$GABA_B$ receptor positive allosteric modulators with different efficacies affect neuroadaptation to and self-administration of alcohol and cocaine

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Running title

$GABA_B$ PAMs and drugs of abuse

ABSTRACT

Drugs of abuse induce widespread synaptic adaptations in the mesolimbic dopamine (DA) neurons. Such drug-induced neuroadaptations may constitute an initial cellular mechanism eventually leading to compulsive drug-seeking behavior. To evaluate the impact of $GABA_B$ receptors on addiction-related persistent neuroplasticity, we tested the ability of orthosteric agonist baclofen and two positive allosteric modulators (PAMs) of $GABA_B$ receptors to suppress neuroadaptations in the ventral tegmental area (VTA) and reward-related behaviors induced by ethanol and cocaine. A novel compound (S)-1-(5-fluoro-2,3-dihydro-1H-inden-2-yl)-4-methyl-6,7,8,9-tetrahydro-[1,2,4]triazolo[4,3-a]quinazolin-5(4H)-one (ORM-27669) was found to be a $GABA_B$ PAM of low efficacy as agonist, whereas the reference compound (R,S)-5,7-di-tert-butyl-3-hydroxy-3-trifluoromethyl-3H-benzofuran-2-one (rac-BHFF) had a different allosteric profile being a more potent PAM in the calcium-based assay and an agonist, coupled with potent PAM activity, in the [^{35}S]GTPγS binding assay in rat and human recombinant receptors. Using autoradiography, the high-efficacy rac-BHFF and the low-efficacy ORM-27669 potentiated the effects of baclofen on [^{35}S]GTPγS binding with identical brain regional distribution. Treatment of mice with baclofen, rac-BHFF or ORM-27669 failed to induce glutamate receptor neuroplasticity in the VTA DA neurons. Pretreatment with rac-BHFF at non-sedative doses effectively reversed both ethanol- and cocaine-induced plasticity and attenuated cocaine i.v. self-administration and ethanol drinking. Pretreatment with ORM-27669 only reversed ethanol-induced neuroplasticity and attenuated ethanol drinking, but had no effects on cocaine-induced neuroplasticity or self-administration. These findings encourage further investigation of $GABA_B$ receptor PAMs with different efficacies in addiction models to develop novel treatment strategies for drug addiction.

Keywords $GABA_B$ receptor positive allosteric modulators, glutamate neuroplasticity, cocaine self-administration, alcohol drinking

INTRODUCTION

Despite the drugs of abuse having different primary molecular targets in the brain, mesolimbic DA neurons in the ventral tegmental area (VTA) and the nucleus accumbens are thought to be their first common cellular targets (Kauer and Malenka, 2007). Indeed, drugs of abuse trigger long-term glutamate receptor neuroplasticity in VTA DA neurons, which in susceptible subjects might initiate neuroadaptations leading eventually to addiction (Korpi et al., 2015; Luthi and Luscher, 2014). Therefore, testing of anti-abuse agents for their efficacy against induction of neuroplasticity in the VTA DA neurons would give important information on their addiction or anti-addiction potential, when appropriately coupled to preclinical behavioral tests, such as drug self-administration and place conditioning.

The $GABA_B$ receptor agonist baclofen is well-known to acutely suppress DA release induced by morphine, nicotine and cocaine (Fadda et al., 2003) and this property of $GABA_B$ receptor agonistic ligands has been proposed to be exploited in the treatment of dependence to multiple classes of abused substances (Filip et al., 2015). To date, baclofen is used off-label in the treatment of alcoholism in some countries, and in France it has been temporarily approved at high maximal doses (de Beaurepaire, 2014). At the cellular level, baclofen mediates neuronal inhibition by activating the metabotropic $GABA_B$ receptor, which activates $G_{i/o}$ proteins leading to stimulation of K^+-channels, inhibition of Ca^{2+}-channels and to diverse downstream signaling pathways (Urwyler, 2011). In mesolimbic DA neurons expressing $GABA_B$ receptors (Bettler et al., 2004), baclofen reduces the firing rate and suppresses the burst firing and spontaneous pacemaker-like firing (Erhardt et al., 2002; Seutin et al., 1994), most likely via hyperpolarization induced by G protein-gated inwardly rectifying K^+-channels (GIRK) (Cruz et al., 2004; Labouebe et al., 2007). At the behavioral

level, in several studies, baclofen has attenuated self-administration of several drugs of abuse, reinstatement of drug-seeking behavior and drug-induced conditioned place preference (CPP), drug-induced hyperlocomotion and locomotor sensitization, and withdrawal signs in animal models of drug dependence (Filip et al., 2015). Noteworthy, several anti-addictive effects of baclofen have been detected after its injection directly into the VTA (Agabio and Colombo, 2015).

In line with numerous animal studies [(Fattore et al., 2002) and references cited therein], high doses of baclofen were efficacious to completely suppress craving and sustain alcohol abstinence (Ameisen, 2005; de Beaurepaire, 2014; Muller et al., 2015). Also lower doses of baclofen have shown efficacy to reduce alcohol drinking in some studies (e.g. (Addolorato et al., 2011; Morley et al., 2014)), but not in others [e.g., (Beraha et al., 2016; Garbutt et al., 2010)]. Despite of its generally good tolerability and efficacy, patients with impaired renal function or with epilepsy or other seizure disorder history are at significant risk to accumulate baclofen to toxic levels (Agabio and Colombo, 2015). Furthermore, baclofen may induce profound sedation, muscle relaxation, memory impairment and sexual dysfunction (Agabio and Colombo, 2015). In alcohol abusers, pharmacokinetics of baclofen is highly variable, its brain penetration poor (which can prevent achieving efficacious exposure), and there is a possibility to develop tolerance and withdrawal syndrome (Marsot et al., 2014). Thus, there is a clear demand for new $GABA_B$ receptor compounds with a higher therapeutic index than what baclofen has.

Positive allosteric modulators (PAMs) of $GABA_B$ receptors act at sites distinct from the orthosteric agonist binding site, at which baclofen acts (Urwyler, 2011). Because ideally PAMs are able to potentiate GABA responses only when and where it is endogenously released (Urwyler, 2011), they may provide a more physiologically relevant $GABA_B$ receptor

activation. Consequently, PAMs may have a different profile of adverse effects and be less harmful than full agonists (Koek et al., 2013). Indeed, in animal models new $GABA_B$ PAMs, rac-BHFF (Malherbe et al., 2008) and GS39783 (Urwyler et al., 2003), given alone, did not display any sedative, cognition-impairing or myorelaxant activities (Cryan et al., 2004), nor did they affect food- and sucrose-maintained responding at doses that attenuated alcohol or nicotine intake and reward (Maccioni et al., 2010; Paterson et al., 2008).

While the acute effects of $GABA_B$ receptor activation on VTA DA neurons have been well-characterized (Cruz et al., 2004), the effects of $GABA_B$ receptor agonists and PAMs on long-term neuroadaptations in the VTA have not been studied. In the present study, we examined whether baclofen and two PAMs of $GABA_B$ receptors (1) induce long-term glutamate synapse adaptations in VTA DA neurons, (2) suppress ethanol- and cocaine-induced neuroadaptations and (3) reverse addiction-related behaviors at non-sedative doses in mice. We used here rac-BHFF and a novel non-benzofuranone compound ORM-27669 [(S)-1-(5-fluoro-2,3-dihydro-1H-inden-2-yl)-4-methyl-6,7,8,9-tetrahydro-[1,2,4]triazolo[4,3-a]quinazolin-5(4H)-one; Fig. 1A] as $GABA_B$ PAMs.

MATERIALS AND METHODS

In vitro $GABA_B$ receptor pharmacology

In vitro $GABA_B$ receptor agonism and PAM pharmacology was studied in CHO cell lines stably expressing human or rat $GABA_{B1A}$ and $GABA_{B2}$ receptor subunits co-transfected with $G_{\alpha 16}$ by using intracellular Ca^{2+} mobilization monitored by FLIPR Tetra system (Molecular Devices, CA, USA) and [^{35}S]GTPγS binding assay, modified from (Malherbe et al., 2008). Potentiation of dose-responses of GABA and baclofen was studied as a leftward shift in the

presence of a 10-μM concentration of test compound. PAM EC_{50} values were determined using a dose-response of test compound in the presence of low concentrations (EC_5 –EC_{11}) of agonists. For detailed information, see Supporting Information Methods.

Animals and *in vivo* manipulations

We used juvenile (22-30 days old) male and female transgenic TH-EGFP mice (Gong et al., 2003) for electrophysiology and adult (8-11 weeks old) male C57BL/6JCrl mice (Charles River Germany, Sulzfield) for ligand autoradiography and behavioral studies. All drug injections (intraperitoneal i.p., intravenous i.v., or intragastric i.g.) and behavioral tests were performed between 08:00 and 19:00 h when the lights were on, except the ethanol drinking study which was performed during the dark phase. All animal procedures were approved by the Southern Finland Provincial Government, and conducted according to the following guidelines: Act on Use of Animals for Experimental Purposes 497/2013; Decree on Use of Animals for Experimental Purposes 564/2013; and Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purpose.

Agonist-stimulated [^{35}S]GTPγS autoradiography

The [^{35}S]GTPγS autoradiography was performed in 14-μm coronal cryosections of mouse brains as previously described in detail (Korpi et al., 2017). The effects of rac-BHFF and ORM-27669 were compared in the presence of baclofen (30 μM) as the agonist (which alone produced a negligible signal over the baseline), and CGP-54626 (10-100 μM) as the antagonist of $GABA_B$ receptors (Olpe et al., 1993). For detailed information, see Supporting Information Methods.

Behavioral experiments

Spontaneous locomotor activity

These experiments were carried out both and at the University of Helsinki (Fig. 2A) and at Orion Pharma (Turku, Finland, presented in Figure S1).

At the University of Helsinki, mouse locomotor activity was analyzed by video-tracking software Ethovision XT (Version 10.1, Noldus Information Technology, Netherlands) for 90 min in polycarbonate 19 x 36 x 15 cm cages (Vashchinkina et al., 2012). The mice received different doses of $GABA_B$ PAMs or vehicle as a pretreatment (i.p.) 15 min before the animals received either saline or baclofen (1 mg/kg; i.p.) and were transferred into a novel cage for measurement of locomotor activity. Distances travelled during successive 15-min periods were calculated and compared between the treatments (Fig. 2A), and the first 30-min were pooled for the Fig. 2B.

Conditioned place preference

Biased place conditioning paradigm consisted of pre-conditioning, conditioning, and post-conditioning phases (Cunningham et al., 2006; Vekovischeva et al., 2004). The pre-conditioning phase consisted of a 15-min presentation to two distinct floor mats (a metal plate perforated with 1-cm holes and a plastic grid with 2-cm wide bars), bisecting a polycarbonate cage (19 x 36 cm) into two equal zones. Analysis of time that the mice spent on each floor mat revealed preferred and non- preferred floor mats. During conditioning phase, the entire cage floor was covered by two identical mats. Each daily session consisted of two 20-min trials, morning and evening trials. During morning sessions (8:00 - 10:00), mice were

pretreated with vehicle and 30 min later with saline and immediately placed on the preferred floor mats. During evening sessions (16:00 – 18:00), mice were pretreated i.p. with either $GABA_B$ PAMs or vehicle and 30 min later with saline, cocaine (10 mg/kg), or ethanol (0.5 g/kg) and placed on the non-preferred floor mats. The daily conditioning procedure was repeated four times. The mats and cage were deodorized with 2% H_2O_2 after each animal. The injection-free post-conditioning test (15 min) was performed 48 h after the last conditioning session. During this test, the cage floor was equally covered with both floor mats used during conditioning. Locomotor activities and locations were determined by the Ethovision software. The difference (‘‘time shift’’) in time spent on the non-preferred material during pre-conditioning and post-conditioning tests was calculated as the measure of conditioned place preference (CPP). Two independent batches of mice were tested.

Ethanol binge-like drinking

We used the drinking-in-the-dark procedure (DID) adapted from Rhodes et al. (Rhodes et al., 2005) facilitating stable limited-access ethanol drinking in mice. Different batches of mice were used for rac-BHFF and ORM-27669 tests. Three-hours into the dark cycle, a water bottle was replaced for 2 or 4 h with a 10-ml plastic tube affixed to a ball-bearing sipper and filled with 20% ethanol solution. During the first two weeks, the mice were given a 2-h access to 20% (v/v) ethanol for three consecutive days, and a 4-h access on the fourth day followed by a 3-day break; thereafter the mice had 4 h of access to ethanol for 4 days a week. After at least two weeks of drinking, the drug testing started. At this point, averaged basal 4-h drinking values were 4.1 ± 0.3 g/kg, n=13, and 4.2 ± 0.3 g/kg, n=16, (mean ± SEM) for mice used in the rac-BHFF and ORM-27669 experiments, respectively. During the following weeks, mice were pretreated with either $GABA_B$ PAMs or vehicle, and 30 min later given a 4-h ethanol

access. Treatment groups were arranged in a Latin square design. Mice had always an injection-free 4-h access to ethanol a day before testing the effects of vehicle or $GABA_B$ PAMs on ethanol intake. The DID paradigm was verified by pretreating mice with the opioid receptor antagonist naltrexone (10 mg/kg) that is known to reduce ethanol drinking (Hwa et al., 2014) (data not shown).

Intravenous drug self-administration

Drug self-administration (SA) procedure based on voluntary nose poking activity of mice was carried out as described in (Vashchinkina et al., 2012). Briefly, the custom-made apparatus (Ritec, Russia) consisted of four identical opaque plastic chambers (8 x 8 x 8 cm) for simultaneous testing of two pairs of mice. Each chamber was fitted with an infrared sensor and a nose poke hole (1.5 cm) on the frontal wall and a vertical slot (5 mm) at the bottom of the back wall for extending and fixing the tail. SA was determined in mouse pairs (active and yoked control) matched based on similarity of nose poke activity during 10-min pretests. Within 1 h after the pretest, matched pairs were pretreated (i.p.) with $GABA_B$ PAMs or vehicle. Thirty minutes later, the animal pairs were placed again into the chambers, and 27G needles connected to 1 ml syringes in a two-syringe infusion pump were inserted into the lateral tail vein of each mouse. During a 15-min test, each nose poke of the active mouse resulted in a simultaneous infusion (1.7 µl; 1 s duration) of cocaine (1 mg/ml) to both active and yoked mice. Each mouse was exposed for drugs only once. As a measure of reinforcement effect, R factor was calculated as the difference between the logarithm (log_{10}) of the ratio between cumulative numbers of nose pokes by active and yoked mice during the SA session and the logarithm of the ratio of their nose pokes during the pretest session (Kuzmin et al., 1994).

Electrophysiological experiments

TH-EGFP mice were injected with either $GABA_B$ PAMs or vehicle and 15 min later with ethanol (2 g/kg), cocaine (10 mg/kg) or vehicle, and decapitated 24 h later. Horizontal 225-μm-thick midbrain slices were prepared and preincubated as described previously (Vashchinkina et al., 2012). VTA DA neurons were visualized using fluorescence microscope (Olympus BX51WI, Hamburg, Germany), and whole-cell voltage-clamp recordings were performed with a Multiclamp 700A amplifier (Molecular Devices, Sunnyvale, CA, USA) (Vashchinkina et al., 2012). The currents were low-pass filtered at 1.6 kHz, and digitized at 20 kHz (Molecular Devices). Electrodes (3-4 MΩ) contained (in mM, pH adjusted to 7.2-7.25 and osmolarity to 280 mOsm): 130 cesium methanesulfonate, 10 HEPES, 0.5 EGTA, 8 NaCl, 5 QX314, 4 MgATP, 0.3 MgGTP, and 10 BAPTA. α-Amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) and N-methyl-D-aspartate (NMDA) receptor-mediated excitatory postsynaptic currents (EPSCs) were induced by stimulating glutamatergic afferents at 0.1 Hz frequency using a bipolar stimulus electrode (Vashchinkina et al., 2012). Neurons were clamped at +40 mV under blockade of $GABA_A$ receptors (picrotoxin, 100 μM), and EPSCs were recorded for 10 min before and after the application of the NMDA receptor blocker D-(-)-2-amino-5-phosphonopentanoic acid (AP5, 50 μM). The ratio was calculated by dividing the peak amplitude of AMPA receptor current with that of NMDA receptor current, averaged from 30 EPSCs. To examine whether NMDAR function was altered after the treatment, we examined kinetics of NMDAR EPSCs (Monyer et al., 1992). The weighted decay time constant (tau, τW) of the NMDAR EPSCs at +40 mV was calculated by fitting a double exponential function to each average EPSC and using the following formula $\tau W = [(A1x\tau1)+($

A2xτ2)]/(A1+A2); where A1 and A2 are the amplitudes and τ1 and τ2 are the decay time constants of the fast and slow components, respectively (Barth and Malenka, 2001).

Drugs

Both rac-BHFF and ORM-27669 (provided by Orion Pharma, Espoo, Finland) were dissolved in 40% (v/v) polyethylene glycol/10% (v/v) polyoxyl-15-hydroxystearate (Solutol HS15) and diluted by one-half with 2% d-α-tocopheryl polyethylene glycol 1000 succinate (TPGS, w/v), in saline, and used for i.g. or s.c. (spontaneous locomotor study at Orion Pharma) or i.p. injections (all other *in vivo* studies) within 20 min of preparation (all from Tocris Bioscience, Bristol, UK). ORM-27669 was synthesized using the protocols that are described in the Supporting Information Methods. Preliminary pharmacokinetics of ORM-27669 in adult male Wistar rats suggested an elimination t½ of about 2.7 h and an AUC_{brain}/AUC_{plasma} of about 0.6 (Supporting Information Table S1). Baclofen and CGP-54626 (Tocris Bioscience), cocaine hydrochloride (Sigma-Aldrich, St. Louis, USA) and ethanol (Altia Oyj, Rajamäki, Finland) were dissolved in saline for i.p. and i.v. injections. Ethanol solutions for the DID were made into tap water (20% v/v). For *in vitro* pharmacology studies, 10 mM stock solutions in DMSO (ORM-27669 and rac-BHFF) and water (baclofen, GABA and CGP-54626) were used.

Statistical analyses

The results are presented as means ± SEM. Data were statistically analyzed with the IBM SPSS Statistics 21 software (IBM, Armonk, New York, NY, USA) or Prism 6 (GraphPad Software, La Jolla, CA, USA), using ANOVA followed by a Bonferroni or Dunnett test ($P < 0.05$) or Student's *t*-test.

RESULTS

$GABA_B$ pharmacology and positive allosteric modulation of rac-BHFF and ORM-27669

In the calcium-based assay, baclofen was found to be a full agonist with EC_{50} values of 1.9 ± 0.4 and 0.80 ± 0.2 μM at human (n = 6) and rat (n = 7) $GABA_B$ receptors, respectively, similarly to GABA (1.0 ± 0.1 and 0.66 ± 0.08 μM, respectively). Rac-BHFF showed low efficacy and potency as an agonist at human and rat $GABA_B$ receptors (EC_{50} > 30 μM, data not shown), while ORM-27669 was similar to rac-BHFF at human receptors, but showed a weak partial agonism at rat receptors (EC_{50} 9.8 ± 0.6 μM, intrinsic activity IA 0.26). ORM-27669 was a less potent PAM compared to rac-BHFF, the difference in potency being slightly more prominent with baclofen than GABA as the agonist (Table 1). At 10 μM concentration, rac-BHFF was more efficient than ORM-27669 in potentiating the GABA and baclofen dose-responses at human and rat $GABA_B$ receptors (fold changes in Table 1). ORM-27669 potentiated the maximal efficacy to a greater extent than rac-BHFF. Rac-BHFF induced a 20% reduction of maximal efficacy of both GABA and baclofen at the rat $GABA_B$ receptors (Table 1).

In the [^{35}S]GTPγS binding assay, GABA increased membrane association of [^{35}S]GTPγS with EC_{50} values of 0.73 ± 0.08 and 2.2 ± 0.1 μM, at human and rat receptors, respectively (Table 1B). Rac-BHFF (10 μM) was also an agonist, with a slightly lower efficacy (0.92 ± 0.04) as compared to 100 μM GABA, which prevented accurate estimation of its allosteric pharmacology in this assay (Table 1B). ORM-27669 showed positive allosteric

effect by potentiating GABA dose-response, i.e., shifting the GABA dose-response to the left, by about 2.5-fold, at human and rat receptors.

Taken together, ORM-27669 was found to be a less potent and efficient $GABA_B$ PAM than rac-BHFF at both human and rat $GABA_B$ receptors. Some differences in the *in vitro* pharmacological profile were found at rat $GABA_B$ receptors between the compounds and the signaling pathways studied. Rac-BHFF was a pure PAM in the calcium-based assay and an intrinsic agonist and PAM in the endogenous signaling [^{35}S]GTPγS binding assay. ORM-27669 showed a similar allosteric profile in both signaling assays, with some tendency to a weak partial agonism at rat $GABA_B$ receptors.

Next, we aimed to find out how brain regional stimulation of [^{35}S]GTPγS binding would be affected by the PAMs (Fig. 1BC). Both Rac-BHFF (10 μM) and ORM-27669 (300 μM) potentiated the stimulation of binding of [^{35}S]GTPγS by a low concentration of baclofen (30 μM) in $GABA_B$ receptor antagonist-sensitive manner widely in mouse brain regions (Fig. 1B; similar data were found also for Wistar rat brain sections, data not shown). Baclofen alone at this low concentration and both PAMs alone had no effects. While the combined effect was clearly greater with rac-BHFF, the regional profiles were very similar for both PAMs, suggesting that they activated the same population(s) of baclofen-stimulated $GABA_B$ receptor subtypes (Fig. 1C). It should be noted that our [^{35}S]GTPγS binding assay was not sensitive enough for consistent quantification of the $GABA_B$ receptor activation in the VTA.

Specificity of ORM-27669 was screened in tissue homogenates or recombinant preparations using orthosteric sites of 121 relevant targets at Eurofins Cerep SA (Celle-Levescault, France). At 10 μM concentration, no affinity was detected to any of the targets

studied (defined by <50 % inhibition of specific binding), except for affinity to human PACAP (PAC1) receptor (52% inhibition, Supporting Information Table S2). Rac-BHFF at 10 μM has also been found to be very selective, as out of the 56 targets tested (Malherbe et al., 2008), only ligand binding to the CCK1 receptor was inhibited by almost 90%.

Effects of $GABA_B$ receptor ligands on locomotor activity of mice

In initial experiments at Orion Pharma with acute administration of baclofen, rac-BHFF and ORM-27669, we tested several doses to find out whether the compounds induced changes in spontaneous locomotor activity counts in mice. When tested separately against the common vehicle-treated group, the data showed that only baclofen at the doses of 10 and 30 mg/kg ($F_{4,33} = 11.4$, $p < 0.0001$), and not rac-BHFF ($F_{4,30} = 1.4$, $p > 0.26$), reduced the activity (Fig. S1: for both drugs, treatment effect $F_{8,56} = 5.7$, $p < 0.0001$), in agreement with (Besheer et al., 2004). ORM-27669 did not affect locomotion within a wide dose range ($F_{4,35} = 1.4$, $p > 0.25$).

Then, we re-tested non-sedative doses of rac-BHFF and ORM-27669 after acute i.p. injections in mice at the University of Helsinki and found that rac-BHFF at 30 mg/kg and ORM-27669 at 100 mg/kg did not significantly alter the distances traveled (Fig. 2AB), but rac-BHFF at 60 mg/kg did reduce the locomotion (Fig. 2B). Baclofen (1 mg/kg) alone did not alter locomotor activity as compared to vehicle treatment, but when co-administered with rac-BHFF (30 mg/kg) and ORM-27669 (100 mg/kg) a clear decrease in locomotor activity was induced (Fig. 2AB, treatment effect for rac-BHFF: $F_{5,52} = 7.4$, $p < 0.001$; for ORM-27669: $F_{5,69} = 4.6$, $p = 0.001$). The locomotion-reducing effect of rac-BHFF at 60 mg/kg was no more enhanced by

baclofen. Higher doses of baclofen were not included in further behavioral experiments due to its sedative effect.

Single *in vivo* doses of $GABA_B$ receptor ligands failed to induce long-lasting potentiation in the VTA DA neurons

In VTA DA neurons of midbrain slices from baclofen-, rac-BHFF- and ORM-27669-treated mice obtained 24 h after the single injections, the AMPA and NMDA receptor-mediated EPSCs and their current ratios were similar to those in neurons from vehicle-treated mice (Fig. 2CD; one-way ANOVA $F_{7,55} = 0.70$, $p = 0.62$). Co-administration of baclofen (1 mg/kg) with rac-BHFF (30 mg/kg) and ORM-27669 (10 mg/kg) also failed to increase the AMPA/NMDA ratio (Fig. 2D; $F_{5,45} = 1.9$, $p = 0.1$).

In addition to amplitude analysis of the EPSCs, we also examined the kinetics of NMDA EPSCs to estimate whether the treatments induced any differences in NMDAR subunit composition (Barth and Malenka, 2001; Flint et al., 1997). We found no difference in the weighted decay time constants (τW) of the NMDA EPSCs from drug-treated mice compared to the decay time constants of neurons from vehicle-treated mice [$F_{9,73} = 0.77$, $p = 0.64$; τW given as mean ± SEM in ms: vehicle 71 ± 19; baclofen (1 mg/kg) 68 ± 9; baclofen (5 mg/kg) 77 ± 17; baclofen (30 mg/kg) 103 ± 50; rac-BHFF (30 mg/kg) 114 ± 24; rac-BHFF (100 mg/kg) 77 ± 9; baclofen (1 mg/kg) + rac-BHFF (30 mg/kg) 105 ± 20; ORM-27669 (10 mg/kg) 66 ± 9; ORM-27669 (100 mg/kg) 63 ± 5; baclofen (1 mg/kg) + ORM-27669 (10 mg/kg) 96 ± 18].

Comparison of pretreatment effects of baclofen and $GABA_B$ PAMs on drug-induced plasticity in VTA DA neurons

We next determined whether the pretreatment with baclofen (1-30 mg/kg), rac-BHFF (30-100 mg/kg) and ORM-27669 (100 mg/kg) affected ethanol- and cocaine-induced increase in AMPA/NMDA ratio (Ungless et al., 2001; Vashchinkina et al., 2012). In this set of experiments, we recorded distinct subpopulations of VTA DA neurons, i.e., those in the dorsomedial posterior VTA in ethanol-treated mice (Mrejeru et al., 2015) and those in the ventrolateral VTA in the cocaine-treated mice (Lammel et al., 2011). Consistent with previous results, ethanol (2 g/kg, i.p.) and cocaine (10 mg/kg, i.p.) increased AMPA/NMDA ratios compared to vehicle (Fig. 3AB).

Ethanol-induced increase in the AMPA/NMDA ratio in the VTA DA neurons was suppressed by all types of pretreatments (except for the low dose of baclofen) given 15 min before the single ethanol dose (Fig. 3A, $F_{6,48} = 7.4$, $p < 0.0001$). Both pretreatments with rac-BHFF (30 mg/kg) and ORM-27669 (100 mg/kg) effectively reversed ethanol-induced AMPA/NMDA ratio at the non-sedative dose, whereas pretreatment with baclofen was effective only at higher sedative dose (30 mg/kg) compared to vehicle-treated mice.

Cocaine-induced increase in the AMPA/NMDA ratio in the VTA DA neurons was suppressed only by pretreatment with rac-BHFF given 15 min before the single cocaine dose (Fig. 3B, $F_{4,37} = 5.2$, $p = 0.002$). Particularly, pretreatment with a non-sedative dose of rac-BHFF (30 mg/kg) markedly reversed cocaine-induced AMPA/NMDA ratio, but pretreatments with baclofen (1 mg/kg) and ORM-27669 (100 mg/kg) were not effective compared to vehicle-treated mice. Thus, rac-BHFF effectively reversed both ethanol- and cocaine-induced neuroplasticity, whereas ORM-27669 seemed to be more potent in reversing ethanol- than cocaine-induced neuroplasticity.

In some mice, the highest dose of rac-BHFF (100 mg/kg) used alone (n = 3 out of 10 mice) or in combination with ethanol (n = 1 out of 8) or cocaine (n = 1 out of 7) induced a deep sedative state documented even at 24 h after the treatment. These mice were sacrificed and excluded from the study.

Pretreatment with non-sedative doses of rac-BHFF and ORM-27669 suppressed reward-related behaviors in mice

We then tested whether the non-sedative doses of rac-BHFF (3-30 mg/kg) and ORM-27669 (100 mg/kg), that counteracted the drug-induced neuroplasticity, also altered the reinforcing properties of ethanol and cocaine. First, we studied the effects of pretreatments with the $GABA_B$ PAMs on the development of the CPP to ethanol and cocaine. In line with previous results (Vekovischeva et al., 2004), within the vehicle-pretreated mice, ethanol- and cocaine-conditioned mice developed the CPP compared to the saline-conditioned mice (Fig. 4A, $F_{6,75} = 7.9$, $p < 0.0001$). When conditioning to ethanol was carried out after pretreatments with either PAM, no statistically significant CPP was expressed as compared to vehicle pretreatment (Fig. 4A; $p > 0.05$ by Bonferroni test), suggesting that the rewarding effects of ethanol were diminished by the PAMs. In contrast, only pretreatment with rac-BHFF, but not with ORM-27669, suppressed expression of cocaine-induced CPP (Fig. 4A). Additional analysis of locomotor activity during the conditioning sessions suggested that only rac-BHFF slightly suppressed cocaine-induced locomotor hyperactivity during the 3rd conditioning session (Fig. 4B: $F_{4,53} = 42.1$, $p < 0.05$ for cocaine vs. rac-BHFF + cocaine).

Then, we studied the effect of pretreatments with the $GABA_B$ PAMs on ethanol drinking and self-administration of cocaine. Using the DID paradigm, we found that ethanol

intake was significantly attenuated by pretreatments with both rac-BHFF (30 mg/kg; $p < 0.05$) and ORM-27669 (100 mg/kg, $p < 0.001$) as compared to ethanol intake after vehicle injections (Fig. 4D). In contrast, acute intravenous self-administration of cocaine was suppressed selectively by rac-BHFF (30 mg/kg: $F_{2,24} = 4.8$, $p < 0.05$) in a dose-dependent manner, but not at all by ORM-27669 (100 mg/kg) (Fig. 4E: $F_{2,13} = 13.3$, $p < 0.01$). Taken together, rewarding effects of ethanol were blunted by both rac-BHFF and ORM-27669, while only rac-BHFF affected the rewarding effects of cocaine.

DISCUSSION

The present results demonstrate for the first time that $GABA_B$ receptor agonists are able to reverse persistent neuroadaptations induced by drugs of abuse in the VTA DA neurons. Prominent suppression of alcohol- and cocaine-induced synaptic plasticity in mice was observed with two different $GABA_B$ PAMs, a potent and highly efficient benzofuranone rac-BHFF and a novel low-potency and low-efficacy non-benzofuranone compound ORM-27669. The effect was comparable to that observed with a sedative dose of the orthosteric agonist baclofen. Furthermore, the non-sedative doses of $GABA_B$ PAMs clearly decreased voluntary drinking of ethanol in mice, while only the more efficient and potent compound reduced acute intravenous self-administration of cocaine. Ethanol- and cocaine-induced place preference was not consistently affected by $GABA_B$ PAMs, although the results suggested that ethanol reward was somewhat sensitive to both PAMs, while cocaine reward again only to rac-BHFF. The lack of sedation during the behavioral tests suggests that $GABA_B$ PAMs may have a wider therapeutic window than baclofen, although we found that in some animals that the administration of the highest dose (100 mg/kg) of the potent rac-BHFF induced prolonged,

heavy sedation.

VTA neuroadaptations

Data obtained from measurements of the AMPA and NMDA receptor currents in VTA DA neurons from juvenile mice demonstrated that *in vivo* administration of baclofen and PAMs alone did not have long-term neuroplasticity effects on DA neurons. Moreover, there were no alterations in the kinetics of NMDA receptor currents, indicating that they did not alter the subunit composition of the receptors (Barth and Malenka, 2001; Brothwell et al., 2008). In line, using c-Fos and ΔFosB expression to estimate neuronal activation, (Lhuillier et al., 2007) demonstrated that neither baclofen nor a PAM GS39783 *per se* altered expression of these immediate early genes in the striatum.

Both baclofen and GS39783 potently reduced cocaine-induced c-Fos expression in the striatum (Lhuillier et al., 2007). However, GS39783 blocked the prolonged ΔFosB expression by subchronic cocaine efficiently in the dorsal striatum, but not in the nucleus accumbens, indicating regional selectivity in its actions. In order to compare the pretreatment effects of distinct $GABA_B$ receptor ligands on plasticity induced by addictive substances, we examined comparable non-sedative doses of rac-BHFF, ORM-27669 and baclofen. We recorded distinct VTA DA neuron subgroups due to well-known regional differences in their responses to alcohol and cocaine. Particularly, VTA DA neurons from the dorsomedial posterior VTA have been found to be highly responsive to alcohol (Mrejeru et al., 2015), while VTA DA neurons located in the ventrolateral VTA are highly responsive to cocaine (Lammel et al., 2011). Standard neuroplasticity-inducing single doses of ethanol (2 g/kg) and cocaine

(10 mg/kg) produced significantly increased AMPA/NMDA receptor current ratios in the DA neurons of the respective regions of the VTA (Fig. 3). At non-sedative doses, rac-BHFF, but not ORM-27669, suppressed cocaine-induced plasticity. The magnitude of the effect produced by rac-BHFF was comparable to baclofen at 30 mg/kg, a dose known to induce profound sedation (Besheer et al., 2004). On the other hand, ethanol effects were suppressed to the same extent by both rac-BHFF and ORM-27669.

Our present and the earlier findings (Lhuillier et al., 2007) indicate that the $GABA_B$ PAMs have the potential to counteract at least some processes of the neuroplasticity/adaptation to drugs of abuse. Further characterization of the binding sites and signaling pathways for the PAMs is thus warranted in order to interpret their differences in modulating VTA neuroplasticity responses to ethanol and cocaine. Our autoradiographic analyses on mouse brain sections anyway indicate a robust difference in the potency and efficacy between rac-BHFF and ORM-27669, but identical brain regional $GABA_B$ receptor populations as targets (Fig. 1BC). Therefore, it is plausible that ethanol-induced neuroadaptation is less stringent than cocaine neuroadaptation, being sensitive to lower stimulation of $GABA_B$ receptors. It should be noted that, as discussed above, different VTA DA neuron populations are sensitive to ethanol and cocaine and only the sensitive ones were recorded here. Therefore, it was not surprising that the two PAMs had different effects. It should be also noted that the poorly soluble ORM-27669 was used in behavioral experiments at its maximal dose, which still appeared to potentiate the low-dose baclofen effect on locomotion, similarly to rac-BHFF (30 mg/kg, Fig. 2AB).

Behavioral characterization of $GABA_B$ PAMs

Dose-dependent hypoactivity was observed in mice with baclofen (Figure S1) and in one experiment with rac-BHFF (Fig. 2A, but not in Figure S1), while ORM-27669 consistently induced no hypoactivity. Co-administration of subthreshold sedative doses of baclofen and $GABA_B$ PAMs resulted in marked enhancement of sedation (Fig. 2AB), confirming that such effects of PAMs are mediated by $GABA_B$ receptor (Filip et al., 2015).

Doses of $GABA_B$ PAMs that were efficacious in neuroplasticity experiments in juvenile mice were used in adult mice in reward-related behavioral paradigms, which were previously linked to VTA adaptations (Dunn et al., 2005; Harris et al., 2004). Rac-BHFF suppressed neuroplasticity and attenuated behavioral responses to cocaine and ethanol, suggesting correlated mechanisms (Figs 3 and 4). Pretreatment with rac-BHFF did not consistently affect cocaine-induced hyperactivity during the conditioning sessions. However, in the present experiments, cocaine did not induce locomotor sensitization after repeated conditioning doses, and thus the effects of $GABA_B$ PAMs on sensitization could not be assessed. The $GABA_B$ PAM GS39783 efficiently blocks cocaine-induced hyperactivity, but affects only slightly the sensitization to cocaine (Lhuillier et al., 2007). Pretreatment with rac-BHFF reduced ethanol drinking, in line with (Maccioni et al., 2010), and it also reduced acute cocaine self-administration. ORM-27669 effectively reduced only ethanol drinking, but not cocaine self-administration and cocaine-induced hyperlocomotion (Fig. 4). These results are in agreement with the effects on VTA DA neuroplasticity, as ORM-27669 also failed to reverse cocaine-induced neuroplasticity (Fig. 3).

Only modest effects were seen in the CPP test by the pretreatments with the $GABA_B$ PAMs. Conditioning with ethanol and cocaine induced significant CPP, and these effects were reduced by the $GABA_B$ PAMs to the level that statistically significant CPPs were

no more detectable. However, the time shifts during preference testing in mice with conditioning pretreatments with rac-BHFF and ORM-27669 did not differ from the corresponding vehicle-pretreated groups. The small suppression of development of ethanol- and cocaine-induced CPP by rac-BHFF and ORM-27669 might be attributed to their inability to also act on brain areas such as the extended amygdala and olfactory tubercle that also regulate conditioned effects of cues associated with cocaine (Tzschentke, 2007) and ethanol (Gremel and Cunningham, 2008) and/or to affect conditioned motivational properties of drug-paired cues. To our knowledge, no study has reported clear effects of rac-BHFF on development or expression of CPP induced by addictive compounds in mice (Filip et al., 2015). Furthermore, there is no evidence for rac-BHFF *per se* to induce conditioned place preference or aversion (Filip et al., 2015). Conditioning with baclofen also fails to induce place preference or aversion (reviewed in (Tzschentke, 2007)), nor does co-treatment with it block ethanol-induced place preference in mice (Chester and Cunningham, 1999). Thus, the CPP test may not be the proper test to reveal any robust anti-addiction effects of $GABA_B$ receptor ligands.

In vitro $GABA_B$ receptor pharmacology

Human and rat $GABA_B$ receptors utilized here responded rather similarly to agonists and PAMs in both intracellular Ca^{2+} mobilization and G protein-coupling assays, and with the $GABA_B$ subunits being conserved in mouse (Bettler et al., 2004), the data suggest high translational potential of studied $GABA_B$ PAMs. Rac-BHFF was found to be a pure allosteric modulator in the intracellular Ca^{2+} mobilization assay, whereas in the [^{35}S]GTPγS binding assay the compound demonstrated agonistic activity at both human and rat $GABA_B$ receptors.

Earlier Malherbe et al. (Malherbe et al., 2008) also reported agonistic activity of rac-BHFF in the absence of exogenous GABA in the stable CHO-$G_{\alpha16}$-h$GABA_{B(1a,2a)}$ cell system. It still remains unclear whether rac-BHFF *per se* is able to directly activate native $GABA_B$ receptors, and whether the allosteric profile differs depending on the signalling pathway studied (calcium-based vs. [^{35}S]GTPγS assay) and/or the level of constitutive activity of receptors in the system studied.

In contrast to rac-BHFF, ORM-27669 had a more pure allosteric pharmacological profile in both assays but with a tendency to increased partial agonistic profile at rat compared to human $GABA_B$ receptors. The limited buffer-solubility of ORM-27669 and rac-BHFF might underestimate the actual *in vitro* potency, with the lipophilicity being often a characteristic of $GABA_B$ receptor ligands (Urwyler, 2011).

Despite the differences in potency and allosteric profile between the $GABA_B$ PAMs, the mouse brain regional profiles were very similar for the PAMs, suggesting that they activate the same population(s) of $GABA_B$ receptor subtypes. ORM-27669 did not display specific affinity to 121 receptors and binding sites studied at 10 μM concentration, indicating selective targeting to $GABA_B$ receptors (Supporting Information Table S2).

Mechanism of suppression of drug-induced neuroplasticity in VTA DA by $GABA_B$ ligands

Suppression of drug-induced neuroplasticity and reward-associated behaviour by $GABA_B$ agonists and PAMs may result from the restoration of inhibition, which may be disturbed by drugs of abuse (Liu et al., 2005; Nugent et al., 2007). In the VTA, there are several putative

targets for $GABA_B$ ligands, including postsynaptic $GABA_B$ receptors on DA neurons (Cruz et al., 2004; Lalive et al., 2014; Munoz et al., 2016) or/and presynaptic $GABA_B$ receptors on the afferent GABAergic projections (Padgett et al., 2012). Indeed, recent reports link both postsynaptic and presynaptic $GABA_B$ receptor mechanisms in the actions of ethanol and psychostimulants in VTA DA neurons. Both ethanol (Herman et al., 2015) and cocaine (Wise et al., 1995) induce bursting mode of firing in VTA DA neurons. It has been shown that the bursting mode, but not the tonic firing mode, potentiates $GABA_B$-GIRK-mediated slow inhibitory postsynaptic currents (IPSCs) in VTA DA neurons (Lalive et al., 2014), which then brings the firing back to baseline. Thus, facilitation of $GABA_B$ receptors on VTA DA neurons by agonists and PAMs can lead to augmentation of GIRK-mediated inhibition that could reduce VTA DA neuron firing and DA efflux in the terminals (Herman et al., 2015). In the VTA, the reduction of DA neuron firing would limit the development of neuroplasticity, as detected when $GABA_B$ PAMs were given before single doses of ethanol or cocaine.

Alternatively, suppression of drug-induced neuroadaptations in VTA DA neurons could be mediated via facilitation of presynaptic $GABA_B$ receptors. Psychostimulants persistently impair $GABA_B$-GIRK slow IPSCs in VTA GABA neurons (Padgett et al., 2012) and DA neurons (Arora et al., 2011; Cruz et al., 2004; Munoz et al., 2016), via different mechanisms. Ethanol affects the excitatory/inhibitory balance by directly activating GIRK3 channels in VTA DA neurons (Federici et al., 2009; Herman et al., 2015). Hence, activation of $GABA_B$ receptors in VTA GABA cells by their agonists or PAMs may restore excitatory/inhibitory balance previously disturbed by drugs of abuse.

In the hippocampus, $GABA_B$-GIRK slow IPSCs have been proposed to transmit only pertinent information to and within the hippocampus. Particularly, $GABA_B$-GIRK slow

IPSCs appear only after high-frequency stimulation and, at least in part, prevent the development of LTP (Hasuo and Akasu, 2001). If the same holds true for the VTA, then the use-dependent activation of $GABA_B$ receptors (seen as the absence of the VTA plasticity after $GABA_B$ PAMs) implies that $GABA_B$ receptors act as a protective regulatory gate to prevent drug-induced overflow of information from the VTA to its targets, including the nucleus accumbens.

In conclusion, the present study demonstrated that use-dependent activation of $GABA_B$ receptors can decrease long-term adaptive synaptic changes in VTA DA neurons and reward-associated behaviour after ethanol and cocaine treatments. Importantly, efficacy of $GABA_B$ PAMs in our models was observed at non-sedative doses that were devoid of their own effects on VTA plasticity, consistent with the therapeutic principle of PAMs. It should be noted that only ethanol effects could be reversed also by the low-efficacy $GABA_B$ PAM ORM-27669, while the high-efficacy ligand rac-BHFF reversed both ethanol and cocaine effects.

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Author contributions

EdM, OV, KK, MM, SKJ, PH, A-ML and ERK were responsible for the study concept and design of experiments. EdM, OV, JK, ML and A-ML contributed to the acquisition of animal

data. KK, MV and NP contributed to the acquisition of *in vitro* data. EdM, OV, NP, KK, MM, SKJ, PH, A-ML and ERK assisted with data analysis and interpretation of findings. EdM drafted the manuscript. KK, SKJ and ERK provided critical revision of the manuscript for important intellectual content. All authors critically reviewed the content and approved the final version for publication.

SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

Supporting Information Methods

Table S1 ORM-27669 Pharmacokinetics

Table S2 ORM-27669 Affinity profile

Figure S1 Locomotor activity

References

Addolorato G, Leggio L, Ferrulli A, Cardone S, Bedogni G, Caputo F, Gasbarrini G, Landolfi R, Baclofen Study G (2011) Dose-response effect of baclofen in reducing daily alcohol intake in alcohol dependence: secondary analysis of a randomized, double-blind, placebo-controlled trial. Alcohol Alcohol 46:312-317.

Agabio R, Colombo G (2015) GABAB receptor as therapeutic target for drug addiction: from baclofen to positive allosteric modulators. Psychiatria polska 49:215-223.

Ameisen O (2005) Complete and prolonged suppression of symptoms and consequences of alcohol-dependence using high-dose baclofen: a self-case report of a physician. Alcohol Alcohol 40:147-150.

Arora D, Hearing M, Haluk DM, Mirkovic K, Fajardo-Serrano A, Wessendorf MW, Watanabe M, Lujan R, Wickman K (2011) Acute cocaine exposure weakens GABA(B) receptor-dependent G-protein-gated inwardly rectifying K+ signaling in dopamine neurons of the ventral tegmental area. J Neurosci 31:12251-12257.

Barth AL, Malenka RC (2001) NMDAR EPSC kinetics do not regulate the critical period for LTP at thalamocortical synapses. Nat Neurosci 4:235-236.

Beraha EM, Salemink E, Goudriaan AE, Bakker A, de Jong D, Smits N, Zwart JW, Geest DV, Bodewits P, Schiphof T, Defourny H, van Tricht M, van den Brink W, Wiers RW (2016) Efficacy and safety of high-dose baclofen for the treatment of alcohol dependence: A multicentre, randomised, double-blind controlled trial. Eur Neuropsychopharmacol 26:1950-1959.

Besheer J, Lepoutre V, Hodge CW (2004) GABA(B) receptor agonists reduce operant ethanol self-administration and enhance ethanol sedation in C57BL/6J mice. Psychopharmacology (Berl) 174:358-366.

Bettler B, Kaupmann K, Mosbacher J, Gassmann M (2004) Molecular structure and physiological functions of GABA(B) receptors. Physiol Rev 84:835-867.

Brothwell SL, Barber JL, Monaghan DT, Jane DE, Gibb AJ, Jones S (2008) NR2B- and NR2D-containing synaptic NMDA receptors in developing rat substantia nigra pars compacta dopaminergic neurones. J Physiol 586:739-750.

Chester JA, Cunningham CL (1999) Baclofen alters ethanol-stimulated activity but not conditioned place preference or taste aversion in mice. Pharmacol Biochem Behav 63:325-331.

Cruz HG, Ivanova T, Lunn ML, Stoffel M, Slesinger PA, Luscher C (2004) Bi-directional effects of GABA(B) receptor agonists on the mesolimbic dopamine system. Nat Neurosci 7:153-159.

Cryan JF, Kelly PH, Chaperon F, Gentsch C, Mombereau C, Lingenhoehl K, Froestl W, Bettler B, Kaupmann K, Spooren WP (2004) Behavioral characterization of the novel GABAB receptor-positive modulator GS39783 (N,N'-dicyclopentyl-2-methylsulfanyl-5-nitro-pyrimidine-4,6-diamine): anxiolytic-like activity without side effects associated with baclofen or benzodiazepines. J Pharmacol Exp Ther 310:952-963.

Cunningham CL, Gremel CM, Groblewski PA (2006) Drug-induced conditioned place preference and aversion in mice. Nat Protoc 1:1662-1670.

de Beaurepaire R (2014) The use of very high-doses of baclofen for the treatment of alcohol-dependence: a case series. Frontiers in psychiatry 5:143.

Dunn JM, Inderwies BR, Licata SC, Pierce RC (2005) Repeated administration of AMPA or a metabotropic glutamate receptor agonist into the rat ventral tegmental area augments

the subsequent behavioral hyperactivity induced by cocaine. Psychopharmacology (Berl) 179:172-180.
Erhardt S, Mathe JM, Chergui K, Engberg G, Svensson TH (2002) GABA(B) receptor-mediated modulation of the firing pattern of ventral tegmental area dopamine neurons in vivo. Naunyn Schmiedebergs Arch Pharmacol 365:173-180.
Fadda P, Scherma M, Fresu A, Collu M, Fratta W (2003) Baclofen antagonizes nicotine-, cocaine-, and morphine-induced dopamine release in the nucleus accumbens of rat. Synapse 50:1-6.
Fattore L, Cossu G, Martellotta MC, Fratta W (2002) Baclofen antagonizes intravenous self-administration of nicotine in mice and rats. Alcohol Alcohol 37:495-498.
Federici M, Nistico R, Giustizieri M, Bernardi G, Mercuri NB (2009) Ethanol enhances GABAB-mediated inhibitory postsynaptic transmission on rat midbrain dopaminergic neurons by facilitating GIRK currents. Eur J Neurosci 29:1369-1377.
Filip M, Frankowska M, Sadakierska-Chudy A, Suder A, Szumiec L, Mierzejewski P, Bienkowski P, Przegalinski E, Cryan JF (2015) GABAB receptors as a therapeutic strategy in substance use disorders: Focus on positive allosteric modulators. Neuropharmacology 88C:36-47.
Flint AC, Maisch US, Weishaupt JH, Kriegstein AR, Monyer H (1997) NR2A subunit expression shortens NMDA receptor synaptic currents in developing neocortex. J Neurosci 17:2469-2476.
Garbutt JC, Kampov-Polevoy AB, Gallop R, Kalka-Juhl L, Flannery BA (2010) Efficacy and safety of baclofen for alcohol dependence: a randomized, double-blind, placebo-controlled trial. Alcohol Clin Exp Res 34:1849-1857.
Gong S, Zheng C, Doughty ML, Losos K, Didkovsky N, Schambra UB, Nowak NJ, Joyner A, Leblanc G, Hatten ME, Heintz N (2003) A gene expression atlas of the central nervous system based on bacterial artificial chromosomes. Nature 425:917-925.
Gremel CM, Cunningham CL (2008) Roles of the nucleus accumbens and amygdala in the acquisition and expression of ethanol-conditioned behavior in mice. J Neurosci 28:1076-1084.
Harris GC, Wimmer M, Byrne R, Aston-Jones G (2004) Glutamate-associated plasticity in the ventral tegmental area is necessary for conditioning environmental stimuli with morphine. Neuroscience 129:841-847.
Hasuo H, Akasu T (2001) Activation of inhibitory pathways suppresses the induction of long-term potentiation in neurons of the rat lateral septal nucleus. Neuroscience 105:343-352.
Herman MA, Sidhu H, Stouffer DG, Kreifeldt M, Le D, Cates-Gatto C, Munoz MB, Roberts AJ, Parsons LH, Roberto M, Wickman K, Slesinger PA, Contet C (2015) GIRK3 gates activation of the mesolimbic dopaminergic pathway by ethanol. Proc Natl Acad Sci U S A 112:7091-7096.
Hwa LS, Kalinichev M, Haddouk H, Poli S, Miczek KA (2014) Reduction of excessive alcohol drinking by a novel GABAB receptor positive allosteric modulator ADX71441 in mice. Psychopharmacology (Berl) 231:333-343.
Kauer JA, Malenka RC (2007) Synaptic plasticity and addiction. Nat Rev Neurosci 8:844-858.
Koek W, Cheng K, Rice KC (2013) Discriminative stimulus effects of the GABAB receptor-positive modulator rac-BHFF: comparison with GABAB receptor agonists and drugs of abuse. J Pharmacol Exp Ther 344:553-560.

Korpi ER, den Hollander B, Farooq U, Vashchinkina E, Rajkumar R, Nutt DJ, Hyytia P, Dawe GS (2015) Mechanisms of action and persistent neuroplasticity by drugs of abuse. Pharmacol Rev 67:872-1004.

Korpi ER, Linden AM, Hytonen HR, Paasikoski N, Vashchinkina E, Dudek M, Herr DR, Hyytia P (2017) Continuous delivery of naltrexone and nalmefene leads to tolerance in reducing alcohol drinking and to supersensitivity of brain opioid receptors. Addict Biol 22:1022-1035.

Kuzmin AV, Patkina NA, Zvartau EE (1994) Analgesic and reinforcing effects of morphine in mice. Influence of Bay K-8644 and nimodipine. Brain Res 652:1-8.

Labouebe G, Lomazzi M, Cruz HG, Creton C, Lujan R, Li M, Yanagawa Y, Obata K, Watanabe M, Wickman K, Boyer SB, Slesinger PA, Luscher C (2007) RGS2 modulates coupling between GABAB receptors and GIRK channels in dopamine neurons of the ventral tegmental area. Nat Neurosci 10:1559-1568.

Lalive AL, Munoz MB, Bellone C, Slesinger PA, Luscher C, Tan KR (2014) Firing modes of dopamine neurons drive bidirectional GIRK channel plasticity. J Neurosci 34:5107-5114.

Lammel S, Ion DI, Roeper J, Malenka RC (2011) Projection-specific modulation of dopamine neuron synapses by aversive and rewarding stimuli. Neuron 70:855-862.

Lhuillier L, Mombereau C, Cryan JF, Kaupmann K (2007) GABA(B) receptor-positive modulation decreases selective molecular and behavioral effects of cocaine. Neuropsychopharmacology 32:388-398.

Liu QS, Pu L, Poo MM (2005) Repeated cocaine exposure in vivo facilitates LTP induction in midbrain dopamine neurons. Nature 437:1027-1031.

Luthi A, Luscher C (2014) Pathological circuit function underlying addiction and anxiety disorders. Nat Neurosci 17:1635-1643.

Maccioni P, Thomas AW, Carai MA, Gessa GL, Malherbe P, Colombo G (2010) The positive allosteric modulator of the GABA(B) receptor, rac-BHFF, suppresses alcohol self-administration. Drug Alcohol Depend 109:96-103.

Malherbe P, Masciadri R, Norcross RD, Knoflach F, Kratzeisen C, Zenner MT, Kolb Y, Marcuz A, Huwyler J, Nakagawa T, Porter RH, Thomas AW, Wettstein JG, Sleight AJ, Spooren W, Prinssen EP (2008) Characterization of (R,S)-5,7-di-tert-butyl-3-hydroxy-3-trifluoromethyl-3H-benzofuran-2-one as a positive allosteric modulator of GABAB receptors. Br J Pharmacol 154:797-811.

Marsot A, Imbert B, Alvarez JC, Grassin-Delyle S, Jaquet I, Lancon C, Simon N (2014) High variability in the exposure of baclofen in alcohol-dependent patients. Alcohol Clin Exp Res 38:316-321.

Monyer H, Sprengel R, Schoepfer R, Herb A, Higuchi M, Lomeli H, Burnashev N, Sakmann B, Seeburg PH (1992) Heteromeric NMDA receptors: molecular and functional distinction of subtypes. Science 256:1217-1221.

Morley KC, Baillie A, Leung S, Addolorato G, Leggio L, Haber PS (2014) Baclofen for the treatment of alcohol dependence and possible role of comorbid anxiety. Alcohol Alcohol 49:654-660.

Mrejeru A, Marti-Prats L, Avegno EM, Harrison NL, Sulzer D (2015) A subset of ventral tegmental area dopamine neurons responds to acute ethanol. Neuroscience 290:649-658.

Muller CA, Geisel O, Pelz P, Higl V, Kruger J, Stickel A, Beck A, Wernecke KD, Hellweg R, Heinz A (2015) High-dose baclofen for the treatment of alcohol dependence (BACLAD study): A randomized, placebo-controlled trial. Eur Neuropsychopharmacol.

Munoz MB, Padgett CL, Rifkin R, Terunuma M, Wickman K, Contet C, Moss SJ, Slesinger PA (2016) A Role for the GIRK3 Subunit in Methamphetamine-Induced Attenuation of GABAB Receptor-Activated GIRK Currents in VTA Dopamine Neurons. J Neurosci 36:3106-3114.

Nugent FS, Penick EC, Kauer JA (2007) Opioids block long-term potentiation of inhibitory synapses. Nature 446:1086-1090.

Olpe HR, Steinmann MW, Ferrat T, Pozza MF, Greiner K, Brugger F, Froestl W, Mickel SJ, Bittiger H (1993) The actions of orally active GABAB receptor antagonists on GABAergic transmission in vivo and in vitro. Eur J Pharmacol 233:179-186.

Padgett CL, Lalive AL, Tan KR, Terunuma M, Munoz MB, Pangalos MN, Martinez-Hernandez J, Watanabe M, Moss SJ, Lujan R, Luscher C, Slesinger PA (2012) Methamphetamine-evoked depression of GABA(B) receptor signaling in GABA neurons of the VTA. Neuron 73:978-989.

Paterson NE, Vlachou S, Guery S, Kaupmann K, Froestl W, Markou A (2008) Positive modulation of GABA(B) receptors decreased nicotine self-administration and counteracted nicotine-induced enhancement of brain reward function in rats. J Pharmacol Exp Ther 326:306-314.

Rhodes JS, Best K, Belknap JK, Finn DA, Crabbe JC (2005) Evaluation of a simple model of ethanol drinking to intoxication in C57BL/6J mice. Physiol Behav 84:53-63.

Seutin V, Johnson SW, North RA (1994) Effect of dopamine and baclofen on N-methyl-D-aspartate-induced burst firing in rat ventral tegmental neurons. Neuroscience 58:201-206.

Tzschentke TM (2007) Measuring reward with the conditioned place preference (CPP) paradigm: update of the last decade. Addict Biol 12:227-462.

Ungless MA, Whistler JL, Malenka RC, Bonci A (2001) Single cocaine exposure in vivo induces long-term potentiation in dopamine neurons. Nature 411:583-587.

Urwyler S (2011) Allosteric modulation of family C G-protein-coupled receptors: from molecular insights to therapeutic perspectives. Pharmacol Rev 63:59-126.

Urwyler S, Pozza MF, Lingenhoehl K, Mosbacher J, Lampert C, Froestl W, Koller M, Kaupmann K (2003) N,N'-Dicyclopentyl-2-methylsulfanyl-5-nitro-pyrimidine-4,6-diamine (GS39783) and structurally related compounds: novel allosteric enhancers of gamma-aminobutyric acidB receptor function. J Pharmacol Exp Ther 307:322-330.

Vashchinkina E, Panhelainen A, Vekovischeva OY, Aitta-aho T, Ebert B, Ator NA, Korpi ER (2012) GABA site agonist gaboxadol induces addiction-predicting persistent changes in ventral tegmental area dopamine neurons but is not rewarding in mice or baboons. J Neurosci 32:5310-5320.

Vekovischeva OY, Semenova SG, Verbitskaya EV, Zvartau EE (2004) Effects of morphine and cocaine in mice with stable high aggressive and nonaggressive behavioral strategy. Pharmacol Biochem Behav 77:235-243.

Wise RA, Newton P, Leeb K, Burnette B, Pocock D, Justice JB, Jr. (1995) Fluctuations in nucleus accumbens dopamine concentration during intravenous cocaine self-administration in rats. Psychopharmacology (Berl) 120:10-20.

Table 1

Positive allosteric modulation pharmacology of rac-BHFF and ORM-27669 at human and rat $GABA_B$ receptors in the calcium-based and [^{35}S]GTPγS binding assay. **A**. In the calcium-based assay, rac-BHFF and ORM-27669 showed similar allosteric pharmacology in PAM EC_{50} values (in μM) in the presence of EC_5-EC_{11} concentration of GABA or baclofen and in potentiation (at 10 μM) of the GABA and baclofen dose-response curves at both human and rat $GABA_B$ receptors. Potentiation was determined as a leftward shift of GABA and baclofen dose-response curves (fold potentiation of agonist EC_{50} values). **B**. Rac-BHFF showed significant intrinsic agonism (IA, relative to 100 μM GABA ± SEM), which prevented accurate estimation of its allosteric pharmacology. ORM-27669 (at 10 μM concentration) showed potentiation of GABA dose-response at both human and rat $GABA_B$ receptors. Results are expressed as means of quadruplicates at each concentration from 4-11 plates ± SEM.

A	PAM EC_{50} (μM) GABA as agonist	PAM EC_{50} (μM) baclofen as agonist	Potentiation at 10 μM with GABA as agonist (fold / E_{max})	Potentiation at 10 μM with baclofen as agonist (fold / E_{max})
human $GABA_B$ receptors				
Rac-BHFF	2.3 ± 0.1	3.1 ± 0.4	13.8 ± 2.6 / 1.1 ± 0.1	16.3 ± 3.3 / 1.1 ± 0.1
ORM-27669	5.3 ± 0.5	10.9 ± 1.8	7.8 ± 1.3 / 1.2 ± 0.1	4.8 ± 0.5 / 1.2 ± 0.1
rat $GABA_B$ receptors				
Rac-BHFF	1.0 ± 0.4	0.6 ± 0.1	14.6 ± 1.4 / 0.8 ± 0.1	8.7 ± 1.7 / 0.8 ± 0.1
ORM-27669	1.9 ± 0.2	2.9 ± 0.2	3.7 ± 1.2 / 1.0 ± 0.1	5.3 ± 0.7 / 1.1 ± 0.1

B	Fold change of potentiation of GABA EC_{50}	Fold change of potentiation of 100 μM GABA	Agonism (IA) as compared to 100 μM GABA

human $GABA_B$ receptors			
Rac-BHFF	N/A	0.90 ± 0.02	0.92 ± 0.04
ORM-27669	2.5 ± 0.2	1.00 ± 0.02	0.06 ± 0.02
rat $GABA_B$ receptors			
Rac-BHFF	N/A	1.57 ± 0.09	0.54 ± 0.06
ORM-27669	2.5 ± 0.2	1.1 ± 0.04	0.06 ± 0.00

Figure legends

Figure 1 **A.** Chemical structures of test compounds. **B.** Representative images of the stimulation of [^{35}S]GTPγS binding in adult mouse brains by baclofen, rac-BHFF and ORM-27669. The [^{35}S]GTPγS binding in the presence of 30-μM baclofen and its robust potentiation by 300-μM ORM-27669 and 10-μM rac-BHFF, respectively, in many brain regions. The effects of both PAMs were fully antagonized in the presence of $GABA_B$ receptor antagonist CGP-54626 (10 μM). **C.** Quantitative data on the stimulation of G protein coupling by rac-BHFF and ORM-27669 at 10 and 300 μM concentrations, respectively, in the presence of 30 μM baclofen, which was almost inactive alone at this concentration. Data are means ± SEM, n = 5 brains. (CbMol, cerebellar molecular layer; CbGr, cerebellar granule cell layer; Ctx, cerebral cortex; SuG, superior colliculus; NAc, nucleus accumbens; CPu-DL, dorsolateral caudate-putamen; Th-MG, thalamic medial geniculate nucleus; CA3, hippocampal CA3 area; CA1, hippocampal CA1 area; DG, hippocampal dentate gyrus).

Figure 2 Effects of single i.p. administrations of $GABA_B$ receptor ligands and their co-administration on spontaneous locomotor activity in C57BL/6J mice, and on AMPA/NMDA receptor current ratios in VTA DA neurons of midbrain slices obtained *ex vivo* 24 h after the drug injection in TH-EGFP mice. **A**. Time course of the effects of $GABA_B$ drugs during the 90-min measuring period for 15-min time bins. Acute i.p. administration of rac-BHFF and ORM-27669 was tested in two separate experiments in adult C57BL/6J mice. On the left, results for mice injected with Veh (n = 8), Bac1 (n = 9), BH30 (n = 5), BH60 (n = 10), co-administration of baclofen and rac-BHFF (BH30+Bac1, n = 8; BH60+Bac1, n = 14). On the right, the results

from the ORM-27669 test: Veh (n = 17), Bac1 (n = 12), ORM10 (n = 11), ORM30 (n = 11), ORM100 (n = 12), co-administration ORM-27669 with baclofen (ORM100+Bac1, n = 12). Data are presented as mean distance traveled + SEM (unless within the symbol). * $p < 0.05$, **$p < 0.01$ for the significance of difference from the vehicle group (ANOVA followed by Bonferroni test). **B**. Cumulative distances traveled during 30 min after the drug administrations. Data are presented as mean distance traveled + SEM. * $p < 0.05$, **$p < 0.01$, ***$p < 0.001$ for the significance of difference from the vehicle group. **C**. Representative examples of AMPA and NMDA receptor-mediated current traces for midbrain slices from mice injected 24 h earlier with vehicle, baclofen, ORM-27669 and rac-BHFF. Calibration: 20 pA/50 ms. **D**. AMPA/NMDA (A/N) receptor current ratios for vehicle (Veh, n = 14, where n is the number of tested mice), Bac1, 1 mg/kg, n = 8; Bac5, 5 mg/kg, n = 7; Bac30, 30 mg/kg, n = 7; BH30, 30 mg/kg, n = 7; BH100, 100 mg/kg, n = 7; BH30+Bac1, n = 9; ORM10, 10 mg/kg, n = 8; ORM100, 100 mg/kg, n = 7; and ORM10+Bac1, n = 4, are shown as means + SEM. No statistically significant differences between the groups ($F_{9,66} = 0.6$, $p = 0.7$).

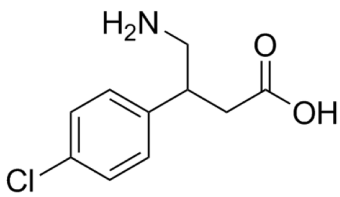
Figure 3 Effects of $GABA_B$ ligands on ethanol- and cocaine-induced neuroplasticity. The AMPA/NMDA receptor current ratio (A/N ratio) in VTA DA neurons of midbrain slices obtained *ex vivo* 24 h after the drug injection in TH-EGFP mice. **A**. Treatment with vehicle (Veh, n = 8, where n is the number of tested mice), ethanol (EtOH, 2 g/kg, n = 14), baclofen + ethanol (Bac1+EtOH, n = 7; Bac30+EtOH, n = 5), rac-BHFF + ethanol (BH30+EtOH, n = 7; BH100+EtOH, n = 8), ORM-27669 + ethanol (ORM100+EtOH, n = 6). **B**. Treatment with vehicle (Veh, n = 9), cocaine (coc, 10 mg/kg, n = 13), baclofen + cocaine (Bac1+Coc, n = 5), rac-BHFF + cocaine (BH30+Coc, n = 7) and ORM-27669 + cocaine (ORM100+Coc, n = 8).

Data are shown as mean + SEM. *p < 0.05, **p < 0.01, ***p < 0.001 for the significance of the difference from ethanol or cocaine group (one-way ANOVA followed by Dunnett test).

Figure 4 Effects of 30-min pretreatment with rac-BHFF and ORM-27669 on ethanol- and cocaine-induced place conditioning and self-administration in adult C57BL/6J mice. **A**. CPP expressed as time shifts between post- and pre-conditioning times spent in the drug-paired compartment at 48 h after the last conditioning session with saline (Sal, n = 17), ethanol (EtOH, 0.5 g/kg, n = 9), rac-BHFF 30 mg/kg + EtOH (BH30+EtOH, n = 6), and ORM-27669 100 mg/kg + EtOH (ORM100+EtOH, n = 12), cocaine (Coc, 10 mg/kg, n = 16), rac-BHFF + cocaine (BH30+Coc, n = 11), ORM-27669 + cocaine (ORM100+Coc, n = 11). Conditioning with ethanol and cocaine induced statistically significant CPP (*** p < 0.001), but not when the animals were pretreated with BH30. ORM100 only reduced conditioning to ethanol. **B**, **C.** Locomotor activity during 20-min conditioning sessions after pretreatments of vehicle, rac-BHFF (B) or ORM-27669 (C). *p < 0.05, a significant reduction of cocaine-induced activity by BH30. **D**. Four-hour intake of ethanol (20%, v/v) after pretreatment with vehicle (Veh, n = 13) and rac-BHFF (BH30, n = 13) or with vehicle (Veh, n = 16) and ORM-27669 (ORM100, n = 16). Data are means ± SEM. *p < 0.05, ***p<0.001 for the significance of the difference from vehicle group (Student t-test). **E**. Reinforcement factor for cocaine i.v. self-administration (SA) 30 min after pretreatment with rac-BHFF and ORM-27669. Drug-naive C57BL/6J mice were allowed to i.v. self-administer saline (Veh+Sal, n = 4, where n is the number of tested pairs of active and yoked-control mice) or cocaine (Coc, 1 mg/ml) during 20-min sessions. On the left, the mice were pretreated with vehicle (Veh+Coc, n = 3) or ORM-27669 (100 mg/kg, ORM100+Coc, n = 9), and, on the right, with vehicle (Veh+Coc, n = 13) or rac-BHFF (3 mg/kg BH3+coc, n = 6; 30 mg/kg BH30+Coc, n = 8). A positive reinforcement factor indicates

positive reinforcement. Data are means ± SEM. **p<0.01, ***p<0.001 for the significance of the differences from vehicle-saline group, *p < 0.05 for the significance of the difference from the corresponding vehicle group (one-way ANOVA followed by Dunnett test).

A



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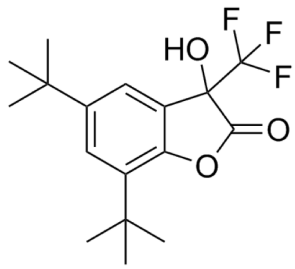

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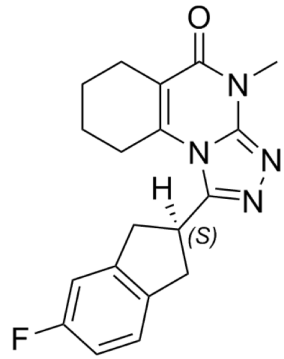


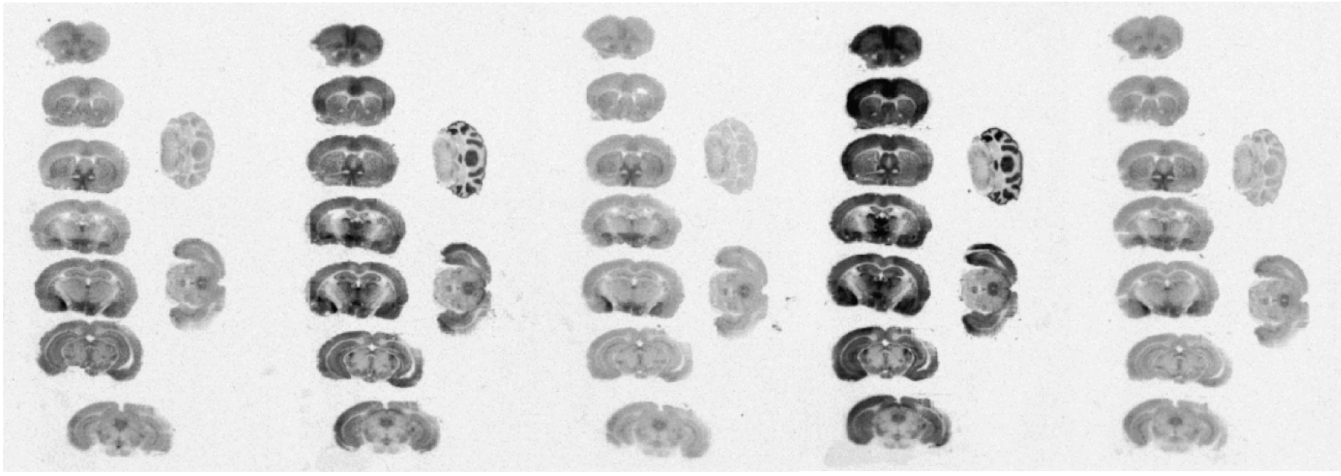
ORM-27669

B

30 μM Baclofen

30 μM Baclofen +
300 μM ORM-27669 +
10 μM CGP-54626

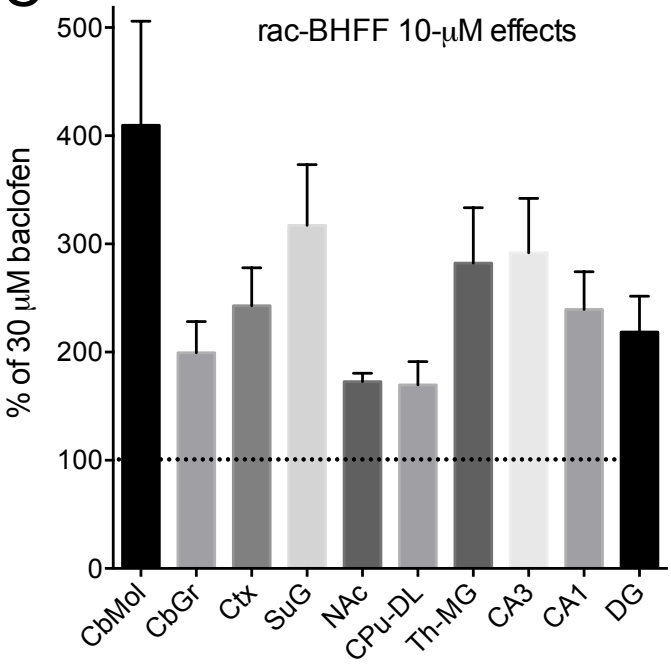
30 μM Baclofen +
10 μM rac-BHFF +
10 μM CGP-54626

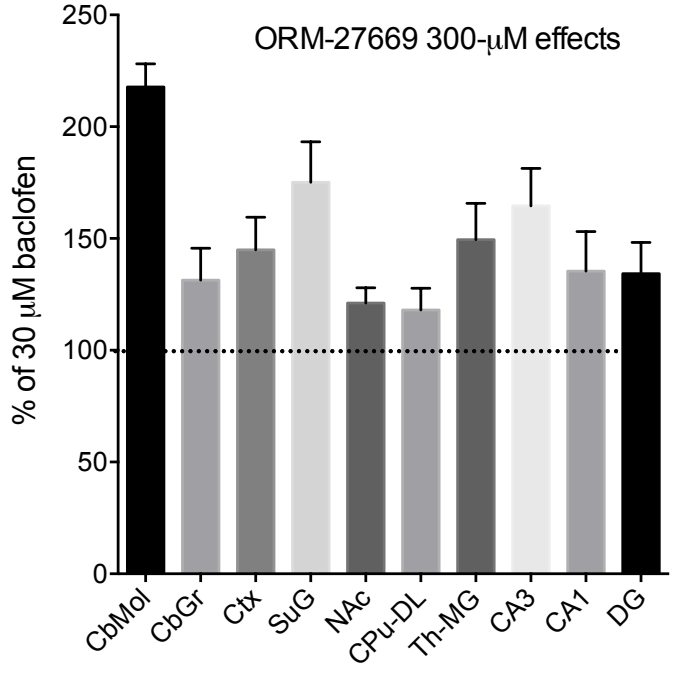


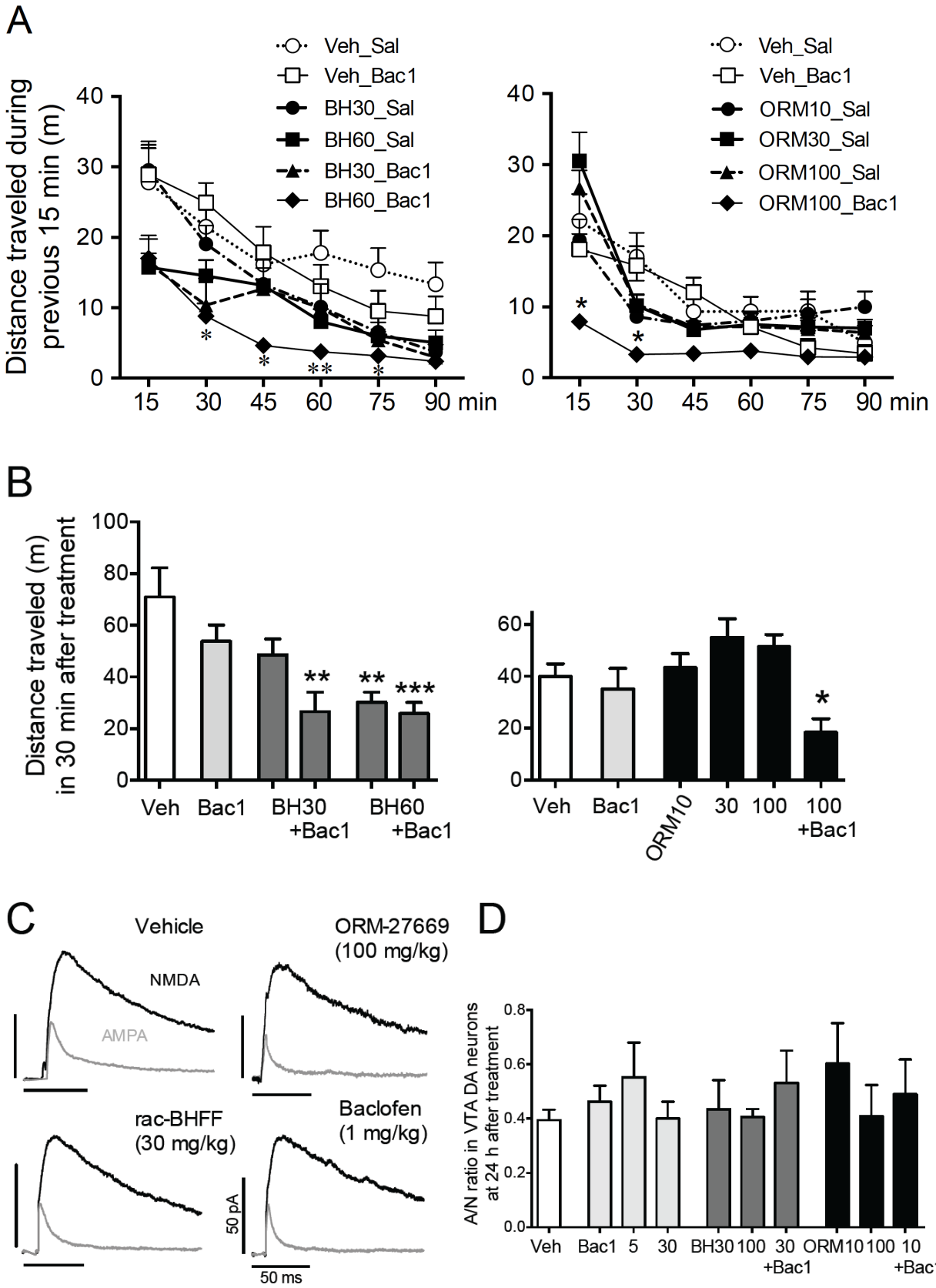
30 μM Baclofen +
300 μM ORM-27669

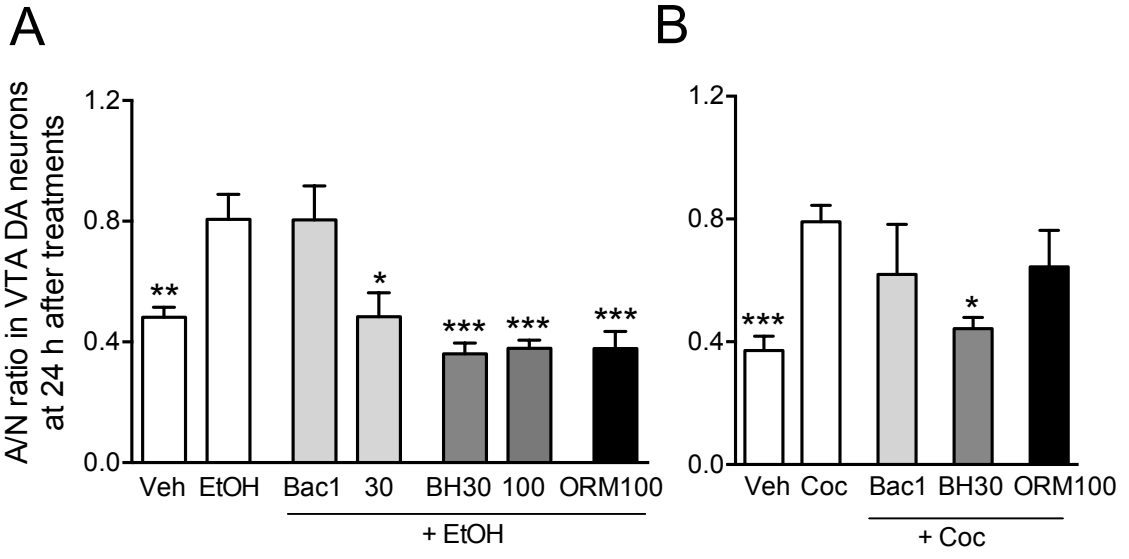
30μM Baclofen +
10 μM rac-BHFF

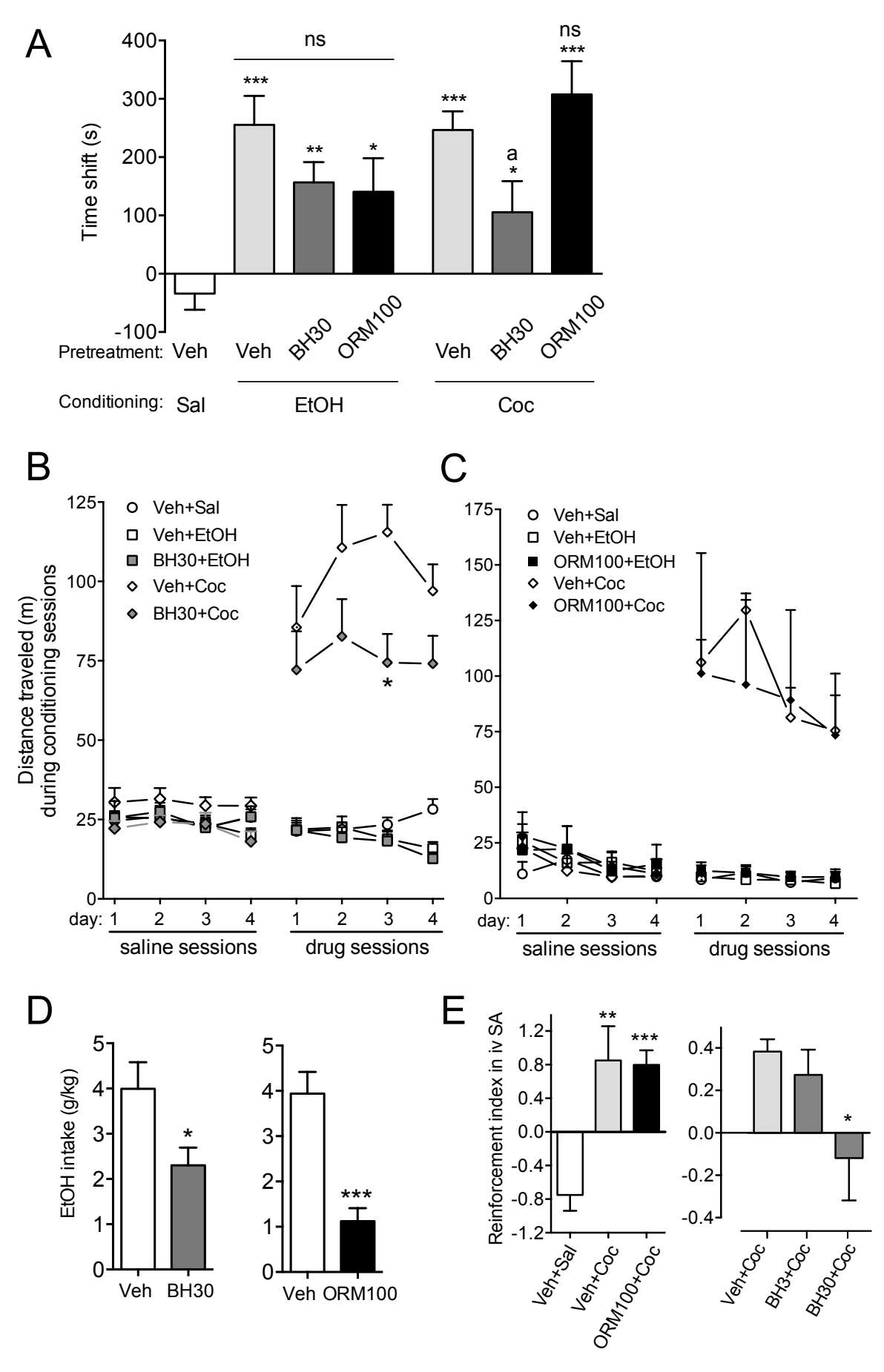
C






A
Distance traveled during previous 15 min (m)
Veh_Sal
Veh_Bac1
BH30_Sal
BH60_Sal
BH30_Bac1
BH60_Bac1
ORM10_Sal
ORM30_Sal
ORM100_Sal
ORM100_Bac1
15 30 45 60 75 90 min
B
Distance traveled (m) in 30 min after treatment
Veh
Bac1
BH30 +Bac1
BH60 +Bac1
ORM10
30
100
100 +Bac1
C
Vehicle
ORM-27669 (100 mg/kg)
NMDA
AMPA
rac-BHFF (30 mg/kg)
Baclofen (1 mg/kg)
50 pA
50 ms
D
A/N ratio in VTA DA neurons at 24 h after treatment
Veh
Bac1
5
30
BH30
100
30 +Bac1
ORM10
100
10 +Bac1


A
B
A/N ratio in VTA DA neurons at 24 h after treatments
1.2
0.8
0.4
0.0
**
*
***
***
***
Veh
EtOH
Bac1
30
BH30
100
ORM100
+ EtOH
***
*
Veh
Coc
Bac1
BH30
ORM100
+ Coc


A
Time shift (s)
400
300
200
100
0
-100
ns
ns
***
***
**
*
***
a
*
***
Pretreatment: Veh Veh BH30 ORM100 Veh BH30 ORM100
Conditioning: Sal EtOH Coc
B
Distance traveled (m) during conditioning sessions
Veh+Sal
Veh+EtOH
BH30+EtOH
Veh+Coc
BH30+Coc
125
100
75
50
25
0
*
day: 1 2 3 4 1 2 3 4
saline sessions
drug sessions
C
Veh+Sal
Veh+EtOH
ORM100+EtOH
Veh+Coc
ORM100+Coc
175
150
125
100
75
50
25
0
day: 1 2 3 4 1 2 3 4
saline sessions
drug sessions
D
EtOH intake (g/kg)
5
4
3
2
1
0
*
Veh BH30
***
Veh ORM100
E
Reinforcement index in iv SA
1.2
0.8
0.4
0.0
-0.4
-0.8
-1.2
**
***
Veh+Sal
Veh+Coc
ORM100+Coc
0.4
0.2
0.0
-0.2
-0.4
*
Veh+Coc
BH3+Coc
BH30+Coc