ABSTRACT

Objectives There is a need to explore novel mechanisms of action of existing/new antipsychotics. One potential candidate is the endocannabinoid system (ECS). The present study tried to elucidate the effects of the antipsychotic paliperidone on stress-induced ECS alterations.

Methods Wistar rats were submitted to acute/chronic restraint stress. Paliperidone (1 mg/kg) was given prior each stress session. Cannabinoid receptors and endocannabinoids (eCBs) synthesis and degradation enzymes were measured in prefrontal cortex (PFC) samples by RT-PCR and Western Blot.

Results In the PFC of rats exposed to acute stress, paliperidone increased CB1 receptor (CB1R) expression. Furthermore, paliperidone increased the expression of the eCB synthesis enzymes N-acylphosphatidylethanolamine-hydrolysing phospholipase D and DAGLα, and blocked the stress-induced increased expression of the degrading enzyme fatty acid amide hydrolase. In chronic conditions, paliperidone prevented the chronic stress-induced down-regulation of CB1R, normalised DAGLα expression and reverted stress-induced down-regulation of the 2-AG degrading enzyme monoacylglycerol lipase. ECS was analysed also in periphery. Acute stress decreased DAGLα expression, an effect prevented by paliperidone. Contrarily, chronic stress increased DAGLα and this effect was potentiated by paliperidone.

Conclusions The results obtained described a preventive effect of paliperidone on stress-induced alterations in ECS. Considering the diverse alterations on ECS described in psychotic disease, targeting ECS emerges as a new therapeutic possibility.

Introduction

The endocannabinoid system (ECS) is a homeostatic system that can be activated by several stimuli, both in the central nervous system (CNS) and the periphery.

The ECS consists of the cannabinoid receptors, their endogenous ligands (endocannabinoids, eCBs), the enzymes involved in the synthesis and degradation of these eCB and their uptake/reuptake mechanisms. The two main endocannabinoid G1/o protein-coupled receptors cannabinoid receptor 1 (CB1R) and cannabinoid receptor 2 (CB2R) were characterised in the early 1990s (Matsuda et al. 1990; Munro et al. 1993). CB1R is located in CNS but also in several peripheral tissues (Mackie 2005). CB2R is primarily expressed in peripheral immune cells and also in some brain areas, predominantly in microglia and neural stem cells (Gong et al. 2006; Morgan et al. 2009). Other receptors related to the ECS are the nuclear receptor peroxisome proliferator-activated receptor alpha (PPARα), that interacts with the eCB-like molecules N-palmitoylethanolamine (PEA) and N-oleoylethanolamine (OEA; Sun et al. 2007); the transient receptor potential vanilloid 1 (TPVR1) and the G protein-coupled receptor 55 (GPR55) orphan receptor (Ryberg et al. 2007; Marco et al. 2014).

The eCBs belong to a family of polyunsaturated fatty acid derivatives that function as lipid signalling molecules released "on demand" (Piomelli 2003). The main eCBs are N-arachidonoylethanolamine (anandamide, AEA) and 2-arachidonoylglycerol (2-AG) (Devane et al. 1992; Mechoulam et al. 1995). AEA is produced by immune cells and neurons and it is more selective for CB1R, whereas 2-AG acts as a full agonist for both receptors (Sugiura et al. 2002). AEA has several possible routes of synthesis, the most important is a two-step enzyme reaction catalysed by N-acylphosphatidylethanolamine-hydrolysing phospholipase D (NAPE-PLD) (Tsuboi et al. 2013). The major degradation enzyme for AEA is fatty acid amide hydrolase (FAAH; McKinney...
and Cravatt 2005). However, 2-AG synthesis is catalysed by diacylglycerol lipase (DAGL; Reisenberg et al. 2012). Several enzymes can hydrolyse 2-AG, but this reaction appears to be principally catalysed by monoacylglycerol lipase (MAGL; Blankman et al. 2007).

The ECS has a crucial role in the modulation of neurogenesis, neurotransmission and neuroprotection and regulates a great number of physiological functions such as anxiety, eating, learning, memory, metabolism, reproduction and hormonal regulation, among others (Mechoulam and Shohami 2007).

The modulation of ECS is emerging as an interesting therapeutic target for the treatment of CNS diseases (Kucerova et al. 2014). In particular, several studies have related the ECS to psychotic disorders. Reduced CB1R expression and activity have been found in different brain areas of patients with schizophrenia (SZ; Eggon et al. 2008). A close relationship has also been reported between a diminished CB2R function (polymorphism Q63R) and an increased susceptibility to the disease (Ishiguro et al. 2010). Symptoms remission has been linked to changes in CB2R mRNA transcripts in peripheral blood mononuclear cells (PBMC; De Marchi et al. 2003). Moreover, deletion of CB2R has been related to SZ-like behaviours in rodents (Ortega-Alvaro et al. 2011).

Regarding other components of the ECS, cerebrospinal fluid (CSF) AEA levels have been found elevated in subjects with schizophrenia (SZ; Giumbrè et al. 2004; Leweke et al. 2008). A close relationship has also been reported between changes in CB2R mRNA transcripts in peripheral blood mononuclear cells and a peripheral ECS deregulation in first-episode psychosis (FEP) has been reported (Bioque et al. 2013). However, ECS is an important modulator of stress response and emotional behaviour (Viveros et al. 2005). Acute/chronic restraint stress exposure modulate eCBs in particular brain areas, prefrontal cortex (PFC) included (McLaughlin et al. 2012; Patel et al. 2009). In the last years it has been postulated a possible regulatory role for CBRs in stress-induced excitotoxicity and neuroinflammation, also in PFC (Zoppi et al. 2011).

Similarly, stress is a major regulator of ECS (Morena et al. 2015). The notion that psychosocial and/or physical stress exposures are main environmental risk factors for psychotic disease is longstanding (Holtzman et al. 2013). Nowadays, there is a pressing need to further explore the molecular mechanisms related to the therapeutic potential of current antipsychotics. One potential candidate is the modulation of the ECS. Previous reports have showed alterations in the peripheral expression of endocannabinoid receptors and synthesis and degradation enzymes in FEP subjects that were mainly treated with paliperidone (Bioque et al. 2013), and the immunomodulatory role of paliperidone in animal models based on acute/chronic stress exposure (MacDowell et al. 2015).

Consequently, this study is aimed to elucidate the potential regulatory effects of the antipsychotic paliperidone on acute/chronic restraint stress-induced specific alterations on the ECS in rat PFC and PBMC.

Material and methods

Animals

Forty-two young-adult (aged 12 weeks) male Wistar Hannover rats (HsdHan:Wist, Harlan Iberica, Spain) weighing 225–250 g were used. All experimental protocols adhered to the guidelines of the Animal Welfare Committee of the Universidad Complutense in accordance with European legislation (D2010/63/UE). The rats were housed with standard temperature and humidity conditions and in a 12-h light/dark cycle (lights on at 08:00 h) with free access to food and water. All the animals were maintained under constant conditions for 7 days prior to the experiment.

Drug administration and experimental designs

The atypical antipsychotic paliperidone (3-[2-[4-(6-fluoro-1,2-benzisoxazol-3-yl)-1-piperidinyl]ethyl]-6,7,8,9-tetrahydro-9-hydroxy-2-methyl-4H-pyrido[1,2-a]pyrimidin-4-one) (molecular weight 426.48) and other chemicals were purchased from Sigma-Aldrich (Spain) or as indicated. Paliperidone was suspended in a saline solution (0.1% Tween 20 (Veh) by sonication for 1 min.

Two experimental designs were performed (see Figure S1 in Supplemental material available online): acute restraint stress model (6 h of restraint) using a plastic rodent restrainer (Decapicone® type) that allowed for a close fit to rats (Leza et al. 1998). Control animals were not subjected to stress, but were handled for a few seconds, and food and water were removed during the period of time that the stressed rats were kept in the restrainer. Vehicle or paliperidone was given by oral gavage (0.5 ml) immediately before placing the animal into the plastic restrainer (Groups 1–4 in Supplemental Figure A1 (a chronic restraint stress model (6h/day during 21 days) (Madrigal et al. 2001) (Groups 5–8).

All protocols started at 9:00 h to avoid circadian changes in the stress response. All groups in the acute stress model contained n = 4 and n = 5–8 in chronic stress model. Samples (blood and brain tissue) were taken after the restraint session using sodium pentobarbital (320 mg/kg, intraperitoneally, i.p., Vetoquinol®,...
Madrid, Spain). The i.p. administration of sodium pentobarbital does not produce changes on brain mRNA content of molecules related to the stress response, such as CRF, NMDA and GABAA receptors and plasma corticosterone levels in a foot shock acute stress model (Wu et al. 2015).

The dose of paliperidone (P1; 1 mg/kg) was chosen on the basis of previous in vivo determinations of signalling pathways related to oxidative stress, cytokines and synaptic plasticity in the prefrontal cortex (PFC) of Wistar rats (Corena-McLeod et al. 2008).

**Preparation of biological samples**

Blood was collected by cardiac puncture and anticoagulated in the presence of trisodium citrate (3.15%, 1 vol citrate/9 vol blood). After decapitation, the brain was removed from the skull and after careful removal of the meninges and blood vessels, PFC from both brain hemispheres were excised and frozen at −80°C until assayed. PBMC were prepared as previously described (Garcia-Bueno et al. 2014) (see Supplemental material available online for details).

**Western blot analysis**

To determine the expression of CB1R, TRPV1 and GPR55 and the synthesis and degradation enzymes of eCBs, brain PFC samples were homogenized by sonication in 400 μl of PBS (pH 7) plus a protease inhibitor cocktail (Complete®, Roche, Spain); followed by centrifugation at 12,000×g for 10 min at 4°C. Similarly, CB2R was also determined in PBMC. PPARα protein was determined in nuclear extracts in PFC and PBMC samples. Nuclear extracts were prepared according previous protocols (MacDowell et al. 2013). Detailed information about sample preparation, protocol, and primary and secondary antibodies used can be found in Supplemental material available online.

The specificity of CB1-CB2R, PPARα, TRPV1 antibodies was tested in previous studies using the respective knockout mice controls (García-Gutierrez et al. 2010; Wu et al. 2010; Zoppi et al. 2011; Barbieri et al. 2012). The validation of NAPE-PLD, DAGLα, FAAH and MAGL antibodies was made using specific blocking peptides (Kallendrusch et al. 2012; Brocato et al. 2013).

**Real time-polymerase chain reaction analysis**

Total cytoplasmic RNA was prepared from samples of PFC using TRIzol reagent (Invitrogen, Grand Island, NY); aliquots were converted to complementary DNA using random hexamer primers (see Table 1 for the set of primers used) quantitative changes in messenger RNA (mRNA) levels were estimated by real time-polymerase chain reaction (see Supplemental material available online for details).

**Protein assay**

Protein levels were measured using Bradford method based on the principle of protein-dye binding (Bradford 1976).

**Statistical analyses**

Data in text and figures are expressed as mean ± SEM. For multiple comparisons, a two-way ANOVA followed by the Bonferroni post-hoc test was used, considering as the first factor the presence or absence of stress and, as second, the presence or absence paliperidone treatment, and by one-way ANOVA following by Newman-Keuls post-hoc test when appropriate. A P value <0.05 was considered statistically significant. Data were analysed using GraphPad Prism version 5.04 (GraphPad Software, San Diego, CA, USA). All the results of the two-way ANOVA analyses (F values and dfs) are included in Tables A1–A3 in Supplemental material available online.

**Results**

Effects of paliperidone on acute and chronic restraint stress on classical and non-classical endocannabinoid receptors in rat PFC

In acute stress conditions, there was a main effect of paliperidone treatment in CB1 mRNA levels. Post-hoc analysis showed that the CB1 mRNA levels up-regulation produced by paliperidone is evident only in stress conditions (P1 + acute stress group compared to Veh + acute stress group (P < 0.05) (Figure 1A). In spite of the lack of a main effect for stress and interaction, this result suggested that paliperidone elicited up-regulation of CB1 mRNA levels only takes place in stress conditions. This idea was confirmed when CB1 mRNA levels of PFC were measured using real time-polymerase chain reaction (see Supplemental material available online for details).

**Table 1. Specific primers for RT-PCR.**

|   | Forward primers (5′-3′) | Reverse primers (5′-3′) |
|---|------------------------|------------------------|
| CB1 | GCTGCAATCTGTTTTCTCGCACT | CACAAGAACAGCAGCACACACT |
| CB2 | ACCCTTGTTCCTGCTGTTCTCTCTT | AAGAGCAGAGGGAGCCTGCTG |
| NAPE | AGAAATGTCGTTGAGAAAAGGTA | AACCTGGTGTCATCCAGAGAGGTT |
| FAAH | AGCACCCTGTTCACCCTGACCCTA | TGGATAGAAGGGAATCAGGCTG |
| DAGL | AGTGAATTGACAGAGCGCCAGCTTTCA | ACTGTTGACAGGCGTTATGGA |
| MAGL | AAGTCCCTACCTGGACCTACCAT | ACTGTTGACAGGCGTTATGGA |
| TRPV1 | AGCTTACATGACGACCTCCCTA | CCTCACAGGGCGAGTATGTA |
| Tubulin | CTCCTGCAGGGTATGAATACAT | AGCTGAGTGTGCTGTTTATG |
| GAPDH | TGACACCAACGTCTTCTAGC | GCCGATGACTGTGTTATG |

The specificity of CB1-CB2R, PPARα, TRPV1 antibodies was tested in previous studies using the respective knockout mice controls (García-Gutierrez et al. 2010; Wu et al. 2010; Zoppi et al. 2011; Barbieri et al. 2012). The validation of NAPE-PLD, DAGLα, FAAH and MAGL antibodies was made using specific blocking peptides (Kallendrusch et al. 2012; Brocato et al. 2013).
Figure 1. Effects of paliperidone on acute and chronic restraint stress on classical endocannabinoid receptors in rat prefrontal cortex. Messenger RNA (mRNA) and protein levels of CB1R (A, B) and CB2R mRNA (C) on rats exposed to acute restraint stress. Messenger RNA (mRNA) and protein levels of CB1R (D, E) and mRNA levels of CB2R (F) on rats exposed to chronic restraint stress. The densitometric data of the respective band of interest are normalised by β-actin (lower band). *p < 0.05, **p < 0.01, ***p < 0.001 vs. Veh + without stress (control); &p < 0.01 vs. 1 mg/kg paliperidone (P1) + without acute stress; ##p < 0.01, ###p < 0.001 vs. Stress (Veh). (Bonferroni post-hoc test or Tukey post-hoc test.) Data represent the mean ± SEM.
protein expression was studied. Two-way ANOVA found main effects of paliperidone treatment, stress exposure and interaction. Further analysis showed that $P_1 + $ acute stress group showed higher levels of CB1 protein in relation to Veh + no acute stress (C), $P_1 + $ without acute stress and Veh + acute stress groups (Figure 1B; $F(3,15) = 12.3; P = 0.006$). The further analysis of the interaction suggests that paliperidone effects on CB1 are potentiated in stress conditions. CB2 mRNA remained unaltered in all groups studied (Figure 1C).

In the chronic stress protocol, there were main effects of treatment and interaction in CB1 mRNA. Chronic restraint stress down-regulated CB1 mRNA and this effect was prevented by paliperidone (Figure 1D; $F(3,26) = 7.52; P = 0.0011$). Regarding CB1 protein, two-way ANOVA analysis also found main effects of treatment and interaction. Further analysis showed that CB1 protein levels were significantly increased in paliperidone pre-treated group compared to the Veh + chronic stress group (Figure 1E; $F(3,26) = 4.94; P = 0.009$). Again, these analyses showed that paliperidone effects on CB1 are modulated by the presence/absence of stress exposure. A main effect of stress was found in CB2 mRNA. Bonferroni post-hoc tests showed that chronic stress reduced CB2 mRNA levels compared to control ($P < 0.01$) (Figure 1F).

Next, we studied the effects of acute/chronic stress and paliperidone pre-treatment on the receptors PPARα, TRPV1 and GPR55. In acute stress conditions, interaction was present for PPARα mRNA levels. Post-test analysis showed that acute stress up-regulated PPARα mRNA levels with/without paliperidone pre-treatments ($P < 0.01$ in both cases), but there are no differences between both groups (Figure 2A). In the case of TRPV1 mRNA expression main effects for stress and treatment were found. As in the previous case of PPARα, Bonferroni post-tests showed that acute stress up-regulated TRPV1 mRNA levels with/without paliperidone pre-treatments ($P < 0.01$ in both cases), but there are no significant differences between both groups (Figure 2B).

In the case of PPARα and TRPV1 proteins, two-way ANOVA showed interaction and a main effect for stress, respectively. Post-test analysis revealed that acute stress reduced PPARα levels (Figure 2C; $F(3,15) = 4.22; P = 0.0296$). Bonferroni post-tests showed that stressed paliperidone animals also presented a significant increase on TRPV1 protein expression compared to $P_1 + $ without stress group (Figure 2D).

Finally, there were no changes after chronic stress exposure in the expression of PPARα and TRPV1 in any of the groups studied, except for TRPV1 mRNA levels in which main effects of stress and treatment were found. TRPV1 mRNA levels increased in the $P_1 + $ chronic stress group in relation to Veh + chronic stress group (Figure 2E). GPR55 protein expression did not change among groups (data not shown).

**Effects of paliperidone on acute and chronic restraint stress-induced alterations of AEA metabolic pathway in rat PFC**

No significant differences were found in NAPE-PLD mRNA in the acute stress model (Figure 3A). At protein level, two-way ANOVA analysis found main effects for treatment and interaction. Further analysis showed that there is an increase of NAPE protein expression in animals treated with paliperidone before acute restraint stress exposure when compared with both $P_1 + $ no acute stress and Veh + acute stress groups (Figure 3B; $F(3,15) = 7.292; P = 0.0048$). These results suggested that paliperidone effects on NAPE protein expression are dependant of the posterior stress exposure. FAAH mRNA remained unchanged between groups (Figure 3C) but, at protein level, main effects for acute stress and treatment were present. Bonferroni post-test showed that protein levels of FAAH increased in animals exposed to acute restraint stress, being this effect prevented by paliperidone pre-treatment (Figure 3D).

In chronic stress conditions, there is a main effect of stress in NAPE mRNA. Stressed paliperidone pre-treated group also presented a significant increase on NAPE mRNA compared to $P_1 + $ without chronic stress group (Figure 3E). Protein levels did not change among groups (Figure 3F). In addition, there is a main effect of repeated restraint stress in FAAH mRNA levels. Chronic restraint stress decreased FAAH mRNA in Veh + chronic stress and $P_1 + $ chronic stress groups compared to their respective controls (Figure 3G). Repeated restraint stress decreased FAAH mRNA in Veh + chronic stress and $P_1 + $ chronic stress groups compared to their respective controls (Figure 3G). Similarly, at protein level there is a main effect of chronic stress. Bonferroni post-tests showed that FAAH protein decreased only in the Veh + chronic stress animals when compared to Veh + without chronic stress (control) group (Figure 3H). In both acute and chronic stress cases paliperidone treatment is not capable to modulate the significant inhibitory effect of stress on FAAH expression.
Figure 2. Effects of paliperidone on acute and chronic restraint stress on non-classical endocannabinoid receptors in rat prefrontal cortex. Messenger RNA (mRNA) and protein levels of PPARα (A, C) and TRPV1 (B, D) on rats exposed to acute restraint stress. mRNA levels of TRPV1 (E) on rats exposed to chronic restraint stress. The densitometric data of the respective band of interest are normalised by β-actin (lower band). *P < 0.05, **P < 0.01, vs. Veh + without stress (control); & & P < 0.05, & & P < 0.01 vs. 1 mg/kg paliperidone (P1) + without acute stress; †P < 0.05, ††P < 0.01 vs. Stress (Veh). (Bonferroni post-hoc test or Tukey post-hoc test.) Data represent the mean ± SEM.
Figure 3. Effects of paliperidone on acute and chronic restraint stress-induced alterations of AEA metabolic pathway in rat prefrontal cortex. Messenger RNA (mRNA) and protein levels of NAPE-PLD (A, B) and FAAH (C, D) on rats exposed to acute restraint stress. mRNA and protein levels of NAPE-PLD (E, F) and FAAH (G, H) on rats exposed to chronic restraint stress. The densitometric data of the respective band of interest are normalised by β-actin (lower band). *P < 0.05, **P < 0.01 vs. Veh + without stress (control); &P < 0.05 vs. 1 mg/kg paliperidone (P1) + without acute stress; #P < 0.05, ##P < 0.01 vs. Stress (Veh). (Bonferroni post-hoc test or Tukey post-hoc test.) Data represent the mean ± SEM.
Effects of paliperidone on acute and chronic restraint stress-induced alterations of 2-AG metabolic pathway in rat PFC

In acute stress exposure, two-way ANOVA showed main effects for stress, treatment and interaction for DAGLα mRNA. Further analysis found that the administration of paliperidone produced a significant increase at mRNA level expression only in stress conditions (when compared to both P+ without acute stress and Veh + acute stress groups) (Figure 4A; F(3,15) = 6.14; P = 0.009). At protein level there are not significant changes on DAGLα (Figure 4B).

The results for MAGL mRNA levels are complex. Main effects for stress (F(1,12) = 12.42; P = 0.004), treatment and interaction existed. Further analysis showed that paliperidone administration prevented the stress-induced increase of MAGL mRNA (Figure 4C; F(3,15) = 9.65; P = 0.0016). At MAGL protein level interaction is present but there were no changes between groups (Figure 4D).

After chronic stress exposure, two-way ANOVA for DAGLα mRNA showed main factors for treatment and interaction. Further analysis revealed a stress-induced decrease of DAGLα mRNA levels, while daily administration of paliperidone normalised DAGLα content to control values (Figure 4E; F(3,26) = 5.35; P = 0.0061). At protein level, a main effect for treatment and interaction also exist. Further intergroup analysis showed that paliperidone induced a marked increase in the protein levels of DAGLα when compared to Veh + chronic stress group (Figure 4F; F(3,26) = 7.6; P = 0.0011). Again, paliperidone effects depended on the presence/absence of stress).

Finally, MAGL mRNA levels remained unaltered in any group studied (Figure 4G). At protein level there were main effects for stress, treatment and interaction. Further analysis showed that repeated restraint stress decreased MAGL protein, and paliperidone pre-treatment normalised values to control (Figure 4H; F(3,26) = 7.16; P = 0.0016).

Paliperidone effects on peripheral ECS after acute/chronic restraint stress

On the acute restraint model the main findings were related to 2-AG metabolism (see Table 2). Two-way ANOVA analysis showed a main effect of stress for CB2 mRNA levels but there are no significant differences between groups. In the case of DAGLα protein main effects were described for stress, treatment and interaction. Further analysis found that restraint stress reduced DAGLα levels, being this effect prevented by paliperidone (Table 2; F(3,15) = 6.99; P = 0.0057). Two-way ANOVA showed a main effect for stress in MAGL protein. Acute restraint stress increased MAGL in PBMC.

Finally, in chronic stress conditions a main effect for stress exposure was found in DAGLα protein. After repeated chronic stress DAGLα were significantly elevated. Interestingly this effect was potentiated by daily administration of paliperidone (Table 2). In our experimental conditions it was not possible to reliably detect CB1R expression in PBMC in the majority of samples.

Discussion

Our results support the idea that the modulation of ECS makes part of the mechanism of action of paliperidone, although further investigation is needed to elucidate whether this ability accounts for their therapeutic profile. However, our results also suggest that the chronic use of antipsychotic medication could be considered a confounding factor to take into account in studies analysing possible alterations in the ECS in psychotic disease (Ferretjans et al. 2012). One of the immediate implications is the importance of studying the ECS status in drug naive samples of patients with psychotic disorders.

Our data are in agreement with previous evidence suggesting that the ECS is very reactive to stress exposure (Crowe et al. 2014). Some authors have found that the effects of stress on endocannabinoid signalling in the brain are mediated by the glucocorticoid receptor (Hill et al. 2011). Although chronic restraint stress exposure is not an animal model of SZ, the use of stress-based animal models is relevant to study possible mechanisms involved in its pathophysiology, considering that stress exposure is present in almost all psychiatric diseases, and its effects on the immune/endocrine system need to be elucidated and controlled (Bradley and Dinan 2010).

In addition, the most used animal models for depression are based on the exposure to chronic stress and many atypical antipsychotics are also used as adjuncts for the treatment of treatment-resistant depression and posttraumatic stress disorder (Rogoz 2013; Wang et al. 2013).

It is important to remark that our results have been obtained in PFC samples. The role of the ECS in this brain area is very important in stress-related disorders, regulating processes such as hypothalamic–pituitary–adrenal (HPA) axis activity, neuroinflammation and stress coping and emotional behaviours (Zoppi et al. 2011; McLaughlin et al. 2014).
Figure 4. Effects of paliperidone on acute and chronic restraint stress-induced alterations of 2-AG metabolic pathway in rat prefrontal cortex. Messenger RNA (mRNA) and protein levels of DAGLα (A, B) and MAGL (C, D) on rats exposed to acute restraint stress. mRNA and protein levels of DAGLα (E, F) and MAGL (G, H) on rats exposed to chronic restraint stress. The densitometric data of the respective band of interest are normalised by β-actin (lower band). *$P < 0.05$, **$P < 0.01$ vs. Veh + without stress (control); &$P < 0.05$ vs. 1 mg/kg paliperidone (P1) + without acute stress; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs. Stress (Veh). (Bonferroni post-hoc test or Tukey post-hoc test.) Data represent the mean ± SEM.
Here we describe particular alterations on ECS produced by the pre-treatment with paliperidone. These alterations are predominantly dependent on stress presence/absence, but also on the duration of the exposition to stress. In the acute stressed rats without paliperidone pre-treatment, CB1R/CB2R expression remained unaffected and there was a decrease in PPARα nuclear protein levels. Moreover, there was an increase in FAAH mRNA and protein expression and in MAGL both at brain PFC and blood level. The immediate stimulatory effects of acute stress exposure on FAAH protein have not been previously described to our knowledge, but they could be related to the existence of putative response elements for glucocorticoids in the FAAH gene promoter (Waleh et al. 2002).

Thus, acute stress seems to decrease the availability of eCBs. However, this coordinated response could represent a mechanism to regulate a putative acute release of AEA and 2-AG during the beginning of stress exposure. This increase on eCB tone is an effect well described by other authors in acute stress conditions (Ross 2003). Recently, a down-regulation of CB2R, NAPE-PLD and DAGLα proteins and a concomitant up-regulation of FAAH and MAGL have been described in PBMCs of FEP patients (Bioque et al. 2013). This synergic response could represent a decrease on the eCB tone or availability during the first psychotic episode, or even a regulatory mechanism against a previous synthesis and release of eCBs. This way, the normalising potential of paliperidone in acute stress could be remarkable in the management of a stress-related psychotic episode.

The chronic stress model also produced specific changes on the ECS elements, down-regulating CB1R and CB2R at mRNA level. These results are in agreement with other that found decreased function and expression of CB1R after chronic stress/corticosterone administration (Bowles et al. 2012; Hong et al. 2011; Hu et al. 2011; Xing et al. 2011). In addition, CB1R knockout mice presented significant increases in depressive-like behaviour (Valverde and Torrens 2012). Similar results have been found with the use of CB1R antagonists, resulting in a depressive-like phenotype (Beyer et al. 2010). However it should be noted that other authors have found exactly the opposite effect: increased CB1 expression has been found in the PFC of rats submitted to chronic unpredictable stress (McLaughlin et al. 2013), and even in the brain of patients diagnosed with post-traumatic stress disorder and depression (Neumeister et al. 2013).

Chronic stress reduced FAAH and MAGL proteins, as well as DAGLα mRNA levels. Some authors have used the same type of homotypic stressor (restraint stress) during 10 days and have reported an increase in 2-AG, while AEA is reduced at this time point (Patel et al., 2003; Patel et al. 2009). Whether our results at 21 days of stress are a consequence or a response to these...
persistent changes is difficult to determine, taking into consideration the great reactivity of the ECS in relation to the duration of stress.

As in acute conditions, paliperidone pre-treatment in stressed animals tended to normalise to control values all these ECS alterations. Briefly, opposite changes in the expression and activity of brain cannabinoid receptors (mostly CB1R up-regulation) and in the eCB levels in CSF, blood and post-mortem tissue (mainly hyperanandaminergia) have been described (Ferretjans et al. 2012). Despite of this potential, the efficacy of current antipsychotics is partial and there is a need to explore new compounds which modulate ECS, providing a novel therapeutic target for the treatment of psychotic diseases.

In this vein, future translational research should corroborate the effects here reported of paliperidone on the main components of the ECS (specially by quantifying the endogenous ligands in different organic compartments) and its regulatory mechanisms in animal models of SZ and, finally, in patients with SZ or psychosis. The use of animal models of SZ will also allow checking whether the effects on the ECS produced by paliperidone correlate with alterations in behavioural SZ-like symptoms.

Furthermore, considering that a great number of the effects here described only take place at mRNA or protein level, more detailed time-course studies are needed to better describe the dynamic of mRNA/protein relationship in all the ECS elements studied.

Most studies performed in rodents reporting direct effects of antipsychotic medication on ECS are focussed on CB1R receptor expression and activity and concluded that antipsychotic drugs likely up-regulate CB1R expression in particular brain areas (striatum, prefrontal cortex) (McPartland et al. 2014). In addition, some cannabinoid CNR1 gene polymorphisms have been related to a differential pharmacological response to atypical antipsychotics (Fernandez-Espejo et al. 2009). Our results, in agreement with others, showed a direct up-regulation of CB1R induced by the pre-treatment with paliperidone, especially in acute stress conditions (Secher et al. 2010). However, other authors have found a down-regulation of CB1R in particular cortical areas of SZ patients related to antipsychotics use (Eggan et al. 2008; Uriguen et al. 2009).

Paliperidone is also capable to modulate PPARx and TPRV-1 receptors. As already commented, PPARx is activated by PEA and OEA, and it has been recently described a predominant role in the modulation of dopamine cell activity through nicotinic receptors, that could be relevant for the treatment of psychotic diseases (Melis et al. 2013). Our results showed that paliperidone also regulated the enzymatic machinery necessary to synthesise OEA and PEA. The production of these molecules has been recently proposed as a homeostatic mechanism of neuroprotection in diverse stress-related CNS pathologies (Fidaleo et al. 2014). The exploration of this pathway in psychotic disease deserves further investigation, considering these molecules are altered both at CNS and peripheral level in SZ (Leweke et al. 1999; Giuffrida et al. 2004; Muguruza et al. 2013).

We are still far from elucidating the precise relevant alterations of the ECS in the pathophysiology of psychotic disease and whether they are cause or consequence of the disease. Moreover, growing evidence from the pre-clinical/clinical arena shows a very complex scenario in which stress exposure plays a main environmental role (Serretti and Fabbri 2013). The need to find new therapeutic strategies or to potentiate certain aspects of the treatments already being used is mandatory. The pharmacological modulation of the ECS is an attractive alternative but its ubiquitous presence and multiple functions warrant a great and coordinated scientific effort for the future.

Acknowledgements
This work was supported by Spanish Ministry of Health, Instituto de Salud Carlos III (PI10/00123 & PI13/01102), CANNAB-CM 20012-15, and Foundation Santander-UCM (GR 58/08). B García-Bueno is a Ramón y Cajal post-doctoral fellow (MEC).

Statement of interest
None to declare.

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