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RESEARCH PAPER

Regulation of Fas receptor/Fas-asssociated protein with death domain apoptotic complex and associated signalling systems by cannabinoid receptors in the mouse brain

M Álvaro-Bartolomé[1,2], S Esteban[1,2], MS García-Gutiérrez[2,3], J Manzanares[2,3], O Valverde[2,4] and JA García-Sevilla[1,2]

[1]*Laboratori de Neurofarmacologia, IUNICS, Universitat de les Illes Balears, Palma de Mallorca, Spain,* [2]*Red Temática de Investigación Cooperativa en Salud (RETICS-Trastornos Adictivos), Madrid, Spain,* [3]*Instituto de Neurociencias de Alicante, Universidad Miguel Hernández-CSIC, Alicante, Spain and* [4]*Grup de Recerca de Neurobiologia del Comportament, Universitat Pompeu Fabra, PRBB, Barcelona, Spain*

Background and purpose: Natural and synthetic cannabinoids (CBs) induce deleterious or beneficial actions on neuronal survival. The Fas-associated protein with death domain (FADD) promotes apoptosis, and its phosphorylated form (p-FADD) mediates non-apoptotic actions. The regulation of Fas/FADD, mitochondrial apoptotic proteins and other pathways by CB receptors was investigated in the mouse brain.

Experimental approach: Wild-type, CB_1 and CB_2 receptor knock-out (KO) mice were used to assess differences in receptor genotypes. CD1 mice were used to evaluate the effects of CB drugs on canonical apoptotic pathways and associated signalling systems. Target proteins were quantified by Western blot analysis.

Key results: In brain regions of CB_1 receptor KO mice, Fas/FADD was reduced, but p-Ser191 FADD and the p-FADD/FADD ratio were increased. In CB_2 receptor KO mice, Fas/FADD was increased, but the p-FADD/FADD ratio was not modified. In mutant mice, cleavage of poly(ADP-ribose)-polymerase (PARP) did not indicate alterations in brain cell death. In CD1 mice, acute WIN55212-2 (CB_1 receptor agonist), but not JWH133 (CB_2 receptor agonist), inversely modulated brain FADD and p-FADD. Chronic WIN55212-2 induced FADD down-regulation and p-FADD up-regulation. Acute and chronic WIN55212-2 did not alter mitochondrial proteins or PARP cleavage. Acute, but not chronic, WIN55212-2 stimulated activation of anti-apoptotic (ERK, Akt) and pro-apoptotic (JNK, p38 kinase) pathways.

Conclusions and implications: CB_1 receptors appear to exert a modest tonic activation of Fas/FADD complexes in brain. However, chronic CB_1 receptor stimulation decreased pro-apoptotic FADD and increased non-apoptotic p-FADD. The multi-functional protein FADD could participate in the mechanisms of neuroprotection induced by CBs.

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Keywords: CB_1 and CB_2 receptor-deficient mice; WIN55,212-2; JWH133; apoptosis; Fas receptor; FADD adaptor; MAPKs; Akt/PEA-15; mouse brain

Abbreviations: AIF, apoptosis-inducing factor; Akt/PKB, v-akt murine thymoma viral oncogene homologue/protein kinase B; CB, cannabinoid; CK1α, casein kinase 1α; ECL, enhanced chemiluminescence; ERK, extracellular signal-regulated kinase; FADD, Fas-associated death domain or Fas (TNFRSF6)-associated via death domain; Fas, FS7-associated cell surface antigen or TNFRSF6, tumour necrosis factor receptor superfamily 6; JNK, c-Jun

Correspondence: Dr JA García-Sevilla, Laboratori de Neurofarmacologia, IUNICS, Universitat de les Illes Balears, Cra. Valldemossa km 7.5, E-07122 Palma de Mallorca, Spain. E-mail: jesus.garcia-sevilla@uib.es
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NH_2-terminal protein kinase or stress-activated protein kinase (SAPK); MAPK, mitogen-activated protein kinase; MEK, MAPK kinase; NF-L, neurofilament-L; p38 MAPK, p38 kinase of 38 kDa; PAR-4, nuclear protein prostate apoptosis response 4; PARP, poly(ADP-ribose)-polymerase; PEA-15, phosphoprotein enriched in astrocytes of 15 kDa; PI3K, phosphoinositide 3-kinase; SDS–PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis; THC, Δ^9-tetrahydrocannabinol

Introduction

Apoptosis (programmed cell death) is an indispensable process for the development of any organism and tissue function, including the normal and diseased brain (Lossi and Merighi, 2003). Programmed cell death is primarily mediated by the extrinsic or death receptor pathway (Algeciras-Schimnich *et al*., 2002), and the intrinsic or mitochondrial pathway (Galluzzi *et al*., 2009), which converge to activate common executioner caspases (caspase-3/7) with the final cleavage of downstream targets that include DNA repair enzymes (Kumar, 2007). The self-associated complex formed by Fas receptor with its adaptor Fas-associated protein with death domain (FADD) has a central role in promoting apoptosis (Chinnaiyan *et al*., 1995; Scott *et al*., 2009). In contrast, phosphorylated FADD (p-FADD; phosphorylated on Ser191 in mouse; phosphorylated on Ser194 in human; Zhang *et al*., 2004) mediates non-apoptotic actions such as cell growth and differentiation (Alappat *et al*., 2005). Recently, the multifunctional Fas/FADD complex and its associated signalling pathways [mitogen-activated protein kinases (MAPK), Akt or protein kinase B, phosphoprotein enriched in astrocytes of 15 kDa (PEA-15)] have also been associated with the induction of non-apoptotic signals (neuroadaptations) in the CNS (García-Fuster *et al*., 2009; Ramos-Miguel *et al*., 2009; 2010).

Natural and synthetic cannabinoids mediate their effects through the activation of at least two forms of $G_{i/o}$ protein-coupled receptors, the CB_1 and CB_2 receptors (nomenclature follows Alexander *et al*., 2009). The CNS expression of CB_1 (mainly in neurones) and CB_2 (glial and neuronal cells) receptors is well documented (Herkenham *et al*., 1991; Gong *et al*., 2006). Among the many effects induced by cannabinoids (Pertwee, 1997; Valverde *et al*., 2005), their beneficial or deleterious actions on neuronal survival remain a controversial topic (Guzmán *et al*., 2002). Although cannabinoids can induce pro-apoptotic activity in different cellular models (Maccarrone and Finazzi-Agró, 2003), recent evidence demonstrates that these compounds, acting through CB_1 receptors (Aguado *et al*., 2007) or CB_2 (Viscomi *et al*., 2009) and associated signalling pathways [anti-apoptotic MAPK–extracellular signal-regulated kinase (ERK) and phosphoinositide-3 kinase (PI3K)/Akt], can also protect neurones from death (Galve-Roperh *et al*., 2008).

The predominant CB_1 receptor is one of the most highly expressed G-protein-coupled receptor in the CNS, which is mainly located on GABA-ergic axon terminals where it regulates GABA release (Katona *et al*., 1999). CB_1 receptors also display a high level of constitutive activity (Gifford and Ashby, 1996) and thus can exert a tonic control (i.e. ligand-independent activity) on its endocytic cycle (Leterrier *et al*., 2004), as well as on the function of other receptors (Canals and Milligan, 2008). This contrasts with the brain CB_2 receptor whose pharmacological activation has been questioned in conscious rats (Chin *et al*., 2008), and therefore the presence of any receptor constitutive activity is uncertain. Recently, the pro-apoptotic Fas/FADD complex was shown to be under the tonic control of δ-opioid receptors (García-Fuster *et al*., 2007b), an inhibitory receptor with a high level of ligand-independent activity (Costa and Herz, 1989). Similarly, the remarkable constitutive activity of the CB_1 receptor could be involved in the tonic control of apoptotic cascades and in the actions of endocannabinoids on neuronal death or survival.

Against this background, the aims of the present study were to: (i) determine the influence of genetic deletion of CB_1 or CB_2 receptor on the basal expression of Fas, FADD and p-Ser191 FADD in mouse brain regions; (ii) assess the effects of WIN55212-2 (CB_1 receptor agonist) and JWH133 (CB_2 receptor agonist) on FADD, p-Ser191 FADD and other components of the canonical apoptotic pathways; and (iii) investigate the modulation by WIN55212-2 of classic (MAPKs) and new (PEA-15 and Akt kinase) pathways mediating apoptotic or survival actions in the brain. A preliminary account of this work has been given at the 20th ECNP Congress (Álvaro-Bartolomé *et al*., 2008).

Methods

Animals and genotyping

All animal care and experimental procedures were conducted according to standard ethical guidelines (European Communities Council Directive 86/609/EEC) and approved by the Local Bioethical Committees. Adult male CB_1 ($n = 9$) or CB_2 ($n = 13$) receptor-deficient [knock-out (KO)] mice and the corresponding wild-type (WT) littermate controls ($n = 21$) were used (23–28 g) for the assessment of basal neurochemical differences between genotypes. Male Swiss albino CD1 mice ($n = 96$; 30–35 g; Charles River, Barcelona, Spain) were used for cannabinoid treatments. CB_1 and CB_2 receptor KO mice were originally generated by homologous recombination as described (Ledent *et al*., 1999; Buckley *et al*., 2000). To homogenize the genetic background, first-generation CB_1 receptor heterozygote mice were bred for 30 generations on a CD1 background, with selection for the mutant CB_1 receptor gene at each generation. CB_2 receptor KO mice (Buckley *et al*., 2000) with C57BL/6J background were backcrossed with CD1 mice for six generations to obtain CB_2 receptor KO mice on the CD1 background. Heterozygote–heterozygote mating of CB_1 or CB_2 receptor KO mice produced homozygous WT and KO littermates, and animals from the same breeding series were finally used in this study. CB_1 and CB_2 receptor KO mice were genotyped (genomic DNA) in the authors' laboratory (O.V. at UPF, Barcelona, Spain and J.M. at UMH-CSIC, Ali-

cante, Spain). The animals were housed under standard conditions (21°C; 12:12 h light/dark cycle), and handled for several days before the experiments to reduce stress.

Treatments with cannabinoid drugs

CD1 mice were acutely treated with WIN55212-2 (0.5, 1 or 8 mg·kg^{-1}, i.p., 1 h), a mixed CB_1/CB_2 receptor full agonist. To assess the specificity of the CB receptor involved, other mice received rimonabant (SR141716; 10 mg·kg^{-1}, i.p.), a selective CB_1 receptor antagonist/inverse agonist (Pertwee, 2005), alone (100 min) or 40 min before WIN55212-2 (1 mg·kg^{-1}). Other groups of mice were acutely treated with JWH133 (1 and 3 mg·kg^{-1}, i.p., 1 h), a selective CB_2 receptor full agonist (Huffman, 2005). Control mice received drug vehicle (Cremophor : ethanol : water, 1:1:18 proportion or DMSO : Tween 80 : water, 1:1:8 proportion; 2 mL·kg^{-1}, i.p., 1 h). The doses of WIN55,212-2, rimonabant and JWH133 were chosen from earlier studies (Moranta *et al*., 2007; see also Barna *et al*., 2009).

For the chronic treatment with WIN55212-2, groups of mice were injected (i.p.) twice daily for 5 days with increasing doses of the agonist (1–8 mg·kg^{-1}; Moranta *et al*., 2007). The first group of mice was killed 1 h after the last dose of WIN55212-2 to assess the brain content of target proteins. The second group of mice was injected with rimonabant (10 mg·kg^{-1}, i.p.) to precipitate and evaluate the cannabinoid withdrawal syndrome (Moranta *et al*., 2007). Finally, the third group of mice was killed 1 h after rimonabant-precipitated withdrawal to quantify various signalling proteins in the brain. The hypothermic response induced by WIN55212-2 (acute, chronic and withdrawal effects) was also assessed (rectal temperature; Moranta *et al*., 2007) to monitor a well-known pharmacological effect of CB_1 receptor agonists (Ledent *et al*., 1999).

Preparation of brain samples and subcellular fractionation

WT, CB_1 and CB_2 receptor KO mice, as well as CD1 cannabinoid-treated mice were killed without anaesthesia by decapitation at the indicated times. The cerebral cortex, corpus striatum (caudate/putamen and nucleus accumbens), cerebellum and thalamus/hypothalamus, regions enriched in CB_1 and/or CB_2 receptors (Herkenham *et al*., 1991; Gong *et al*., 2006), were dissected on ice, frozen in liquid nitrogen and stored at –80°C. Brain samples (total homogenate) were prepared for Western blot analysis of proteins as described (García-Fuster *et al*., 2007a; 2008a,b).

Additional experiments were carried out to assess at the subcellular level (cerebral cortex) the regulation of FADD and p-Ser191 FADD in WT, CB_1 and CB_2 receptor KO mice, as well as the modulation of FADD forms and casein kinase (CK) 1α after the acute treatments with WIN55212-2 in CD1 mice. Protein localization was monitored by use of the subcellular proteome extraction kit (ProteoExtract, Calbiochem, Darmstadt, Germany) as described previously (García-Fuster *et al*., 2007a; 2008a). This method yields four sub-proteomes enriched in cytosolic (F1), membrane and membrane organelle-localized (F2), soluble and DNA-associated nuclear (F3) and cytoskeletal (F4) proteins. The selectivity of this procedure was assessed by immunodetection of PEA-15 in F1, Fas receptor in F2, nuclear protein prostate apoptosis response 4 (PAR-4) in F3 and neurofilament-L (NF-L) in F4 (see García-Fuster *et al*., 2008a).

Functional assay for apoptotic cell death

The potential of genetic deletion of CB receptors and that of WIN55212-2 treatments to induce abnormal brain cell death was monitored by measuring the cleavage of the nuclear enzyme poly(ADP-ribose)-polymerase (PARP-1; Putt *et al*., 2005) using the PARP cleavage detection kit (Calbiochem). The death substrate PARP-1 (116 kDa) is cleaved into specific 85 and 29 kDa fragments after activation of caspases-3/7 (Kumar, 2007). The pattern of PARP-1 cleavage in the cerebral cortex of WT, CB_1 and CB_2 KO mice, as well as in vehicle- and WIN55-212-2-treated CD1 mice, was assessed by Western blot analysis (García-Fuster *et al*., 2008b).

SDS–PAGE, Western blots and quantification of target proteins

Target proteins were quantified by Western blot as described (García-Fuster *et al*., 2007a; 2008a,b). Briefly, brain proteins (40 or 60 μg of total homogenate; 15 μg of each subcellular fraction) were resolved by electrophoresis on 10–12% SDS–PAGE minigels, and then electrotransferred to nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA, USA). In some experiments, the transfer buffer also contained 0.1% SDS to better resolve Fas receptor aggregates. After blocking non-specific protein-binding sites, the membranes were incubated overnight at 4°C in blocking solution with the primary antibody (1:500–1:10 000 dilution), and the blots were developed with peroxidase-conjugated secondary antibodies (anti-rabbit, anti-goat or anti-mouse IgG; 1:5000 dilution for 1 h at 24°C). Blots were subjected to enhanced chemiluminescence (ECL detection system, Amersham, Buckinghamshire, UK), and the signal of bound antibody was visualized by exposure to autoradiographic film (Amersham ECL Hyperfilm). The primary antibodies (immunoaffinity-purified epitopes) used are described in Supporting Information Table S1.

The autoradiograms were quantified as described (García-Fuster *et al*., 2007b). For a direct comparison, samples from CB_1 or CB_2 receptor KO mice and the corresponding WT control mice were run together in the same gel to assess for basal differences in CB receptor genotypes. The amount of target proteins in brain samples of CD1 mice treated with cannabinoid drugs was compared in the same gel with that of control mice that received drug vehicle. These experiments were repeated four to eight times in different gels. Percentage changes in immunoreactivity with respect to control samples (100%) were calculated in the various gels, and the mean value was used as a final estimate. As a control for sample loading and protein transfer, the blots were stripped (Boronat *et al*., 2001) and re-probed with anti-β-actin monoclonal antibody, and target protein content was normalized to that of β-actin. Drug activation of MAPKs and Akt1 are expressed as the ratio of the phosphorylated enzyme (p-ERK, etc.) to the corresponding total enzyme, and reported as a percentage of that in control mice.

Data analyses and statistics

Results are expressed as means ± SEM values. All series of data were analysed with the program GraphPad Prism, version 4.0 (GraphPad Software, Inc., San Diego, CA, USA). Neurochemical differences between WT mice and CB receptor KO mice were assessed by a Student's two-tailed *t*-test. Drug effects and global cannabinoid withdrawal scores were analysed by one-way ANOVA followed by a Bonferroni *post hoc* test for multiple comparisons. Pearson's correlation coefficients were calculated to test for possible association between variables. The level of significance chosen was $P \leq 0.05$.

Materials

WIN55212-2 and JWH133 were purchased from Tocris Cookson Ltd (Avonmouth, UK). Rimonabant (SR141716A) was a gift from Sanofi Recherche (Montpellier, France). Drug and molecular target nomenclature in this report follows Alexander *et al*. (2009).

Results

Basal Fas and FADD in brains of WT and CB receptor KO mice: cleavage of the nuclear enzyme PARP

In CB_1 receptor KO mice, compared to their WT littermate controls, Fas aggregates showed a trend towards a decrease in all three brain regions examined, but this decrease was significant only in the cerebellum (Figure 1). Native and glycosylated Fas forms did not differ between receptor genotypes (data not shown). FADD content was reduced more consistently across the brain regions (14–33%) (Figure 1). In contrast, p-Ser191 FADD was increased (17–27%) in mutant mice (Figure 1). Thus, genetic deletion of CB_1 receptors resulted in a net increase in the p-Ser191 FADD/FADD ratio in all three

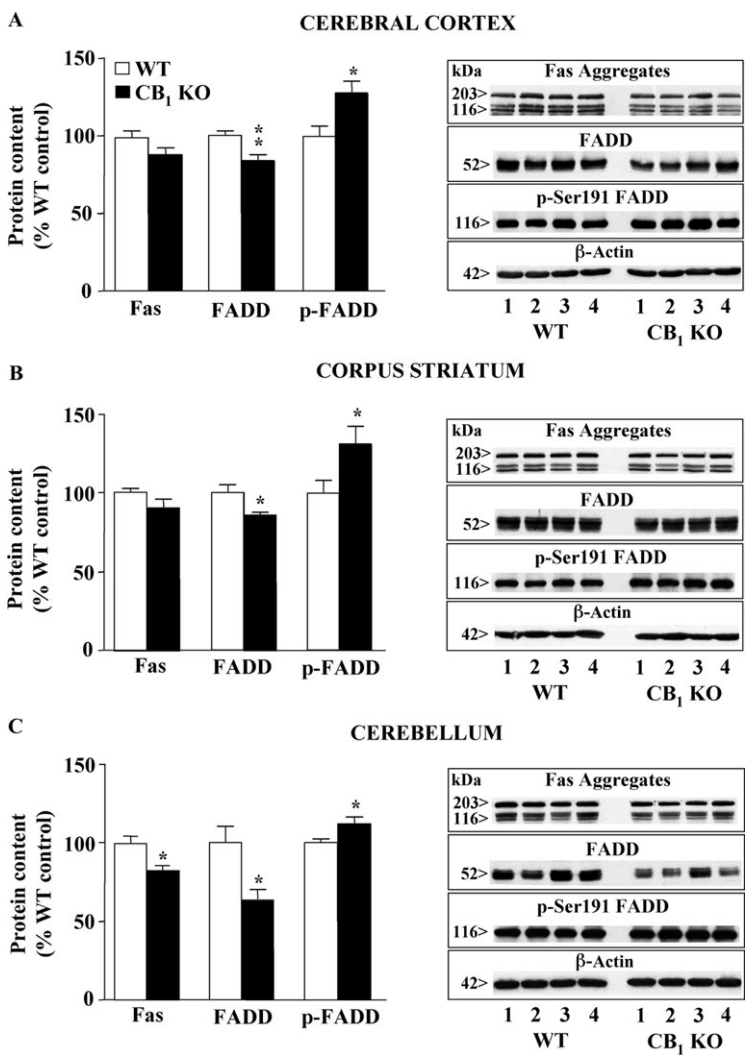

Figure 1 Effects of constitutive deletion of CB_1 receptors (WT, $n = 9$; CB_1 KO, $n = 9$) on the basal contents of Fas receptor (~116/203 kDa aggregated forms), FADD (~52 kDa dimeric form) and p-Ser191 FADD (~116 kDa oligomeric form) in the mouse cerebral cortex (A), corpus striatum (B) and cerebellum (C). The columns are means ± SEM values (% immunoreactivity) expressed as percentage of WT control mice. *$P < 0.05$; **$P < 0.01$ when compared with the corresponding WT group (two-tailed Student's *t*-test). Right: representative immunoblots for Fas, FADD, p-FADD and β-actin in the various brain regions of WT ($n = 4$) and CB_1 KO ($n = 4$) mice. Protein molecular masses (kDa) were estimated from referenced standards.

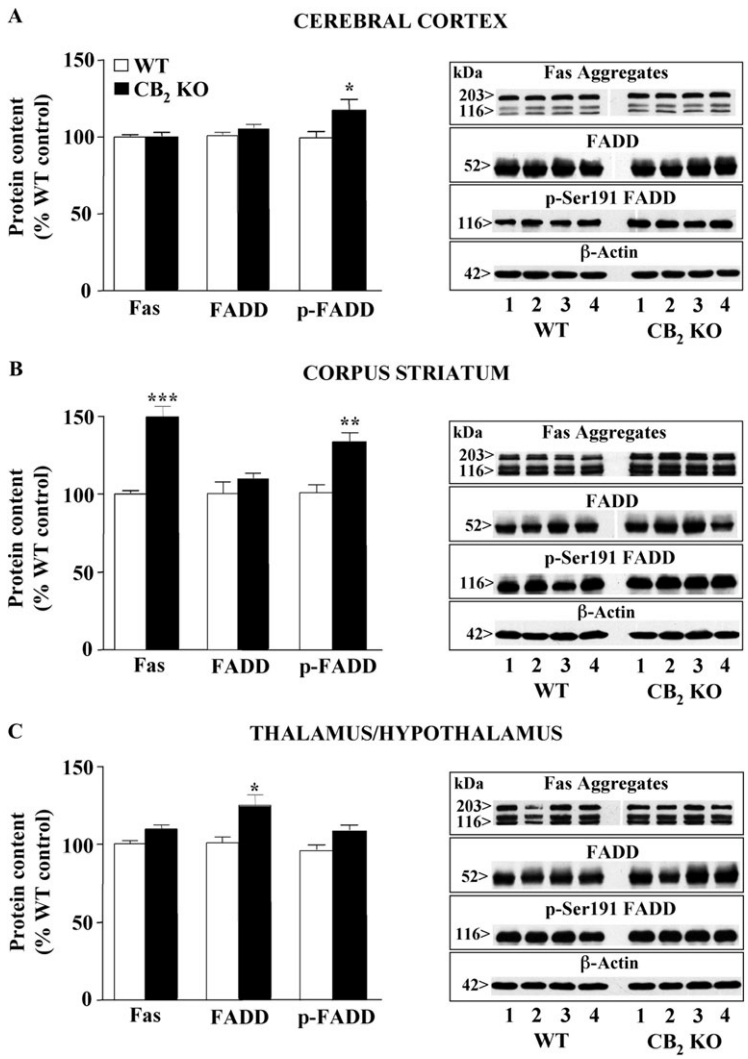

Figure 2 Effects of constitutive deletion of CB_2 receptors (WT, $n = 12$; CB_2 KO, $n = 13$) on the basal contents of Fas receptor (~116/203 kDa aggregated forms), FADD (~52 kDa dimeric form) and p-Ser191 FADD (~116 kDa oligomeric form) in the mouse cerebral cortex (A), corpus striatum (B) and thalamus/hypothalamus (C). The columns are means ± SEM values (% immunoreactivity), expressed as percentage of WT control mice. *$P < 0.05$; **$P < 0.01$; ***$P < 0.0001$ when compared with the corresponding WT group (Student's *t*-test). Right: representative immunoblots for Fas, FADD, p-FADD and β-actin in the various brain regions of WT ($n = 4$) and CB_2 KO ($n = 4$) mice. Protein molecular masses (kDa) were estimated from referenced standards.

regions: cerebral cortex (WT: 0.99 ± 0.05; KO: 1.44 ± 0.14; $P < 0.01$), corpus striatum (WT: 1.00 ± 0.07; KO: 1.42 ± 0.10; $P < 0.005$) and cerebellum (WT: 1.06 ± 0.11; KO: 2.08 ± 0.42; $P < 0.05$).

In CB_2 receptor KO mice, brain Fas forms were similar to those in WT controls, except for Fas aggregates which were clearly increased in striatum (Figure 2). In these mutant mice, FADD was increased in thalamus/hypothalamus and p-Ser191 FADD in cortex and striatum (Figure 2). CB_2 receptor deletion did not alter the p-Ser191 FADD/FADD ratio in cortex (WT: 1.02 ± 0.06; KO: 1.12 ± 0.07), striatum (WT: 1.08 ± 0.12; KO: 1.17 ± 0.07) or thalamus/hypothalamus (WT: 0.96 ± 0.04; KO: 0.90 ± 0.06; all $P > 0.05$).

Consistent with these findings using total homogenates, in key subcellular compartments (cytosol; F1/membranes; F2) of a representative CB_1 KO mouse, cortical FADD was reduced (48–67%), and p-Ser191 FADD was augmented (15–157%) (Figure 3A). In a CB_2 receptor KO mouse, minimal increases of FADD (3–20%) and marked up-regulations of p-Ser191 FADD (21–113%) were measured in the cytosol and membrane fractions (Figure 3B).

Notably, PARP-1 cleavage to the 85 kDa main fragment (a marker of apoptosis) in brains of CB_1 and CB_2 receptor KO mice was not different from that measured in WT animals (Figure 3C).

Acute effects of WIN55212-2 and JWH133 on brain FADD and p-Ser191 FADD: interaction of FADD and CK1α

Acute treatments of CD1 mice with WIN55212-2 (0.5, 1 and 8 $mg \cdot kg^{-1}$), compared with vehicle administration, did not alter Fas (native, glycosylated and aggregated forms) in the cerebral cortex (data not shown). WIN55212-2 (0.5 $mg \cdot kg^{-1}$) increased cortical FADD, whereas higher doses (1 and

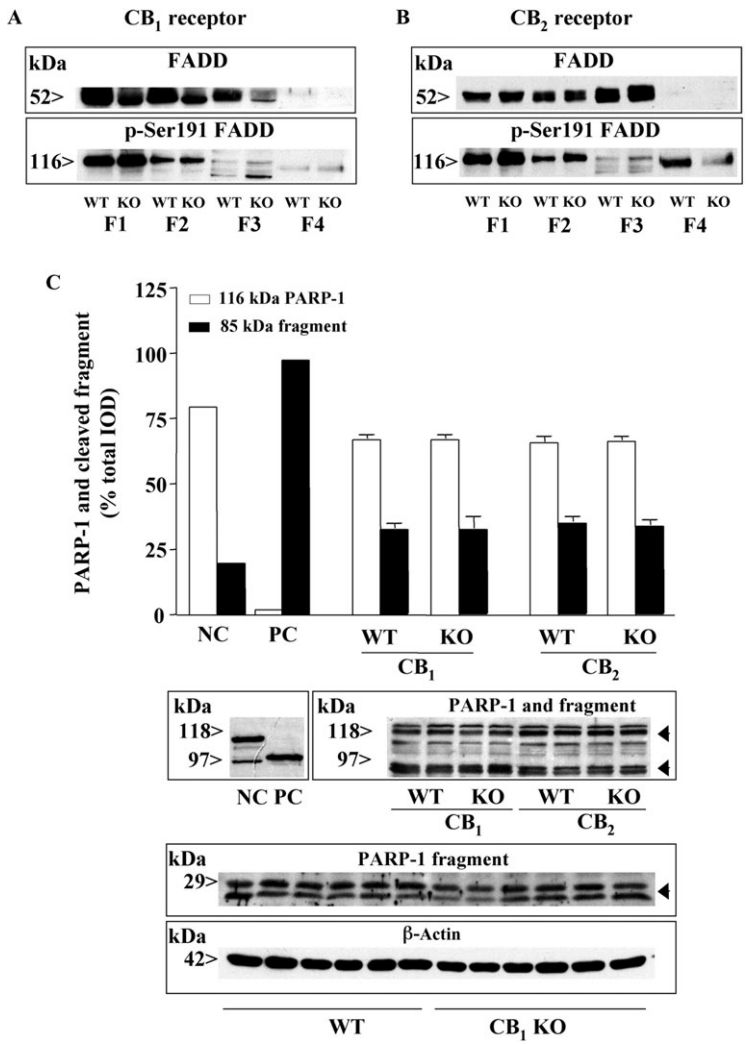

Figure 3 (A and B) Effects of constitutive deletion of CB_1 (A) or CB_2 (B) receptors on the subcellular content of FADD (~52 kDa dimeric form) and p-Ser191 FADD (~116 kDa oligomeric form) in the cerebral cortex of representative WT and KO mice (F1: cytosolic fraction; F2: membrane/organelle fraction; F3: nuclear fraction; F4: cytoskeletal fraction). The immunoblots of selective subcellular markers (F1: PEA-15; F2: Fas receptor; F3: PAR-4; F4: NF-L) are shown in Figure 6E. (C) Effects of constitutive deletion of CB_1 receptor or CB_2 receptor on the content of PARP-1 (~116 kDa) and its cleaved fragment (~85 kDa) in the cerebral cortex of WT (n = 6–12) and KO (n = 7–13) mice. The columns are means ± SEM values expressed as percentage of total immunodensity (IOD units) for each group (PARP plus fragment). NC: negative control; whole cell extract of human HL60 leukemia cells. PC: positive control; etoposide-induced apoptosis in HL60 cells. Two-tailed Student's t-tests did not detect significant differences between WT and KO mice for PARP-1 and its cleaved fragment. Bottom: representative immunoblots for the pattern of PARP-1 cleavage in HL60 cells and the cerebral cortex of WT/KO mice (n = 2 for each group). A representative immunoblot for the 29 kDa PARP-1 fragment and β-actin in the cortex of WT (n = 6) and CB_1 KO (n = 6) mice is included (negative results). (A–C) Protein molecular masses (kDa) were estimated from referenced standards.

8 mg·kg^{-1}) decreased this protein in cortex (Figure 4A). WIN55212-2 also induced bell-shaped dose effects on p-Ser191 FADD, but in the opposite direction, with no changes at the lowest dose and increases at the higher doses (Figure 4B). Rimonabant abolished the opposite effects of WIN55212-2 (1 mg·kg^{-1}) on FADD and p-Ser191 FADD (Figure 5), indicating a CB_1 receptor-related mechanism. The CB_2 receptor agonist JWH133 (1 and 3 mg·kg^{-1}) did not induce significant changes of FADD or p-Ser191 FADD in cortex (Figure 4C,D). As expected, WIN55212-2, but not JWH133, induced hypothermia in mice (data not shown).

Notably, WIN55212-2 (0.5–8 mg·kg^{-1}) modulated in opposite directions the content of FADD and p-Ser191 FADD in the same cortical samples (r = –0.80) (Figure 6A). Thus, the activation of CB_1 receptors can decrease (lower dose) or increase (higher doses) the ratio of p-Ser191 FADD/FADD in the mouse brain (vehicle: 1.02 ± 0.05; 0.5 mg·kg^{-1} WIN: 0.63 ± 0.09, P < 0.001; 1 mg·kg^{-1} WIN: 1.66 ± 0.18, P < 0.005; 8 mg·kg^{-1} WIN: 3.66 ± 0.47, P < 0.001). At the subcellular level, WIN55212-2 (1 and 8 mg·kg^{-1}) increased p-Ser191 FADD in cytosol (77 and 120%) and membranes (158 and 166%) and to a lesser extent in nucleus (14 and 40%) (Figure 6B). In contrast, WIN55212-2 decreased FADD in membranes (20 and 71%) and nucleus (5 and 31%), and increased its content in cytosol (20 and 30%) (Figure 6C). WIN55212-2 also increased levels of the kinase CK1α in cytosol (28 and 66%) (Figure 6D), which was coincident with the marked enhancement of p-Ser191 FADD in this compartment (Figure 6C,D).

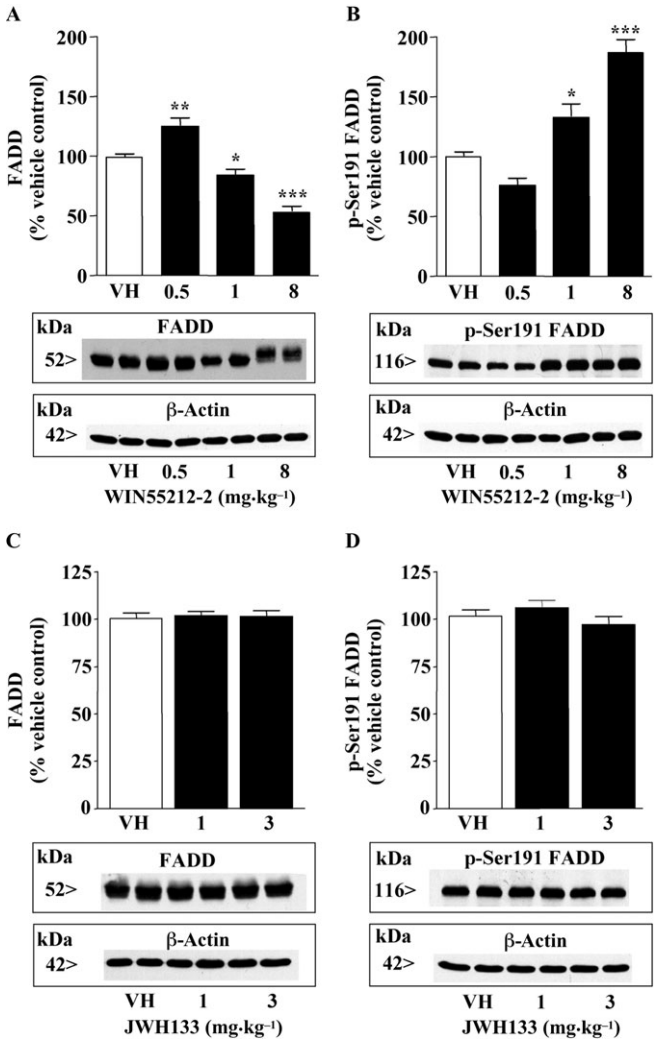

Figure 4 (A and B) Acute effects of the CB_1 receptor agonist WIN55212-2 on the content of (A) FADD (~52 kDa dimeric form) and (B) p-Ser191 FADD (~116 kDa oligomeric form) in the cerebral cortex of CD1 mice. Groups of mice were treated (i.p.) with drug vehicle (VH, $n = 13$) or WIN (0.5, 1 and 8 mg·kg^{-1}, 1 h, $n = 5$–10). The columns are means ± SEM values of protein immunoreactivity and expressed as percentage of the corresponding VH-treated group. One-way ANOVA detected significant differences between the groups of treatments for FADD [$F(3,29) = 26.1$, $P < 0.0001$] and p-FADD [$F(3,29) = 21.7$, $P < 0.0001$]. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ when compared with the corresponding VH group (ANOVA followed by Bonferroni's test). Bottom: representative immunoblots for the effect of WIN on FADD, p-FADD and β-actin in the mouse cerebral cortex. (C and D) Acute effects of the CB_2 receptor agonist JWH133 on the content of (C) FADD and (D) p-Ser191 FADD in the cerebral cortex of CD1 mice. Groups of mice were treated (i.p.) with drug vehicle (VH, $n = 6$) or JWH133 (1 and 8 mg·kg^{-1}, 1 h, $n = 6$ each group). The columns are means ± SEM values of protein immunoreactivity and expressed as percentage of the corresponding VH-treated group. One-way ANOVA did not detect significant differences between the groups of treatments for FADD [$F(2,15) = 0.072$, $P = 0.93$] and p-FADD [$F(2,15) = 1.40$, $P = 0.29$]. Bottom: representative immunoblots for the effect of JWH on FADD, p-FADD and β-actin in the mouse cerebral cortex. (A–D) Protein molecular masses (kDa) were estimated from referenced standards.

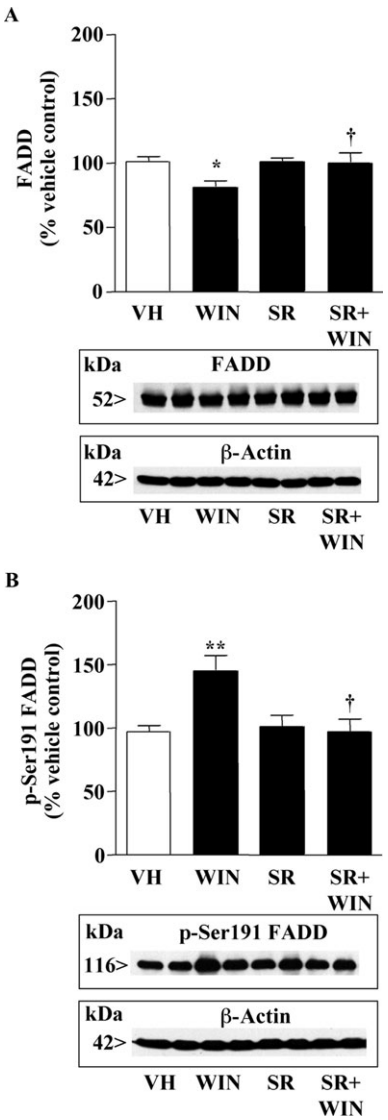

Figure 5 Effects of the selective CB_1 receptor antagonist rimonabant (SR141716A) on WIN55212-2-induced changes in (A) FADD (~52 kDa dimeric form) and (B) p-Ser191 FADD (~116 kDa oligomeric form) in the cerebral cortex of CD1 mice. The cannabinoid drugs were administered alone (WIN; 1 mg·kg^{-1}, i.p., 1 h, $n = 6$; SR, 10 mg·kg^{-1}, i.p., 100 min, $n = 5$) or in combination 40 min apart (SR + WIN, $n = 6$). Control mice received drug vehicle (VH, 2 mL·kg^{-1}, i.p., $n = 8$). The columns are means ± SEM values of protein immunoreactivity, and expressed as percentage of the corresponding VH-treated group. One-way ANOVA detected significant differences between the groups of treatments for FADD [$F(3,21) = 3.10$, $P = 0.048$] and p-FADD [$F(3,21) = 7.34$, $P = 0.0015$]. *$P < 0.05$, **$P < 0.01$ when compared with the corresponding VH group; †$P < 0.05$ when compared with the corresponding WIN group (ANOVA followed by Bonferroni's test). Bottom: representative immunoblots for the effects of WIN, SR and SR + WIN on FADD, p-FADD and β-actin in the mouse cerebral cortex. (A and B). Protein molecular masses (kDa) were estimated from referenced standards.

Effects of chronic WIN55212-2 and rimonabant-precipitated withdrawal on brain FADD and p-Ser191 FADD

Chronic treatment (5 days) of CD1 mice with WIN55212-2 and rimonabant-induced withdrawal did not alter Fas (native, glycosylated and aggregated forms) in the cerebral cortex (data not shown). As expected, acute WIN55212-2 (8 mg·kg^{-1})

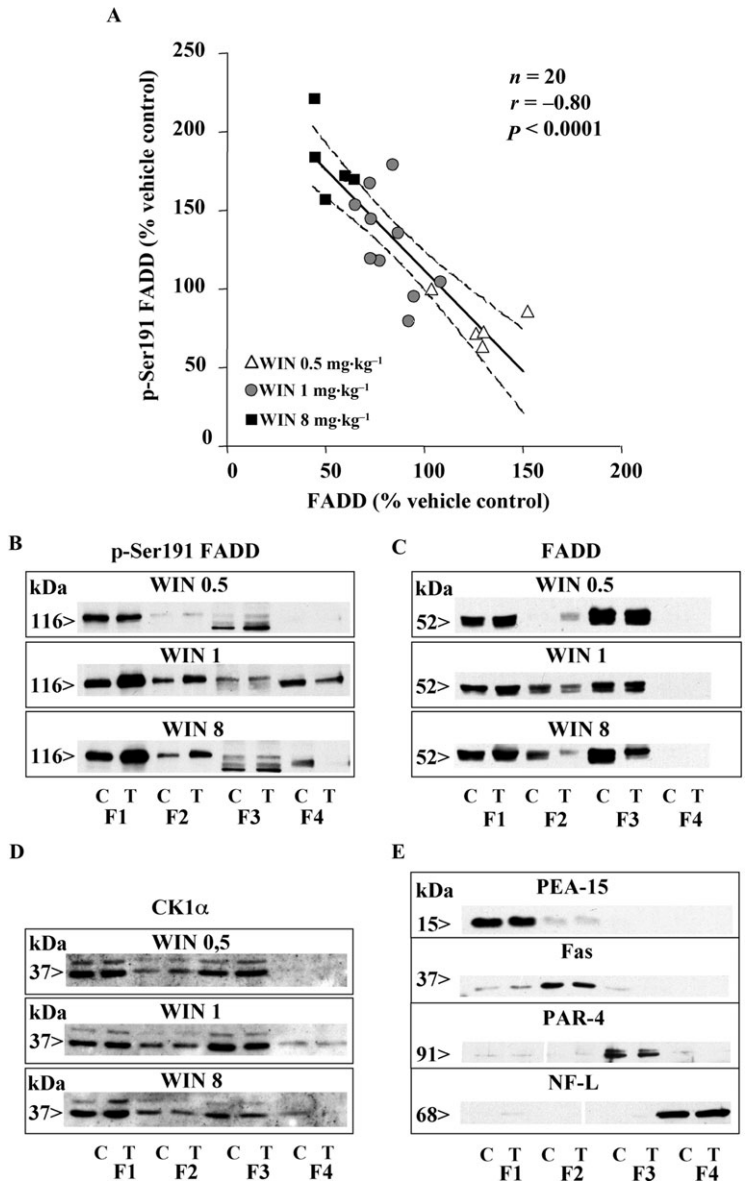

Figure 6 (A) Scatterplot depicting a significant inverse correlation between the immunodensities of p-Ser191 FADD (~116 kDa oligomeric form) and FADD (~52 kDa dimeric form) in the mouse cerebral cortex (same samples) after acute treatments (1 h) with the CB_1 receptor agonist WIN55212-2, and expressed as percentage of the corresponding vehicle-treated CD1 mice (controls). Each symbol represents a different WIN-treated mouse (0.5 mg·kg^{-1} WIN, n = 5; 1 mg·kg^{-1} WIN, n = 10; 8 mg·kg^{-1} WIN, n = 5). The solid line is the best fit of the correlation (r = –0.80, F = 32.40, n = 20, P < 0.0001). The dotted curves indicate the 95% confidence interval for the regression line. (B–D) Effects of WIN55,212-2 (0.5, 1 and 8 mg·kg^{-1}, 1 h) on the subcellular content of p-Ser191 FADD, FADD and CK1α in the cerebral cortex of representative vehicle-treated (C) and WIN-treated (T) CD1 mice. F1: cytosolic fraction; F2: membrane/organelle fraction; F3: nuclear fraction; F4: cytoskeletal fraction. (E) Immunoblots of selective subcellular markers (F1: PEA-15; F2: Fas receptor; F3: PAR-4; F4: NF-L).

decreased FADD and increased p-Ser191 FADD in cortex (Figure 7A,B). Chronic agonist treatment was also associated, although to a lesser extent, with down-regulation of FADD and up-regulation of p-Ser191 FADD, which suggested the development of adaptive changes (p-FADD/FADD ratio; vehicle: 1.01 ± 0.05, chronic WIN55212-2: 2.29 ± 0.24, P < 0.005; Figure 7). Tolerance to the acute hypothermic effect of WIN55212-2 was also observed after chronic treatment (data not shown). In chronically WIN55212-2-treated mice, rimonabant induced the rapid expression of somatic signs of withdrawal (data not shown), indicating the induction of dependence on this agonist. In WIN55212-2-withdrawn mice, the content of FADD and p-Ser191 FADD returned, partially or completely, to basal values (Figure 7).

Effects of WIN55212-2 and JWH133 on mitochondrial apoptotic proteins and PARP cleavage

The acute and chronic treatments of CD1 mice with WIN55212-2, as well as rimonabant-precipitated withdrawal, did not alter the contents of cytochrome *c*, apoptosis-inducing factor (AIF) or the cleavage of PARP-1 in the cerebral cortex (Supporting Information Figure S1). Similar negative results were obtained after acute treatments with JWH133 (data not shown).

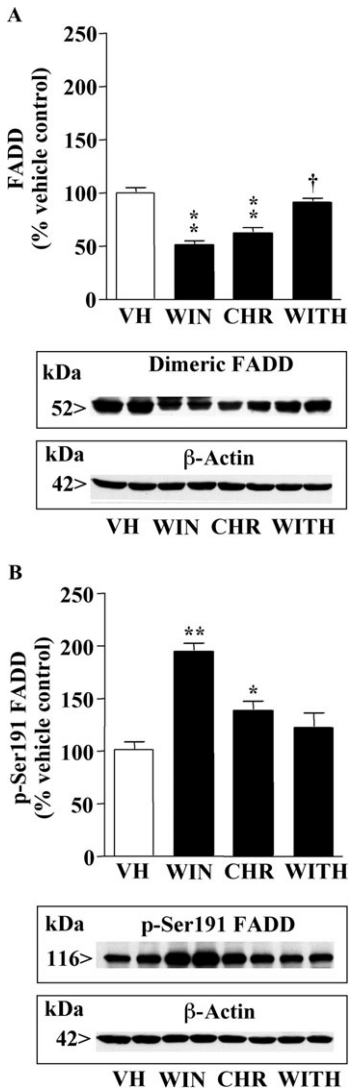

Figure 7 Acute, chronic and withdrawal effects of the CB_1 receptor agonist WIN55212-2 on the content of (A) FADD (~52 kDa dimeric form) and (B) p-Ser191 FADD (~116 kDa oligomeric form) in the cerebral cortex of CD1 mice. Groups of mice were treated (i.p.) with drug vehicle (VH, $n = 5$), acute WIN (WIN, 8 mg·kg^{-1}, 1 h, $n = 5$), chronic WIN (CHR, 1–8 mg·kg^{-1}, increasing doses for 5 days, $n = 5$) and chronic WIN followed 4 h later by the CB_1 receptor antagonist rimonabant (WITH, 10 mg·kg^{-1} for 1 h, $n = 5$) and killed at the indicated times. The columns are means ± SEM values of protein immunoreactivity and expressed as percentage of the corresponding VH-treated group. One-way ANOVA detected significant differences between the groups of treatments for FADD [$F(3,16) = 27.10$, $P < 0.0001$] and p-FADD [$F(3,16) = 18.80$, $P < 0.0001$]. *$P < 0.05$, **$P < 0.001$ when compared with the corresponding VH group; †$P < 0.001$ when compared with the corresponding CHR group (ANOVA followed by Bonferroni's test). Bottom: representative immunoblots for the effects of WIN, CHR and WITH on FADD, p-FADD and β-actin in the mouse cerebral cortex. (A and B). Protein molecular masses (kDa) were estimated from referenced standards.

Acute, chronic and withdrawal effects of WIN55212-2 on the activation of brain MAPKs and regulation of Akt and PEA-15

Acute treatment of CD1 mice with WIN55212-2 markedly stimulated activation of ERK1/2 (shown as the ratio of pERK1/2 : total ERK1/2) in the cerebral cortex, although the activation of this anti-apoptotic kinase was lost after the chronic treatment (Figure 8A). The effect of WIN55212-2 on the phosphorylation of pro-apoptotic JNK1/2 and p38 MAPKs showed a similar time-course to that of ERK, with acute activations after acute treatment, followed by loss of effect after chronic treatment (Figure 8B,C). In WIN55212-2-withdrawn (rimonabant) mice, activation of JNK1/2 and p38 kinase was increased to levels above those after chronic agonist treatment and above values after vehicle (Figure 8). The corresponding ratio for ERK1/2 was unaffected by withdrawal of WIN55212-2.

Activation of Akt1 has been shown to induce the phosphorylation of PEA-15 (p-Ser116) promoting anti-apoptotic actions. Acute WIN55212-2 activated Akt1 (increased p-Akt : total Akt ratio) and markedly increased p-Ser116 PEA-15 in the cerebral cortex (Figure 9A,B). However, chronic WIN55212-2 treatment and rimonabant-precipitated withdrawal were not associated with significant changes in Akt1 activation and PEA-15 phosphorylation (Figure 9). The acute, chronic and withdrawal effects of WIN55212-2 did not alter the content of total PEA-15 (un-phosphorylated PEA-15 can also regulate the ERK pathway) (Figure 9B, tPEA-15 immunoblot).

Discussion

Role of CB receptors in the tonic control of apoptotic Fas/FADD complex in the mouse brain

The association of Fas receptor with the cytosolic adaptor FADD is the initial step in the activation of the extrinsic apoptotic pathway (Algeciras-Schimnich *et al*., 2002). Notably, the over-expression of FADD results in Fas-independent triggering of apoptosis (Chinnaiyan *et al*., 1995), and its phosphorylation (p-Ser191, mouse) is associated with non-apoptotic actions (Alappat *et al*., 2005). Therefore, Fas and the multifunctional FADD might participate in the deleterious or beneficial actions of natural and synthetic cannabinoids on neuronal survival (see Introduction).

In this context, WT and CB receptor KO mice were first compared to assess if an endogenous cannabinoid tone regulates the basal expression of Fas and/or FADD in various brain regions (total homogenate and subcellular compartments). In CB_1 receptor KO mice, the contents of Fas aggregates (relevant receptor forms in transmitting the death signal; Algeciras-Schimnich *et al*., 2002) and total FADD (non-phosphorylated/phosphorylated forms; García-Fuster *et al*., 2007a) were reduced, which suggested that endocannabinoids acting on CB_1 receptors stimulate the expression of Fas/FADD complexes (pro-apoptotic action). CB_1 receptor-deficient mice also showed an increased brain content of oligomeric p-Ser191 FADD (p-FADD/FADD ratio increased), suggesting that CB_1 receptors tonically inhibit the phosphorylation of FADD, which could also favour the induction of pro-apoptotic actions. Consistent with these findings, a low dose (0.5 mg·kg^{-1}) of the CB_1 receptor agonist WIN55212-2 increased brain FADD and decreased p-Ser191 FADD (p-FADD/FADD ratio decreased; see further discussion below), which was opposite to the changes of FADD in CB_1 KO mice. Therefore, CB_1 receptors, which exhibit a high level of constitutive activity (Gifford and Ashby, 1996; Leterrier *et al*., 2004), appear to exert a tonic activation of the

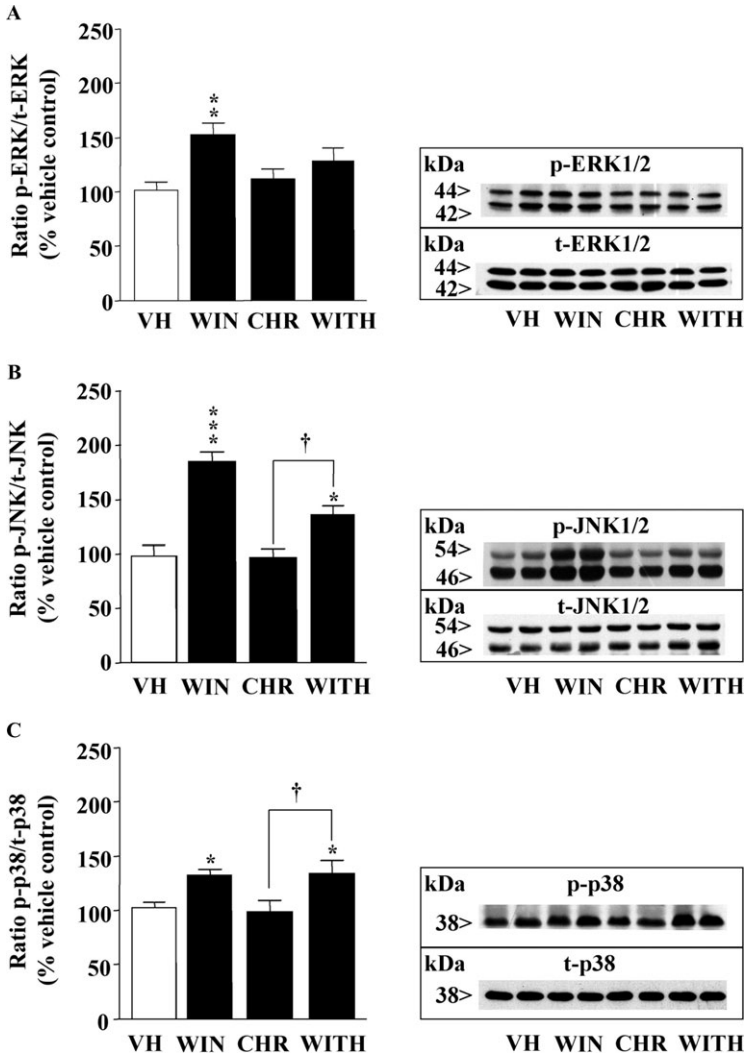

Figure 8 (A–C) Acute, chronic and withdrawal effects of the CB_1 receptor agonist WIN55212-2 on the activation of (A) ERK1/2 (B) JNK1/2 and (C) p38 MAPKs (expressed as the ratio of phosphorylated enzyme to the corresponding total enzyme) in the cerebral cortex of CD1 mice. Groups of mice were treated (i.p.) with drug vehicle (VH, $n = 5$), acute WIN (WIN, 8 mg·kg^{-1}, 1 h, $n = 5$), chronic WIN (CHR, 1–8 mg·kg^{-1}, increasing doses for 5 days, $n = 5$) and chronic WIN followed 4 h later by the CB_1 receptor antagonist rimonabant (WITH, 10 mg·kg^{-1} for 1 h, $n = 5$) and killed at the indicated times. The columns are means ± SEM values of protein immunoreactivity, and expressed as percentage of the corresponding VH-treated group. One-way ANOVA detected significant differences between the groups of treatments for ERK1/2 [$F(3,16) = 5.30$, $P = 0.01$], JNK1/2 [$F(3,16) = 22.56$, $P < 0.0001$] and p38 kinase [$F(3,16) = 4.40$, $P = 0.02$], *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ when compared with the corresponding VH group; †$P < 0.05$ when compared with the corresponding CHR group (ANOVA followed by Bonferroni's test). Right: representative immunoblots for the effects of WIN, CHR and WITH on MAPKs in the mouse cerebral cortex. (A–C) Protein molecular masses (kDa) were estimated from referenced standards.

pro-apoptotic Fas/FADD complexes. However, rimonabant (10 mg·kg^{-1}), a selective CB_1 receptor antagonist/inverse agonist did not alter FADD or p-Ser191 FADD in brains of CD1 mice, suggesting that the postulated receptor tonic control on this system is moderate. On the other hand, CB_1 receptor KO mice showed no differences in the brain content of anandamide and 2-arachidonoylglycerol compared with WT mice (Maccarrone *et al.*, 2001), indicating that the modulation of Fas/FADD complexes observed in CB_1 receptor mutant mice was not the net result of an altered endogenous activation of CB_2 receptors.

In brain regions of CB_2 receptor KO mice, the contents of Fas aggregates and total FADD were normal or showed increases (changes opposite to those in CB_1 receptor KO mice), and p-Ser191 FADD/FADD ratio was not modified in any brain region. These results indicate that endocannabinoids acting on CB_2 receptors do not appear to tonically regulate the expression of pro-apoptotic Fas/FADD complex. In line with these negative findings, the selective CB_2 receptor agonist JWH133 did not alter FADD in the cerebral cortex of CD1 mice. In the striatum of CB_2 receptor KO mice, a marked increase in Fas aggregates was quantified, but the adaptor FADD was unaltered. Therefore, the degree of constitutive activity of CB_2 receptors regulating the apoptotic Fas/FADD complex, if any, is low (but see Viscomi *et al.*, 2009; CB_2 agonists and antagonists induced opposite effects on various effectors).

The alterations of Fas/FADD in brains of CB receptor KO mice did not appear to result in an increased cell death. Thus,

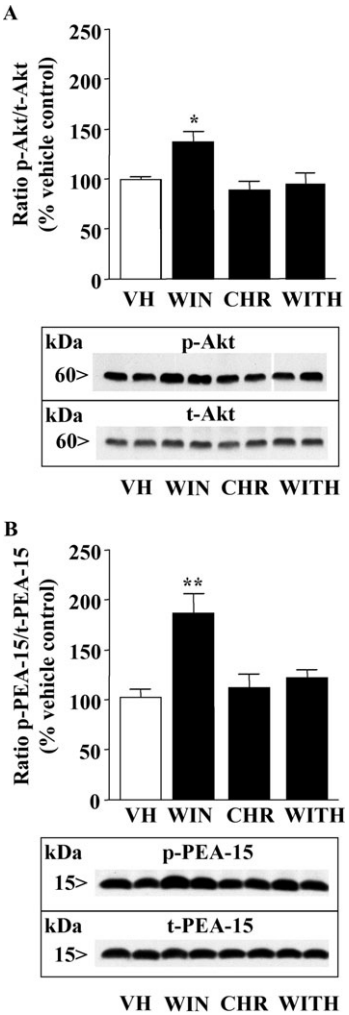

Figure 9 (A and B) Acute, chronic and withdrawal effects of the CB_1 receptor agonist WIN55212-2 on the activation of (A) Akt-1 (expressed as the ratio of phosphorylated enzyme to total enzyme) and (B) PEA-15 (expressed as the ratio of phosphorylated PEA-15 to total PEA-15) in the cerebral cortex of CD1 mice. Groups of mice were treated (i.p.) with drug vehicle (VH, $n = 5$), acute WIN (WIN, 8 mg·kg^{-1}, 1 h, $n = 5$), chronic WIN (CHR, 1–8 mg·kg^{-1}, 5 days, $n = 5$) and chronic WIN followed 4 h later by the CB_1 receptor antagonist rimonabant (WITH, 10 mg·kg^{-1} for 1 h, $n = 5$) and killed at the indicated times. The columns are means ± SEM values of protein immunoreactivity, and expressed as percentage of the corresponding VH-treated group. One-way ANOVA detected significant differences between the groups of treatments for Akt-1 activation [$F(3,16) = 5.88$, $P = 0.007$] and PEA-15 activation [$F(3,16) = 8.01$, $P = 0.0018$]. *$P < 0.05$, **$P < 0.01$ when compared with the corresponding VH group (ANOVA followed by Bonferroni's test). Bottom: representative immunoblots for Akt-1 and PEA-15 in the mouse cerebral cortex, after WIN, CHR or WITH treatments. (A and B). Protein molecular masses (kDa) were estimated from referenced standards.

the pattern of cleavage of the nuclear enzyme PARP-1 (a marker of apoptosis; Putt *et al.*, 2005) in the cerebral cortex of CB_1 or CB_2 receptor KO mice was not different to that in WT mice, which would discount an abnormal activation of executioner caspases-3/7 (Kumar, 2007).

Recently, Fas/FADD content was found to be markedly increased in brains of δ-opioid receptor KO mice, indicating that this constitutively activated receptor (Costa and Herz, 1989) tonically inhibited the pro-apoptotic complex (García-Fuster *et al.*, 2007b). Given the functional interactions between cannabinoids and opiates, and particularly between CB_1 and δ-opioid receptors (Urigüen *et al.*, 2005), the Fas/FADD complex could be, in part, under the opposite tonic control of these inhibitory receptors (δ-receptor: significant reduction in FADD signalling; CB_1 receptor: modest increase in FADD signalling). These mechanisms could be involved in the neuroprotective actions, including the prevention of apoptosis, induced by stimulation of δ-opioid receptors (Narita *et al.*, 2006).

Modulation of FADD and other apoptotic proteins by CB receptors in CD1 mice: role of CB_1 receptors in the regulation of non-apoptotic p-Ser191 FADD

Many *in vitro* and *in vivo* studies have reported neurotoxic or neuroprotective effects induced by natural and synthetic cannabinoids acting at CB receptors (Downer *et al.*, 2003; Maccarrone and Finazzi-Agró, 2003; Aguado *et al.*, 2007). Thus, the agonist WIN55212-2 was shown to induce CB_1 receptor-mediated apoptosis in cultured cells (Pozzoli *et al.*, 2006) and CB_2 receptor-mediated neuroprotection in mice (Price *et al.*, 2009).

In the current study, acute treatment with WIN55212-2 (1 and 8 mg·kg^{-1}) reduced FADD and increased p-Ser191 FADD (p-FADD/FADD ratio also increased) in the cerebral cortex of CD1 mice (effects prevented by rimonabant), clearly indicating that CB_1 receptor stimulation (with moderate/high agonist doses) not only results in attenuation of apoptotic FADD signalling (possible neuroprotection; García-Fuster *et al.*, 2007a; 2008b), but also in the activation of p-Ser191 FADD, the protein form associated with non-apoptotic actions (Alappat *et al.*, 2005) including the induction of neuroplasticity (García-Fuster *et al.*, 2009; Ramos-Miguel *et al.*, 2009; 2010). In contrast, relevant doses of JWH133, a selective CB_2 receptor agonist, did not alter the content of brain FADD or p-Ser191 FADD, suggesting the specific involvement of CB_1 receptors in the regulation of the extrinsic apoptotic pathway.

Notably, acute WIN55212-2 (0.5, 1 and 8 mg·kg^{-1}) modulated in opposite directions FADD and p-Ser191 FADD in the same cortical samples (total homogenate and subcellular compartments), most probably through the interaction of FADD with CK1α, the relevant phosphorylating kinase (Alappat *et al.*, 2005; García-Fuster *et al.*, 2008a). Similar inverse relationships for FADD and p-FADD (with increased p-FADD/FADD ratio) were observed for the effects of opiate drugs (García-Fuster *et al.*, 2007a; 2008a) and cocaine (García-Fuster *et al.*, 2009) in rat brain. These results indicate that different drugs of abuse share the capacity to induce the interconversion between non-phosphorylated FADD and p-FADD, which appears to favour the non-apoptotic (neuroplastic) activities of this multifunctional protein (Ramos-Miguel *et al.*, 2009; 2010). As observed with opiate drugs and cocaine (García-Fuster *et al.*, 2007a; 2009), WIN55212-2 also induced bell-shaped dose effects on brain FADD and p-Ser191 FADD, although the inhibition of FADD and the stimulation of p-FADD were more marked. Various biphasic effects induced by endocannabinoids (Sulcova *et al.*, 1998) and synthetic cannabinoids (Rubino *et al.*, 2008) have been reported,

which could be related to the fact that CB_1 receptors are activated under basal conditions (see above; FADD stimulation at a low dose of WIN55212-2, and FADD inhibition at larger agonist doses that would neutralize receptor constitutive activity when drug concentration is greater than that of endogenous ligand; see Barna *et al*., 2009).

It is noteworthy that chronic WIN55212-2 administration (5 days) also resulted in down-regulation of FADD and up-regulation of p-Ser191 FADD in the brain, which indicate a sustained attenuation of apoptotic signalling in spite of the induction of some tolerance (tachyphylaxis) upon the repeated stimulation of CB_1 receptors (Sim-Selley, 2003). Rimonabant-precipitated WIN55212-2 withdrawal did not cause a rebound of FADD or p-Ser191 FADD over control values. However, similar acute and chronic treatments of rats with WIN55212-2 did not alter brain FADD (García-Fuster *et al*., 2007a), indicating important species differences in total FADD regulation by the CB receptor agonist.

Interestingly, neither acute/chronic WIN55212-2 (and withdrawal effects) nor acute JWH133 altered the brain contents of cytochrome *c* (a mitochondrial activator of caspase-3; Galluzzi *et al*., 2009) and AIF (the main mitochondrial mediator of caspase-independent apoptosis and a key cell death effector in neurones; Galluzzi *et al*., 2009), suggesting a lack of abnormal activation of the intrinsic apoptotic pathway after the stimulation of CB receptors. In brains of CD1 mice, moreover, the cleavage of PARP-1 (mediated in part by caspases-3/7 and AIF) was not altered by the various WIN55212-2 treatments. In contrast to these *in vivo* data, Δ^9-THC was reported to promote, through the activation of CB_1 receptors, apoptotic effects (increased cytochrome *c* and caspase-3, and DNA fragmentation) in cultured cortical neurones (Downer *et al*., 2003). However, treatment of adult rats with Δ^9-THC did not result in activation of the mitochondrial apoptotic pathway (Downer *et al*., 2007). These results highlight the relevance of *in vivo* studies in the assessment of programmed cell death regulation by cannabinoids.

Regulation of MAPKs and Akt1/PEA-15 pathways by CB_1 receptors in the mouse brain

Numerous intracellular signalling systems are involved in mediating the effects of CB receptors (Demuth and Molleman, 2006). In particular, the *in vivo* stimulation of CB_1 receptors with Δ^9-THC has been shown to induce the phosphorylation (activation) of ERK1/2 (Valjent *et al*., 2001) and PI3K/Akt (Ozaita *et al*., 2007) in rodent brain regions. Therefore, the activation of these anti-apoptotic cascades could participate in the neuroprotection induced by cannabinoids (see Ozaita *et al*., 2007). However, the acute sequential stimulation of Raf-MEK1/2-ERK1/2 induced by WIN55212-2 in the rat cerebral cortex was no longer observed after chronic agonist treatment, indicating that the activation of this neuroprotective system by CB_1 receptors is short lasting (Moranta *et al*., 2007). Interestingly, the activation of Akt1 stimulates p-Ser116 PEA-15 to promote, together, anti-apoptotic actions (see Ramos-Miguel *et al*., 2009).

In the present study, acute, but not chronic, treatment with WIN55212-2 markedly stimulated the activation of anti-apoptotic ERK1/2 and Akt1/p-Ser 116 PEA-15, as well as pro-apoptotic JNK1/2 and p38 MAPK in the mouse cerebral cortex. This suggests that the acute *in vivo* neuroprotection induced by some cannabinoids (see above) could be the result of a favourable balance between the relative activation of anti- and pro-apoptotic signalling pathways. In contrast to FADD and p-Ser191 FADD, the lack of a sustained stimulation of anti- and pro-apoptotic cascades upon chronic WIN55212-2 treatment probably reflects the rapid induction of CB_1 receptor desensitization (Sim-Selley, 2003) in the regulation of these systems. Also at variance with FADD, rimonabant-precipitated withdrawal after chronic WIN55212-2 was associated with significant rebounds in the phosphorylation of JNK1/2 and p38 MAPK, but not of ERK1/2 and Akt1/PEA15, demonstrating the rapid activation of pro-apoptotic cascades (without apparent functional repercussion; see above) during the expression of the cannabinoid withdrawal syndrome in mice. This reactivation of JNK1/2 and p38 MAPK could be related to the increase in the content/release of anandamide observed in brains of cannabinoid-withdrawn rats (González *et al*., 2004), these pro-apoptotic cascades being more sensitive to the endogenous agonist.

The present *in vivo* study indicates that the acute and chronic stimulation of CB_1 receptors is associated with a marked down-regulation of brain FADD, a major pro-apoptotic molecule of the extrinsic cell death pathway. This may represent a relevant molecular mechanism to explain, in part, the neuroprotective effects induced by natural and synthetic cannabinoids (Guzmán *et al*., 2002). In addition, the acute and chronic stimulation of CB_1 receptors was also associated with up-regulation of p-Ser191 FADD, the protein form that mediates non-apoptotic actions including brain plasticity (Ramos-Miguel *et al*., 2009; 2010). The link between CB_1 receptors and the multifunctional protein FADD provides new insights into the complex neurobiology of the cannabinoid system.

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Conflict of interest

The authors declare no conflict of interest.

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Supporting information

Additional Supporting Information may be found in the online version of this article.

Figure S1 Acute effects of the CB_1 receptor agonist WIN55212-2 on the contents of (A) cytochrome *c* and (B) AIF in the cerebral cortex of CD1 mice. Groups of mice were treated (i.p.) with drug vehicle (VH, $n = 12$) or WIN (0.5, 1 and 8 $mg \cdot kg^{-1}$, 1 h, $n = 5$ for each group). The columns are means ± SEM values of protein immunoreactivity and expressed as percentage of the corresponding VH-treated group. One-way ANOVA did not detect significant differences between the groups of treatments for cytochrome *c* [$F(3,23) = 3.00$, $P = 0.054$] or AIF [$F(3,23) = 0.77$, $P = 0.523$]. Bottom: representative immunoblots for the effects of WIN on cytochrome *c*, AIF and β-actin in the mouse cerebral cortex. Similarly, chronic WIN55212-2 treatment (1–8 $mg \cdot kg^{-1}$, 5 days) was not associated with significant changes in cytochrome *c* or AIF (data not shown) (C) Effects of vehicle (VH, $n = 5$), acute WIN55212-2 (WIN, $n = 5$), chronic WIN (CHR, $n = 5$) and chronic WIN followed by withdrawal (WITH, $n = 5$) on the content of PARP-1 and its cleaved fragment in the cerebral cortex of CD1 mice. One-way ANOVA did not detect significant differences between the groups of treatments for PARP-1 [$F(3,16) = 2.2$, $P = 0.12$] and its fragment [$F(3,16) = 2.37$, $P = 0.11$]. Bottom: representative immunoblot for the effects of VH, WIN, CHR and WITH on the pattern of PARP-1 cleavage in the mouse cerebral cortex ($n = 2$ for each group of treatment). (A–C) Protein molecular masses (kDa) were estimated from referenced standards.

Table S1 Antibodies used for the detection and quantification of apoptotic proteins and other signalling molecules in the mouse brain.

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