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# Role of the satiety factor oleoylethanolamide in alcoholism

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## ABSTRACT

Oleoylethanolamide (OEA) is a satiety factor that controls motivational responses to dietary fat. Here we show that alcohol administration causes the release of OEA in rodents, which in turn reduces alcohol consumption by engaging peroxisome proliferator-activated receptor- $\alpha$  (PPAR- $\alpha$ ). This effect appears to rely on peripheral signaling mechanisms as alcohol self-administration is unaltered by intracerebral PPAR- $\alpha$  agonist administration, and the lesion of sensory afferent fibers (by capsaicin) abrogates the effect of systemically administered OEA on alcohol intake. Additionally, OEA is shown to block cue-induced reinstatement of alcohol-seeking behavior (an animal model of relapse) and reduce the severity of somatic withdrawal symptoms in alcohol-dependent animals. Collectively, these findings demonstrate a homeostatic role for OEA signaling in the behavioral effects of alcohol exposure and highlight OEA as a novel therapeutic target for alcohol use disorders and alcoholism.

**Keywords** Alcohol self-administration, alcoholism, oleoylethanolamide, PPAR- $\alpha$ , relapse.

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## INTRODUCTION

Oleoylethanolamide (OEA) is a bioactive lipid mediator that belongs to the fatty acid ethanolamide (FAE) family. Although OEA is a structural analogue of the endocannabinoid arachidonoyl ethanolamide (AEA or anandamide), it neither binds to nor activates cannabinoid receptors (Rodríguez de Fonseca *et al.* 2001; Fu *et al.* 2003). Indeed, OEA exerts a number of pharmacological effects, including the induction of satiety, the reduction of body weight gain and the stimulation of lipolysis, through the activation of the peroxisome proliferator-activated receptor- $\alpha$  (PPAR- $\alpha$ ) (Rodríguez de Fonseca *et al.* 2001; Fu *et al.* 2003; Guzmán *et al.* 2004). OEA binds to

this nuclear receptor with high affinity and the majority of its effects are absent in mice lacking PPAR- $\alpha$  (Fu *et al.* 2003; Guzmán *et al.* 2004).

There is a growing body of evidence supporting the idea that OEA might serve as a homeostatic signal that controls multiple aspects of the intake and metabolism of highly caloric fat-containing foods (for review, see Piomelli 2003). OEA regulates energy expenditure and fat utilization primarily through its involvement in the modulation of fat appetite (Schwartz *et al.* 2008), lipid and glucose metabolism (Fu *et al.* 2003; González-Yanes *et al.* 2005; Serrano *et al.* 2006, 2011; Martínez de Ubago *et al.* 2009) and the reward associated with high-calorie intake (Tellez *et al.* 2013). OEA is generated in the

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intestine upon the arrival of fat-containing food through a process controlled by the sympathetic nervous system (Fu *et al.* 2007, 2011). By engaging PPAR- $\alpha$ , the OEA formed in the intestine activates peripheral sensory systems located in the vagus nerve that convey information to the nucleus of the solitary tract and gut peptides that are released into the blood stream to regulate the hypothalamic integrative networks that control energy expenditure (Gaetani *et al.* 2010; Serrano *et al.* 2011). Additionally, *in vitro* and *in vivo* studies have demonstrated that OEA stimulates lipolysis and fatty acid oxidation through a mechanism that involves PPAR- $\alpha$  (Guzmán *et al.* 2004; Suárez *et al.* 2014).

In addition to its role as a homeostatic signal that controls energy expenditure, growing evidence suggests that OEA might influence motivational processes through its ability to modulate reward systems in the brain. Recent reports (Tellez *et al.* 2013) have shown that, by modulating gut sensory nerve terminals of the vagus nerve, intestinal OEA affects the dopaminergic activity that is induced by infusion of either low- or high-calorie emulsions. Indeed, exogenously administered OEA might restore the dopaminergic deficiency that is associated with high-fat diets, which indicates that, in addition to the control of appetite, OEA might serve as a homeostatic signal that is associated with the motivational drives involved in food selection (Tellez *et al.* 2013). This finding aligns with the recent description of the ability of OEA to modulate the dopaminergic responses associated with nicotine self-administration (Melis *et al.* 2008; Mascia *et al.* 2011). Indeed, OEA not only modulates mesocorticolimbic dopaminergic neurons but also blocks both nicotine-induced reward and nicotine-induced nicotine self-administration through a PPAR- $\alpha$ -dependent mechanism (Mascia *et al.* 2011). Considering the presence of the OEA-signaling machinery in the brain, particularly in the hippocampus (Rivera *et al.* 2014) and the description of OEA's ability to modulate memory consolidation (Campolongo *et al.* 2009), the homeostatic role of this bioactive lipid extends to both motivational and cognitive processes that are associated with the hedonic valence of actions.

Given that ethanol is both a high-calorie food and a drug of abuse that is capable of modulating reward, we hypothesized that OEA might interfere with the endogenous signals activated by alcohol. There is compelling evidence that lipid mediators of the FAE family are involved in alcohol dependence (Rodríguez de Fonseca *et al.* 2005). Studies have shown that acute administration of intoxicating doses of ethanol inhibits the *in vivo* formation of AEA in the rat brain (Ferrer *et al.* 2007), whereas low to moderate doses enhance its release *in vivo* and *in vitro* (Basavarajappa, Ninan & Arancio 2008; Ceccarini *et al.* 2013). Chronic ethanol exposure is asso-

ciated with increases in the formation of AEA and FAE precursors. Although AEA and CB<sub>1</sub> receptors have been shown to mediate some of the pharmacological and behavioral aspects of alcohol use (Basavarajappa & Hungund 2002; Rodríguez de Fonseca *et al.* 2005; Ferrer *et al.* 2007), the effects of non-cannabinoid FAEs such as OEA have been poorly characterized, and direct experimental evidence regarding the behavioral significance of the OEA-PPAR- $\alpha$  interaction on ethanol-related behaviors is still lacking. To fill this knowledge gap, we evaluated the potential roles of OEA and PPAR- $\alpha$  in alcohol-seeking behavior (ethanol self-administration and relapse) and monitored the changes in OEA production during acute and chronic ethanol exposure.

## MATERIALS AND METHODS

### Animals

The experiments were performed on male Wistar rats (Charles River, Barcelona, Spain). *In vivo* microdialysis studies were performed on adult C57Bl/6 male mice (Jackson Laboratory, Bar Harbor, ME, USA). All of the procedures involving the care and use of animals were conducted in adherence with the European Directive 2010/63/EU on the protection of animals used for scientific purposes and Spanish regulations (Real Decreto 53/2013 and 178/2004, Ley 32/2007 and 9/2003 and Decreto 320/2010) for the care and use of laboratory animals.

### Drugs

OEA was synthesized as previously described (Rodríguez de Fonseca *et al.* 2001). OEA was dissolved in a vehicle of 5 percent Tween-80 (Sigma Aldrich Co., St. Louis, MO, USA) and 95 percent sterile saline and administered *i.p.* in a volume of 1 ml/kg body weight. The following doses of OEA were used: (1) 1, 5 and 20 mg/kg for the self-administration and reinstatement studies; (2) 1 and 5 mg/kg for the ethanol deprivation study; and (3) 5 mg/kg for the evaluation of alcohol withdrawal signs.

Wy-14643 (Tocris Bioscience, Bristol, UK) was mixed in a vehicle of 10 percent DMSO, 10 percent Tween-80 and 80 percent sterile saline. Wy-14643 was administered *i.p.* in a volume of 1 ml/kg body weight and by *i.c.v.* injection in a total volume of 5  $\mu$ l. The following doses were used: (1) 5, 20 and 40 mg/kg for the self-administration and reinstatement studies; (2) 20 mg/kg for the ethanol deprivation study; (3) 5 and 20 mg/kg for the locomotor and anxiety tests; and (4) 1 and 10  $\mu$ g for central administrations.

The PPAR- $\alpha$  antagonist GW6471 (Tocris Bioscience) was dissolved in a vehicle of 5 percent Tween-80 and 95 percent sterile saline and administered *i.p.* at a dose of 1 mg/kg in a volume of 1 ml/kg body weight.

The orphan G-protein coupled receptor 119 (GPR119) antagonist PSN375963 (Tocris Bioscience) was dissolved in a vehicle of 5 percent Tween-80 and 95 percent sterile saline and administered i.p. at doses of 0.1 and 10 mg/kg in a volume of 1 ml/kg body weight.

The cannabinoid CB<sub>1</sub> receptor antagonist SR141716A (rimonabant) was purchased from Sanofi-Aventis (Sanofi S.A., Paris, France). Rimonabant was suspended with two to three drops of Tween-80 in sterile saline as the vehicle. Rimonabant was administered i.p. at a dose of 3 mg/kg in a volume of 1 ml/kg body weight.

The neurotoxin capsaicin was purchased from Sigma-Aldrich Co. and dissolved in 5 percent Tween-80, 5 percent propylene glycol and 90 percent sterile saline.

### Deafferentation

Capsaicin was administered subcutaneously (12.5 mg/ml) (Kaneko, Kaunitz & Tache 1998) to rats that were anesthetized with ethyl ether. The total dose of capsaicin (125 mg/kg) was divided into three injections (25 mg/kg in the morning, 50 mg/kg in the afternoon and 50 mg/kg on the following day). The control rats received vehicle injections. The experiments were performed 10 days after capsaicin treatment in rats that had lost the corneal chemosensory reflex (i.e. eye wiping for 1–3 minutes after the application of 0.1 percent ammonium hydroxide into one eye).

### Intracerebral infusion cannulas and intra-NAC OEA testing

For the intracerebral infusions, stainless steel guide cannulas aimed at the NAc were implanted in the rats. Animals that had previously been trained to self-administer 10 percent ethanol were anesthetized with an isoflurane/oxygen vapor mixture (1.5–2.0 percent) and implanted with bilateral microinfusion guide cannulas (12 mm, 22 gauge, stainless steel) that terminated 3 mm above the ventral surface of the NAc [from bregma: anteroposterior (AP), +1.6 mm; mediolateral (ML),  $\pm 2.0$  mm; and dorsoventral (DV), –5.0 mm from dura]. These coordinates provided injector placements at the interface between the shell and core of the NAc. A minimum of 5 days of post-operative recovery was allowed before the evaluation of the effect of the local administration of OEA on ethanol self-administration. After the establishment of stable self-administration behavior, the animals received an initial microinjector insertion (no liquid infusion) immediately before self-administration to acclimate them to the procedure and to produce the initial tissue damage from the insertion of the injector. Subsequently, vehicle or OEA infusions were made via bilateral 33-gauge microinjectors that extended 2 mm beyond the tips of the guide cannulas. Infusions of

1  $\mu$ l per side were made over a 2-minute period, and the removal of the injector was delayed by an additional 1 minute to allow for drug diffusion. The stylets were then replaced in the guide cannulas, and the rats were allowed immediate access to the ethanol self-administration paradigm. The effects produced by vehicle or 1  $\mu$ g OEA per side were evaluated.

### Surgery procedure and i.c.v. drug injection

For the i.c.v. injections, stainless steel guide cannulas aimed at the lateral ventricle were implanted in the rats. The animals were anesthetized with an isoflurane/oxygen vapor mixture (1.5–2.0 percent) and placed on a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA, USA) with the incisor bar set at 5 mm above the interaural line. A guide cannula (7 mm, 23 gauge) was secured to the skull with two stainless steel screws and dental cement and was closed with 30-gauge obturators. The implantation coordinates calculated relative to bregma as follows: AP +0.6 mm, ML  $\pm 2.0$  mm and DV –3.2 mm from the surface of the skull. These coordinates placed the cannula 1 mm above the ventricle. After 7 days of a post-surgical recovery period, cannula patency was confirmed by the gravity-fed flow of isotonic saline through a stainless steel injector (8 mm, 30 gauge) that was inserted within the guide to 1 mm beyond its tip. This procedure allowed the animals to become familiar with the injection technique.

For the i.c.v. administration of Wy-14643, the stylet was removed from the guide cannula of each rat and an injector connected to 70 cm of calibrated polyethylene-10 tubing was lowered into the ventricle. The tubing was then raised until the flow began, and 5  $\mu$ l of drug solution at doses of 1 and 10  $\mu$ g was infused over a 30- to 60-second period. The injector was left in the guide cannula for an additional 30 seconds and then removed. The stylet was immediately replaced. The animals were tested 5 minutes after injection. The i.c.v. cannula placements were evaluated after each experiment via dye injection. Only rats with proper i.c.v. placements were included in the data analysis.

### Acute ethanol administration: blood and tissue sampling

Ethanol was dissolved in sterile 0.9 percent (w/v) saline and administered i.p. at a dose of 4 g/kg.

For the blood and visceral sampling, a group of animals was anaesthetized with methoxyflurane at 0, 45, 90 or 240 minutes after the injection of ethanol. Blood (2 ml) was collected from the heart of the animal with a syringe filled with 1 ml of Krebs–Tris buffer (136 mM NaCl, 6 mM KCl, 1.2 mM MgCl<sub>2</sub>, 2.5 mM CaCl<sub>2</sub>, 10 mM glucose and 20 mM Trizma base, pH 7.4). The blood

samples were centrifuged in Accuspin tubes (Sigma-Aldrich Co.) at 800× g for 10 minutes at 22°C. The samples were obtained from the small intestine and the liver, and were frozen immediately on solid CO<sub>2</sub>.

The brains were collected from a second group of animals after rapid decapitation and frozen immediately on solid CO<sub>2</sub>. The frozen brains were placed in acrylic rat brain matrices, and 2-mm thick slices were obtained using brain matrix razor blades. The NAc and cerebellum were dissected in 2-mm thick frozen coronal slices that corresponded to plates 9–13 (NAc) and 56–65 (cerebellum) of the atlas of Paxinos & Watson (1998).

#### **Chronic ethanol administration and ethanol withdrawal: collection of rat plasma**

The regular chow diet was removed and replaced with a liquid diet consisting of chocolate-flavored boost liquid nutritional supplement fortified with vitamins and minerals. The rats were separated into two groups: the ethanol group received a diet containing 10 percent (w/v) ethanol and the control group received an ethanol-free diet supplemented with sucrose to equalize the caloric intake across the two groups. The diet was available 24 hours for 21 days. On the final liquid diet day, the animals were maintained in their home cage with access to the regular chow diet and water. Blood samples were drawn at 0, 6 and 12 hours after the removal of the liquid diet.

#### *Ethanol withdrawal behaviors*

Beginning 6 hours after the diet with ethanol was withdrawn, the animals were evaluated at hourly intervals. The rats were observed for 5 minutes, and ethanol withdrawal signs, including general activity, shakes, tremors, mobility, spontaneous convulsive seizures and rigidity, were scored.

#### **Plasma ethanol levels**

The plasma ethanol levels were enzymatically measured using a commercial kit (Sigma-Aldrich Co.). The assays were run according to the manufacturer's instructions.

#### **High-performance liquid chromatography–mass spectrometry (HPLC-MS) analyses of the tissue content of OEA**

OEA was extracted with methanol/chloroform (1:2, v/v) from the plasma, peripheral tissues (small intestine and liver) and brain areas (NAc and cerebellum). Samples were injected into an HP 1100 Series HPLC-MS system equipped with a Hewlett-Packard OctaDecyl Silica Hypersil column (100 × 4.6 mm i.d., 5 μm). The estimated recovery of OEA was 99.7 ± 0.3 percent. Reversed

phase separations were performed using linear increases of methanol in water at a flow rate of 0.5 ml/min as previously described (Giuffrida, Rodriguez de Fonseca & Piomelli 2000). The MS analyses were performed in the positive ionization mode with an electrospray ion source. For the quantitative analyses, diagnostic fragments corresponding to the protonated molecules [(M + H)<sup>+</sup>] and the sodium adducts of the molecular ions [(M + Na)<sup>+</sup>] were followed in the selected ion monitoring mode. System control and data evaluation were conducted using an on-line system software (HP Chemstation Software, Hewlett-Packard, Palo Alto, CA, USA).

#### ***In vivo* microdialysis studies**

Mice received i.p. injections of ethanol (2 g/kg) every other day over 2 weeks (see the experimental design in the Supporting Information). The day before the last injection, the mice were each implanted with a single microdialysis probe into the NAc that was secured to the skull with dental cement. The stereotaxic coordinates from bregma were +1.5 mm AP, ±0.8 mm ML and –5.0 mm DV (from the skull) (Paxinos & Watson 1998), and the microdialysis probes employed in 1-mm length active membranes (polyethyl sulfone membrane, 15 kDa cut off from SciPro Inc., Sanborn, NY, USA).

The probes were perfused with artificial cerebrospinal fluid, which was delivered at a flow rate of 0.6 μL/min and was composed of the following (in mM): 149 NaCl, 2.8 KCl, 1.2 CaCl<sub>2</sub>, 1.2 MgCl<sub>2</sub>, 0.25 ascorbic acid, 5.4 D-glucose and 30 percent (w/v) hydroxypropyl-β-cyclodextrin (HP-β-CD). The inclusion of the HP-β-CD in the perfusate provided a substantial increase in the dialysis recovery of OEA. Approximately 12–16 hours after probe implantation, dialysate samples were collected at 10-minute intervals. Following a baseline dialysate collection (60 minutes), mice were injected with ethanol (2 g/kg; i.p.) and dialysates collected for 120 minutes. Dialysate samples were frozen on dry ice and stored at –80°C until analysis for OEA content using liquid chromatography coupled with mass spectrometry (Caillé *et al.* 2007).

Proper placement of the active dialysis membrane within the NAc was histologically verified for each experiment. The brains were sliced (20 μm), and the placements of the microdialysis probes were verified using the atlas of Paxinos (Paxinos & Watson 1998).

#### **Operant training for liquid reinforcers**

Training and testing were conducted in standard operant chambers that were located in sound-attenuating, ventilated environmental cubicles. Each chamber was equipped with a drinking reservoir (volume capacity: 0.10 ml) positioned 4 cm above the grid floor in the



center of the front panel of the chamber, and two retractable levers were located 3 cm to the right and left of the drinking receptacle. Auditory and visual stimuli were presented via a speaker and a light located on the front panel. A microcomputer controlled the delivery of fluids, presentation of auditory and visual stimuli, and recording of the behavioral data. The rats were trained to self-administer 10 percent (v/v) ethanol, 10 percent sucrose or water in 30-minute daily sessions on a fixed ratio 1 schedule of reinforcement in which each response resulted in the delivery of 0.1 ml of fluid as previously described (Weiss *et al.* 1993). Briefly, for the first 3 days of training, water availability in the home cage was restricted to 2 hours per day to facilitate the acquisition of operant responding for a liquid reinforcer. During this time, lever pressing that was reinforced with a 0.2 percent (w/v) saccharin or a 10 percent (w/v) sucrose solution was established. At this point, water was made freely available, and saccharin/sucrose self-administration training continued until the animals reached stable baseline responding. The rats in the saccharin-trained group were then trained to self-administer ethanol using a modification of the sucrose-fading procedure (Samson 1986) that employed saccharin rather than sucrose (Weiss *et al.* 1993). During the first 6 days of this ethanol initiation phase, a 5 percent (w/v) ethanol solution containing 0.2 percent saccharin (w/v) was available to the rats. Beginning on day 7, the concentration of ethanol gradually increased from 5 to 8 percent and finally to 10 percent (w/v), whereas the concentration of saccharin correspondingly decreased to 0 percent. At the beginning of the saccharin-fading procedure, a second, inactive lever was introduced. During all training and testing phases, the responses on this lever were recorded as a measure of non-specific behavioral activation, but they had no programmed consequences (Cippitelli *et al.* 2005).

#### Sucrose self-administration

Following completion of the sucrose training, Wistar rats were used to study the effects of OEA (0, 1, 5 and 20 mg/kg) when administered 30 minutes prior to a self-administration session. These experiments were conducted every fourth day using a Latin square counterbalanced design. Responding at the inactive lever was recorded throughout the experiment to monitor non-specific behavioral effects.

#### Ethanol self-administration

Following the completion of the saccharin-fading procedure, Wistar rats were trained in sessions of 30 minutes/day to lever press for 10 percent ethanol (0.1 ml per

response) until stable baseline responding was reached. We studied the effects of OEA (0, 1, 5 and 20 mg/kg) or Wy-14643 (0, 5, 20 and 40 mg/kg) when administered 30 minutes prior to the self-administration sessions. These experiments were conducted every fourth day using a Latin square counterbalanced design. Responding at the inactive lever was recorded throughout the experiment to monitor non-specific behavioral effects.

#### Alcohol deprivation

Rats were trained to self-administer 10 percent ethanol. Following 3 days of baseline responding sessions, the rats were deprived of ethanol for five consecutive days (no operant sessions). At the end of the fifth day of the deprivation phase, the rats were distributed into four groups that were treated with vehicle, OEA (1 and 5 mg/kg) or Wy-14643 (20 mg/kg) 30 minutes before the test session.

#### Reinstatement of ethanol-seeking behavior

##### Conditioning phase

At the completion of the fading procedure, the animals were trained to discriminate between 10 percent ethanol and water in 30-minute daily sessions. Beginning with self-administration training at the 10 percent ethanol concentration, discriminative stimuli that were predictive of ethanol or water availability were presented during the ethanol and water self-administration sessions, respectively. The discriminative stimulus for ethanol consisted of an orange extract odor ( $S^+$ ), and the water availability (i.e. no reward) was signaled with an anise extract ( $S^-$ ). The olfactory stimuli were generated by placing six to eight drops of the respective extract into the bedding of the operant chamber. Additionally, each lever press resulting in the delivery of ethanol was paired with the illumination of the chamber's house light for 5 seconds ( $CS^+$ ). The corresponding cue during the water sessions was a 5-second tone (70 dB;  $CS^-$ ). During the presentation of these stimuli, a 5-second time out period was in effect; during this period responses were recorded but not reinforced. The olfactory stimuli that served as the  $S^+$  or  $S^-$  for ethanol or water availability were introduced 1 minute before the extension of the levers and remained present throughout the 30-minute sessions. The bedding of the chamber was changed, and the bedding trays were cleaned between sessions. The rats were only given ethanol sessions during the first 3 days of the conditioning phase. Subsequently, ethanol and water sessions were conducted in a random order across training days with the constraint that all of the rats received totals of 10 ethanol and 10 water sessions.

### Extinction phase

After the last conditioning day, the rats were subjected to 30-minute extinction sessions for 15 consecutive days. During this phase, the sessions began with the extension of the levers without the presentation of the discriminative stimuli. Responses at the lever activated the delivery mechanism but did not result in the delivery of liquids or the presentation of the response-contingent cues (i.e. the house light or tone).

### Reinstatement testing

The reinstatement tests began the day after the last extinction session. These tests lasted 30 minutes, and the conditions were identical to those of the conditioning phase with the exception that the alcohol and water were not available. The sessions were initiated by the extension of both levers and the presentation of either the ethanol (S<sup>+</sup>)- or water (S<sup>-</sup>)-paired stimulus. The respective discriminative stimulus remained present during the entire session, and responses at the previously active lever were followed by the activation of the delivery mechanism and a 5-second presentation of the CS<sup>+</sup> in the S<sup>+</sup> condition or the CS<sup>-</sup> in the S<sup>-</sup> condition. The animals were tested under in the S<sup>+</sup>/CS<sup>+</sup> condition on day 1 and in the S<sup>-</sup>/CS<sup>-</sup> condition on day 2. Subsequently, reinstatement experiments were conducted every fourth day (on days 6, 10 and 14), and OEA or Wy-14643 was administered 30 minutes prior to these sessions. Responding at the inactive lever was constantly recorded to monitor possible non-specific behavioral effects.

### Two-bottle choice test

The two-bottle choice method was used to determine the voluntary ethanol consumption of rats. The animals were individually housed in standard cages and allowed to acclimate for 1 week. Each rat had continuous access to two identical bottles, one of which always contained tap water. Food was available *ad libitum*. After 4 days of water consumption (both bottles), a choice between 10 percent (v/v) ethanol and water was offered for several days until reaching a stable baseline. The bottle positions were changed every day to avoid position preferences. On the test day, animals received an administration of vehicle, PSN375963, OEA and GW6471 or a combination of OEA and GW6471 30 minutes prior to ethanol and water access, and consumption was recorded over 24 hours. Both ethanol and water intake (g/kg) were calculated at different time intervals.

### Statistical analyses

All data in the graphs are expressed as the mean  $\pm$  SEM. The different experiments included 6–10 animals per treatment according to the assay. Statistical analyses of

the results were performed with one- and two-way ANOVA followed by multiple comparison tests (Dunnett's test). A *P*-value below 0.05 was considered statistically significant. All analyses were performed with the computer program GraphPad Prism version 5.04 (GraphPad Software Inc., San Diego, CA, USA).

## RESULTS

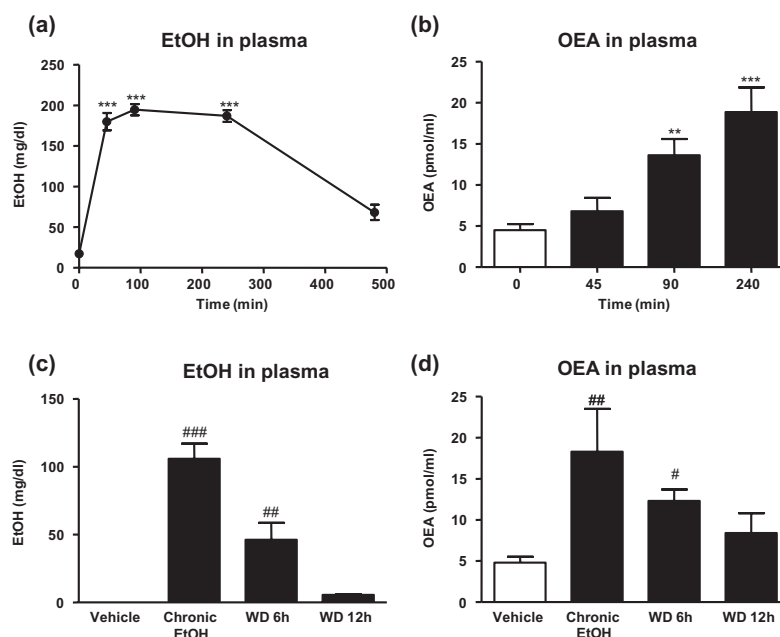
### Effects of ethanol administration on plasma and tissue levels of OEA

To determine whether ethanol influenced OEA mobilization, we evaluated the time course of the effects of acute ethanol administration on the OEA levels in the plasma and various peripheral tissues and brain areas over a period of 240 minutes. Single ethanol injections (4 g/kg; i.p.) resulted in rapid and marked elevations in plasma ethanol levels ( $F_{4,50} = 131.6$ ,  $P < 0.001$ ) that were significant at 45, 90 and 240 minutes after administration compared with the zero time control group ( $***P < 0.001$ ) (Fig. 1a). Decreases in plasma ethanol concentrations were evident 8 hours after the administrations, and the control levels were re-established within the first 24 hours (data not shown). Because plasma ethanol levels remained stable for 240 minutes, we selected the times of 0, 45, 90 and 240 minutes for the analyses of OEA in the plasma and tissues. A one-way ANOVA detected a significant effect of the plasma OEA response to ethanol administration ( $F_{3,41} = 11.55$ ,  $P < 0.001$ ) due to significant increases at 90 ( $**P < 0.01$ ) and 240 minutes ( $***P < 0.001$ ) (Fig. 1b).

Additionally, we evaluated whether OEA influenced ethanol levels. We found similar plasma ethanol concentrations after a single ethanol administration (4 g/kg; i.p.) to rats pre-treated with vehicle or OEA (5 mg/kg) (Supporting Information Fig. S1), indicating that OEA administration does not result in alterations in ethanol pharmacokinetics in the 0–240 minutes post-injection interval.

As shown in Table 1, acute ethanol also led to elevations in the OEA levels in the peripheral tissues and brain areas (small intestine:  $F_{3,41} = 6.65$ ,  $P = 0.001$ ; liver:  $F_{3,41} = 4.29$ ,  $P = 0.011$ ; NAc:  $F_{3,41} = 3.21$ ,  $P = 0.034$ ; and cerebellum:  $F_{3,41} = 9.44$ ,  $P < 0.001$ ). In the small intestine, the OEA increase was apparent at 45 minutes ( $**P < 0.01$ ) after ethanol administration, and the OEA levels returned to the control value at 240 minutes. In the liver, OEA remained at control levels for the first 45 minutes, peaked at 90 minutes ( $*P < 0.05$ ) and returned to normal levels at 240 minutes. Similarly, ethanol resulted in increases in OEA in the NAc at 45 and 90 minutes after its administration ( $*P < 0.05$ ) and in the cerebellum at 90 minutes ( $***P < 0.001$ ). These results

**Figure 1** Effects of acute and chronic ethanol administration on the plasma levels of ethanol and oleylethanolamide (OEA) in rats. Time course of the effects of acute ethanol (4 g/kg; i.p.) at 0, 45, 90 and 240 minutes on plasma levels of ethanol (a) and OEA (b). Effects of a 21-day exposure to an ethanol-liquid diet and withdrawal (measured at 6- and 12-hour time points following the removal of the ethanol-liquid diet) on plasma levels of ethanol (c) and OEA (d). The data are presented as the mean  $\pm$  standard error of the mean ( $n=6-8$  determinations per group).  $**P < 0.01$  and  $***P < 0.001$  denote significant differences compared with the control zero time group.  $#P < 0.05$ ,  $##P < 0.01$  and  $###P < 0.001$  denote significant differences compared with the vehicle group. EtOH = ethanol; WD = withdrawal



**Table 1** Effects of ethanol administration on OEA levels in various tissues.

Tissue	Time after administration (min)			
	0	45	90	240
Small intestine	148.9 $\pm$ 41.8	595.4 $\pm$ 66.2**	398.1 $\pm$ 124.4	128.1 $\pm$ 25.3
Liver	38.4 $\pm$ 5.1	34.6 $\pm$ 2.0	82.5 $\pm$ 17.9*	38.4 $\pm$ 9.1
Nucleus accumbens	128.1 $\pm$ 67.4	610.7 $\pm$ 108.8*	582.6 $\pm$ 209.4*	265.7 $\pm$ 21.6
Cerebellum	248.2 $\pm$ 19.4	420.7 $\pm$ 75.5	610.8 $\pm$ 96.6***	164.4 $\pm$ 17.9

Effects of acute intraperitoneal administration of ethanol (4 g/kg) on OEA levels (pmol/g of tissue) in small intestine, liver and brain sections (nucleus accumbens and cerebellum) were measured at 0, 45, 90 and 240 minutes after injection. Data are means  $\pm$  standard error of the mean (six to eight determinations per group).  $*P < 0.05$ ,  $**P < 0.01$  and  $***P < 0.001$  denote significant differences compared with zero time group. OEA = oleylethanolamide.

suggest that the intestine and NAc were the tissues in which the ethanol-associated increases in OEA occurred most quickly.

To evaluate the effects of chronic ethanol exposure and withdrawal on plasma OEA levels, Wistar rats were maintained on an ethanol-containing liquid diet for 21 days. The animals displayed a significant increase in plasma ethanol concentration at the end of the liquid diet exposure ( $###P < 0.001$ ) (Fig. 1c). This rise remained evident 6 hours into withdrawal ( $##P < 0.01$ ). Regarding the plasma OEA levels, long-term ethanol exposure produced a significant increase ( $##P < 0.01$ ) (Fig. 1d). In the plasma, this elevation was also evident following 6 and 12 hours of withdrawal, but was only significantly different from the control group at 6 hours ( $#P < 0.05$ ).

#### Effects of OEA on ethanol consumption

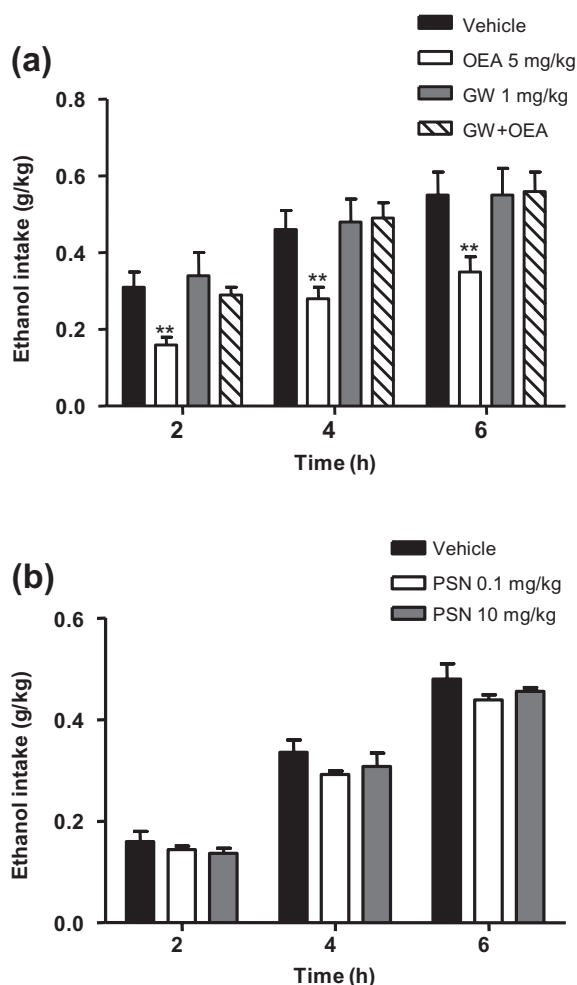
To examine whether OEA affects the ethanol consumption, we used the two-bottle choice paradigm. OEA

(5 mg/kg) administration significantly decreased ethanol intake relative to the vehicle group ( $F_{1,60} = 26.50$ ,  $P < 0.001$ ), and significant decreases were observed at all time points ( $**P < 0.01$ ) (Fig. 2a). Next, because OEA is a PPAR- $\alpha$  agonist, we tested whether the PPAR- $\alpha$  antagonist GW6471 was able to reverse the decrease in ethanol consumption caused by OEA. As shown in Fig. 2a, GW6471 antagonized the decrease in ethanol consumption that was produced by OEA ( $F_{3,21} = 2.81$ ,  $P = 0.025$ ).

Because GPR119 has been reported as a novel OEA target, we also evaluated whether the GPR119 receptor agonist PSN375963 produced similar effects than OEA on ethanol consumption. However, as shown in Fig. 2b, PSN375963 (0.1 and 10 mg/kg) did not affect ethanol intake at all time points evaluated. Similarly, this compound had no effect on ethanol self-administration (Supporting Information Fig. S2).

In addition, water consumption was not affected by none of the treatments tested (Supporting Information Fig. S3).





**Figure 2** The effects of oleoylethanolamide (OEA) on alcohol intake are mediated by peroxisome proliferator-activated receptor- $\alpha$  (PPAR- $\alpha$ ). The effects of OEA (5 mg/kg) on voluntary alcohol intake in a two-bottle choice paradigm were prevented by pre-treatment with the synthetic PPAR- $\alpha$  antagonist GW6471 (1 mg/kg) (a). The GPR119 receptor agonist PSN375963 (0.1 and 10 mg/kg) had no effect on voluntary alcohol intake (b). The data are presented as the mean  $\pm$  standard error of the mean ( $n=8-10$  determinations per group). \*\* $P < 0.01$  denotes significant differences compared with the control group. GW = GW6471; PSN = PSN375963

#### Effects of OEA on ethanol self-administration

Next, we examined whether OEA affects ethanol self-administration. Wistar rats trained to self-administer 10 percent ethanol received injection of various doses of OEA (1, 5 and 20 mg/kg). OEA administration significantly decreased ethanol self-administration relative to the control condition ( $F_{3,31} = 3.42$ ,  $P = 0.031$ ), and significant decreases were observed after the administration of both 5 and 20 mg/kg of OEA ( $*P < 0.05$ ) (Fig. 3a). These effects were also observed for sucrose ( $F_{3,31} = 10.71$ ,  $P < 0.001$ ) after the administration of both 5 ( $*P < 0.05$ ) and 20 mg/kg of OEA ( $***P < 0.001$ ) (Fig. 3b). Similarly,

systemic administration of Wy-14643 reduced ethanol self-administration at doses of 20 and 40 mg/kg ( $*P < 0.05$ ) (Fig. 3c).

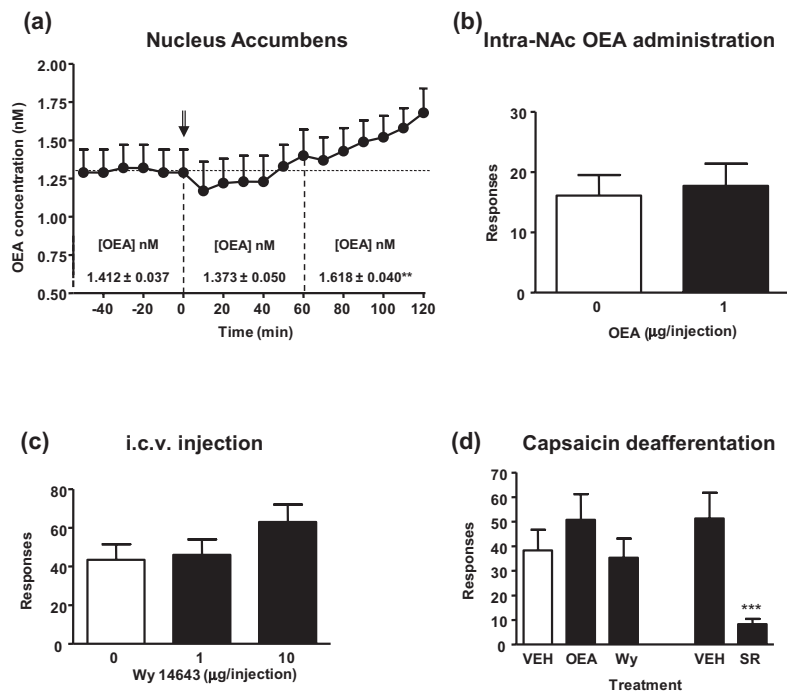
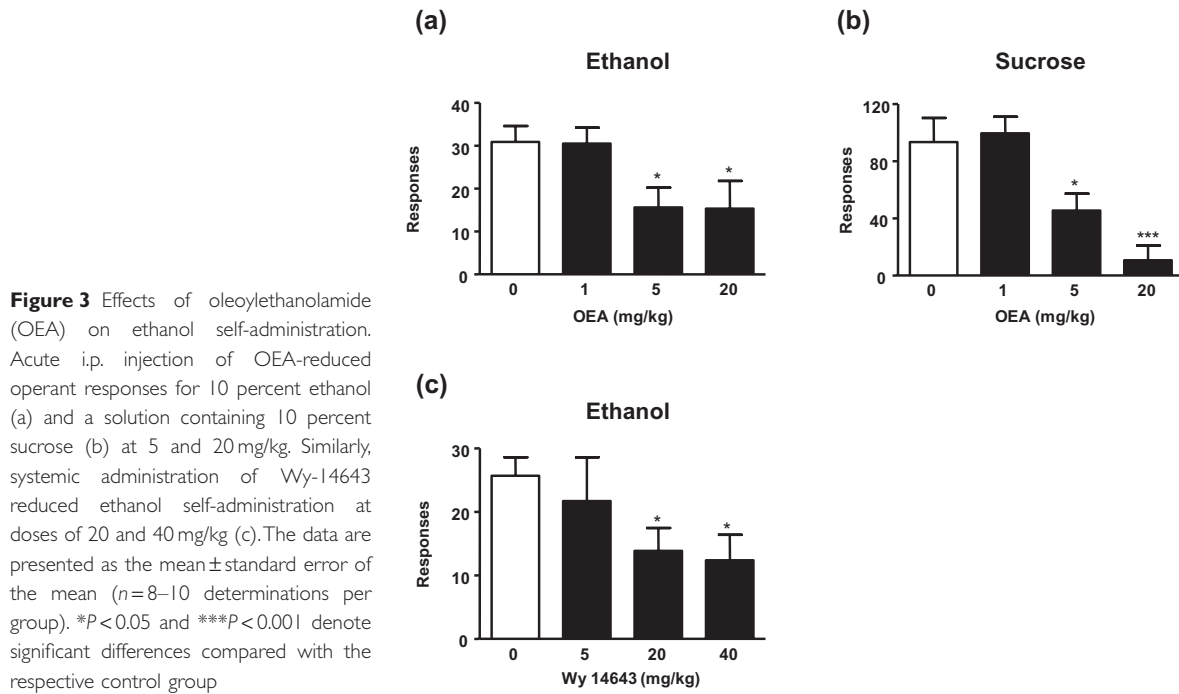
Additionally, Wy-14643 treatment was evaluated in behavioral tests and doses of 5 and 20 mg/kg did not affect locomotion and did not induce anxiety-like behaviors (Supporting Information Fig. S4).

Because OEA reduces food intake through a peripheral mechanism, but reports exist about its ability to modulate central dopaminergic pathways, we studied the effects of OEA when injected centrally and in animals with chemical sensory deafferentation. Because NAc was found to display rapid variations in OEA levels following ethanol injection, we first evaluated the effects of repeated ethanol administration on the OEA response to ethanol challenge. The effects of repeated ethanol treatment (2 g/kg; i.p., and every other day for 14 days) on NAc OEA levels are shown in Fig. 4a. During baseline sample collection dialysate OEA levels were  $1.412 \pm 0.037$  nM, and no trend toward an increase or decrease in levels was observed during this period. Relative to these baseline levels, dialysate OEA content was significantly enhanced after an acute ethanol challenge (2 g/kg; i.p.) ( $F_{1,2,43} = 2.04$ ,  $P = 0.025$ ). This increase began approximately 60 minutes after ethanol challenge and progressively increased over the course of the subsequent 60 minutes. During the final hour of sampling, OEA levels were significantly higher as compared with baseline ( $**P < 0.01$ ), reaching a maximum of 134 percent of baseline 120 minutes after injection.

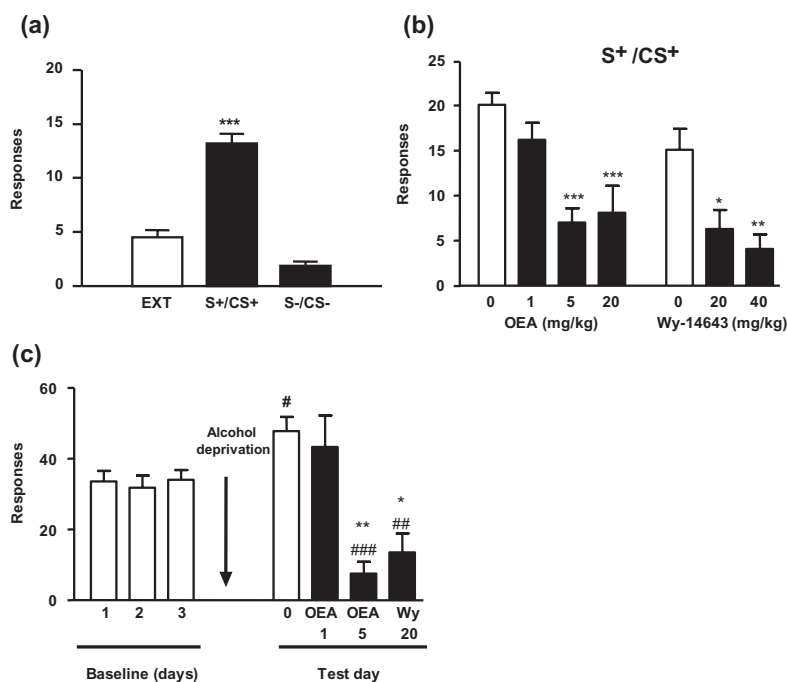
Next, we evaluated the effects of the injection of OEA into NAc on ethanol self-administration. As shown in Fig. 4b, the injection of OEA into the NAc did not modify ethanol self-administration. Similarly, central infusions into the lateral ventricles of different doses of Wy-14643 (1 and 10  $\mu$ g) had no effects on ethanol self-administration (Fig. 4c). However, sensory deafferentation with the neurotoxin capsaicin abolished both OEA- and Wy14643-induced reduction of operant responding for ethanol but not the reduced responding induced by the cannabinoid receptor antagonist/inverse agonist rimonabant (Fig. 4d) that acts centrally to reduce alcohol self-administration.

#### Effects of OEA on conditioned reinstatement and alcohol deprivation

We next examined the effects of OEA and Wy-14643 as modulators of the operant responses elicited by the contextual stimuli associated with ethanol. Reinstatement was induced by presenting cues that were associated with ethanol delivery during training. The operant responses induced by the ethanol-associated stimuli ( $S^+/CS^+$ ) were robustly increased to  $19.4 \pm 2.6$  presses per session, which was significantly different from the behaviors elicited on the last day of extinction ( $4.5 \pm 0.7$  responses;



**Figure 4** Site of action of oleoylethanolamide (OEA) on alcohol self-administration. Dialysate OEA content was significantly enhanced after an acute ethanol challenge (2 g/kg; i.p.). In NAc of mice previously exposed to repeated ethanol treatment (2 g/kg every other day for 14 days). Ethanol injection is indicated by arrow at time point zero (a). Acute intra-accumbens injection of OEA had no effect on ethanol self-administration (b). This lack of a central effect was also observed when the synthetic peroxisome proliferator-activated receptor- $\alpha$  agonist Wy-14643 was injected into the lateral ventricles (c). The effect of OEA on ethanol self-administration was abolished by the deafferentation of the vagal terminals with capsaicin (d). Capsaicin pre-treatment abolished the effects of OEA and Wy-14643 on ethanol self-administration but did not affect the actions of the CB<sub>1</sub> cannabinoid receptor antagonist rimonabant (SR141716A), which acts centrally to reduce alcohol self-administration. The data are presented as the mean  $\pm$  standard error of the mean ( $n=8-10$  determinations per group). \*\* $P < 0.01$  denotes significant differences compared with baseline OEA concentration; \*\*\* $P < 0.001$  denotes significant differences compared with the control group. SR=rimonabant; VEH=vehicle; Wy=Wy-14643



**Figure 5** Effects of oleoylethanolamide (OEA) on conditioned reinstatement and the effects of alcohol deprivation. Conditioned responses to ethanol-associated cues (a) were reduced by the administration of both OEA (1, 5 and 20 mg/kg) and the peroxisome proliferator-activated receptor- $\alpha$  receptor agonist Wy-14643 (20 or 40 mg/kg). (b) i.p. administration of OEA or Wy-14643 reduced the enhancement of the operant ethanol responses that were observed after a 5-day ethanol deprivation period (c). The data are presented as the mean  $\pm$  standard error of the mean ( $n=8-10$  determinations per group). \* $P < 0.05$ , \*\* $P < 0.01$  and \*\*\* $P < 0.001$  denote significant differences compared with the respective control group. # $P < 0.05$ , ## $P < 0.01$  and ### $P < 0.001$  denote significant differences compared with the last day of baseline period

\*\*\* $P < 0.001$ ) and under the S-/CS- condition ( $4.4 \pm 1.1$  responses; \*\*\* $P < 0.001$ , Fig. 5a). As shown in Fig. 5b, OEA pre-treatment produced a dose-dependent reduction in S<sup>+</sup>-induced ethanol seeking relative to the control group ( $F_{3,31} = 9.54$ ,  $P < 0.001$ ); significant reductions were observed at OEA doses of 5 and 20 mg/kg (\*\*\* $P < 0.001$ ). Similarly, Wy-14643 pre-treatment produced a dose-dependent reduction in S<sup>+</sup>-induced ethanol seeking compared with the controls ( $F_{2,23} = 8.29$ ,  $P = 0.002$ ), and significant reductions were observed at Wy-14643 doses of 20 (\* $P < 0.05$ ) and 40 mg/kg (\*\* $P < 0.01$ ).

We also evaluated the effects of PPAR- $\alpha$  agonists in an alcohol deprivation model (Fig. 5c). During the final three self-administration sessions (baseline) before ethanol deprivation, the animals obtained an average of  $33.1 \pm 3.0$  ethanol reinforcers per session. Ethanol deprivation increased the operant responses for ethanol to  $47.8 \pm 4.0$ . OEA administration decreased ethanol self-administration following the ethanol deprivation phase ( $F_{2,23} = 13.79$ ,  $P < 0.001$ ), and significant decreases were observed after the administration of 5 mg/kg OEA (\*\* $P < 0.01$ ). Similar results were observed after the administration of 20 mg/kg Wy-14643 (\* $P < 0.05$ ). The numbers of operant responses observed after either treatment were even lower than those observed in the baseline condition (OEA: ### $P < 0.001$ ; Wy-14643: ## $P < 0.01$ ).

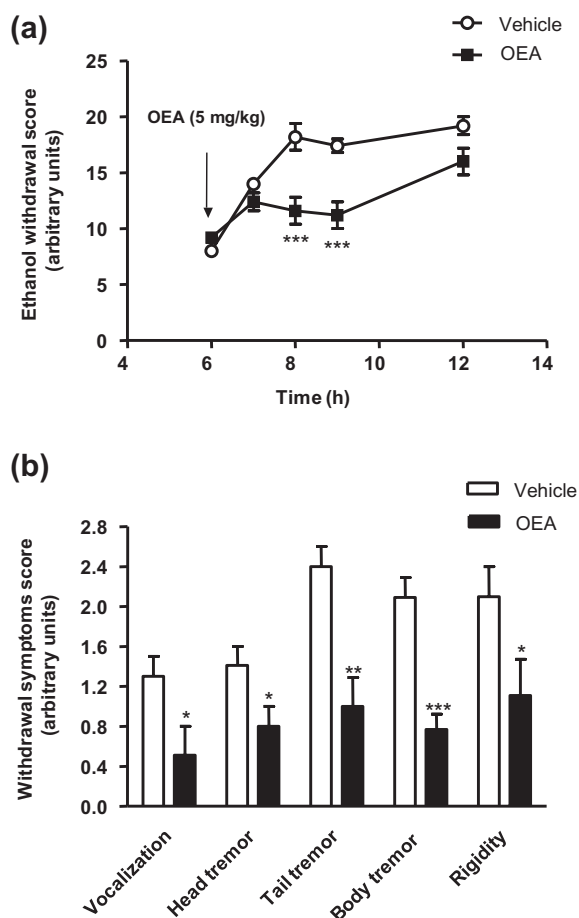
#### Effects of OEA on ethanol withdrawal symptoms

To further explore the effects of OEA on ethanol-related behaviors, several signs of withdrawal were scored in

ethanol-dependent rats after the withdrawal of ethanol. As shown in Fig. 6a, OEA treatment had a significant effect on ethanol withdrawal symptoms ( $F_{1,70} = 34.13$ ,  $P < 0.001$ ). This analysis also revealed a significant interaction between treatment and time ( $F_{4,70} = 6.73$ ,  $P < 0.001$ ). Thus, OEA administration induced significant decreases in the total scores for ethanol withdrawal signs at 8 and 9 hours into withdrawal (\*\*\* $P < 0.001$ ) compared with the vehicle-treated animals. Indeed, the administration of OEA produced significant decreases in each of the withdrawal symptoms that were evaluated; i.e. vocalization, head tremor and rigidity (\* $P < 0.05$ ); tail tremor (\*\* $P < 0.01$ ); and body tremor (\*\*\* $P < 0.001$ ) (Fig. 6b).

## DISCUSSION

The present results identified a prominent role for OEA as a homeostatic signal that controls multiple aspects of the physiological adaptations to alcohol exposure. OEA production is triggered by alcohol administration and contributes to the regulation of alcohol intake, the acute motivational response to alcohol and the onset of withdrawal symptoms after cessation of chronic alcohol consumption. Because OEA has been identified as an intestinal satiety signal that controls hedonic value of food (mainly fat), we cannot consider the observed response as specific for ethanol. Moreover, OEA has been identified already as an important mediator of the reinforcing properties of nicotine (Mascia *et al.* 2011) supporting this general role of control of the hedonic homeostasis.



**Figure 6** Effects of oleylethanolamide (OEA) on ethanol withdrawal symptoms. Acute administration of OEA reduced the global ethanol withdrawal scores that were measured between 6 and 12 hours into withdrawal (a). This effect was observed on each of the symptoms that were evaluated (b). The data are presented as the mean  $\pm$  standard error of the mean ( $n=8-10$  determinations per group). \* $P < 0.05$ , \*\* $P < 0.01$  and \*\*\* $P < 0.001$  denote significant differences compared with the vehicle-treated group

The first interesting finding of the present study was the induction of OEA mobilization following acute exposure to alcohol. This effect was observed in different tissues and was detected in plasma samples. The intensities and time courses of alcohol-induced OEA production were time and tissue dependent, and the small intestine (jejunum) and the NAc were the first tissues in which OEA accumulation was detected. In the NAc, the time course of OEA release (Fig. 4a) paralleled that of classical transmitters activated by ethanol. These transmitters can activate receptors capable of triggering OEA formation through the NAPE-PLD pathway. Alcohol might recruit either catecholaminergic, cholinergic or glutamatergic neurons (Spanagel 2009), which in turn might trigger receptor-dependent OEA activation (Stella & Piomelli 2001). A contribution of a modification of FAAH activity induced by the presence of ethanol was discarded in pre-

vious studies (Ferrer *et al.* 2007). The mechanisms by which alcohol triggers OEA mobilization in the periphery might be related to the dependence of OEA production on sympathetic activity (Fu *et al.* 2011). Alcohol is an activator of the sympathetic nervous system (for review see Spanagel 2009) and sympathetic activation has been found to increase OEA production not only in the intestine but also in metabolically relevant organs such as adipose tissue (Guzmán *et al.* 2004; LoVerme *et al.* 2006; Fu *et al.* 2011). Thus, alcohol-enhanced activation of sympathetic output might be responsible for the observed increases in OEA. Because the plasma and liver OEA elevations were delayed relative to the intestinal accumulation, we infer that this effect is secondary to the activation of primary targets of sympathetic innervation. In the brain, the rapid response of the NAc, which was observed by monitoring tissue contents, is suggestive of a direct action of alcohol on the brain. In this organ, alcohol might recruit either catecholaminergic, cholinergic or glutamatergic neurons (Spanagel 2009), which in turn might trigger receptor-dependent OEA activation (Stella & Piomelli 2001). This hypothesis needs to be tested in future studies. Finally, plasma OEA levels were found to be constantly elevated during chronic alcohol consumption. The removal of alcohol from the diet induced a decrease in OEA levels that paralleled the decrease in alcohol level, thus indicating a tight association between OEA formation and the presence of alcohol in the body. As we discuss later, the disappearance of OEA is related to overt alcohol withdrawal symptoms, which supports the adaptive nature of OEA as an alcohol-driven homeostatic signal that is necessary to adapt the body to the presence of ethanol.

OEA has been linked to physiological responses that are associated with high-calorie intake, including the control of motivational aspects of eating and metabolic adaptations to high-calorie foods (Piomelli 2003; Schwartz *et al.* 2008). Based on this hypothesis, we studied whether alcohol-induced OEA regulated alcohol intake. Confirming this hypothesis, we found that the administration of either OEA or PPAR- $\alpha$  agonists reduced alcohol intake and self-administration. The action of OEA effect was dependent on the presence of PPAR- $\alpha$ , because it was blocked by a selective PPAR- $\alpha$  antagonist. This effect was extended to sucrose and was not related to the activation of an additional OEA target, the GPR119 receptor (Overton *et al.* 2006).

Overall, these findings indicate that, as has been described for fats and high-calorie foods (Rodríguez de Fonseca *et al.* 2001; Fu *et al.* 2003, 2007; Piomelli 2013), OEA is capable of modulating both the consumption and motivational responses to alcohol, which is also highly caloric. These motivational aspects extend to the control of the effects of contextual memories associated

with alcohol consumption in alcohol relapse. Interestingly, both OEA and PPAR- $\alpha$  agonists reduced cue-induced reinstatement of alcohol consumption, which suggests that, following extinction, OEA has the ability to curb motivational memories that are associated with the effects of alcohol. Although OEA has been demonstrated to strengthen memory consolidation (Campolongo *et al.* 2009), the present results suggest that it can also modulate the impact of a recalled memory on motivational drives. However, this hypothesis needs to be confirmed as a peripheral-driven satiety/anhedonic response derived of the activation of the vagus nerve by OEA might counteract the motivational contextual responses associated with alcohol intake.

Following the strategy used to determine the site of action of FAEs in the suppression of food intake (Rodríguez de Fonseca *et al.* 2001; Gómez *et al.* 2002), we searched for the site of action of OEA on alcohol self-administration. As described for food intake, the effects of OEA on alcohol self-administration and cue-induced reinstatement were found to be dependent on the integrity of the peripheral sensory system. Capsaicin-induced deafferentation of the small intestine abrogated these effects and left the centrally mediated effects of compounds, such as the CB<sub>1</sub> receptor antagonist rimonabant (which reduces alcohol self-administration by targeting the prefrontal cortex) intact (Hansson *et al.* 2007). This finding was confirmed by the lack of effects of i.c.v. injections of PPAR- $\alpha$  agonist into alcohol self-administering animals. Thus, we hypothesize that alcohol, as has been described for high-calorie foods, might activate ascending sensory pathways that ultimately inhibit the motivational aspects of alcohol intake by releasing OEA in the intestine (Rodríguez de Fonseca *et al.* 2001; Piomelli 2003). The participation of the nucleus of the solitary tract and its connections with the oxytocinergic (Gaetani *et al.* 2010) and histaminergic (Provinsi *et al.* 2014) systems that further mediate OEA-induced feeding inhibition, will be addressed in future studies.

Following the observation of the decrease in plasma OEA with ethanol withdrawal, we observed that this decline paralleled the onset of alcohol withdrawal symptoms. OEA injection at the beginning of withdrawal (i.e. the time at which OEA levels dropped) induced a clear reduction in the severity of the behavioral symptoms associated with withdrawal. At the present time, we do not know whether this finding reflects an intrinsic ability of OEA to reduce the hyper-excitability associated with ethanol withdrawal. Although OEA has been found to interact with ion channels to regulate excitability, we cannot rule out further actions in targets that are recruited by alcohol withdrawal including the peptidergic and classical neurotransmitter systems. However, the reductions of the motivational effects of ethanol-

associated cues and reductions of the severities of alcohol withdrawal symptoms by the single molecule OEA provide a unique profile for the future design of therapies for alcoholism.

In conclusion, our results revealed that OEA is an endogenous signal that participates in the homeostatic adaptation to alcohol. The actions of OEA cover multiple physiological aspects and open a potential new path for the development of effective therapies for alcoholism.

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### DISCLOSURES/CONFLICT OF INTEREST

The authors state no competing financial interests or other interests that might be perceived to influence the results and discussion reported in this article.

### Authors Contribution

AB performed operant self-administration experiment as well as i.c.v. administration, ethanol treatments and open field and elevated plus-maze studies. AS did the two-bottle choice experiments, the intra-NAc infusion studies and wrote the manuscript. AC was responsible of operant self-administration and reinstatement of ethanol-seeking experiments. FJP did the *in vivo* microdialysis, the mass spectrometry measures and ran all statistical analyses. AG measured OEA in both tissues and plasma samples. JS and EB synthesized the OEA. RGH and NG-M were responsible for ethanol withdrawal studies. LO and FA did the GPR119 experiments. RC, BFC and LHP helped with experimental design and provided methodological support for relapse studies. DP contributed to experimental design and supervision and corrected the manuscript. FRF designed the study, coordinated the experiments and wrote the manuscript.



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## SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

**Figure S1** Effects of OEA pre-treatment on the plasma levels of ethanol

**Figure S2** Effects of the GPR119 receptor agonist on ethanol self-administration

**Figure S3** Effects of OEA and synthetic ligands on water intake

**Figure S4** Effects of Wy-14643 on locomotion and anxiety-like behaviors