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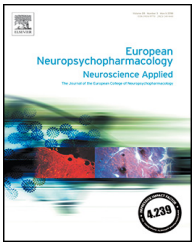

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Bupropion, a possible antidepressant without negative effects on alcohol relapse

Antonio Ballesta[a], Laura Orio[a], Rocío Arco[b], Antonio Vargas[b], Pablo Romero-Sanchiz[b,c,d], Raquel Nogueira-Arjona[c,e], Raquel Gómez de Heras[a], María Antón[a], Mayte Ramírez-López[a], Antonia Serrano[b], Francisco Javier Pavón[b], Fernando Rodríguez de Fonseca[a,b,*], Juan Suárez[b,**], Francisco Alen[a,b,*]

[a] *Departamento de Psicobiología y Metodología en Ciencias del Comportamiento, Facultad de Psicología, Universidad Complutense de Madrid, 28224 Spain*
[b] *Laboratorio de Medicina Regenerativa, Instituto de Investigación Biomédica de Málaga (IBIMA), UGC Salud Mental, Hospital Regional Universitario de Málaga, Av. Carlos Haya 82, sótano, Málaga 29010, Spain*
[c] *Unidad de Salud Mental, Hospital Universitario Regional de Málaga, Instituto de Investigación Biomédica de Málaga (IBIMA), Spain*
[d] *Departamento de Personalidad, Evaluación y Tratamientos Psicológicos. Universidad de Málaga, Málaga, Spain*
[e] *Department of Psychology and Neuroscience, Dalhousie University, Canada.*

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Abstract
Rationale: the role that antidepressants play on alcohol consumption is not well understood. Previous studies have reported that treatment with a Selective Serotonin Reuptake Inhibitor (SSRIs) increases alcohol consumption in an animal model of relapse, however it is unknown whether this effect holds for other antidepressants such as the atypical dopamine/norepinephrine reuptake inhibitors (SNDRI).
Objectives: the main goal of the present study was to compare the effects of two classes of antidepressants drugs, bupropion (SNDRI) and fluoxetine (SSRI), on alcohol consumption during

* Corresponding authors at: Laboratorio de Medicina Regenerativa, Instituto de Investigación Biomédica de Málaga (IBIMA), UGC Salud Mental, Hospital Regional Universitario de Málaga, Av. Carlos Haya 82, sótano, Málaga 29010, Spain.
** Corresponding author.
E-mail addresses: fernando.rodriguez@ibima.eu (F.R. de Fonseca), juan.suarez@ibima.eu (J. Suárez), p_alen@yahoo.es, f.alen@psi.ucm.es (F. Alen).

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relapse. Since glutamatergic and endocannabinoid signaling systems plays an important role in alcohol abuse and relapse, we also evaluated the effects of both antidepressants onthe expression of the main important genes and proteins of both systems in the prefrontal cortex, a critical brain region in alcohol relapse.
Methods: rats were trained to self-administered alcohol. During abstinence, rats received a 14d-treatment with vehicle, fluoxetine (10 mg/kg) or bupropion (20 mg/kg), and we evaluated alcohol consumption during relapse for 3 weeks. Samples of prefrontal cortex were taken to evaluate the mRNA and protein expression of the different components of glutamatergic and endocannabinoid signaling systems.
Results: fluoxetine treatment induced a long-lasting increase in alcohol consumption during relapse, an effect that was not observed in the case of bupropion treatment. The observed increases in alcohol consumption were accompanied by distinct alterations in the glutamate and endocannabinoid systems.
Conclusions: our results suggest that SSRIs can negatively impact alcohol consumption in relapse while SNDRIs have no effects. The observed increase in alcohol consumption are accompanied by functional alterations in the glutamatergic and endocannabinoid systems. This finding could open new strategies for the treatment of depression in patients with alcohol use disorders.

1. Introduction

Alcohol is the most consumed psychoactive substance in the world and in European countries including Spain, where the last 12 months prevalence of use in the population from 15 to 64 years of age was 94.1% (Drogas 2016; Winstock 2017). It is known that alcohol is associated with depression in humans, and depression-like symptoms can be found I animals chronically exposed to ethanol (Fergusson et al., 2009; Hauser et al., 2011; Pietraszek et al., 1991). In addition, primary depression is considered to predispose to alcohol consumption (Khantzian 1997; Paré et al., 1999). In consonance with that, a great number of alcohol use disorder (AUD) patients complain from depressive symptoms (Davidson 2018; Naranjo and Knoke 2001; Schuckit et al., 1997) and around 46% of them are prescribed antidepressant treatment (Mark et al., 2003). However, a relevant number of these patients relapse to alcohol use and, since it is advised to avoid combining alcohol and antidepressants, most of them discontinue antidepressant treatment during relapse (Haw and Stubbs 2011). Although initial studies suggest that Selective Serotonin Reuptake Inhibitors (SSRIs), the most commonly used class of antidepressants, reduce alcohol consumption in heavy drinkers by 15–20% (Naranjo and Bremner 1993), subsequent placebo controlled trials have not found a reduction in alcohol drinking (Kranzler et al., 1995). Moreover, several studies suggest a possible negative role of antidepressant treatment on alcohol consumption since some depressed patients show an enhanced craving for alcohol or develop alcohol-dependence after using these drugs (Atigari et al., 2013; Brookwell et al., 2014; Charney et al., 2015; Menkes and Herxheimer 2014).

The variability in clinical trials as well as in the mentioned clinical studies may be explained by hidden variables, such as the heterogeneity of the antidepressants used in the studies, which display different pharmacological mechanisms (Torrens et al., 2005).

The potential negative effects of antidepressant treatment cessation on alcohol relapse have been explored in preclinical models. Previous studies have identified that antidepressant cessation-induced increased vulnerability to alcohol consumption and relapse. This increased vulnerability has been observed using antidepressants with different mechanisms of action. We observed that fluoxetine, an SSRI with no appreciable effects on norepinephrine or dopamine uptake, when administered sub-chronically increased alcohol consumption in relapse, an effect that also held for venlafaxine, a mixed serotonin/noradrenaline reuptake inhibitor- This effect was of a more shorter lived when atomoxetine, a norepinephrine/dopamine reuptake inhibitor was used (Alen et al. 2014a,2014b). These results indicate that antidepressants with different pharmacological profiles might have different impact on alcohol relapse. Antidepressants based on the blockade of dopamine uptake, such as bupropion, are not common in therapeutics, but they might offer an innovative opportunity for avoiding these unwanted effects.

In this context, the main aim of the present study was to compare the effects of bupropion a selective dopamine and norepinephrine reuptake inhibitor with no serotonergic activity (Wilkes 2008) with that of the selective serotonin reuptake inhibitor fluoxetine on alcohol relapse, taking in consideration that this ISSRI dramatically increased alcohol relapse in animal models (Alen et al. 2014a).

Because growing evidence suggests that alterations of both glutamatergic and endocannabinoid systems in the prefrontal cortex (PFC) may contribute to excessive alcohol consumption during relapse (Cippitelli et al., 2005; Eisenhardt et al., 2015; Hansson et al. 2007; Hermann et al. 2012; Karlsson et al. 2012), we also assessed the expression of selected genes and proteins related to glutamate and endocannabinoid function in this brain area. The PFC is a brain region that receives extensive projections from ascending mesolimbic dopaminergic neurons, as well as serotonergic input. Thus, PFC might be a crucial brain area to analyze the impact of either bupropion or fluoxetine treatment along a 2-week withdrawal period following relapse (Farley et al., 2012; Zarate et al., 2008). In addition to the involvement of both glutamatergic and endocannabinoid systems in AUD, both signaling systems play a critical role in mood disorders

(Hwa et al., 2017; Rodríguez-Muñoz et al., 2016; Trullas and Skolnick 1990). Thus, the characterization of the effects of these different antidepressants on alcohol relapse as well as their effects on glutamatergic and endocannabinoid systems in the PFC might help to understand why the association of depression and AUD is a severe clinical complication with poor therapeutic outcomes.

2. Experimental procedures

2.1. Animals

Considering that drugs that are used to treat mental disorders are best studied in animal models of the disorder (Russell et al., 2005; Soeters et al., 2008), rats were exposed to a passive model of depression, based on isolation, which is considered to have greater etiological validity than those based on lesions or monoamine depletion (Barrot et al. 2005; Deussing 2006; Willner 1991). According to this, rats were single-housed from adolescence up to the time of the experiment. Twenty-four adult male Wistar rats (8 per group) (ENVIGO, Barcelona, Spain), kept under a 12-h light/dark cycle (lights off at 12:00 p.m.) in a room at constant temperature (23 ± 1 °C) and weighted 375–425 g at the beginning of the experiments. Standard food and tap water were available *ad-libitum* at the home-cage. All experimental animal procedures were performed in compliance with the European Directive 2010/63/EU on the protection of animals used for scientific purposes and with Spanish regulations (RD 53/2013 and 178/2004). All protocols were approved by the Ethics Committee of the Universidad Complutense de Madrid. Special care was taken to minimize the suffering and number of animals to achieve our research goals.

2.2. Drugs

Bupropion HCl was purchased from Sandoz (Spain). Fluoxetine HCl was obtained from Eli Lilly (Spain). Bupropion and fluoxetine solutions were prepared daily by dissolving in 0.9% saline, and were injected intraperitoneally (i.p.) at a dose of 20 mg/kg (bupropion) and 10 mg/kg (fluoxetine) in a volume of 2 mL/kg as previously described (Alen et al. 2014a; Hall et al., 2015). Alcohol solution (10% ethanol w/v solution) was prepared daily from 99% ethanol.

2.3. Alcohol self-administration and relapse model

We used an alcohol relapse model based on the alcohol deprivation effect, which is considered to have excellent face and predictive validity in relation to alcohol consumption (Vengeliene et al., 2014). Then, all the animals were trained to lever-press for alcohol self-administration for the same amount of time, and, after they had reached a steady level of self-administration (less than 15% variation in 2 consecutive days), they were sub-chronically treated with fluoxetine or bupropion. After that, antidepressant treatment ceased, and alcohol self-administration sessions were re-introduced and changes in the patterns of alcohol consumption were monitored for three consecutive weeks.

Specifically, animals were trained to self-administer alcohol in operant chambers (Letica, LE 850 model; Panlab, Barcelona, Spain) enclosed in sound-attenuating boxes and fitted with an exhaust fan. The chambers were equipped with two retractable levers (one being the active lever, and the other being the inactive lever), located on either side of a drinking dipper. The side of the active lever was counterbalanced between sessions to avoid development of location preferences. Pressing the active lever resulted in the delivery of 0.1 mL of the solution, which was present to the animal followed by a 2.5 s. time-out, while pressing of the inactive lever had no consequences. All the alcohol operant sessions lasted 30 min per day over 5 days/week (Monday to Friday) schedule for the entire study. The number of responses and dipper presentations in both levers were registered automatically by computer software. Animals were weighed daily before the alcohol self-administration sessions. Training was carried out using a modification of the traditional saccharine fading procedure (Samson et al., 1999) described in Alen et al. (2009). Once saccharine is eliminated, ethanol can reinforce lever presses in the same way as other reinforcers and, indeed, sucrose self-administration in the later phases of the procedure has been found to be unrelated to saccharine self-administration (Rogowski et al., 2002; Samson and Czachowski 2003).During the first 3 days of training, the animals received 0.2% saccharin solution in the dipper to facilitate the acquisition of lever pressing. Thereafter, the following sequence on a fixed-ratio 1 schedule was used: 0.16% saccharine and 2% alcohol for three sessions, 0.12% saccharine and 4% alcohol for three sessions, 0.08% saccharine and 6% alcohol for four sessions, 0.04% saccharine and 8% alcohol for four sessions, 0.02% saccharine plus 10% alcohol, and finally 10% alcohol alone for the rest of sessions. The experiments began once a relatively constant level of alcohol consumption had been reached, following a period of at least 6 weeks of access to alcohol (10%w/v). Then, the 24 animals were randomly assigned to one of the three experimental groups: 1) vehicle; 2) bupropion (20 mg/kg); 3) fluoxetine (10 mg/kg)], receiving the corresponding treatment for 14 days. After that, animals were let to rest for a period of 24 h, and then the daily 30 min. ethanol self-administration sessions were reintroduced and monitored. See Fig. 1(A) for a schematic representation of the experimental procedure.

2.4. Tissue collection

One hour after the last alcohol self-administration session, all animals were euthanized by decapitation and their brains were immediately dissected out, frozen on dry ice, and stored at −80 °C. The brains were dissected in 2-mm thick coronal brain slices on dry ice using razor blades and a rat brain slicer matrix (Zivic Instruments, Pittsburgh, PA, USA). The medial prefrontal cortex (mPFC or prelimbic cortex) was precisely removed from Bregma 4.70 mm to 2.20 mm with fine surgical instruments (Paxinos and Franklin 2004). The samples were stored at −80 °C until further use for gene and protein analyses.

2.5. RNA isolation and RT-qPCR analysis

Real-time PCR was used to quantify the relative mRNA levels of different receptors, transporters, and synthesis/degradation enzymes involved in the glutamatergic [liver-type glutaminase isoforms (LGA or GLS2), kidney-type glutaminase isoforms (KGA or GLS1), metabotropic receptors (mGlu3/5), ionotropic glutamate N-methyl D-aspartate (NMDA) receptor subunits (GluN1/2A/2B/2C), ionotropic glutamate a-amino-3-hydroxy-5-methyl-4- isoxazolepropionic acid (Birch et al.) receptor subunits (GluA1/2/3/4), and the glutamate transporter (Slc1a1 or EAAC1)] and endocannabinoid [cannabinoid receptor type 1 (CB_1) and cannabinoid receptor type 2 (CB_2), peroxisome proliferator-activated receptor-α (PPAR-α) and peroxisome proliferator-activated receptor-γ (PPAR-γ); N-acyl phosphatidylethanolamine phospholipase D (NAPE-PLD), diacyl-glycerol lipase alpha/beta (DAGLα/β), fatty acid amide hydrolase (FAAH), and monoacylglycerol lipase (MAGL)] signaling.

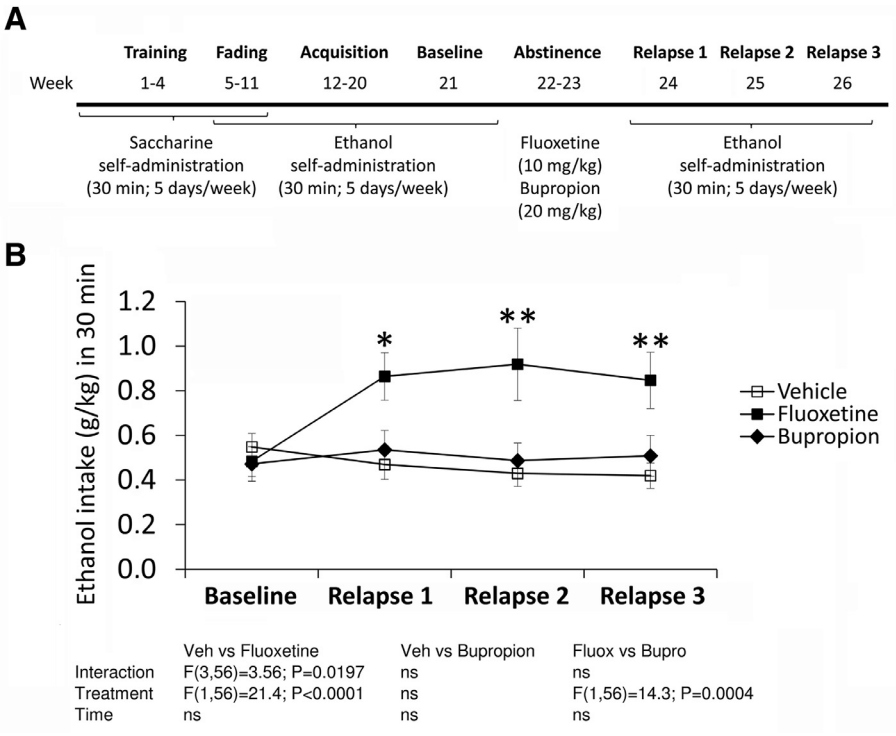

Fig. 1 Experimental design of the model of ethanol consumption and treatment with fluoxetine and bupropion during ethanol deprivation (A). Weekly average of ethanol intake by operant self-administration in the rats treated with vehicle, fluoxetine and bupropion along three consecutive weeks (B). Data are the average (5-days) of daily ethanol intake, ± SEM ($n = 8$). *Post hoc* analysis: $^{\#/\#\#}p<0.05/0.01$ *vs*. baseline of their respective group; $^{*/**}p<0.05/0.01$ *vs*. vehicle group.

We performed real-time PCR (TaqMan, ThermoFisher Scientific, Waltham, MA, USA) as described previously (Serrano et al., 2012) using specific sets of primer probes from TaqMan® Gene Expression Assays (Table S1). Total RNA was extracted from mPFC samples using Trizol® method, according the manufacturer's instructions (ThermoFisher Scientific). RNA samples were isolated with RNAeasy minelute cleanup-kit including digestion with DNase I column (Qiagen, Hilden, Germany) and quantified using a spectrophotometer to ensure A260/280 ratios of 1.8-2.0. After the reverse transcript reaction from 1 μg of mRNA, a quantitative real-time reverse transcription polymerase chain reaction (qPCR) was performed in a CFX96TM Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) and the FAM dye labeled format for the TaqMan® Gene Expression Assays (ThermoFisher Scientific). A melting curve analysis was performed to ensure that only a single product was amplified. After analyzing several control genes, values obtained from the brain samples were normalized in relation to β-actin gene (*Actb*) levels, which was found not to vary significantly between groups.

2.6. Western blot analysis

Western blotting was used to measure the protein levels of the endocannabinoid-related signaling system and the glutamate receptors GluN1, GluA1 and mGlu5 in the mPFC as previously described (Suarez et al., 2008). The samples were homogenized in 50 mM Hepes buffer (pH 8) and 0.32 M sucrose buffer to obtain the membrane protein extracts. For immunoblotting, the protein samples (40 μg) were separated on 10% (w/v) SDS-PAGE gels, transferred to nitrocellulose membranes (BioRad, Hercules, CA, USA) and controlled by Ponceau Red staining. After blocking with 5% (w/v) bovine serum albumin in PBST (0.1% Tween 20 in PBS) at room temperature for 1 h, the membranes were incubated with the following primary antibodies overnight at 4 °C: anti-CB_1 (#ab23703, Abcam, 1:200), anti-CB_2 (#ab3561, Abcam, 1:200), anti-PPARα (#RDI-PPARAabrx, Fitzgerald, 1:500), anti-PPARγ (#sc-7273, Santa Cruz, 1:100), anti-DAGLα (1:100), anti-DAGLβ (1:50), anti-MAGL (Cayman, 100035, 1:200), GluN1 (#M-207, Sigma-Aldrich, 1:100), GluR1 (#PA5-77416, ThermoFisher, 1:200), and mGluR5 (#PA1-24637, ThermoFisher, 1:500) (see Table S2 for further information). A HRP-conjugated anti-rabbit or anti-mouse IgG ($H + L$) secondary antibodies (Promega, Madison, WI, USA) diluted 1:10,000 was added for 1 h at room temperature. The specific protein bands were visualized and quantified by chemiluminescence using an imaging AutoChemi™ UVP BioImaging System (LTF Labortechnik, Bodensee, Germany). β-actin was quantified and used as a loading control (anti-β-actin diluted 1:1000 from Sigma-Aldrich, cat. no. A5316).

2.7. Data analysis

Data are means ± SEM ($n = 8$/group for behavior; $n = 6$-8/group for mRNA analysis; $n = 3$/group for protein analysis). The operant alcohol self-administration results were analyzed by means of a two-way analysis of variance (ANOVA) with repeated measures (time and treatment as factors), and multiple comparison *post-hoc* test (Bonferroni) when appropriate. Regarding mRNA and protein levels, results were also analyzed using a one-way ANOVA, and multiple comparison *post-hoc* test (Bonferroni) when appropriate. The statistical analysis of all results was performed using the computer program GraphPad Prism v6 (GraphPad Software, San Diego, CA, USA). A *p*-value less than 0.05 was considered to be statistically significant.

3. Results

3.1. Effects of bupropion and fluoxetine on weekly alcohol self-administration after relapse

After achieving a relatively constant level of active lever pressing and alcohol self-administration during the last week of acquisition period (baseline), rats were exposed to an alcohol deprivation period of 14 days (Fig. 1(A)). During alcohol deprivation, rats were daily treated with vehicle, bupropion or fluoxetine. The general repeated measures ANOVA indicated a treatment effect on ethanol self-administration in fluoxetine-treated rats ($F_{1, 56}$=21.4, p<0.0001) after relapse for 3 weeks (Fig. 1(B)). No treatment effect on ethanol self-administration was found in bupropion-treated rats. Interaction between time and treatment was also observed in the fluoxetine-treated rats ($F_{3, 56}$=3.56, p = 0.019), indicating that fluoxetine affected differently ethanol self-administration along the 3 weeks of relapse. *Post hoc* analysis showed an increase in the weekly average of ethanol consumption in fluoxetine-treated rats in the first week ($^{*}p$<0.05), the second week ($^{**}p$<0.01) and the third week ($^{**}p$<0.01) of relapse compared to vehicle-treated rats (Fig. 1(B)). When they were compared to baseline levels of ethanol consumption, fluoxetine also increased ethanol self-administration along the 3 weeks after relapse (#/##p<0.05/0.01; Fig. 1(B)). No baseline changes in ethanol intake by self-administration were found in the bupropion group. No differences were found in inactive-lever pressing when compared to the different groups.

3.2. Effects of bupropion and fluoxetine on mRNA levels of endocannabinoid system-related components in mPFC

One-way ANOVA indicated significant changes in the mRNA levels of *Cnr2* ($F_{2, 19}$= 4.59; p = 0.023), *Ppar*α ($F_{2, 19}$= 3.37; p = 0.043), *Nape-pld* ($F_{2, 19}$= 5.38; p = 0.014), *Dagl*α ($F_{2, 19}$= 5.99; p = 0.009) and *Dagl*β ($F_{2, 19}$= 4.34; p = 0.027) in the mPFC of ethanol-exposed rats. Bonferroni's test for multiple comparisons indicated that both bupropion and fluoxetine administrations increased the mRNA levels of *Nape-pld* ($^{*}p$<0.05) and decreased the mRNA levels of *Dagl*α and *Dagl*β ($^{*}p$<0.05) in the mPFC of ethanol-exposed rats compared to those of the vehicle group (Fig. 2(A)). Specifically, fluoxetine also increased the mRNA levels of *Cnr2* and decreased the mRNA levels of *Ppar*α in the mPFC of ethanol-exposed rats compared to vehicle group ($^{*}p$<0.05; Fig. 2(A)). No changes in *Cnr1*, *Ppar*γ, *Faah* and *Mgll* mRNA levels were found in the mPFC of the treated rats.

3.3. Effects of bupropion and fluoxetine on mRNA levels of glutamate system-related elements in mPFC

One-way ANOVA indicated significant changes in the mRNA levels of *Grin1* ($F_{2, 19}$= 6.66; p = 0.006), *Grin2b* ($F_{2, 19}$= 5.64; p = 0.011) and *Gria2* ($F_{2, 19}$= 4.30; p = 0.028) in the mPFC of ethanol-exposed rats. Bonferroni's test for multiple comparisons indicated that both bupropion and fluoxetine administrations reduced the mRNA levels of *Grin1* ($^{*}/^{**}p$<0.05/0.01) in the mPFC of ethanol-exposed rats compared to those of the vehicle group (Fig. 2(B)). Specifically, fluoxetine also decreased the mRNA levels of *Grin2b* ($^{**}p$<0.01) and increased the mRNA levels of *Gria2* and *Gria3* ($^{*}p$<0.05) in the mPFC of ethanol-exposed rats compared to vehicle-treated rats (Fig. 2(B)). No changes in mRNA levels of *Gls*, *Gls2*, *Grin2a*, *Grin2c*, *Gria1*, *Gria4*, *Grm3*, *Grm5* and *Slc1a1* were found in the mPFC of the treated rats.

3.4. Effects of bupropion and fluoxetine on the expression of selected proteins of the endo cannabinoid system and glutamate receptors in mPFC

Following the previous results of mRNA levels, we evaluated the protein expression of selected elements of the endocannabinoid system and glutamate receptors in the mPFC of ethanol-exposed rats. Regarding the endocannabinoid system, one-way ANOVA indicated significant changes in the protein levels of CB_1 ($F_{2, 6}$ = 4.58; p = 0.033), CB_2 ($F_{2, 6}$ = 12.19; p = 0.0005), PPARα ($F_{2, 6}$ = 5.47; p = 0.020), DAGLα ($F_{2, 6}$ = 21.01; p = 0.001) and MAGL ($F_{2, 6}$ = 10.04; p = 0.0007) in the mPFC of ethanol-exposed rats. Bonferroni's test for multiple comparisons indicated that bupropion decreased the protein levels of CB_1, PPARα, DAGLα and MAGL in the mPFC of ethanol-exposed rats compared to those of the vehicle-treated rats ($^{*}/^{**}/^{***}p$<0.05/0.01/0.001; Fig. 3(A) and (C)). Fluoxetine specifically increased the CB_2 protein levels and decreased the DAGLα protein levels in the mPFC of ethanol-exposed rats compared to the vehicle group. ($^{*}/^{**}p$<0.05/0.01; Fig. 3(A) and (C)). Regarding the glutamatergic receptors, one-way ANOVA indicated significant changes in the protein levels of GluN1 ($F_{2, 6}$ = 31.67; p = 0.0006), GluA1 ($F_{2, 6}$ = 35.47; p = 0.0005) and mGlu5 ($F_{2, 6}$ = 7.23; p = 0.025). Bonferroni's test for multiple comparisons indicated that both bupropion and fluoxetine decreased the protein levels of GluN1 and GluA1 in the mPFC of the ethanol-exposed rats compared with vehicle-treated animals ($^{**}/^{***}p$<0.01/0.001; Fig. 3(B) and (D)). Specifically, the mPFC of the ethanol-exposed rats treated with bupropion also had reduced protein levels of mGlu5 compared to vehicle-treated rats ($^{*}p$<0.05; Fig. 3(B) and (D)).

4. Discussion

The present study indicates that subchronic treatment with bupropion did not induce the sustained increase in alcohol consumption observed after cessation of fluoxetine administration, in accordance with our previous results (Alen et al., 2013, 2014. In addition, the increase in alcohol consumption found in fluoxetine-exposed animals is associated with specific changes in the mRNA and protein expression of endocannabinoid-signaling elements such as the cannabinoid CB2 receptor, the PPARα receptor or the endocannabinoid-degrading enzyme MAGL. Overall, the present data supports the notion that antidepressants that

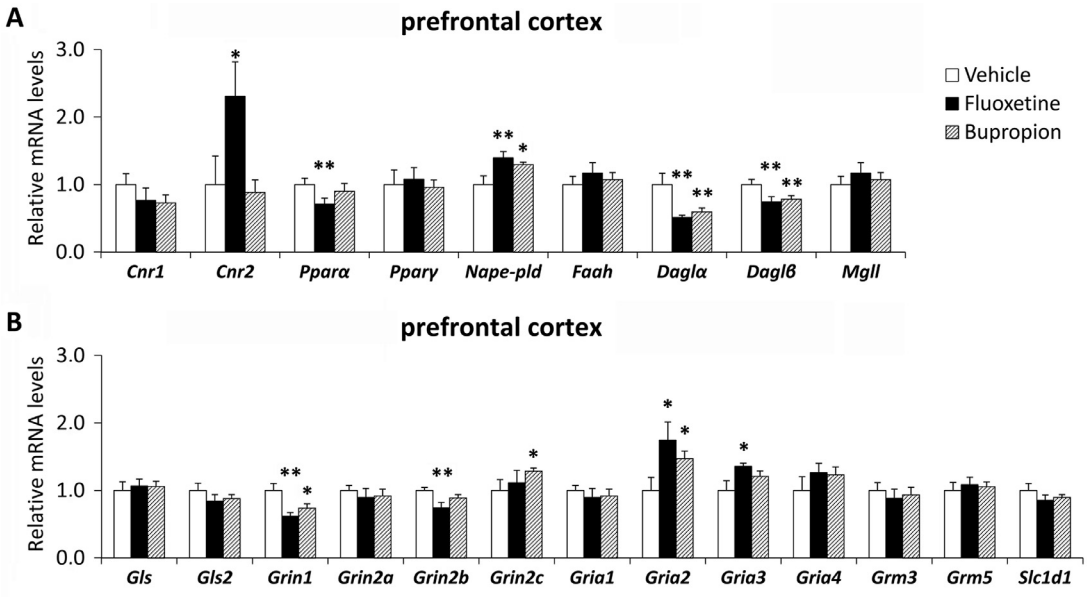

Fig. 2 Relative mRNA levels of endocannabinoid (A) and glutamate (B) signaling system-related genes in the mPFCx of adult rats exposed to ethanol self-administration and treated with vehicle, fluoxetine or bupropion. Bars represent the means ± SEM (Vehicle, $n=8$; Fluoxetine, $n=6$; Bupropion, $n=8$). Bonferroni analysis: $^{*}p<0.05$ *vs*. vehicle group.

do not target serotonin neurotransmission might offer a more secure profile than classical ISSR ones.

The pharmacological profile of bupropion observed in the present study was clearly different from that of other antidepressants such as fluoxetine, venlafaxine and atomoxetine, that induce an increase in alcohol consumption after relapse (Alen et al. 2014a,2014b). Bupropion not only does not affect alcohol consumption but also has a less disruptive impact on the endocannabinoid/*N*-acylethanolamine and glutamatergic and systems than that observed after fluoxetine. This differential impact might contribute to the lack of alcohol relapse-enhancing effects of this drug. Despite the fact that the functionality of the serotonergic, noradrenergic or dopaminergic systems was not directly assessed, based on the pharmacological properties of the drugs used in this study we can state that the serotonergic profile of fluoxetine is related to the observed increases in alcohol consumption in relapse, while the noradrenergic/dopaminergic profile of bupropion not associated with this unwanted side effect. Further research using selective noradrenergic uptake blockers such as reboxetine might be needed to clarify whether they offer a profile devoid of side effects such as that observed with mixed dopamine/noradrenaline antidepressants

In any case, the present results would be in agreement with early studies using the intracranial self-stimulation (ICSS) paradigm that show that noradrenaline and serotonin antidepressants have a negative impact in the reward system, assessed in the intracranial self-stimulation paradigm, while bupropion had no effect (McCarter and Kokkinidis 1988). Despite the fact that we did not assess the integrity of the dopaminergic/opioidergic reward system, it may play an important role in the observed results, possibly explaining the long-lasting nature of the increases in alcohol consumption, somehow resembling the phenomenon of cross tolerance between drugs of abuse and this could be mediated by the cannabinoid and glutamatergic systems (Fattore et al., 2004; Gardner, 2005; Konradi et al., 2017). Concerning the specific findings of fluoxetine on the endocannabinoid and glutamatergic systems after alcohol relapse, it is important to note that despite the long history and widespread use of antidepressants, there is scarce information is regarding the way in which antidepressants modulates those signaling systems in the brain systems (Cruceanu et al., 2017; Rodríguez-Muñoz et al., 2017). In our experimental approach, the most prominent signal identified is the enhanced expression of cannabinoid CB2 receptors in fluoxetine-treated animals. Cannabinoid CB2 receptors have been identified as receptors expressed by glial cells and neurons (Onaivi et al., 2012). That control alcohol self-administration in rodents (Navarrete et al., 2018; Ortega-Álvaro et al., 2015). Whether this differential expression contribute to the enhanced alcohol consumption observed after cessation of fluoxetine treatment or it is just a marker of alcohol-induced alterations in the PFC cannot be clearly established with the present exploratory design. In this respect, animals previously treated with fluoxetine drank almost double than control animals, so alcohol might induce greater neuroinflammation associated to enhanced glial reactivity affecting cannabinoid CB2 receptor expression, than that observed in bupropion or control relapsing animals Additionally, It is possible that withdrawal from fluoxetine treatment might induce a negative affective state that promotes drinking or adaptive changes in glutamate transmission that again might enhance drinking. The later hypothesis is currently being explored in our laboratory. We have identified that in central amygdala circuits there are plastic changes in glutamate transmission induced by fluoxetine cessation in a context of alcohol self-administration. Increases in the gene expression of CB_2 receptor in the whole brain seems to be induced also by other drugs of abuse, since they have been observed after chronic treatment with

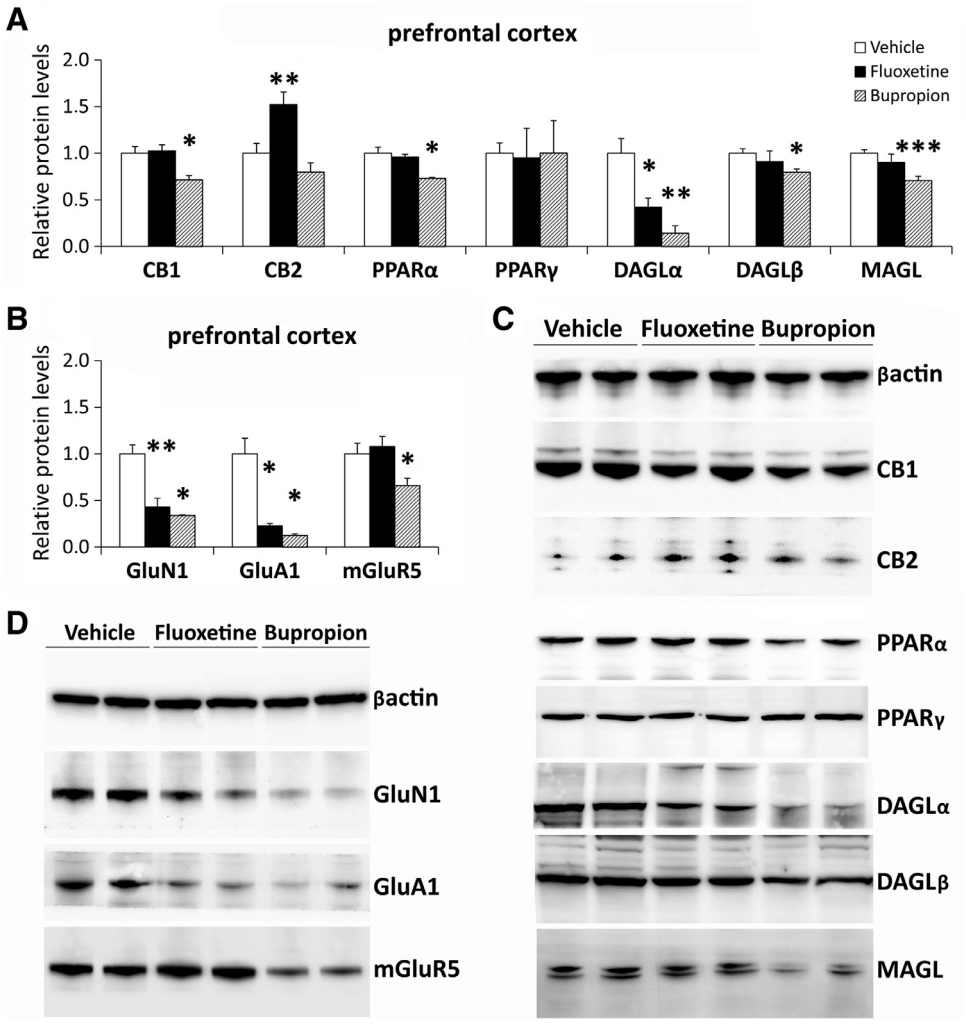

Fig. 3 Relative protein levels of selected components of the endocannabinoid (A) and glutamate (B) signaling system in the mPFCx of adult rats exposed to ethanol self-administration and treated with vehicle, fluoxetine or bupropion. Representative immunoblots of respective proteins were also shown (C) and (D). Bars represent the means ± SEM ($n = 3$/group). Bonferroni analysis: */**/***$p<0.05/0.01/0.001$ *vs*. vehicle group.

heroin or cocaine in the C57Bl/6 mouse, which displays a natural preference for alcohol (Onaivi et al. 2008). Brain CB_2 receptors appear to be associated with neuropsychiatric disorders such as AUD or depression and are supposed to explain some common phenotypes in these disorders (Onaivi et al., 2012; Roche and Finn 2010). In agreement with that, recent studies have found that animals lacking CB_2 receptors exhibit alcohol preference derived of dysregulated endogenous opioid transmission (Ortega-Álvaro et al., 2015). It is known that the expression of CB_2 in the brain is increased under inflammatory conditions, which are also present in neuropsychiatric disorders, including AUDs and depression, and they are supposed to constitute a protective response to excitotoxicity induced by excessive glutamatergic activity (Roche and Finn 2010).

Additionally, markers of reduced N-acylethanolamine activity such as PPARα, DAGLα, DAGLβ or MAGL expression have been reported in models of inflammation or alcohol exposure (Birch et al. 2016). Other alterations in the endocannabinoid system that were found in both the fluoxetine and the bupropion-treated groups are also compatible with a neuroprotective response of the brain by the endocannabinoid and *N*-acylethanolamine systems (see Pistis and Muntoni 2017; Sanchez-Marin et al. 2017). However, the observed alterations in gene expression of *Cnr2* and *Ppar*α, which were observed only in the fluoxetine-treated group, suggest a greater extent of cannabinoid disruption in this group, and this could be related to the increased alcohol consumption during relapse observed in this group (see Anderson 2018; Atigari et al., 2013; Brookwell et al., 2014; Charney et al., 2015; Menkes and Herxheimer 2014).

The endocannabinoid system plays an important regulatory role of glutamatergic neurotransmission acting as retrograde signals (see Eisenhardt et al., 2015; Hermann et al. 2012; Rodríguez-Muñoz et al., 2016). Specifically, glutamatergic hyperfunction and related excitotoxicity may be landmarks of depressive states and vulnerability to alcohol consumption (Lakhan et al., 2013; Vengeliene et al., 2008).

Thus, the greater alterations in the endocannabinoid system observed in the fluoxetine group may be compatible with a poorer negative control over glutamatergic activity which would result in increased excitotoxicity and alterations in neuroplasticity conductive to alcohol relapse (Vengeliene et al., 2008).

The present results are in agreement with previous studies that suggest a tendency towards increases in the AMPA balance with respect to NMDA or mGlu receptors in the glutamatergic system, both in depression and in the progression to AUDs (Hwa et al., 2017; Rubio-Casillas and Fernández-Guasti 2016). Thus, we observed a reduction in gene and protein expression related to various NMDA receptors such as *Grin1, Grin2b*, GluN1 in the fluoxetine group, as well as a marked increase in markers of AMPA function such as the mRNA levels of *Gria2* and *Gria3*. By contrast, we found that the protein expression of GluA1 was reduced in the fluoxetine group, and a similar trend was also observed in bupropion-treated rats. In agreement with previous reports (Cruceanu et al., 2017) we did not observe any significant alteration in the gene expression of the mGlu receptors (*Grm3* and *Grm5*). All these data are coherent with the presence of alterations in neuroplasticity in those animals that showed an enhanced relapse to alcohol drinking after the fluoxetine treatment, similar to those present in inflammatory processes and that have also been reported for mood disorders and in the establishment of addiction (Leclercq et al., 2017). Alterations in brain functions similar to those observed in our study could be involved in the numerous clinical cases and reports that describe negative effects of antidepressants on alcohol consumption and/or depression (see Atigari et al., 2013; Brookwell et al., 2014; Charney et al., 2015; Menkes and Herxheimer 2014).

Numerous authors have called attention to the lack of basic and clinical studies in comorbid populations with AUD and mood disorders, which could inform the currently sketchy practice, where the prescription rate of antidepressants is high and they are considered over-prescribed (Anderson 2018; Foulds et al., 2015; Mark et al., 2003; Petrakis et al., 2012). Moreover, it is often suggested that maximally tolerated doses of the antidepressants should be used in these patients, what would reasonably be expected to magnify the negative effects on relapse to alcohol (Ciraulo et al., 1988; Pettinati et al., 2015). and eventually could worsen drinking outcomes in these patients. The bupropion dose used here, as in the case of fluoxetine, has been shown to have antidepressant properties in rats and could, therefore be comparable to human doses used for these same effects (Machado et al. 2012; Weiss et al., 2012).

The possibility that a higher dose of bupropion could share the negative effects on alcohol consumption should be investigated in the future. Also, the peculiar animal model used here limits the generalizability of the results and different models should be explored in the future, in animals and humans, to ascertain the validity of our results. Another limitation of this study is that differences in alcohol consumption found between the experimental groups cannot be ruled out to play some role in the observed brain alterations, although alcohol was present in both groups. Further research is needed to clarify the contribution of monoaminergic transmission to the unwanted effects of fluoxetine, exploring ventral striatum and amygdalar circuits that might contribute to the enhanced alcohol consumption derived of ISSR treatment cessation.

Incidentally, bupropion is deemed effective in the treatment of nicotine addiction with or without comorbid depression, though its effectiveness for smoking dependence may be related to its actions on nicotine receptors (Torrens et al., 2005). Some issues should, however, be considered with respect to the use of bupropion for the treatment of depression in the case of AUD: for example, its abuse potential or the possibility that bupropion could lower seizure thresholds when combined with alcohol (Silverstone et al., 2008; Wu and Juurlink 2016).Despite the possible danger that some antidepressants may pose over the success of the alcohol abstinence, it is generally considered that concurrent substance abuse should not be a barrier to treating depression (Nunes and Levin 2004). People with comorbid mood disorder and alcohol or other substance use disorder are hard to treat and for this reason, more research is needed until we can reach safe interventions for depression and/or alcohol consumption in these patients in order to achieve the best results. We are aware that the use of a single dose of each drug represents a limitation and the possible existence of a dose-dependent effect of both fluoxetine and bupropion on alcohol consumption in relapse will need to be addressed in future studies.

Statements of interest

There are no actual or potential conflicts of interest.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.euroneuro.2019.03.012.

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