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Systemic blockade of $LPA_{1/3}$ lysophosphatidic acid receptors by ki16425 modulates the effects of ethanol on the brain and behavior

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ABSTRACT

The systemic administration of lysophosphatidic acid (LPA) $LPA_{1/3}$ receptor antagonists is a promising clinical tool for cancer, sclerosis and fibrosis-related diseases. Since LPA_1 receptor-null mice engage in increased ethanol consumption, we evaluated the effects of systemic administration of an $LPA_{1/3}$ receptor antagonist (intraperitoneal ki16425, 20 mg/kg) on ethanol-related behaviors as well as on brain and plasma correlates. Acute administration of ki16425 reduced motivation for ethanol but not for saccharine in ethanol self-administering Wistar rats. Mouse experiments were conducted in two different strains. In Swiss mice, ki16425 treatment reduced both ethanol-induced sedation (loss of righting reflex, LORR) and ethanol reward (escalation in ethanol consumption and ethanol-induced conditioned place preference, CPP). Furthermore, in the CPP-trained Swiss mice, ki16425 prevented the effects of ethanol on basal c-Fos expression in the medial prefrontal cortex and on adult neurogenesis in the hippocampus. In the c57BL6/J mouse strain, however, no effects of ki16425 on LORR or voluntary drinking were observed. The c57BL6/J mouse strain was then evaluated for ethanol withdrawal symptoms, which were attenuated when ethanol was preceded by ki16425 administration. In these animals, ki16425 modulated the expression of glutamate-related genes in brain limbic regions after ethanol exposure; and peripheral LPA signaling was dysregulated by either ki16425 or ethanol. Overall, these results suggest that $LPA_{1/3}$ receptor antagonists might be a potential new class of drugs that are suitable for treating or preventing alcohol use disorders. A pharmacokinetic study revealed that systemic ki16425 showed poor brain penetration, suggesting the involvement of peripheral events to explain its effects.

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1. Introduction

Alcohol is vastly consumed in first world countries, but its harmful use causes ~5.9% of all global deaths plus numerous diseases and injuries, most notably alcohol dependence, liver cirrhosis and cancer (World Health Organization, 2014). Since current pharmacotherapy for alcohol use disorders is not successful in all patients, there is a clear need to develop more effective treatments (Swift and Aston, 2015), such as the identification of novel molecules as putative targets for ethanol-related behaviors.

Lysophosphatidic acid (LPA, 1-acyl-2-hydroxy-sn-glycero-3-phosphate) is an endogenous lysophospholipid that has multiple biological actions and plays prominent roles in central nervous system processes as well as in peripheral organs (Choi et al., 2010; Yung et al., 2014). The blood is the most abundant source of LPA in mammals (serum: ~4–15.5 μM; plasma: ~0.17–0.63 μM), but LPA is also present in other body fluids, including the cerebrospinal fluid, saliva and semen (Choi et al., 2010; Riaz et al., 2016; Yung et al., 2014). LPA is synthetized from cell membrane phospholipids (mainly those derived from activated platelets in the serum) by two different metabolic pathways that are mediated by phospholipase enzymes (Aoki et al., 2002; Choi et al., 2010; Riaz et al., 2016). One of these metabolic routes involves the action of autotaxin (ATX), which is the primary enzyme responsible of LPA production by converting lysophospholipids into LPA (Choi et al., 2010; Riaz et al., 2016). The synthesis processes produce different variations of the LPA molecule (LPA species), depending on the acyl group, that may differ in their biological actions (Bandoh et al., 2000). The 16:0-, 18:0-, 18:1-, 18:2- and 20:4-acyl LPA forms are the most abundant in the blood (Baker et al., 2001).

LPA acts through six G-coupled cell membrane receptors (LPA_{1-6}) that are ubiquitously distributed throughout the body (Choi et al., 2010; Riaz et al., 2016; Yung et al., 2014); of these receptors, the LPA_1 receptor is the most well studied. In humans and mice, the LPA_1 receptor is abundant in both the brain and periphery and is expressed in glial cells, neurons, neural progenitors, the lung, the heart, the kidney, skeletal muscle, digestive apparatus and reproductive system (Choi et al., 2010; Goldshmit et al., 2010; Walker et al., 2016). In these tissues, the LPA_1 receptor activates the Rho, phospholipase C, MAPK and Akt downstream signaling pathways to trigger cellular responses such as cell proliferation, migration, growth, adhesion and survival/apoptosis (Choi et al., 2010; Yung et al., 2014). In the central nervous system, LPA_1–mediated signaling is critical for neurodevelopment (Estivill-Torrus et al., 2008; Garcia-Diaz et al., 2015) and hippocampal neurogenesis in adulthood (Matas-Rico et al., 2008; Walker et al., 2016). However, LPA_1–mediated signaling has also been involved in promoting many disease states, such as fibrosis, inflammation, cancer, systemic sclerosis, hepatic illnesses and cardiovascular diseases (Choi et al., 2010; Lin et al., 2010; Yung et al., 2014), which are usually associated with increased circulating ATX and/or LPA concentrations (Crack et al., 2014; Kurano et al., 2015; Li et al., 2015; Watanabe et al., 2007). In contrast, compounds that block the LPA_1 receptor have emerged as valuable pharmacological tools to treat the aforementioned pathologies (Kihara et al., 2015; Lin et al., 2010; Llona-Minguez et al., 2015; Stoddard and Chun, 2015; Tigyi, 2010; Yung et al., 2014). Currently, at least three orally administered antagonists for the LPA_1 or $LPA_{1/3}$ receptors are available and are in advanced stages (Phase I or Phase II) of clinical development against systemic sclerosis and idiopathic pulmonary fibrosis (Stoddard and Chun, 2015).

Nevertheless, there is scarce experimental evidence regarding the pharmacological actions of peripherally administered LPA_1 or $LPA_{1/3}$ receptor antagonists on normal or pathological behavior. Most pre-clinical research studies have focused on mutant mice lacking the LPA_1 receptor (LPA_1-null mice) that display wide emotional, exploratory and memory impairments (Blanco et al., 2012; Castilla-Ortega et al., 2016a, 2012; 2010; Harrison et al., 2003; Moreno-Fernández et al., 2017; Santin et al., 2009). However, considering that the LPA_1-null mice suffer from severe neurodevelopmental abnormalities (Estivill-Torrus et al., 2008; Garcia-Diaz et al., 2015) that yield vast neuroadaptations in brain limbic regions (Castilla-Ortega et al., 2016a; Matas-Rico et al., 2008; Musazzi et al., 2011; Pedraza et al., 2014), it is not possible to elucidate whether any of their behavioral alterations correspond to altered LPA signaling in adulthood. A key discovery for the potential role of systemic LPA signaling in neuropsychiatric disorders (substance abuse) was provided by our recent work (Castilla-Ortega et al., 2016a). The LPA_1-null mice submitted to voluntary ethanol drinking (i.e., two-bottle choice protocols) consumed high quantities of ethanol, and importantly, acute systemic administration of ki16425 [a non-lipidic dual $LPA_{1/3}$ competitive antagonist widely used in pre-clinical research (Llona-Minguez et al., 2015; Ohta et al., 2003)] in voluntary ethanol-drinking animals replicated this effect by rapidly increasing ethanol consumption in both Wistar rats and normal C57BL/6J×129X1/SvJ mice (Castilla-Ortega et al., 2016a).

Albeit these preliminary results, the effects of the peripheral blockade of $LPA_{1/3}$ receptors on ethanol-related behaviors are still largely unknown. For example, an animal may increase voluntary ethanol drinking as a consequence of either augmented or reduced ethanol reward (in that latter case, greater quantities of the drug need to be ingested to obtain effects) (Olive et al., 2003). Other relevant behaviors, such as ethanol-induced motivation, sedation or withdrawal also remain to be investigated. Therefore, considering the increasing clinical relevance of $LPA_{1/3}$ receptor antagonists for systemic diseases, the present work aimed to describe the effect of peripheral ki16425 administration on a compelling number of ethanol-induced responses. This evidence would guide future investigation on the potential usefulness of this class of antagonists in the pharmacotherapy of alcoholism. Wistar rats were used for ethanol self-administration, while mouse experiments (i.e., loss of righting reflex, two-bottle choice, conditioned place preference, ethanol withdrawal symptoms) were performed in two commonly used mouse strains (Swiss and/or c57BL/6J mice). Two mouse strains were studied because both physiological responses and ethanol-related behaviors may strongly vary in mice depending on their genetic background (Tambour et al., 2006; Yoneyama et al., 2008). For example, mice from the Swiss lineage usually show less voluntary ethanol drinking (Yoneyama et al., 2008) but enhanced ethanol-induced stimulatory/hyperlocomotor effects (Tambour et al., 2006) compared to c57BL6/J mice. Different ethanol-related traits are usually present in high and low-alcohol preferring mice, providing genetic backgrounds with different sensitivity to ethanol (Breit and Chester, 2016). In addition to behavioral data, we explored whether repeated ki16425 could modulate ethanol-induced neuroadaptations in limbic brain regions [the medial prefrontal cortex (mPFC) and the hippocampus] in both mouse strains; additionally, ethanol-induced regulation of peripheral LPA signaling (plasma LPA and ATX levels) was investigated in c57BL/6J mice. Finally, pharmacokinetics of systemically administered ki16425 are reported for plasma and brain.

2. Materials and methods

2.1. Animals

Male Wistar rats were acquired from Harlan (Barcelona, Spain), and male Swiss (RjOrl:SWISS) or c57BL/6J (C57BL/6JRj) mice were acquired from Janvier (Le Genest-Saint-Isle, France). The animals were housed individually under a 12-h light/dark cycle (cycle was reversed for the self-administration experiments in rats) in standard cages with water and food provided *ad libitum,* and the experiments were initiated at 3–4 months of age. The number of animals used for each experiment is indicated in each figure.

The procedures were performed in accordance with the European (Directive 2010/63/UE) and Spanish (Real Decreto 53/2013, Ley 32/2007) regulations on animal research and were approved by the research ethics committee of the University of Málaga (code: CEUMA N°80 2015-A).

2.2. Ki16425 administration

Ki16425 (ApexBio, Houston, USA) was dissolved in a vehicle solution of 3% fatty acid free bovine serum albumin (Sigma, St. Louis, USA) or 2% DMSO in saline (Sal, 0.9% NaCl) and administered intraperitoneally (i.p.) at a dose of 20 mg/kg, which was based on our previous study (Castilla-Ortega et al., 2016a).

2.3. Experiment I

2.3.1. Chronic oral self-administration of ethanol and saccharin

Wistar rats were trained in operant chambers to self-administer oral ethanol (ETOH, 10%) [supplementary methods (Alen et al., 2013);] or saccharin (0.1%) solutions until they reached a stable baseline performance. Then, the rats were maintained for six weeks under a self-administration schedule [a daily 30 min self-administration session, 5 days per week (Monday-Friday)]. On the day of the experiment, the rats were acutely administered vehicle or ki16425, and 45 min later, the rats were placed in the operant chamber for a 30 min self-administration session (Fig. 1A).

2.4. Experiment II

2.4.1. Loss of righting reflex

Swiss or c57BL/6J mice were acutely administered vehicle or ki16425, and 20 min later, the mice received a 3.4 gr/kg ethanol dose (20% w/v in saline; i.p.) (Blednov and Harris, 2009) (Fig. 2A and B). The mice where then immediately placed on their backs, and the latency to the loss of the righting reflex (LORR) was evaluated. The LORR was defined as the inability of the mouse to right itself three times during a 30-s period. Sleep time was defined as the time the mouse required to regain its righting reflex (Castilla-Ortega et al., 2016a).

2.4.2. Two-bottle choice

The effects of either acute or chronic ki16425 administration on voluntary ethanol consumption were assessed in both Swiss and c57BL/6J mice subjected to a two-bottle choice paradigm (Castilla-Ortega et al., 2016a). For the acute experiment (Fig. 2C and D), mice were habituated to drink from either a water bottle or a bottle containing a 3% (days 1–4) or a 6% (days 5–8) ethanol solution (v/v), which was placed in their home-cage. On day 7, mice received a single vehicle or ki16425 administration at 9:00 a.m., and the bottles were weighed at intervals of 90 min, 3 h, 6 h and 24 h to assess fluid consumption. For the chronic experiment (Fig. 2E and F), naïve mice received a daily vehicle or ki16425 dose in their home-cage and were allowed to freely drink from two bottles containing water or ethanol. Three increasing ethanol concentrations [3, 6, and 12% (v/v)] were offered for 4 days each, and the bottles were weighed at 24-h intervals. Fresh drinking solutions were provided every two days.

Water intake (g/kg) and ethanol intake (g/kg) were calculated for each animal after controlling for evaporation/spillage, which was estimated by an empty cage with two bottles containing water and the appropriate ethanol solution.

2.5. Experiment III

2.5.1. Ethanol-induced conditioned place preference (CPP)

According to the results of the free ethanol drinking experiments, the Swiss mouse strain was used to further investigate the effect of

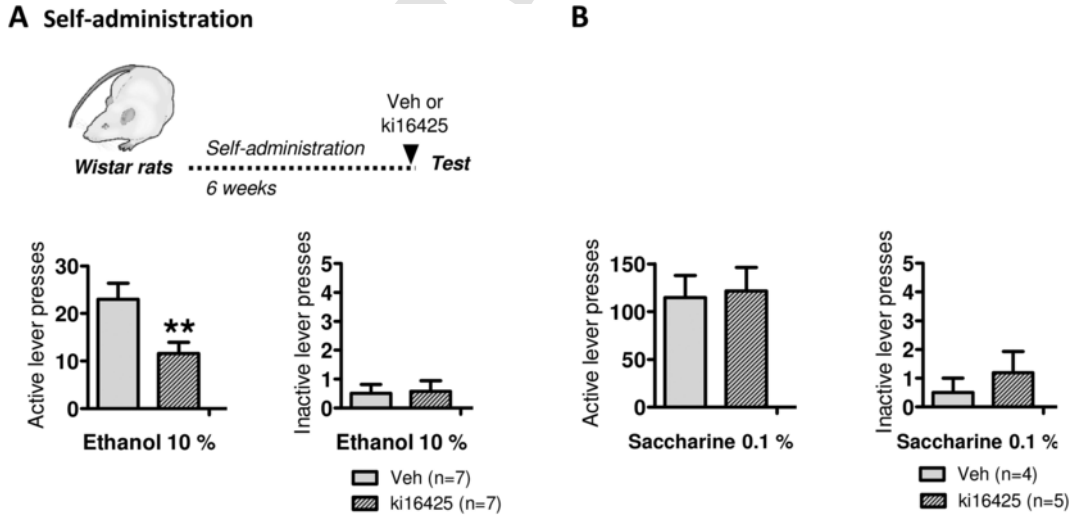


Fig. 1. Experiment I. Acute ki16425 administration reduced oral ethanol self-administration in chronically ethanol self-administering rats (A) but did not change the motivation for saccharine (B). Difference vs the other group: $^{**}P < 0.001$ (Student's *t*-test). Data are expressed as the mean ± SEM.

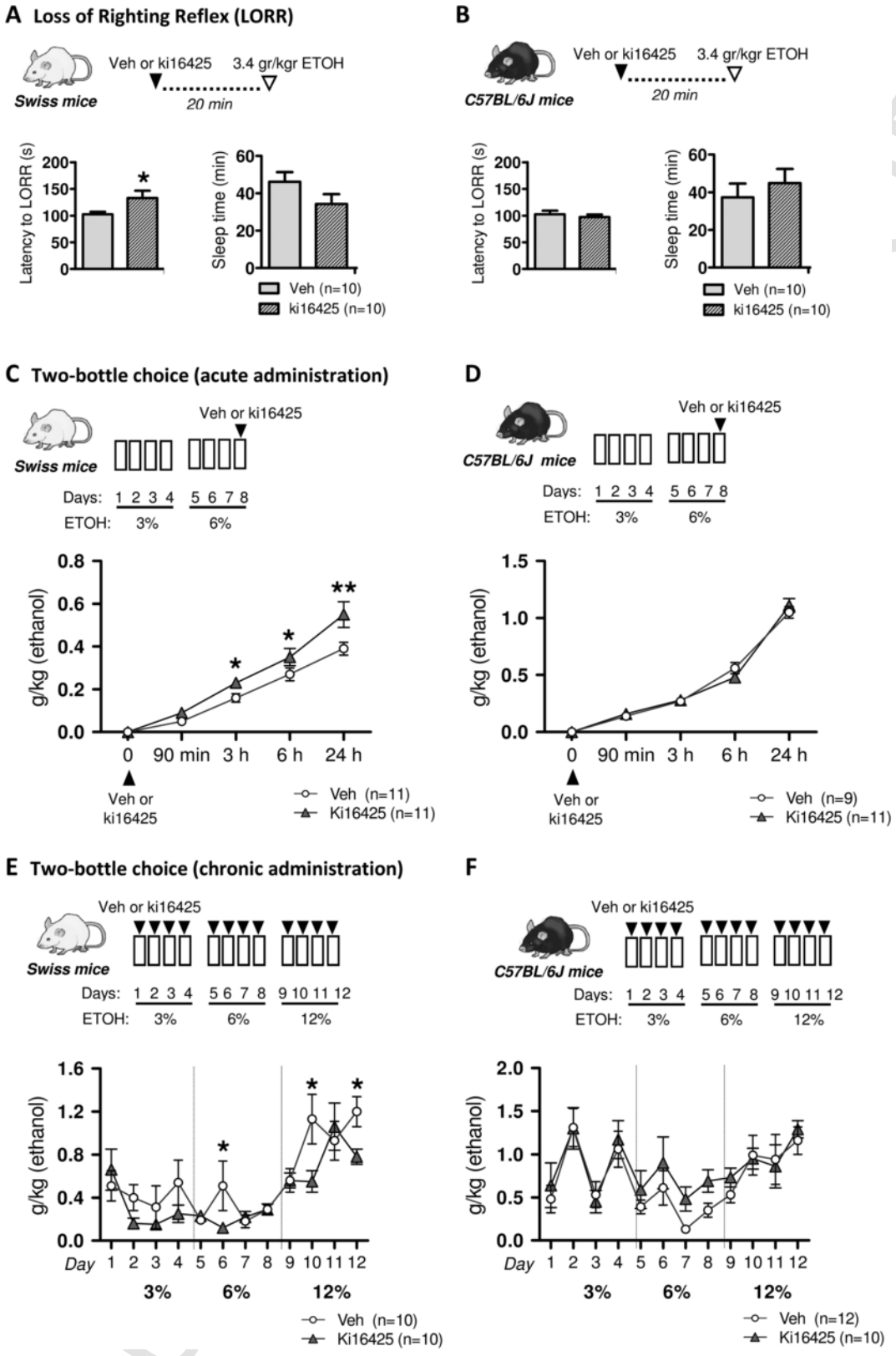


Fig. 2. Experiment II. Strain-dependent effects of ki16425 on ethanol-induced sedation and voluntary ethanol drinking. Acute ki16425 administration increased latency to LORR in the Swiss mouse strain only (A, B). In the two-bottle choice experiments, acute ki16425 increased ethanol consumption in Swiss mice that were previously drinking ethanol (C), while repeated ki16425 administration in ethanol naïve Swiss mice reduced their escalation of ethanol intake (E). No effects were observed in the c57BL/6J mouse strain (D, F). Difference vs the other group: $*P < 0.05$, $**P < 0.001$ with the Student's t-test (A) or LSD post hoc analysis (C, E). Data are expressed as the mean ± SEM.

ki16425 on ethanol reward using an ethanol-induced CPP paradigm (Ledesma et al., 2013). The CPP protocol is detailed in the supplementary methods and in Fig. 3A. Briefly, the mice were first subjected to one habituation session during which they freely explored the whole apparatus. Each subsequent conditioning day consisted of two daily 5-min sessions (separated by 5 h; their daily order was counterbalanced) in which the mice were confined to either the 'conditioned' or the 'non-conditioned' compartment. The confinement to the 'conditioned' compartment was preceded by two i.p. injections (separated by 20 min) of vehicle and saline (Veh-Sal group), ki16425 and saline (ki16425-Sal group), vehicle and ethanol (Veh-ETOH group) or ki16425 and ethanol (ki16425-ETOH group) (Table S1). Ethanol was administered at a subthreshold dose of 0.5 gr/kg (5% w/v in saline; i.p.) on conditioning days 2–5 and at a moderate-high dose of 1.5 gr/kg (Groblewski et al., 2008) (15% w/v in saline; i.p.) on conditioning days 7–10. The confinement in the 'non-conditioned' compartment was always preceded by two i.p. injections of vehicle and saline (Table S1). A test session was performed on day 6 (Test 1) and day 11 (Test 2) to evaluate the conditioning. A significant preference for the 'conditioned' compartment over the 'non-conditioned' compartment was indicated by a positive CPP Score (supplementary methods) that was significantly different from zero.

2.5.2. Immunohistochemistry and cell quantification

After the mice had completed the CPP Test 2 session, they received an additional 1.5 gr/kg ethanol or saline preceded by ki16425 or vehicle (according to their assigned treatment group; Fig. 3A) in their home cage and were sacrificed by intracardiac perfusion 24 h later for brain tissue processing for immunohistochemistry (supplementary methods).

The basal neuronal activity was studied by the expression of the early immediate gene *c-fos* in the mPFC [anterior cingulate cortex (Cg), prelimbic cortex (PL), and infralimbic cortex (IL)] and the hippocampus [dentate gyrus (DG) granule cell layer and *cornu ammonis* (CA3 or CA1) pyramidal cell layer]. Additionally, adult hippocampal neurogenesis was studied by the numbers of proliferating cells [expressing the proliferating cell nuclear antigen (PCNA)] and immature neurons [expressing doublecortin (DCX)] in the DG granule cell layer. Positive cells were quantified and expressed as their average number per unit area (mm^2) in the region of interest (supplementary methods). To assess the basal functional connectivity among regions with c-Fos data, each set of interregional correlations was plotted in color-coded correlation matrices using MATLAB software (MathWorks, Massachusetts, USA) (Moreno-Fernández et al., 2017).

2.6. Experiment IV

2.6.1. Ethanol withdrawal behavioral symptoms

Because the voluntary ethanol drinking experiments did not suggest a modulation of ethanol reward by ki16425 in the c57BL/6J mouse strain, this genotype was further assessed for ethanol-withdrawal behaviors as previously reported (Perez et al., 2015; Perez and De Biasi, 2015). For a total of 15 days, the mice received a daily injection of ki16425 or vehicle solution, and 20 min later, they received an intraperitoneal injection of saline or ETOH at 2 gr/kg (20% w/v in saline). Both the ETOH and saline solutions contained 9 mg/kg of the alcohol dehydrogenase inhibitor 4-methylpyrazole (4MP, Sigma) to elevate blood ethanol levels for longer durations and potentiate the neurobehavioral effects (Paez et al., 2004; Perez et al., 2015; Perez and De Biasi, 2015). The experimental groups were as follows: Veh-Sal, ki16425-Sal, Veh-ETOH and ki16425-ETOH, Fig. 4A.

On treatment day 8, the ethanol withdrawal severity was assessed using the nest building method (Greenberg et al., 2016a, 2016b). A brand-new nestlet (a 5-cm square of compressed cotton-like material; Ancare, New York, USA) was placed in the mouse's home cage 5 h after ETOH/saline administration, and the quality of the nest built was assessed at 1 h, 4 h and 24 h time points (supplementary methods). Behavior was further analyzed from days 9–12 during spontaneous ethanol withdrawal [24 h after ETOH/Sal administration (Perez and De Biasi, 2015)] using the following tasks: observation of physical-somatic withdrawal symptoms (day 9), elevated plus maze (day 10), marble burying (day 11), open-field exploration (day 12) and Y-maze spontaneous alternation (day 12) (Fig. 4A; supplementary methods). One hour after the daily behavioral assessment, the mice received their corresponding pharmacological treatment to continue testing in subsequent days.

Body weights and food consumption were measured daily on days 1–9. The plasma ethanol concentrations were determined using the alcohol oxidase method (Castilla-Ortega et al., 2016a) on the last day of treatment (day 15) (Fig. 4A). The mice were gently sampled via the facial vein by a trained experimenter 1 h after ETOH or saline administration.

2.6.2. Quantitative real-time PCR for glutamate-related genes and plasma analysis

On day 16 (Fig. 4A), the c57BL/6J mice were removed from their home cages and rapidly sacrificed by decapitation. Trunk blood was collected in EDTA tubes centrifuged at 2000 × g for 10 min at room temperature to obtain plasma, and brains were quickly removed and frozen on dry ice. The samples were stored at −80 °C until analysis.

Quantitative real-time PCR of the frozen brain tissue (mPFC and hippocampus) revealed the expression of genes related to the glutamate neurotransmission system (supplementary methods; Table S2): the glutamate-synthesis enzyme kidney-type glutaminase isoforms (*Kga*), the type II metabotropic receptor mGluR3 (inhibits glutamate release in presynaptic terminals) (Simonyi et al., 2004) and the glutamate transporter (*Eaac1*).

The plasma concentrations of corticosterone, ATX and LPA were determined using commercially available enzyme-linked immunoassay kits (catalog no K014-H1, Arbor Assays, Michigan, USA; no K-2800S and no K-5600, Echelon Biosciences, Utah, USA) according to the manufacturer's instructions. Additionally, the major LPA species (16:0, 18:0 and 18:1) were quantified by time-of-flight mass spectrometry (MALDI-TOF) (supplementary methods).

2.7. Pharmacokinetics of systemic ki16425 in plasma and brain

The determination of Ki16425 pharmacokinetics in mouse plasma and brain after an acute i.p. administration (20 mg/kg) was performed using a liquid chromatography-mass spectrometry (LC-MS/MS) method (supplementary methods). This study was carried out in Swiss mice since this mouse strain was the most responsive to ki16425 in the previous experiments (i.e., they showed behavioral effects after both acute and repeated ki16425 dosage). All pharmacokinetic parameters were estimated from the mean concentration values determined at each time point (n = 3) and were evaluated by noncompartmental analysis using PKSolver (Version 2.0) (Zhang et al., 2010).

2.8. Statistical analysis

Group comparisons were carried out with Student's t-tests or factorial ANOVAs as appropriate, followed by post hoc Fisher's least

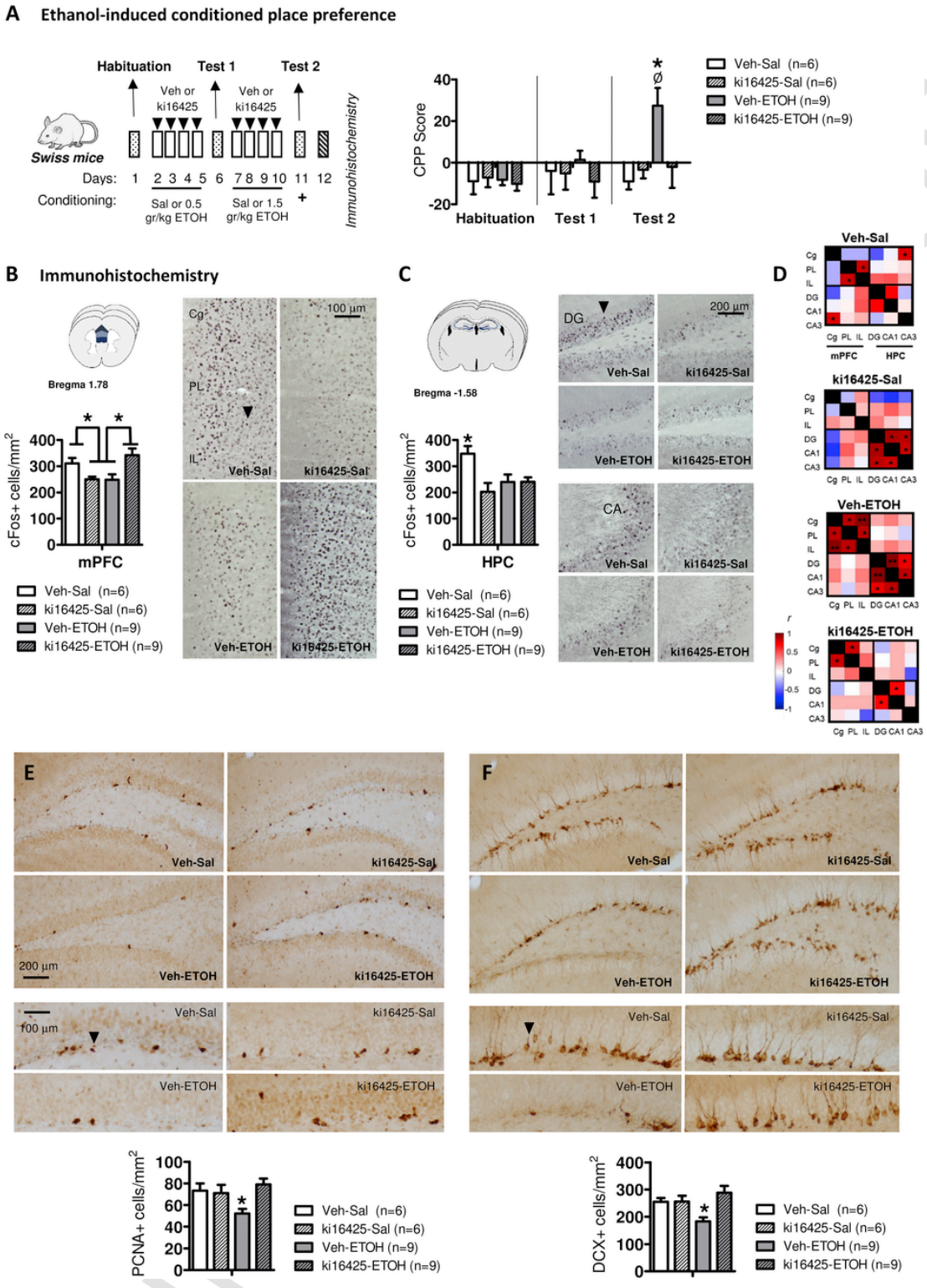


Fig. 3. Experiment III. Ki16425 administration reduced ethanol reward and modulated ethanol-induced neuroadaptations in Swiss mice subjected to a CPP paradigm. (A) Ki16425 administered 20 min before ETOH impeded the acquisition of ethanol-induced conditioned place preference. (B) Resting-state neuronal activity was measured by the expression of the early immediate gene *c-fos*, which was reduced in the mPFC of the ethanol and the ki16425-treated mice, but not after the combined ki16425 and ethanol administration. (C) However, all groups showed reduced basal c-Fos activity in the hippocampus compared with the control group (Veh-Sal). (D) Inter-region correlations of c-Fos expression (a measure of basal functional connectivity) were strengthened by ethanol but normalized in the ki16425-ETOH mice. Asterisks indicate significant Pearson's correlations (*P < 0.05;

**P < 0.001), while their r value is represented by a color scale. (E, F) Ki16425 administration prevented the ethanol-induced reduction in adult hippocampal neurogenesis, as shown by the numbers of proliferating cells and young neurons. Medial prefrontal cortex (mPFC) regions: Cg: cingulate cortex, PL: prelimbic cortex, IL: infralimbic cortex; hippocampus (HPC) regions: CA: Cornu Ammonis; DG: dentate gyrus. C-Fos quantification per region is shown in Fig. S3. Post hoc LSD difference vs the other groups: *P < 0.05. Difference vs zero CPP Score (i.e., absence of preference) with the one-sample *t*-test: Ø$P < 0.05$ (in A). Arrows indicate positive cells. Scale in (E) is valid for (F). +: On day 11 (experimental protocol in A), the mice received an additional pharmacological treatment (Veh-Sal, ki16425-Sal, Veh-ETOH, or ki16425-ETOH; according to their assigned experimental group) after the completion of the test session. Data are expressed as the mean ± SEM. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

significant difference (LSD) analysis when a significant interaction was found. A one-sample *t*-test was used to compare means of a single measure. Correlations were conducted using Pearson's method. Only statistically significant differences ($P < 0.05$) are reported.

3. Results

3.1. Experiment I: acute ki16425 reduces the oral self-administration of ethanol, but not saccharine, in chronically self-administering rats

Wistar rats acutely administered ki16425 showed reduced active lever presses for ethanol (ANOVA effects: 'ki16425': $F(1,24) = 7.986$, $P = 0.009$; 'lever': $F(1,24) = 61.856$, $P = 0.000$; 'ki16425 x lever': $F(1,24) = 6.873$, $P = 0.015$, Fig. 1A), but not for saccharin ('lever': $F(1,14) = 46.813$, $P = 0.000$; Fig. 1B). Therefore, reduced ethanol self-administration after ki16425 cannot be merely explained by a reduction of general activity that would compromise lever-pressing behavior since the saccharin self-administering rats exposed to ki16425 showed a high frequency of active lever-pressing (Fig. 1B).

3.2. Experiment II: strain-dependent effects of ki16425 on the sedative effects of ethanol and voluntary ethanol-drinking behavior

The results revealed the effects of ki16425 in the Swiss mouse strain only. Swiss mice pretreated with ki16425 showed an increased latency to ethanol-induced LORR ($t(18) = -2.115$, $P = 0.049$), while sleep time was unaltered (Fig. 2A). In the two-bottle choice experiments, a single ki16425 administration in free ethanol-drinking Swiss mice acutely increased ethanol consumption ('ki16425': $F(1,14) = 46.813$, $P = 0.000$; 'ki16425 x time': $F(3,60) = 3.907$, $P = 0.013$; post hoc analysis is shown in Fig. 2C). However, when ethanol-naïve Swiss mice were repeatedly treated with ki16425, their escalation of ethanol intake was reduced ('day': $F(11,198) = 11.335$, $P = 0.000$; 'ki16425 x day': $F(11, 198) = 2.8$, $P = 0.029$; post hoc analysis is shown in Fig. 2E). Importantly, ki16425 did not modify water consumption in any of these experiments (data not shown).

In contrast, ki16145 did not affect ethanol-induced LORR (Fig. 2B) or free ethanol drinking (Fig. 2D,F) in the c57BL/6J genetic background.

3.3. Experiment III: Ki16425 effects on ethanol CPP and brain parameters in Swiss mice

3.3.1. Ki16425 pretreatment blocks ethanol reward in a CPP paradigm

The effect of ki16425 on ethanol reward was further investigated in the Swiss mouse strain using an ethanol-induced CPP paradigm in which ethanol was preceded either by ki16425 or vehicle administration (Fig. 3A). Four conditioning sessions with the 0.5 gr/kg ethanol dose did not induce conditioning (Test 1, Fig. 3A), but the Veh-ETOH mice showed a significant preference for the 'conditioned' (ethanol-paired) compartment after four additional conditioning sessions with a 1.5 gr/kg ethanol dose. Interestingly, this preference was absent in the ki16425-ETOH mice (Test 2: 'ETOH': $F(1,26) = 4.936$, $P = 0.035$; 'ETOH x ki16425': $F(1,26) = 4.219$, $P = 0.050$; post hoc analysis is shown in Fig. 3A). The ethanol naïve mice that were treated with ki16425 were indifferent to the 'conditioned' (ki16425-paired) compartment (ki16425-Sal group; Fig. 3A); thus, this compound did not induce either reinforcing or aversive effects. Excluding the first ki16425 exposure, there were no remarkable differences in locomotion across the tests (Fig. S2).

3.3.2. Repeated administration of ki16425 modulates ethanol-induced neuroadaptations in the mPFC and the hippocampus

The basal neuronal activity (c-Fos expression) revealed unique neuroadaptations after each treatment. In the mPFC, either ki16425 or ETOH reduced basal c-Fos activity, but this measure was normal in the ki16425-ETOH group ('ETOH x ki16425': $F(1,26) = 12.747$, $P = 0.001$; post hoc analysis is shown in Fig. 3B; Fig. S3). In the hippocampus, however, all treatments reduced basal c-Fos expression compared with the control (Veh-Sal) mice ('ki16425': $F(1,26) = 7.847$, $P = 0.009$; 'ETOH x ki16425': $F(1,26) = 4.447$, $P = 0.045$; post hoc analysis is shown in Fig. 3C; Fig. S3). The pattern of interregional basal functional connectivity was strengthened in the Veh-ETOH mice but was normalized in the ki16425-ETOH animals (Fig. 3D). Regarding adult hippocampal neurogenesis, the ki16425 pretreatment protected the mice from the ethanol-induced reduction in numbers of DG proliferating cells (PCNA: 'ETOH x ki16425': $F(1,26) = 4.930$, $P = 0.035$) and young neurons (DCX: 'ki16425': $F(1,26) = 5.561$, $P = 0.026$; 'ETOH x ki16425': $F(1,26) = 5.406$, $P = 0.028$) (post hoc analysis is shown in Fig. 3E and F; respectively).

3.4. Experiment IV: Ki16425 effects on ethanol-withdrawal symptoms, brain and plasma parameters in c57BL/6J mice

3.4.1. Ki16425 pretreatment reduces ethanol withdrawal behavioral symptoms

Repeated systemic ki16425 did not affect body weight gain, food consumption or blood ethanol levels (Fig. S4).

No notable effects on behavior were found in the ki16425-treated ethanol naïve mice (ki16425-Sal group) (Fig. 4, Fig. S5). However, ki16425 protected the mice from some of the ethanol withdrawal behavioral symptoms. The ki16425-ETOH mice showed normal nesting behavior at the 4-h interval (1 h: 'ETOH': $F(1,32) = 6.717$, $P = 0.014$; 4 h: 'ETOH': $F(1,32) = 25.174$, $P = 0.000$; 'ki16425': $F(1,32) = 13.829$, $P = 0.001$; 'ETOH x ki16425': $F(1,32) = 9.063$, $P = 0.005$; post hoc analysis is shown in Fig. 4B). Furthermore, the ki16425-ETOH mice, unlike the Veh-ETOH mice, did not display ethanol withdrawal-induced physical symptoms (chewing: 'ETOH x ki16425': $F(1,32) = 4.363$, $P = 0.045$; shaking: 'ETOH': $F(1,32) = 5.985$, $P = 0.020$; 'ki16425': $F(1,32) = 17.632$, $P = 0.000$; 'ETOH x ki16425': $F(1,32) = 8.640$, $P = 0.006$; activity: 'ETOH x ki16425': $F(1,32) = 5.731$, $P = 0.022$; post hoc analysis is shown in Fig. 4C; no significant effects were observed for grooming or scratching), and they were not impaired in the Y-maze spatial working memory task compared to the Veh-ETOH group (spontaneous alternation score: 'ki16425': $F(1,32) = 4.158$, $P = 0.4976$; 'ETOH x ki16425': $F(1,32) = 4.907$, $P = 0.034$; post hoc analysis is shown in Fig. 4D; no differences were found in total arm entries). Nevertheless, ki16425 administration did not prevent other ethanol withdrawal

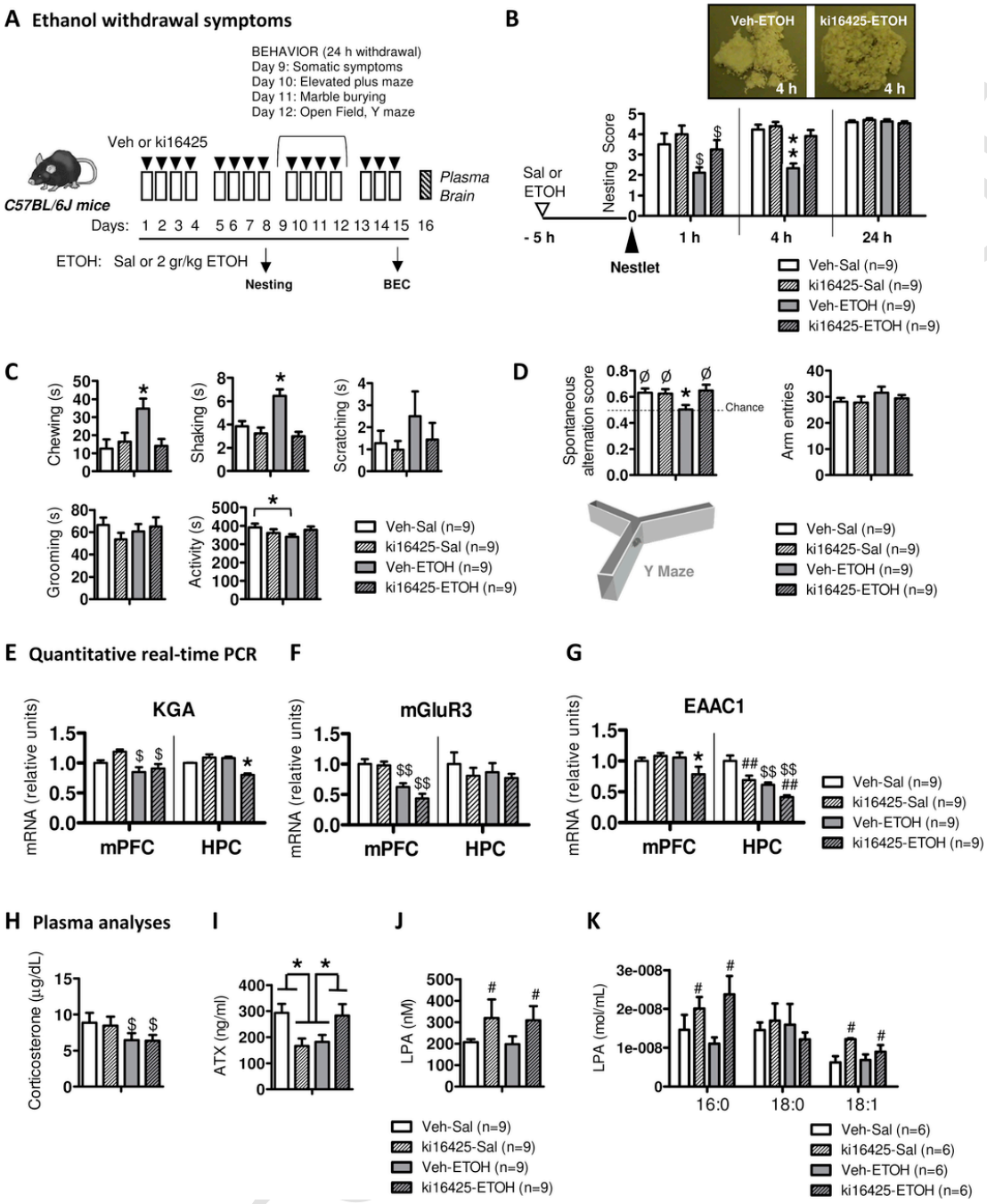


Fig. 4. Experiment IV. Ki16425 administration attenuates ethanol withdrawal symptoms, induces glutamate-related brain changes and regulates peripheral LPA signaling in c57BL/6J mice. (A) Experimental protocol. Ethanol or saline i.p. administrations were preceded by 20 min with ki16425 or vehicle treatment. During the days of the behavioral assessment (days 9–12), the corresponding pharmacological treatment was administered 1 h after the behavioral evaluation. Ethanol deteriorated nest building (B), induced somatic symptoms (C) and impaired continuous spontaneous alternation in the Y-maze (D), but these deleterious effects were attenuated or prevented by the ki16425 pretreatment. Other behaviors were not affected by ki16425 administration (Fig. S5). (E–G) In the medial prefrontal cortex (mPFC) and the hippocampus (HPC), ki16425 modulated ethanol-induced changes in the expression of glutamate-related genes. (H–K). In the plasma, circulating ATX levels were reduced by ethanol but normalized by ki16425 co-administration (I), and ki16425 increased plasma LPA concentrations (J, K) independently of ethanol. ANOVA effects of 'ethanol': $$P < 0.05$; ANOVA effects of 'ki16425': #$P < 0.05$. Post hoc LSD difference vs the other groups: *$P < 0.05$; **$P < 0.001$; difference vs chance performance (0.5) with the one-sample *t*-test: Ø$P < 0.05$ (in D). BEC: blood sampling for the ethanol concentration measurement (1 h after the ethanol or saline dose). Data are expressed as the mean ± SEM.

symptoms such as a disinhibited plus maze behavior and increased anxiety-like state (marble burying) (Fig. S5).

3.4.2. Repeated ethanol or ki16425 administration dysregulates glutamate transmission and peripheral LPA signaling

Quantitative real-time PCR showed that ki16425 modulated the effects of ethanol on glutamate-related transmission genes. In the mPFC, ethanol reduced the expression of both the glutamate-synthesis enzyme KGA ('ETOH': $F(1,32)=13.670$, $P=0.001$; Fig. 4E) and the mGluR3 receptor ('ETOH': $F(1,32)=44.462$, $P=0.000$; Fig. 4F), while the glutamate transporter EAAC1 was reduced only in the mice that received both ki16425 and ETOH treatments ('ETOH x ki16425': $F(1,32)=8.107$, $P=0.008$; post hoc analysis is shown in Fig. 4G). In the hippocampus, the glutamate transporter EAAC1 was reduced either by the ETOH or ki16425 administration ('ETOH': $F(1,32)=25.779$, $P=0.000$; 'ki16425': $F(1,32)=43.051$, $P=0.000$; Fig. 4G), but the KGA enzyme was only reduced in the ki16425-ETOH mice ('ETOH': $F(1,32)=15.313$, $P=0.000$; 'ki16425': $F(1,32)=12.724$, $P=0.001$; 'ETOH x ki16425': $F(1,32)=49.098$, $P=0.000$; post hoc analysis is shown in Fig. 4E).

Regarding the plasma analyses, ethanol reduced the basal corticosterone levels ('ETOH': $F(1,32)=4.169$, $P=0.049$; Fig. 4H). However, plasma ATX was reduced in both the ki16425-Sal and the Veh-ETOH mice, but it was normal in the ki16425-ETOH mice that received both treatments ('ETOH x ki16425': $F(1,32)=11.547$, $P=0.002$; post hoc analysis is shown in Fig. 4I). The total plasma LPA concentration was increased by ki16425 independently of the presence of ethanol ('ki16425': $F(1,32)=4.299$, $P=0.046$; Fig. 4J), particularly the LPA species 16:0 ('ki16425': $F(1,20)=4.507$, $P=0.046$) and 18:1 ('ki16425': $F(1,20)=5.897$, $P=0.025$) (Fig. 4K). Interestingly, in the ETOH-treated groups, the increased plasma concentrations of LPA and ATX were positively correlated to each other and predicted a better cognitive performance in the Y-Maze (Fig. S6).

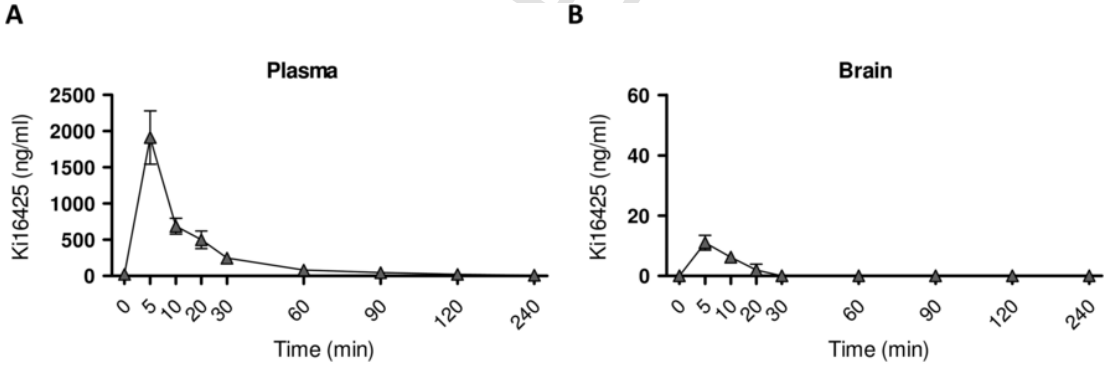
3.5. Ki16425 pharmacokinetics

After intraperitoneal administration of Ki16425 at a dose of 20 mg/kg, plasma and brain concentrations were determined by a LC–MS/MS method. The mean plasma concentration–time profile and the main plasma pharmacokinetic parameters are presented in Fig. 5A. Following intraperitoneal administration of Ki16425, only low concentrations of active drug were able to enter the brain (Fig. 5B), and the brain:plasma ratio was 0.02.

4. Discussion

This study reports that systemic administration of the $LPA_{1/3}$ antagonist ki16425 acts as a modulator (mainly an inhibitor) of ethanol-induced behaviors, and it also modulates ethanol-induced neuroadaptations in limbic brain regions.

A main finding of this study is that acute ki16425 reduced oral self-administration of ethanol in chronically self-administering Wistar rats. This finding apparently contradicts the increased voluntary ethanol drinking reported in this rat strain when they are acutely administered ki16425 in a home cage two-bottle choice test (Castilla-Ortega et al., 2016a). Nevertheless, there are remarkable differences between these two behavioral paradigms. Since ethanol is effortlessly available in the voluntary-drinking models, only the consummatory (drinking) component of ethanol consumption is assessed. However, in an operant self-administration paradigm, the animal is required to perform a response to obtain the drug, which allows for a separate analysis of appetitive (ethanol-seeking responses, i.e., lever pressings) and consummatory (drinking) behaviors (Ferraro et al., 2006; Lopez and Becker, 2014). Thus, operant self-administration behavior



Pharmacokinetic parameters in plasma	
C_{max} (ng/mL)	1913 ± 638.9
T_{max} (min)	5 ± 0
$t_{1/2}$ (min)	60 ± 11.4
$AUC_{0\text{-}240}$ (ng/ml*min)	43767 ± 8778.9
$AUC_{0\text{-}\infty}$ (ng/ml*min)	44553 ± 9010.1
Cl (mg/kg)/(ng/ml)	4E-04 ± 9.4E-05
V_Z (mg/kg)/(ng/ml)	2E-02 ± 6.1E-03

Pharmacokinetic parameters in brain	
C_{max} (ng/mL)	38.5 ± 14.9
T_{max} (min)	5 ± 0
$t_{1/2}$ (min)	NA
$AUC_{0\text{-}10}$ (ng/ml*min)	418.06 ± 208.159
$AUC_{0\text{-}\infty}$ (ng/ml*min)	NA
Cl (mg/kg)/(ng/ml)	NA
V_Z (mg/kg)/(ng/ml)	NA

Fig. 5. Concentration-time profile and pharmacokinetic parameters of Ki16425 in plasma (A) and brain (B) after a single intraperitoneal administration of 20 mg/kg in Swiss mice (n = 3 per time interval). C_{max}: maximum observed concentration; T_{max}: time of maximum observed concentration; $T_{1/2}$: the terminal elimination half-life; $AUC_{0\text{-}240}$ or $AUC_{0\text{-}10}$: the area under the curve from zero to the last sampling time; $AUC_{0\text{-}\infty}$: the area under the curve from zero extrapolated to infinity; Cl: the plasma clearance; V_z: volume of distribution. NA: Not available due to low ki16425 concentration. Data are expressed as the mean ± SD.

measures motivation for ethanol (i.e., how hard the subject is willing to work to obtain this substance, which is most likely driven by drug desire or '*craving*'), while voluntary-drinking indicates the quantity of the substance that the subject may consume once it is freely available (Czachowski et al., 2002). Accordingly, animals frequently show differences in their appetitive and consummatory ethanol-directed behaviors, which apparently rely on different neural pathways (Czachowski et al., 2002; Ferraro et al., 2006; Koros et al., 1999; Wilson et al., 1997). Assuming that ki16425 reduces the motivation for ethanol in Wistar rats, the higher amount of ethanol they may consume by voluntary drinking (Castilla-Ortega et al., 2016a) can then be explained by factors other than increased motivation. For example, either an increased tolerance to the sedative or the aversive effects of ethanol (Blednov et al., 2007; Lopez et al., 2012) or a reduced ethanol reward (Olive et al., 2003) could result in animals ingesting larger ethanol quantities. Any of these factors may be modulated by ki16425.

Accordingly, the effects of ki16425 on voluntary ethanol drinking, ethanol-induced sedation, and ethanol reward and withdrawal were further investigated. Two different mouse strains, outbred Swiss (RjOrl:SWISS) or inbred c57BL/6J (C57BL/6JRj) mice, were used for these studies since it is well established that genetics strongly influence ethanol-related behavior. Therefore, while the results reported in one mouse strain may not be comparable to other strains, the use of both Swiss and c57BL/6J mice is advantageous to test the pharmacological effects of ki16425 in two different genetic backgrounds. Genetic variability is a relevant issue in therapy for alcoholism, as genetics would determine the effectiveness of a particular pharmacological treatment for a given individual (Cservenka et al., 2017).

Swiss mice are low ethanol drinkers (Yoneyama et al., 2008) though they express a reliable ethanol-induced CPP behavior (Ledesma et al., 2013). In the present study, the Swiss mice mimicked the response we have previously described for hybrid C57BL/6J × 129X1/SvJ mice and Wistar rats (Castilla-Ortega et al., 2016a) since ki16425 increased voluntary ethanol consumption when it was acutely administered to ethanol-drinking animals. Importantly, additional data showed that ki16425 reduced both ethanol-induced sedation (latency to LORR) and ethanol reward in the Swiss mouse strain. As discussed above, these conditions could probably account for the acute increase in free ethanol drinking since an animal may need to consume larger ethanol quantities until it experiences its sedative and/or rewarding properties (Blednov et al., 2007; Olive et al., 2003). The assumption that ki16425 reduced ethanol reward in Swiss mice was deduced from two different paradigms. First, ki16425 was repeatedly administered to Swiss mice that –in contrast to the mice used in the acute experiment-had not been previously habituated to ethanol drinking and thus were naïve to the properties of ethanol at the beginning of the experiment. Under this condition, ki16425 reduced the escalation in voluntary ethanol intake. Second, Swiss mice co-administered ki16425 and ethanol did not show a preference for the ethanol-paired compartment in a CPP task, which is a widely used paradigm to measure drug reward (Tzschentke, 2007).

It is also interesting to note that in the Swiss mice subjected to the CPP experiment, each pharmacological treatment yielded different neuroadaptations. C-Fos immunohistochemistry was used to map brain activity and functional connectivity under basal resting conditions in both the mPFC and the hippocampus, which are two limbic regions involved in ethanol-induced behavioral symptoms, ethanol seeking and relapse (Bell et al., 2016; Geil et al., 2014; Kutlu and Gould, 2016; Pickering et al., 2015). This 'resting' 24-h time point was chosen since basal brain activity and connectivity is frequently assessed in addicts because it predicts their behavioral performance (Sutherland et al., 2012). Specifically, alcoholic patients show profound alterations in resting state brain synchrony (Fein and Cardenas, 2015), and repeated ethanol administration in animal models yield to an altered pattern of basal c-Fos expression that is reduced in the mPFC and the hippocampus (Ryabinin et al., 1997). Swiss mice that were administered both ki16425 and ethanol showed normalization of the reduced c-Fos activity in the mPFC -although not in the hippocampus- and of the aberrant inter-region functional connectivity found in the ethanol-treated group. Furthermore, the ki16425 treatment also counteracted the ethanol-induced reduction in adult hippocampal neurogenesis, in which downregulation is associated with impaired cognition and addiction vulnerability (Castilla-Ortega et al., 2016b; Geil et al., 2014).

In contrast, the experiments in the c57BL/6J mice revealed notable strain-dependent effects. Ki16425 in this genotype did not modify ethanol-induced LORR or voluntary ethanol drinking, which did not support a modulation of ethanol reward by ki16425 in this mouse strain. Therefore, c57BL/6J mice were not evaluated in the ethanol CPP paradigm but they were tested for ethanol-withdrawal severity instead. While the c57BL/6J mice are resistant to express handling-induced convulsions after ethanol (Metten et al., 2010), they show affective and somatic withdrawal signs (Perez and De Biasi, 2015). Results revealed that ki16425 attenuated certain withdrawal symptoms. Nest building is an intrinsically motivated behavior for mice that is highly sensitive to disruption by ethanol, likely indicating anhedonia or a depression-like state during withdrawal (Greenberg et al., 2016a, 2016b). In mice repeatedly treated with both ki16425 and ethanol, nest building behavior was normal shortly after an ethanol dose. At 24-h intervals, they showed less physical dependence on ethanol (absence of somatic withdrawal signs) and performed correctly on a Y-maze continuous spontaneous alternation task. The impairment in this task is associated with prefrontal and hippocampal damage in mice withdrawn from dependence-inducing drugs (Ladron de Guevara-Miranda et al., 2017; Pickering et al., 2015). Regarding brain analyses, only the combined ki16425 and ethanol treatment reduced the expression of the glutamate-synthesis enzyme KGA and the glutamate transporter EAAC1 in the hippocampus and mPFC, respectively, suggesting a unique modulation of the glutamate-related transmission system in these limbic regions.

Finally, peripheral LPA signaling was investigated since dysregulated serum concentrations of LPA species (increased: 18:2, 22:6; reduced: 16:0, 18:0) have been reported in c57BL/6J mice subjected to an ethanol-enriched diet for 4 weeks (Zhao et al., 2011). While the ethanol-treated c57BL/6J mice in our study showed normal LPA concentrations in the plasma (probably due to the shorter ethanol protocol we used), they showed reduced levels of the LPA-synthesis enzyme ATX, confirming that peripheral LPA signaling was dysregulated by ethanol. It is also noteworthy that the ki16425 treatment increased the plasma LPA concentrations independently of whether the mice received ethanol. The increased levels of circulating LPA species could contribute to some of the neurobehavioral effects described in the c57BL/6J mice. While LPA-mediated signaling is usually associated with central nervous system pathologies (Crack et al., 2014; Santos-Nogueira et al., 2015) and acute central LPA administration is anxiogenic (Castilla-Ortega et al., 2014; Yamada et al., 2015), the increased LPA may be beneficial under certain conditions, including ethanol exposure. In this regard, repeated central *in vivo* administration of LPA increased adult hippocampal neurogenesis (Walker et al., 2016), and in an *in vitro* study, LPA protected astrocytes from ethanol-induced injury (Guasch et al., 2003; Tomas et al., 2003). Furthermore, chronic oral treatment with the ginseng-derived LPA_1 receptor agonist *gintonin* improved neuroprotection and hip-

pocampal memory in a mouse model of Alzheimer-like disease (Kim et al., 2015) and induced antidepressant-like behaviors in both normal and ethanol withdrawn c57BL/6J mice (Kim et al., 2017). Accordingly, it is interesting to note that the circulating LPA and ATX levels predicted better Y-maze continuous alteration performance in the ethanol-treated c57BL/6J mice in this study.

The mechanisms through which ki16425 interacts with the pharmacological actions of ethanol remain to be elucidated. Importantly, ki16425 does not apparently affect the metabolism of ethanol as indicated by the blood ethanol concentrations [(Castilla-Ortega et al., 2016a); supplementary]. Pharmacokinetic analysis of ki16425 after acute systemic administration revealed that this compound peaked in plasma, and then its concentration was immediately reduced eight-fold in the following 30 min (from 4 μM to 0.5 μM). These results supported a rapid clearance from plasma and a short half-life. In the brain, the compound exhibited poor penetration, being detected only in the first 10 min after administration. A peak concentration was reached at approximately 80 nanomoles/kg of brain tissue in the first 10 min post-injection. Ki16425 is an LPA receptor antagonist with selectivity for the LPA_1 and LPA_3 receptors and exhibits K_i values of 0.34 and 0.93 μM for these receptors, respectively (Ohta et al., 2003). With the present data, we could confirm that the plasma concentrations were sufficient to guarantee full occupation of both LPA_1 and LPA_3 receptors in vascular-opened tissue, but very limited occupation in the brain, where the blood-brain barrier (BBB) prevent its diffusion. However, we could not eliminate the possibility that the concentration in BBB-open areas of the brain (such as the arcuate nuclei, area postrema and circumventricular organs) could be sufficient to fully block these receptors in these brain areas involved in homeostatic behaviors. Another possibility could be related to the peripheral generation of active metabolites that are able to cross the BBB, which might mediate the observed effects. Currently, this hypothesis cannot be discarded because we do lack information for the metabolic pathways and main metabolites generated from ki16425. Further research is needed to understand the site of action of ki16425.

Antagonist ligands for the lysophosphatidic acid $LPA_{1/3}$ receptors are interesting pharmacological targets for innovative therapies for a wide range of systemic pathologies such as fibrosis, sclerosis and cancer (Kihara et al., 2015; Lin et al., 2010; Llona-Minguez et al., 2015; Stoddard and Chun, 2015; Tigyi, 2010; Yung et al., 2014). In summary, the present results indicate that these ligands may also be promising tools for the treatment or prevention of alcohol-related disorders since ki16425 modulates both ethanol-induced behaviors and neuroadaptations in different mouse strains and in rats (Castilla-Ortega et al., 2016a). Moreover, it is relevant that repeated systemic ki16425 administration was innocuous for both general health and behavior in ethanol naïve animals across all experiments, which is indicative of a safe pharmacological profile. Nevertheless, this latter result should be confirmed in studies using additional behavioral tasks requiring mPFC and hippocampal function because the ki16425 treatment induced neuroadaptations in these brain regions, as evidenced by c-Fos immunohistochemistry (in Swiss mice) and quantitative PCR (in c57BL/6J mice) analyses.

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The authors declare no conflicts of interest.

Author contributions

F.R.d.F., L.J.S., A.S. and E.C.-O. designed the experiments. L.S.-M., D.L.d.G.-M., M.C.M.-P., R.D.M.-F., F.A., M.G.-F., C.P., F.J.P., L.J.S., A.S. and E.C.-O. performed the experiments and contributed to the data collection, analysis and/or interpretation. C.D.-N. and J.P.-d.P. performed the pharmacokinetic study. F.R.d.F., L.J.S., A.S. and E.C.-O. wrote the manuscript, which was revised and approved by all authors.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.neuropharm.2018.01.033.

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