficacy to reduce ethanol self-administration

As expected, we found a peak concentration at 30 minutes and then a rapid decrease at 60 and 180 minutes (Figure 5A). The analysis of the data indicated a main effect of the factor time ($F_{(2,44)} = 250.12$, $P < .001$). The Tukey test revealed a significant difference between each of all the three time points ($P < .001$). It is striking to note that at 30 minutes, there was a significant correlation between the plasma Baclofen concentrations and the efficacy to reduce ethanol self-administration at both doses ($R^2=0.35$ and $P < .01$ at 1 mg/kg and $R^2=0.27$ and $P < .05$ at 2 mg/kg) (Figures 5B and 5C).

### 4 DISCUSSION

Market authorization of RS(±)-Baclofen has been decided in France by the National Agency for the Safety of Medicines (ANSM) in October 2018 with the maximum dose requirement of 80 mg per day and use after failure of other treatments. However, the international debate about its benefit/risk balance is still open as well as some questions about the great variability in the clinical response and the lack of a dose response.\(^5\) Further studies are thus required in order to better understand the reason of the observed variability.\(^6\)

It is established that both RS(±)-Baclofen and R(+)−Baclofen reduce ethanol intake in rodents, while S(−)-Baclofen has no effect or increases ethanol intake.\(^{36,51,52}\) Studies in high-alcohol-prefering mice have showed that RS(±)-Baclofen has stereospecific effect with the more active enantiomer, R(+)−Baclofen, suppressing alcohol intake and the less active enantiomer, S(−)-Baclofen, stimulating alcohol intake.\(^{51,52}\) Furthermore, RS(±)-Baclofen (3 mg/kg) and R(+)−Baclofen (0.75-3 mg/kg), but not S(−)-Baclofen (6-24 mg/kg), reduced 15% alcohol operant self-administration in selectively bred Sardinian alcohol-prefering rats.\(^{36}\) We confirmed this assumption in outbred Long Evans rats with our results showing that one administration of both RS(±)-Baclofen and R(+)−Baclofen reduce ethanol intake, while S(−)-Baclofen can either increase or have no effect in the alcohol post-dependent state model of AD. In addition, we found that S(−)-Baclofen can also decrease ethanol intake, and to our knowledge, this effect has not been reported in the literature. Our results showed that both RS(±)-Baclofen and R(+)−Baclofen reduced 1% sucrose intake. We observed that R(+)−Baclofen is more potent to reduce
ethanol intake than RS(±)-Baclofen at both doses 1 and 2mg/kg. At 1.5mg/kg, R(+)-Baclofen induces a strong decrease of ethanol intake without any sedative side effects. R(+)-Baclofen may also be more potent on sedation because alteration of locomotor activity is observed at 2mg/kg, whereas RS(±)-Baclofen has no effect at the same dose. At 1.5 mg/kg, R(−)-Baclofen was more effective in reducing seeking behavior and relapse than 2mg/kg RS(±)-Baclofen.

As observed in humans, our study demonstrated a large variability in the response to the different enantiomers. We identified subgroups of responders, and the most striking finding shows that the efficacy of RS(±)-Baclofen used in humans may be limited by the effects of S(−)-Baclofen that can induce an increase in ethanol intake (~50% increase) in about 30% of the population.

### 4.1 Efficacy and variability in the response to the different enantiomers

As found in humans, we found a difference of response to RS(±)-Baclofen that is associated with a different exposure despite a similar dosage. Thus, our results confirm that the efficacy to reduce ethanol self-administration is linked to pharmacokinetic factors. We found that R(+)-Baclofen is more effective than RS(±)-Baclofen at both 1 and 2mg/kg. Interestingly, we observed a rebound effect for the 1.5mg/kg dose of R(+)-Baclofen thus further suggesting that the R(+) enantiomer may display better efficacy highlighted by the “deprivation-like” effect. Because we observed a reduction in locomotion at the 2mg/kg dose of R(+)-Baclofen, we then tested the 1.5 mg/kg dose. The latter dose induces a strong decrease in ethanol intake without having any motor side effect. Also, when looking at the mean of the population, the S(−)-Baclofen has no effect on ethanol intake. However, the analysis of individual differences revealed unexpected results with both responder and nonresponder rats. S(−)-Baclofen, at the two doses, induced an increase of ethanol intake (~50%) in about 30% of the population and a decrease of about 30% in 30 to 40% of the population. At the 1mg/kg dose, the distribution of responders to RS(±)-Baclofen is similar to that of S(−)-Baclofen, whereas at the 2mg/kg dose, the distribution of responders to S(−)-Baclofen is similar to that of R(+)-Baclofen. The number of responders showing a reduction in ethanol intake is largely increased by doubling the dose of RS(±)-Baclofen and R(+)-Baclofen. Interestingly, at 1mg/kg, the proportion of rats displaying a decrease in ethanol intake is higher in the R(+) Baclofen groups compared with the one of RS(±)-Baclofen and S(−)-Baclofen in which the proportion of rats displaying an increase in ethanol intake is still important (25 to 30%). At 1mg/kg, 29% of the population treated by S(−)-Baclofen and 30% of the population treated by RS(±)-Baclofen increased their consumption by about 50%, while a smaller increase (about 35%) is observed in less than 10% of rats treated with R(+)-Baclofen. Thus, the limited efficacy of RS(±)-Baclofen may be linked to the increasing effect of S(−)-Baclofen on ethanol intake and this opposite effects of S(−)-Baclofen and R(+)-Baclofen may explain, at least in part, the variability of the response to RS(±)-Baclofen and the reduced efficacy of RS(±)-Baclofen (~56% for RS(±)-Baclofen versus ~95% for R(+)-Baclofen at the 2mg/kg dose).

Comparing our preclinical data to those obtained in patients is of interest. First, at 2.0mg/kg of RS(±)-Baclofen, we observed a decrease of about 56% (in about 80% of the population) in ethanol drinking and it is striking to note that the same level of decrease (61.8%) is observed in humans on the drinks per drinking day criterion after 30mg/day Baclofen in a 12-week trial. Interestingly, in the above cited study, when looking at the individual changes in the drinks per drinking day of the 12 patients, three subgroups are observed: nonresponders (n=3), responders with decrease in drinking (n=6), and responders with an increase in drinking (n=3). Thus, the different subgroups identified in animals are also observed in patients and these results confirm that the efficacy of the RS(±)-Baclofen compound may be limited by the fact that patients are not responding but also by the fact that some patients display an increase in ethanol drinking. The same study also showed that the three female participants were among the best responders and thus suggests that looking for subgroups of responders is of great interest regarding the efficacy of RS(±)-Baclofen. It is also important to point out that when looking at the outcome of drink per drinking days at the end of treatment, a recent meta-analysis showed that Baclofen has an increasing effect (mean difference 1.55, 95% CI, 1.32 to 1.77; two studies 72 participants) compared with the placebo group, but this result displays a low level of certainty of evidence. Finally, our results demonstrate that the efficacy to reduce ethanol intake is correlated to plasma Baclofen concentrations. We show that a similar dosage can lead to an exposure varying from simple to triple and could explain treatment outcome as suggested in human studies.

### 4.2 Efficacy depending on baseline ethanol intake

Given the only moderate effect sizes of AD drugs and because in general patients with AD are very heterogeneous populations, more recent research tried to identify “target population” and predictive factors such as genetic polymorphisms. Thus, we also investigated if the effectiveness of Baclofen enantiomers can be linked to basal drinking level because reducing the drinking risk level in patients has been recognized as a target for developing new medications of AD by the EMA in its 2010 guideline. Some studies have suggested that AD drugs are likely to be more beneficial for more severe patients and/or patients with high or very high drinking risk level, for example for Baclofen. Nalmefene is approved in the European Union and other countries for the reduction of alcohol consumption in alcohol-dependent patients with a high drinking risk level according to World Health Organization (“target population”). Identification of “target population” has led us to analyze our data on drug efficacy depending on the basal level of intake. Our results do not show a better efficacy in rats displaying higher ethanol intake since the efficacy of 1.5mg/kg R(+)-Baclofen is inversely correlated with ethanol intake before treatment (data not shown). Nevertheless, AD is a heterogeneous
phenotype and acquired operant alcohol intake in rats could be considered a good model of behavioral dependence, while the post-dependent state resulting from ethanol vapor inhalation could be regarded as a model of AD with physical withdrawal symptoms. It is noteworthy that the effectiveness of RS(±)-Baclofen to reduce alcohol intake in dependent animals may also be explained by its ability to decrease alcohol withdrawal-induced anxiety.

4.3 Efficacy on ethanol seeking and on relapse after abstinence

Ethanol-associated cues are powerful mediators of craving in ethanol-dependent patients. Cue reactivity and cue-induced craving predict, to some degree, relapse in ethanol-dependent patients. Baclofen has been shown to reduce craving in ethanol-dependent patients and possibly through a decrease in brain reactivity to ethanol-associated cues. In animals, ethanol seeking could reflect ethanol craving observed in humans as ethanol-associated cues are still present while lever-responding still unreinforced. In absence of ethanol, we found that both R(+)-Baclofen and RS(±)-Baclofen reduced ethanol seeking behavior and our results are in line with those obtained in Sardinian alcohol-prefering rats with RS(±)-Baclofen (1-3mg/kg). We found that both 1.5mg/kg R(+)-Baclofen and 2.0mg/kg RS(±)-Baclofen were very effective in reducing reacquisition of ethanol intake after abstinence. This result is in line with our previous findings in an animal model of chronic binge drinking showing that 1.0mg/kg R(+)-Baclofen is also very effective in reducing reacquisition of ethanol intake after abstinence. Other studies in ethanol-prefering S rat and outbred Wistar rats, which used operant self-administration procedure, have shown that RS(±)-Baclofen (1-3mg/kg) was very effective in reducing ethanol seeking induced by ethanol-associated cue after a period of extinction of lever- or nose-poke responding for ethanol. Altogether, preclinical data show that both R(+)-Baclofen and RS(±)-Baclofen are effective to reduce ethanol seeking and reacquisition.

Interestingly, when comparing the results obtained on seeking with 1.5mg/kg R(+)-Baclofen to those with 2.0mg/kg RS(±)-Baclofen, we observed that some rats are less responding to the treatment in the RS(±)-Baclofen group, while a majority of good responders was seen in the R(+)-Baclofen group; thus, the difference between the two treatments approached the level of significance (P=0.056). These results are in line with those obtained in humans showing that two subpopulations of patients have been described based on their speed of response to the decreasing effect of Baclofen on craving.

4.4 Effects on sucrose intake

We found that 1.5mg/kg R(+)-Baclofen completely abolished 1% sucrose responding and this result is in line with those of another study in mice showing that R(+)-Baclofen reduced saccharin intake. At 2mg/kg, RS(±)-Baclofen did not significantly reduce 1% sucrose intake and this is in contrast with other studies that showed for example that 1 to 5.6mg/kg RS(±)-Baclofen reduced sucrose responding. Altogether, the studies show that both R (+)-Baclofen and RS(±)-Baclofen display lack of selectivity with the reduction of ethanol intake but also of alternative and nondrug reinforcers such as sucrose, saccharin, and food pellets.

4.5 Mechanism of action of Baclofen of the different enantiomers

S(−)-Baclofen is less potent than R(+)−Baclofen. At the pharmacological level, R(+)−Baclofen and RS(±)-Baclofen may have different behavioral outcome because S(−)-Baclofen without being itself active, especially at low dose, may increase or decrease the response to R (+)-Baclofen. Furthermore, one human study reported a stereoselective metabolic difference between R(+)- and S(−)-Baclofen with no metabolites observed after oral administration of the R-enantiomer, whereas an oxidative deamination metabolite was observed after the administration of the R(+)− and S(−)-mixture. The effect of the two enantiomers cannot be explained by a pharmacokinetic basis since the R(+)−Baclofen dose-dependently decreased ethanol consumption, whereas the high S(−)-Baclofen dose increased ethanol consumption when injected directly into the nucleus accumbens shell of C57Bl/6J mice. It would be very interesting to test the effect of the different enantiomers on striatal dopamine release after chronic alcohol intake. Nowadays, no clear explanation has been proposed to understand the bidirectional effect and the difference of efficacy of Baclofen depending on its enantiomers. One factor could be a difference in the binding affinity of the two enantiomers and the existence of low- and high-affinity GABAB receptors with low-affinity sites more selective for S(−)-Baclofen. Another possibility is that the different enantiomers may have different neurotransmitter targets. R (+)-Baclofen may interact with nongabaergic ones, while S (−)-Baclofen may interact with norepinephrine.

4.6 Limitations

Our study has some limitations that have to be pointed out. It should be noted that the level of ethanol intake of postdependent rats in the present study is lower than the one seen in other studies using Wistar rats; thus, rats of the present study may not display a severe phenotype of postdependent state. However, this low level of ethanol intake (below the 1.0g/kg threshold) has been reported in other studies that used the Long Evans strain. We used only male rats that received multiple acute treatments. Multiple treatments may not have impacted our results because of the short half-life of Baclofen and because we show that the recovery is complete and very quick. Since we tested only the effect of single injection, the potential development of tolerance or sensitization after repeated drug injections remains unknown but seems unlikely because we used low doses and other study has already showed no tolerance after repeated injections. Testing only acute treatments is a clear limitation of most of
the studies in the psychiatry field. It is noteworthy that because of its short half-life (3-4 h), Baclofen treatment needs to be repeated every day, and importantly, since it can be used in a reduction goal (not abstinence), some clinicians recommends to take it as an “as needed” treatment 1 to 2 hours before the usual peak of craving depending on the time of day and on each patient. Thus, it is likely that some patients take Baclofen as needed and not every day. In France, Baclofen has a marketing authorization approval for doses up to 80 mg daily to reduce ethanol intake (not abstinence) to low drinking risk level in patients with high drinking risk level (>60 and >40g ethanol/day in men and women, respectively). We also did not carry out full dose-response curves of the different enantiomers and thus are not able to directly compare the responses at all doses, partly because locomotor side effects appeared at lower dose of the R(+) enantiomer.

4.7 Conclusion

In summary, we provide here new and original data demonstrating that a single administration of R(+) Baclofen is more effective than RS(±)-Baclofen and is also more effective in nondependent (heavy drinker) animals compared with dependent animals. These results are encouraging and reinforce the idea of testing R(+) Baclofen in a clinical trial to explore if low doses may be efficient to reduce alcohol craving and intake and may also limit the occurrence of side effects as seen in patients with the racemic form of Baclofen, thus increasing the benefit/risk balance of the drug. This strategy could also avoid the lack of response or the increase of alcohol intake seen with the S(−) enantiomer. Recently, the STX209 drug comprising the single, active R-enantiomer of Baclofen and the novel prodrug of the R(+) Baclofen, arBaclofen placarbil, have been developed and are currently under clinical study in other diseases.

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CONFLICT OF INTEREST

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AUTHOR’S CONTRIBUTION

MN, VAE, and JJ designed the experiments. VEA, PS, and JJ contributed to the acquisition of animal data. LL and MS conducted experiments to measure plasmatic levels of Baclofen. MN, VAE, and JJ assisted with data analysis and interpretation of findings. MN, VAE, and JJ wrote the paper. All authors critically reviewed content and approved final version for publication.

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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SUPPORTING INFORMATION
Additional supporting information may be found online in the Supporting Information section at the end of this article.

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Supplemental Materials and Methods

2.3. Self-Administration of High Levels of Ethanol in dependent rats
Specifically, individually housed rats (n=24) were given intermittent access to one bottle of 20% ethanol and one bottle of tap water for three 24-hour-sessions per week (Mondays, Wednesdays and Fridays). At the end of the session, the bottles were weighed to assess ethanol consumption (g of pure ethanol / kg body weight) and preference (ethanol consumed / total fluid intake). Rats had unlimited access to two bottles of water between the ethanol-access periods. The placement of the ethanol bottle was alternated each ethanol drinking session to avoid side preferences. This procedure has been shown to induce escalation of ethanol consumption in several strains of rats. It should be noticed that individual differences have been demonstrated using this procedure in Wistar rats (Fredriksson et al., 2017). Rats were maintained on the 20% ethanol intermittent-access (IAE) 2-bottle-choice drinking procedure for 3 weeks.

Following 3 weeks of the IAE, operant ethanol self-administration training started (Jeanblanc et al., 2014, 2018b). Self-administration sessions were conducted in twelve standard operant chambers (Med Associates Inc. Fairfax VT, USA), as previously described. Briefly, the chambers were equipped with two levers (designated as active or inactive) located on either side of a drinking reservoir positioned in the center of the front panel of the chamber. Responding in the active lever resulted in the delivery of a 0.1 mL volume of 20% ethanol solution paired with a 2-second light-cue and 2-second time-out period following the lever press (in this time-out period, lever pressing was without consequences). During the whole session, inactive lever pressing was recorded, but had no consequences. The number of lever presses and ethanol solution deliveries were automatically recorded by the software.

Rats were randomly assigned to one operant chamber and were trained to press one of the two levers (the active lever) for 20% ethanol solution delivery. After two overnight sessions (16h) and four 1-h sessions under a fixed ratio 1 (FR-1) schedule of reinforcement, rats were trained on a FR-3 schedule for five 1-h sessions. After, the operant sessions lasted 30 min under a FR-3 schedule, 5 days a week for the entire study. Training sessions were run at the same time of the day. Finally, when stable levels of responding were observed over at least three consecutive self-administration sessions (±20%), drug treatment started.

After 5 weeks of stable levels of responding in operant ethanol self-administration rats were submitted to the period of ethanol vapors inhalation period. Ethanol vapor was generated by introducing pressured air into a 96% ethanol reservoir (60-80% of hygrometry). Ethanol vapor concentration and exposition time were gradually increased for 4 weeks to reach stable blood ethanol concentrations (BECs; between 150 and 250 mg/dl). Ethanol was evaporated for 14 h each day (from 06:00 pm to 08:00 am). At the end of the 4-weeks period, the 20% ethanol self-administration sessions were performed between 02:00 pm and 04:00 pm, corresponding to 6-8 h of withdrawal period from ethanol vapor when BECs returned to 0 (Rimondini et al., 2002; O’Dell et al., 2004). Rats chronically exposed to such intermittent ethanol vapor displayed a physical dependence, shown by the appearance of somatic withdrawal signs (Schulteis et al., 1995; Gilpin et al., 2008).

2.5. Locomotor activity
The motor activity chamber consists in a 40 × 40 × 30 cm box with opaque acrylic walls, transected with infrared photocell beams 2 cm above the floor at 16 sites along each side. Activity chambers were illuminated with indirect white light (20 lux). Horizontal locomotion was measured from photocell beam interruptions using ActiTrack software (Bioseb, Vitrolles, France) as we previously described (Lebourgeois et al., 2018b; González-Marín et al., 2018). Results are expressed as the total distance travelled (cm) during the 30 min session. In order to study the effect of R(+)-Baclofen or RS(±)-Baclofen on locomotor activity, rats (n=24) were exposed to saline, R(+)-Baclofen (1.5 and 2 mg/kg) and 2 mg/kg of RS(±)-Baclofen in counterbalanced order by a Latin square design, on non-consecutive days. On test days, rats received an i.p. injection of vehicle or Baclofen, and 30 min after animals were placed into the open field and allowed to freely explore the apparatus for 30 min. For
each animal, the total distance traveled during the 30-min test period was quantified using ActiTrack software (Bioseb).

2.6. Baclofen quantification in plasma
Sample preparation involved protein precipitation of plasma (100 μL) with internal solution of baclofen-d4 (10 μL, 100 μg/mL) with acetonitrile and 5% formic acid. Separation was carried out on an Accucore PFP column (100 × 2.1 mm, 2.6 μm, Thermo Fisher Scientific, Villebon-sur-Yvette, France) maintained at 30 °C. The mobile phase consisted of 0.1% formic acid in water and acetonitrile with gradient program at a flow rate of 500 μL/min. The following stepwise gradient conditions were used as follows: at 0 min: 5% of B kept for 1.5 min; at 7.5 min: 95% of B; at 7.6 initial conditions for 2.4 min. The total run time was 10 min. Mass Spectrometric analysis was performed on a TSQ Quantum Ultra® (Thermo Fisher Scientific). Detection in triple quadrupole mass spectrometer used an electrospray ionization probe and operated in positive ion mode. The multiple reaction monitoring transitions used for quantification were m/z 213.9/196.9 for baclofen and 217.9/200.9 for baclofen-d4. The lower and upper limits of quantification were 10 ng/mL and 2000 ng/mL, respectively. A full method validation was performed according to the guidelines for bioanalytical method validation defined by the EMA (European Medicines Agency). The intra- and inter-day precisions for three quality control levels (10, 1000 and 1500 ng/mL) were between 2.9 and 11.5% and between 1.4 and 11.2%, respectively, as described previously (Labat et al., 2016).

Supplemental Results

Supplemental Figure 1: One day recovery after Baclofen treatment
The analysis of the data obtained after the injection if S(-)-Baclofen (Supplemental Fig. 1A) by a Two-way ANOVA with repeated measures (within subject design) indicated no effect of the factor doses (F(3,138) = 0.81, ns) and of the factor time point (F(2,138) = 1.43, ns) and revealed no interaction between both factors (F(6,138) = 0.65, ns). In regards with the R(+)-Baclofen (Supplemental Fig. 1B), the analysis (two-way ANOVA with repeated measures in a within subjects design) revealed a main effect of the factor doses (F(3,138) = 18.88, p < 0.001) of the factor timepoint (F(2,138) = 77.64, p < 0.001) and indicated an interaction between both factors (F(6,138) = 20.05, p < 0.001). The post hoc analysis revealed a significant difference between the pre-injection and the injection timepoints for the doses of 0.5, 1 and 2 mg/kg (p < 0.01 for the dose of 0.5 mg/kg and p < 0.01 for the doses of 1 and 2 mg/kg). Similar differences were observed between the injection and the post-injection timepoints (p < 0.05 for the dose of 0.5 and p < 0.01 for the other doses except the 0 mg/kg dose). Concerning the RS(±)-Baclofen (Supplemental Fig. 1C), the Two-way ANOVA with repeated measures (within subject design) indicated a significant effect of the factor doses (F(3,138) = 4.00, p < 0.05), of the factor timepoints (F(2,138) = 15.01, p < 0.001) and an interaction between both factors (F(6,138) = 6.41, p < 0.001). The post hoc analysis revealed a significant difference only between the timepoint injection and the 2 others for the dose of 2 mg/kg (p < 0.001).

Supplemental Figure 3: Effect of R(+)-Baclofen (1.5mg/kg), RS(±)-Baclofen (2mg/kg) and homemade mixture 1:1 of R(+)-Baclofen/S(-)-Baclofen (2mg/kg) on ethanol consumed in dependent rats.
The one-way ANOVA conducted revealed a significant main effect of the factor treatment (F(3,69) = 12.28, p < 0.001). The post hoc analysis indicated that all the treatments were significantly different from the vehicle group (R(+)-Baclofen at 1.5 mg/kg, p < 0.001; R(+) Baclofen/S(-)-Baclofen at 2 mg/kg, p < 0.01). There is no difference between each treatment (p > 0.05).

Supplemental Fig. 4: Transient effect of the of R(+)-Baclofen (1.5mg/kg) and homemade mixture 1:1 of R(+)-Baclofen/S(-)-Baclofen (2mg/kg) on ethanol consumed in dependent rats.
We then tested a lower dose of R(+)-Baclofen (1.5 mg/kg) in comparison with an homemade mixture of R(+)-Baclofen and S(-)-Baclofen (2 mg/kg). The analysis (Two-way ANOVA with repeated measures
in a within subject design) for the dose of 1.5 mg/kg of R(+)-Baclofen and the homemade mixture of R(+)/S(-)-Baclofen (Supplemental Fig. 1D) revealed a significant main effect of the factor timepoint ($F_{(2,92)} = 30.21, p < 0.001$), no effect of the factor treatment ($F_{(2,92)} = 2.53, p = 0.088$), but an interaction between both factors ($F_{(4,92)} = 15.14, p < 0.001$). The post hoc analysis revealed that all the timepoints were different from each other for the dose of 1.5 mg/kg of R(+)-Baclofen suggesting a rebound effect the day after the injection ($p < 0.001$). In regards with the homemade racemic mixture of baclofen, the injection timepoint was significantly different from the 2 other timepoints ($p < 0.001$).

**Supplemental Figure Legends**

**Supplemental Figure 1:** Short lasting effect of Baclofen on the reduction of ethanol consumed. The number of active lever presses are depicted at 3 different timepoints of the Experiment 1: the session the day before the injection, the day of the injection and the data obtained during the session of the day after the injection day. The effects are shown for all the different doses (0, 0.5, 1 and 2 mg/kg) for the S(-)-Baclofen (A), R(+)-Baclofen (B) and RS(±)-Baclofen (C). Results are expressed as mean ± SEM of active lever presses. $n = 24$ (within subject design).

**Supplemental Figure 2:** Individual representation of the variation in ethanol consumption between the vehicle and the Baclofen treatments

For each treatment, the data are depicted in comparison with the vehicle injection in order to observe the individual responses (non responders, increased intake or decreased intake). S: S(-)-Baclofen, R: R(+)-Baclofen, RS: RS(±)-Baclofen. Doses: 1 = 1 mg/kg, 2 = mg/kg.

**Supplemental Figure 3:** Effect of R(+)-Baclofen (1.5mg/kg), RS(±)-Baclofen (2mg/kg) and homemade mixture 1:1 of R(+)-Baclofen/S(-)-Baclofen (2mg/kg) on ethanol consumed in dependent rats ($n = 24$). Because the 2mg/kg dose of R(+)-Baclofen had locomotor effect we tested the 1.5mg/kg dose. In order to check that the commercial RS(±)-Baclofen has a 50/50 ratio of the R(+) and the S(-) enantiomers we compared its effects with those of our home made racemic Baclofen. ** p < 0.05, *** p < 0.001 compared with vehicle (Veh).

**Supplemental Figure 4:** Short lasting effect of an intermediate dose of R(+)-Baclofen and of the homemade mixture of the RS(±)-Baclofen on the reduction of ethanol consumed. The number of active lever presses are depicted at 3 different timepoints of the Experiment 2: the session the day before the injection, the day of the injection and the data obtained during the session of the day after the injection day. The effects are shown for all the 1.5 mg dose of R(+)-Baclofen and 2 mg/kg of the homemade RS(±)-Baclofen (C). $n = 24$ (within subject design).
Number of active lever presses

Pre-inj. Day  Inj. day  Post-inj. Day

R(+)-Baclofen and R(+) + S(-)-Baclofen