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Role of Multifunctional FADD (Fas-Associated Death Domain) Adaptor in Drug Addiction

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1. Introduction

Human drug addictions are chronic medical disorders characterized by tolerance and dependence to the abused substance, incentive sensitization, loss of control over drug use that becomes compulsive, relapse (Belin & Everitt, 2010), and in some cases high mortality. A large body of research has established that the majority of drugs leading to addiction stimulate dopamine release through the meso-cortico-limbic circuit in laboratory animals and humans (e.g. see Badiani et al., 2011). Brain neuroadaptations along the reward system are a focus of current research, especially those induced in the prefrontal cortex of human addicts (Goldstein & Volkow, 2011). These persistent neuroplastic events appear to be major causes for compulsive drug-seeking behavior despite the negative effects (e.g., neurotoxicity) induced by drugs of abuse in humans (Nutt et al., 2007).

It is generally accepted that some addictive drugs can induce cell death in the human brain, following observations that neurons and astrocytes die when exposed to drugs of abuse (Cunha-Oliveira et al., 2008; Büttner, 2011). Neurotoxicity and neuroplasticity acting together in the addicted brain might explain the dampened cognition and the reinforced behaviors driving to drug consumption. The best-studied cell-killing machinery is the so-called programmed cell death or apoptosis (Galluzzi et al., 2011). *In vivo* studies have reported controversial data for drugs of abuse regulating the apoptotic machinery in the brain (Tegeder & Geisslinger, 2004). Moreover, other findings have revealed important roles of pro-apoptotic proteins in the molecular mechanisms mediating synaptic and structural plasticity in the brain (Gilman & Mattson, 2002). Indeed, proteins belonging to the extrinsic apoptotic pathway have gained special interest in the study of neuroplastic machinery for their functional duality, promoting either apoptosis or cell survival and differentiation (Park et al., 2005; Tourneur and Chiocchia, 2010). Thus, Fas-associated death domain (FADD) protein is the most proximal adaptor molecule that mediates the signaling of death receptors belonging to the tumor necrosis factor receptor superfamily (TNFRSF), such as Fas or TNFRSF6 receptor (Tourneur and Chiocchia, 2010). Although the main role of FADD adaptor is to engage cell death through the extrinsic apoptotic pathway (Galluzzi et al., 2011), it also mediates non-apoptotic actions in cell systems *in vitro* (Park et al., 2005) and has a critical role in embryogenesis (Imtiyaz et al., 2009). In the CNS, Fas receptor

dysregulation is associated with a number of disease states, including neurodegenerative disorders (Sharma et al., 2000). Fas stimulation can also promote neurite outgrowth and neuronal branching, which suggests the induction of neuroplastic responses in neurons (Lambert et al., 2003; Reich et al., 2008). Notably, FADD can translocate to the nucleus, a process favoured by its phosphorylation, and regulate nuclear factors, possibly altering the genetic profile of the cell, and promoting differentiation, neuroplasticity, and/or other anti/non-apoptotic actions.

All these features made of FADD an intriguing molecule for the study of brain neurotoxicity and/or neuroplasticity induced by drugs of abuse. This chapter reviews current evidence on the new roles of brain FADD in the complex neurobiology of drug addiction. After a brief overview on Fas/FADD complex and specific features of FADD protein, the involvement of multifunctional FADD and associated signalling in the acute and chronic effects of opiates, cocaine and cannabinoids are summarized from biochemical and behavioral studies performed in rat, mouse and human brains.

2. Relevant features of FADD protein

2.1 Fas/FADD complex: Pro-apoptotic function

In the standard model of Fas-mediated cell death (binding of FasL resulting in receptor trimerization; Algeciras-Schimmich et al., 2002), Fas and FADD are bound through homotypic death domain (DD) interactions (Fas/FADD complex) (Fig. 1A). Then FADD can recruit death effector domain (DED)-containing initiator pro-caspase 8 (and other molecules such as FLIP and PEA-15) to form a death inducing signalling complex (DISC), which finally promotes the activation of death-effector caspases (mainly caspase-3) with the final cleavage of downstream vital cellular substrates. Recently, two models of Fas/FADD-DISC (Scott et al., 2009; Wang et al., 2010) and a likely 5 Fas:5 FADD stoichiometry (Fig. 1A; Wang et al., 2010) have been proposed based on the crystal structures of the proteins. Therefore, FADD

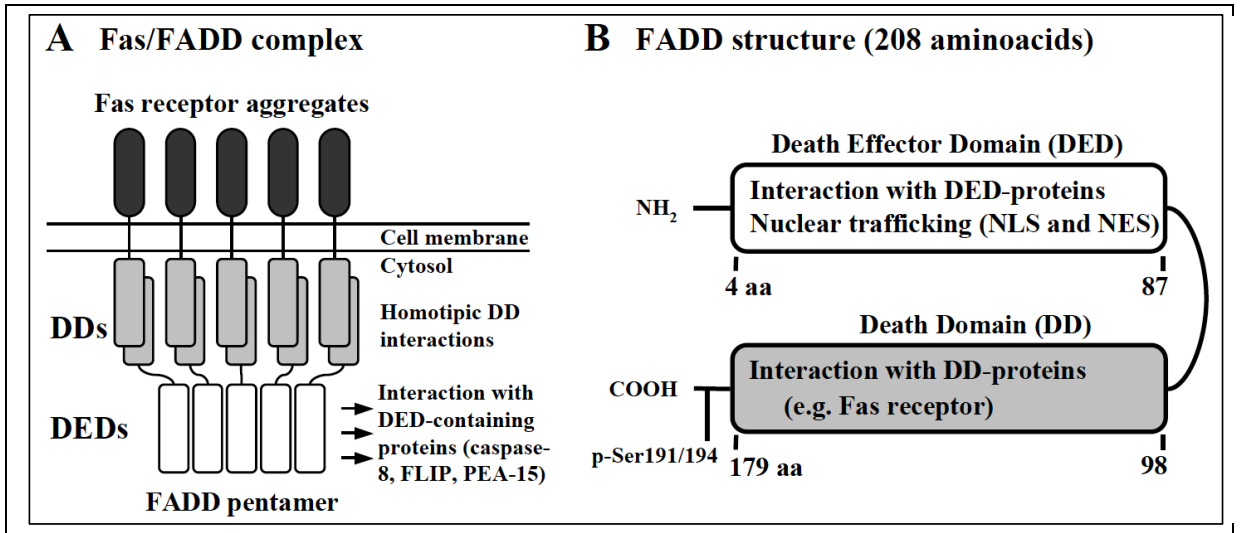


Fig. 1. (A) Fas/FADD-DISC complex. (B) FADD protein structure and domains (DD and DED). NLS: nuclear localization signals; NES: nuclear export signals. FLIP: FADD-like interleukin-1 β -converting enzyme-inhibitory protein; PEA-15: phosphoprotein enriched in astrocytes of 15 kDa.

can form functional homo-oligomers of high molecular mass (oligomeric signalling complexes) which have been shown to increase the efficiency of Fas apoptotic signalling in normal and cancer cells (Sandu et al., 2006). Cell death in the CNS shares the same basic mechanisms operating in peripheral cells. Thus, brain apoptosis can be initiated through the extrinsic (Fas receptor) and intrinsic (mitochondrial) pathways, which converge to the activation of executioner caspases (Sastry and Rao, 2000).

2.2 FADD phosphorylation: Nuclear localization and functional implications

The structure of FADD displays, outside its C-terminal DD region (Fig. 1B), a single serine phosphorylation site (p-Ser191 in mouse, p-Ser194 in human; p-Ser194 or p-Ser195 in rat; Zhang et al., 2004; García-Fuster et al., 2008a). This phosphorylation of FADD, mainly mediated by casein kinase 1 α (CK1 α), is essential for the non-apoptotic actions of this multifunctional protein, such as the regulation of cell growth and differentiation (Alappat et al., 2005).

Although FADD was initially thought to be a cytoplasmic protein, it contains nuclear localization and export signals (NLS/NES; Fig. 1B) that allow its nuclear translocation (Gómez-Angelats and Cidlowski, 2003). Some studies have even reported that FADD is predominantly stored in the nucleus of resting cells, being redistributed to the cytoplasm upon Fas receptor activation (Föger et al., 2009). In any case, p-FADD is the main protein species translocated to the nucleus (Screaton et al., 2003). Nuclear p-FADD is involved in the anti-apoptotic actions of the molecule through the modulation of critical factors (Screaton et al., 2003; Alappat et al., 2005).

2.3 FADD adaptor in the brain: Immunodetection of protein forms and regional distribution

In rat, mouse and human brain tissue, various commercially available antibodies tested against FADD (up to seven) readily immunolabeled a \approx 51-kDa band corresponding to its dimeric form (Fig. 2A, left panel). To a lesser extent, these antibodies also reacted against the monomeric (\approx 20-23 kDa) and other FADD species of higher magnitude (\approx 92-116 kDa) (García-Fuster et al., 2008a). In contrast, different antibodies against p-FADD recognized 92-116-kDa bands corresponding to oligomeric p-FADD species (Fig. 2A, right panel). Noteworthy, these higher FADD structures fit well with the recently proposed pentameric model of DISC association (see Fig. 1A; Wang et al., 2010). In addition, some of these antibodies immunodetected the monomeric p-FADD species (García-Fuster et al., 2008a; Ramos-Miguel et al., 2009) (Fig. 2A, right panel). The ability of these phospho-directed antibodies to label p-FADD species was challenged with the alkaline phosphatase assay, which demonstrated the specificity of these antibodies to bind to the p-sites of the protein (Fig. 2A, right panel). Therefore, in brain tissue, it is likely that non-p-FADD is more stable as a dimer, and its phosphorylation switches FADD self-associative properties. Thus, these FADD (dimers) and p-FADD (monomers and oligomers) forms were initially selected to assess the role of multifunctional FADD protein in the molecular mechanisms of drug addiction. To note that some p-FADD species (e.g. \approx 45 kDa form; Fig. 2A, right) most probably represent degradation products of higher mass p-oligomers. These and other technical issues are largely discussed in previous reports (García-Fuster et al., 2008a).

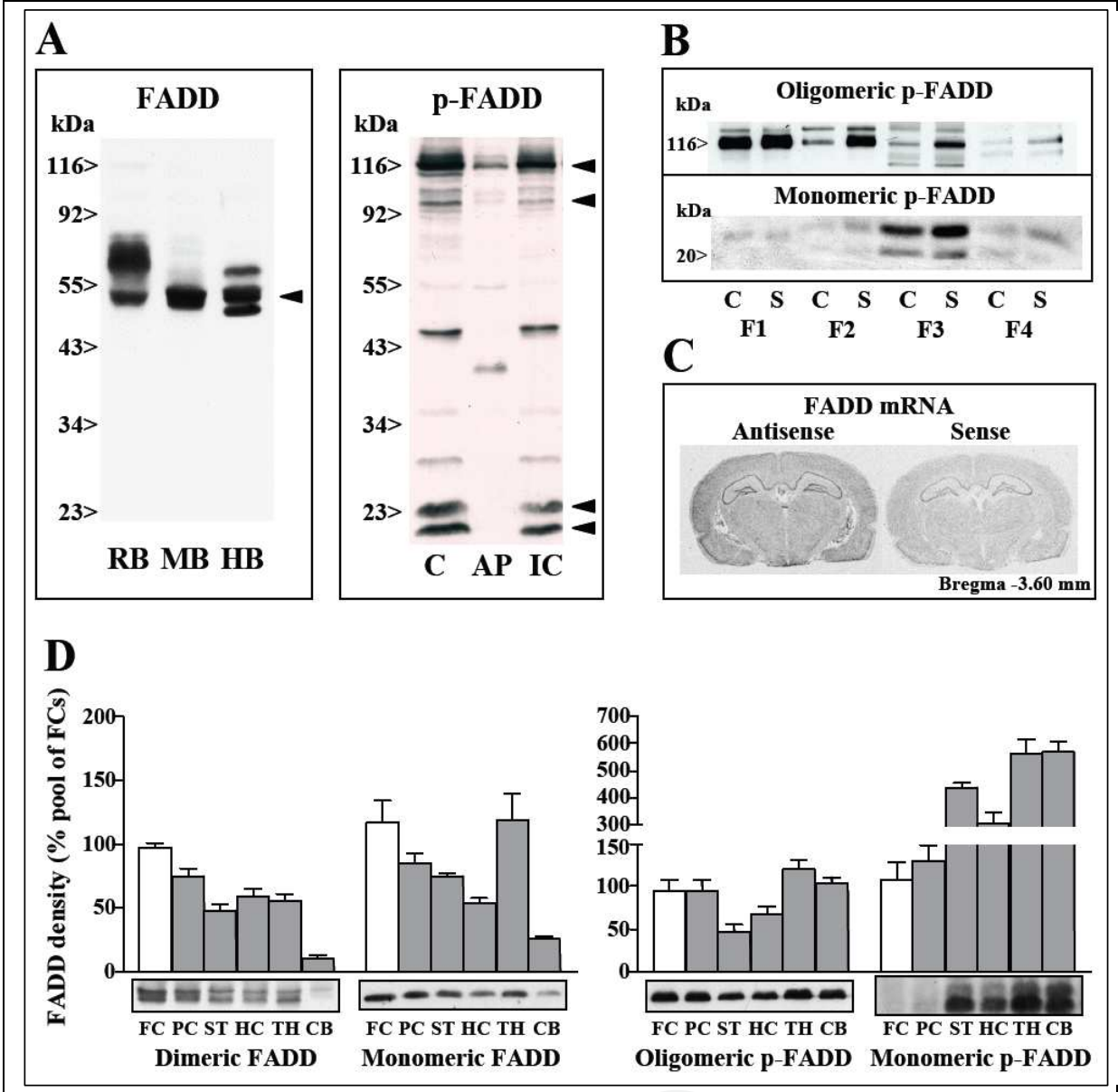


Fig. 2. (A/B) Immunodetection of FADD protein forms (arrow heads: monomeric, dimeric and oligomeric nonphosphorylated and phosphorylated species) in brain total homogenate (RB: rat cortex; MB: mouse cortex; HB: human cortex; C: rat striatum, control sample; AP: alkaline phosphatase; IC: inhibited control, alkaline phosphatase plus sodium pyrophosphate) and subcellular compartments (rat cortex; F1: cytosol; F2: membranes; F3: nucleus; F4: cytoskeleton), in which the acute effect of sufentanil (S: 0.015 mg/kg, s.c., 30 min) on p-FADD is shown (C: control saline). Protein sizes (kDa) as visualized in Western blots. (Modified from García-Fuster et al., 2007a, 2008a). (C) Detection of FADD mRNA in rat brain (anatomical level: Bregma -3.60 mm) by in situ hybridization. Note that FADD mRNA (antisense probe) showed very low expression in the brain, except for hippocampal regions and cortex (see García-Fuster et al., 2009). To verify specificity of binding a sense control probe was hybridized in test tissue. (Modified from García-Fuster et al., 2006). (D) Regional distribution of FADD protein forms in the rat brain (FC: frontal cortex, region of reference; PC: parietal cortex; ST: corpus striatum; HC: hippocampus; TH: thalamus; CB: cerebellum).

FADD is expressed in neurons and glial cells (Hartmann et al., 2002; Bi et al., 2008; Tewari et al., 2008). FADD mRNA expression is homogeneous along the brain tissue, as visualized by *in situ* hybridization (Fig. 2C), with slight increased labeling in cortical areas and hippocampus. However, the distribution of FADD protein (monomeric and dimeric species) in rat brain regions is uneven, with a greater content in the cerebral cortex than in cerebellum (Fig. 2D, left). In contrast, p-FADD (monomeric and oligomeric p-species) is highly expressed in cerebellum (Fig. 2D, right). Thus, the ratio of p-FADD to FADD (monomeric species) was much greater in the cerebellum (CB: 22.4) than in cortical areas (FC: 0.91; PC: 1.58) (Fig. 2D). Subcortical regions also display high p-FADD/FADD ratios (ST: 5.87; HC: 5.61; TH: 4.98) (Fig. 2D). The physiological relevance of the marked variation of p-FADD/FADD ratio across brain regions remains to be determined. To note that FADD and p-FADD are well expressed in brain regions (e.g., the frontal cortex and corpus striatum) more closely associated with the behavioral effects of drug of abuse (Fig. 2D). In the human brain, a dynamic relationship between monomeric and oligomeric p-FADD forms has been observed (Ramos-Miguel et al., 2009). Notably, some opiate and cannabinoid drugs, but not cocaine, have been shown to induce the interconversion between FADD and p-FADD (increasing the ratio p-FADD/FADD), which may favor the induction of non-apoptotic (neuroplastic) actions (see below and Fig. 6).

At the subcellular level, FADD and p-FADD (rat, mouse and human brains) are expressed in cytosol and nucleus, and to a lesser extent in membranes (García-Fuster et al., 2007a, 2008a; Ramos-Miguel et al., 2009; Álvaro-Bartolomé et al., 2010) (Fig. 2B and 11D). To note that the monomeric form of p-FADD is particularly well expressed in the nucleus (Fig. 2B) (Ramos-Miguel et al., 2009). Nuclear p-FADD has been reported to play important roles in the molecular mechanisms of opiate addiction in humans (Ramos-Miguel et al., 2009), possibly by regulating nuclear factors such as methyl-CpG binding domain protein 4 (Screaton et al., 2003) and nuclear factor kappaB (Schinske et al., 2011).

2.4 FADD adaptor: Apoptotic and non-apoptotic signalling pathways

Besides the role of FADD in the cascades of apoptotic signaling in drug addiction (García-Fuster et al., 2007a, 2008b; Ramos-Miguel et al., 2009; Álvaro-Bartolomé et al., 2011), several pathways have been postulated to link FADD with some forms of behavioral plasticity induced by drugs of abuse, especially heroin/morphine (Ramos-Miguel et al., 2009, 2010, 2011) and cocaine (García-Fuster et al., 2009, 2011; Álvaro-Bartolomé et al., 2011) (Fig. 3).

These signalling pathways involve, *inter alia*, the extracellular signal-regulated kinase (ERK), the kinase Akt1 or protein kinase B (PKB), and phosphoprotein enriched in astrocytes of 15 kDa (PEA-15), which interactions with FADD are discussed below (see section 3.3) in the context of the acute/chronic effects of opiates, cocaine and cannabinoids (Fig. 3).

3. Role of FADD adaptor in opiate addiction

Opiate addiction is associated with various forms of neurotoxicity, which can result in serious brain dysfunction in most subjects (Yücel et al., 2007; Bütnner, 2011). Moreover, heroin addicts often develop severe immunodeficiencies that could be the result of apoptotic cell death in the immune system (Kreek, 1990; Govitrapong et al., 1998). In fact, morphine was reported to increase, through a naloxone-sensitive mechanism, the expression of Fas receptor mRNA in

mouse splenocytes and in human blood lymphocytes (Yin et al., 1999). However, the possibility of opiate-induced cell death in the mature brain, including the brains of human addicts, still is a debated issue (Boronat et al., 2001; Tegeder and Geisslinger, 2004; Liao et al., 2005; Cunha-Oliveira et al., 2008; García-Fuster et al., 2008b; Tramullas et al., 2008; Zhang et al., 2008).

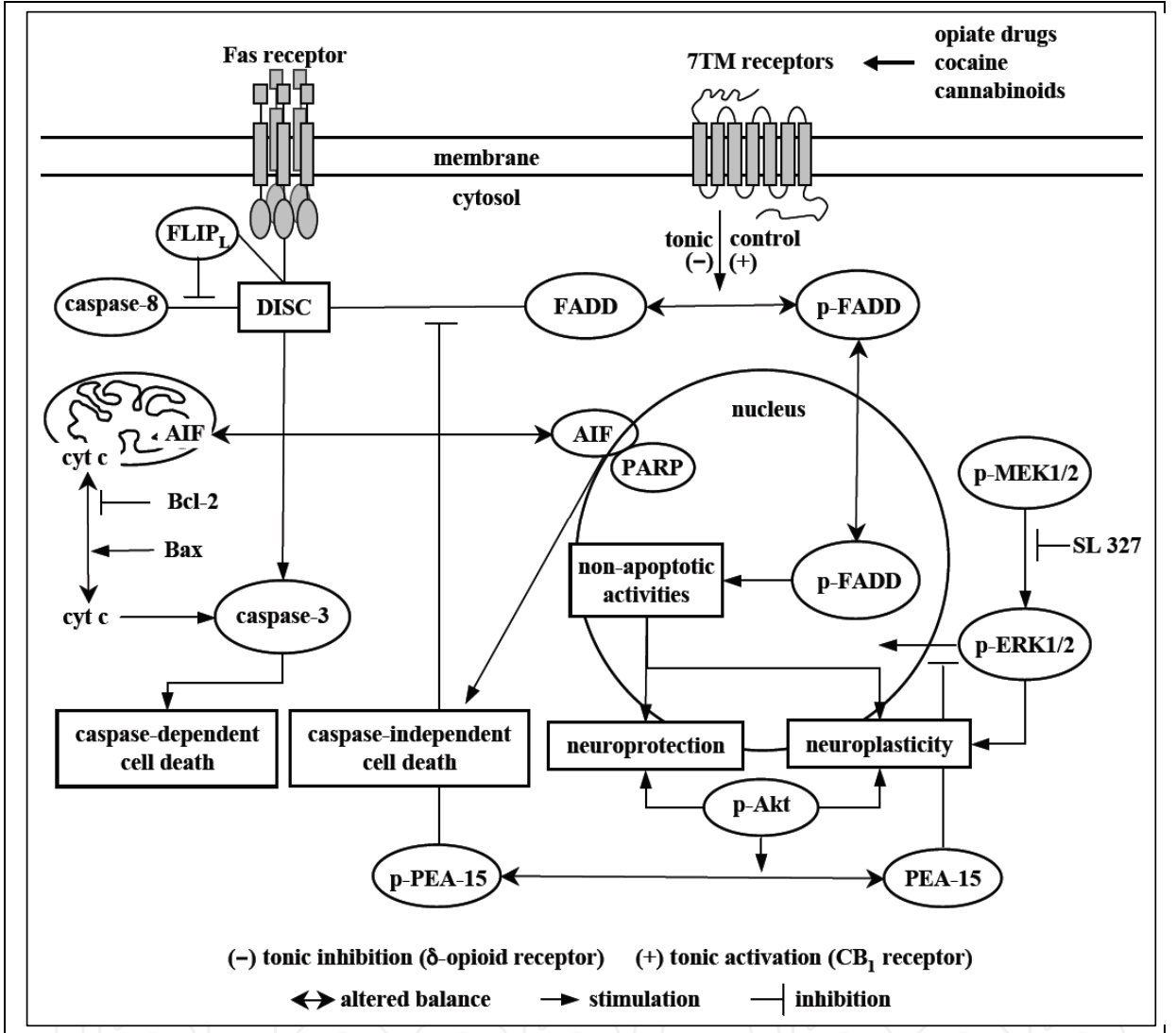


Fig. 3. Schematic diagram illustrating the complex interactions between the multifunctional protein FADD (pro-apoptotic, anti-apoptotic and/or neuroplastic actions) and pro-survival MAP kinases (MEK-ERK) and Akt1/PEA-15 signalling in opiate, cocaine and cannabinoid addiction. See the main text for specific details and comments.

3.1 Regulation of basal Fas/FADD complex by opioid receptors: Anti-apoptotic δ -opioid receptor tone

A relevant interaction between the opioid system and Fas/FADD complex in the brain was disclosed using gene-targeted mice lacking μ -, δ -, or κ -opioid receptors (García-Fuster et al., 2007b). Thus, wild-type (WT) and knock-out (KO) mice were compared to investigate the existence of endogenous opioid tones regulating the basal contents of Fas receptor and FADD adaptor in the brain.

The results indicated that μ - and κ -receptors do not exert a significant tonic control on Fas/FADD complex expression levels in the mouse brain (i.e., no major target changes in μ - and κ -KO mice). In δ -KO mice, however, Fas aggregates (Fas forms triggering receptor signalling) and FADD adaptor were markedly increased in the cortex (Fig. 4) and corpus striatum. Moreover, the basal content of monomeric p-FADD (the FADD species implicated in non-apoptotic signals) was also up-regulated in the cortices of δ -KO mice, which is in line with the observed increase of FADD in these animals (Fig. 4). In this context, it is worth mentioning that inhibitory δ -opioid receptors possess a high level of constitutive (ligand-independent) activity (Costa and Herz, 1989; Neilan et al., 1999), which could control the basal level of some associated signalling molecules such as the Fas/FADD complex.

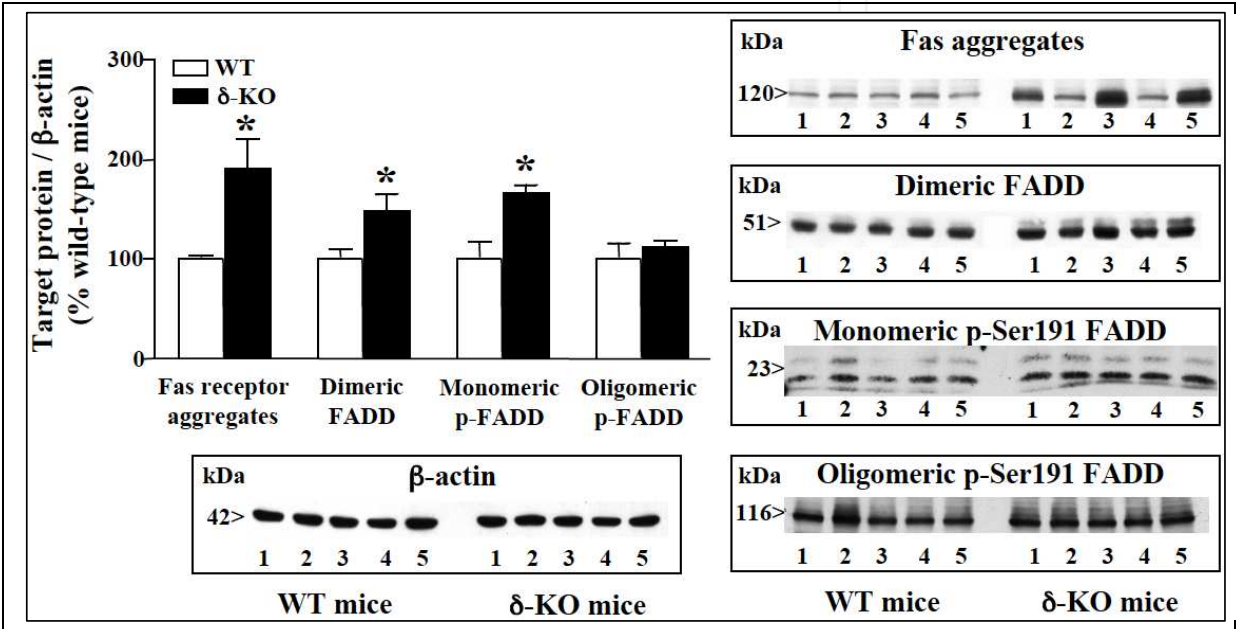


Fig. 4. Fas receptor aggregates, FADD adaptor, and p-FADD (monomeric and oligomeric forms) in the cerebral cortex of WT mice and δ -opioid receptor KO mice. *At least $p<0.05$ versus WT. (Modified from Garca-Fuster et al., 2007b).

Taken together, the findings in δ -KO mice strongly suggest that the functioning of pro-apoptotic Fas/FADD complex *in vivo* is partly under an inhibitory tonic control of brain δ -opioid receptors (i.e., removal of a negative endogenous opioid tone results in Fas/FADD up-regulation; see Fig. 3) (Garca-Fuster et al., 2007b). The anti-apoptotic δ -opioid receptor tone on Fas/FADD complex could play an important role in the neuroprotection afforded by δ -opioid receptor agonists (Narita et al., 2006).

3.2 Acute, chronic and withdrawal effects of opiate drugs on FADD and associated signalling in the brain

Acute and chronic treatments of rats with various opiate drugs (heroin, morphine, SNC-80, U-50488-H, pentazocine), as well as the induction of opiate withdrawal states, were initially shown to result in increases or decreases of various Fas receptor forms in the brain (Boronat et al., 2001q; Garca-Fuster et al., 2003, 2004). Thus, heroin/morphine addiction in rats was associated with up-regulation of both native and aggregated forms, thereby suggesting the

induction of pro-apoptotic actions in the brain (García-Fuster et al., 2003, 2004). In contrast, similar treatments with morphine and selective μ -(fentanyl, sufentanil), δ -(SNC-80) and κ -(U-50488-H) opioid receptor agonists were associated with receptor-specific reductions of FADD, except for the chronic treatments that show tachyphylaxis to the acute drug effects in the brain (Fig. 5) (García-Fuster et al., 2007a).

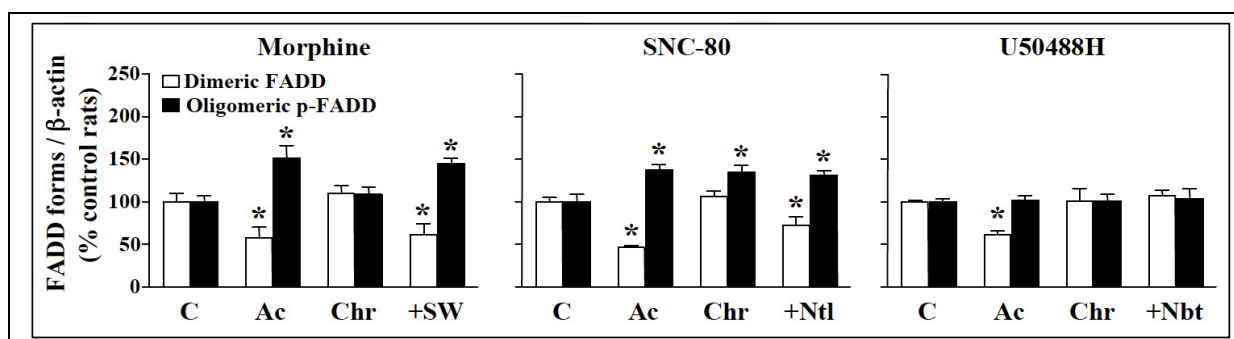


Fig. 5. Acute (Ac), chronic (Chr) and spontaneous (+SW) or antagonist precipitated (naltrindole, Ntl; nor-binaltorphimine, Nbt) withdrawal effects of μ -(morphine), δ -(SNC-80) and κ -(U50488H) opioid receptor agonists on FADD and p-FADD in rat brain cortex. *At least $p < 0.05$ versus control (C). (Modified from García-Fuster et al., 2007a, 2008a).

As a matter of fact, the modulation of FADD by opiate drugs is opposite to that of Fas receptor, which suggests that possible apoptotic signals engaged by Fas activation would be offset by a lesser signal transduction through FADD adaptor. Indeed, μ/δ -opiate agonists increased the content of p-FADD in the brain (Fig. 5; see also Fig. 2B for the acute effect of sufentanil on p-FADD in subcellular compartments), which suggests the induction of non-apoptotic (neuroplastic) effects by these drugs (see Fig. 3) (García-Fuster et al., 2007a, 2008a). On the other hand, SNC-80-induced down-regulation of FADD in rat brain (cortex and striatum) was blunted after the inhibition of the MEK-ERK pathway *in vivo*, which demonstrates the direct involvement of this anti-apoptotic signalling in FADD regulation (García-Fuster et al., 2007a). On the other hand, the molecular mechanism by which seven transmembrane (7TM) receptors interact with FADD (i.e., G protein dependent or independent process; see Fig. 3) remains to be fully determined (see García-Fuster et al., 2008a).

Remarkably, morphine, sufentanil and SNC-80 (acute, chronic and/or withdrawal effects) up-regulated the content of p-FADD with a concomitant decrease of total FADD in rat brain cortex (Fig. 6A), indicating that these drugs promote an increase in the ratio of p-FADD to FADD (a proposed index of non-apoptotic activity). The inverse relationship between p-FADD and FADD is likely to be due to changes in the phosphorylation status, possibly mediated by CK1 α , of the adaptor molecule induced by opiate drugs (García-Fuster et al., 2008a; Ramos-Miguel et al., 2009). These findings support the concept of an interconversion between non-phosphorylated FADD and phosphorylated FADD after exposure to opiate drugs, which appears to be a relevant molecular mechanism in morphine-induced neuroplasticity (see below). A similar inverse correlation between p-FADD and FADD has been observed for the acute effects of the CB₁ receptor agonist WIN55212-2 (Fig. 6B), but not for the psychostimulant cocaine (Fig. 6C).

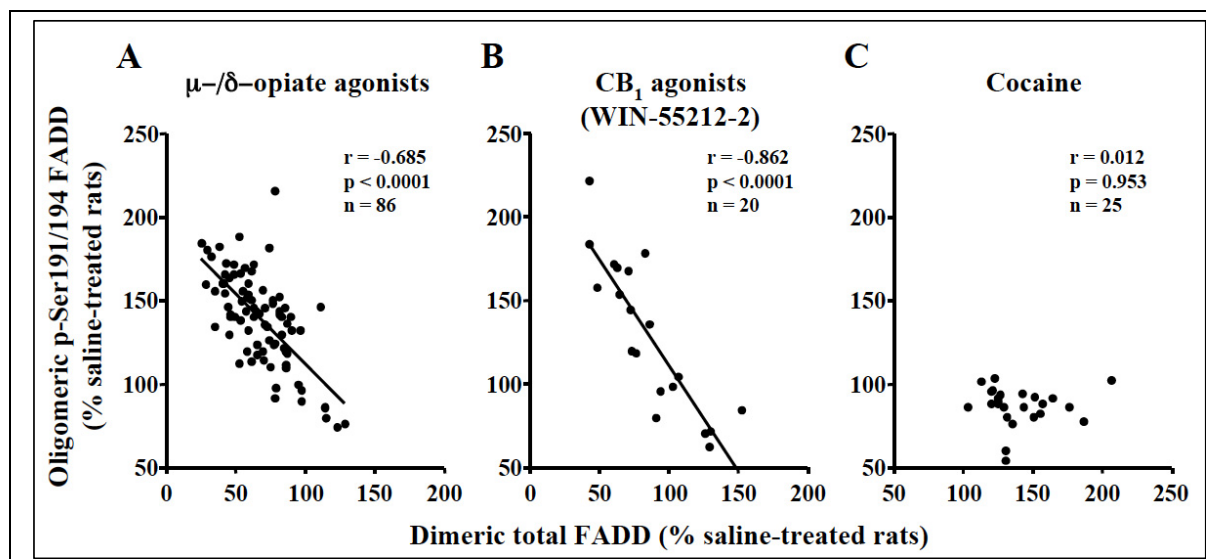


Fig. 6. Inverse correlation between the contents of p-FADD and FADD in rat or mouse brain cortex (each point corresponds to an animal). (A) Opiate agonists: effects of acute (30 and 100 mg/kg) and chronic (10-100 mg/kg; 6 days) morphine, and spontaneous morphine withdrawal (1-3 days); effects of acute sufentanil (15-30 mg/kg); effects of acute SNC-80 (10-30 mg/kg). (B) CB_1 receptor agonist: effects of acute WIN55212-2 (0.5-8 mg/kg). (C) Effects of acute cocaine (3-30 mg/kg). (Modified from García-Fuster et al., 2008a, 2009; Álvaro-Bartolomé et al., 2010).

3.3 FADD phosphorylation correlates with morphine-evoked behaviors

Recent findings have revealed a direct role of p-FADD in the molecular mechanisms leading to the expression of unconditioned morphine-induced psychomotor sensitization (Ramos-Miguel et al., 2010) and to the expression of spontaneous morphine abstinence syndrome (Ramos-Miguel et al., 2011) in rats.

To develop sensitization to morphine (Ramos-Miguel et al., 2010), rats were subjected to a standard treatment protocol (Fig. 7A, left) in which they received saline (controls) or morphine (10 mg/kg/day) for 5 days in absence of environmental cues. After 3 (day 8 of the treatment; Fig. 7A) or 14 days of spontaneous saline/morphine withdrawal (SW3 and SW14, respectively), all rats received a morphine challenge (10 mg/kg) to assess the expression of locomotor sensitization, which was observed at SW3 (Fig. 7A) but not at SW14 (Ramos-Miguel et al., 2010). In parallel to morphine-induced behavioral sensitization, striatal FADD was modulated at SW3, but not at SW14. Thus, p-FADD was up-regulated (Fig. 7A, right) whereas FADD content was decreased (not shown) at SW3. Therefore, the ratio p-FADD/FADD (a postulated marker of neuroplasticity) was increased (2.6-fold) in rat striatum. Similarly, ERK activity was also enhanced in the same striatal samples (Fig. 7A, right). Notably, inhibition of MEK-ERK signalling attenuated the expression of morphine-induced psychomotor sensitization and fully prevented the up-regulation of p-FADD at SW3 (Fig. 7A). The Akt1/PEA-15 pathway, which may link ERK and FADD functions (see Fig. 3), was also activated at SW3, being dependent on the integrity of MEK-ERK signalling (Fig. 7A, right). Taken together, these findings reveal a major role of p-FADD, interacting with MEK/ERK and Akt1/PEA-15, in mediating the short-lasting expression of unconditioned psychomotor sensitization induced by morphine in rats.

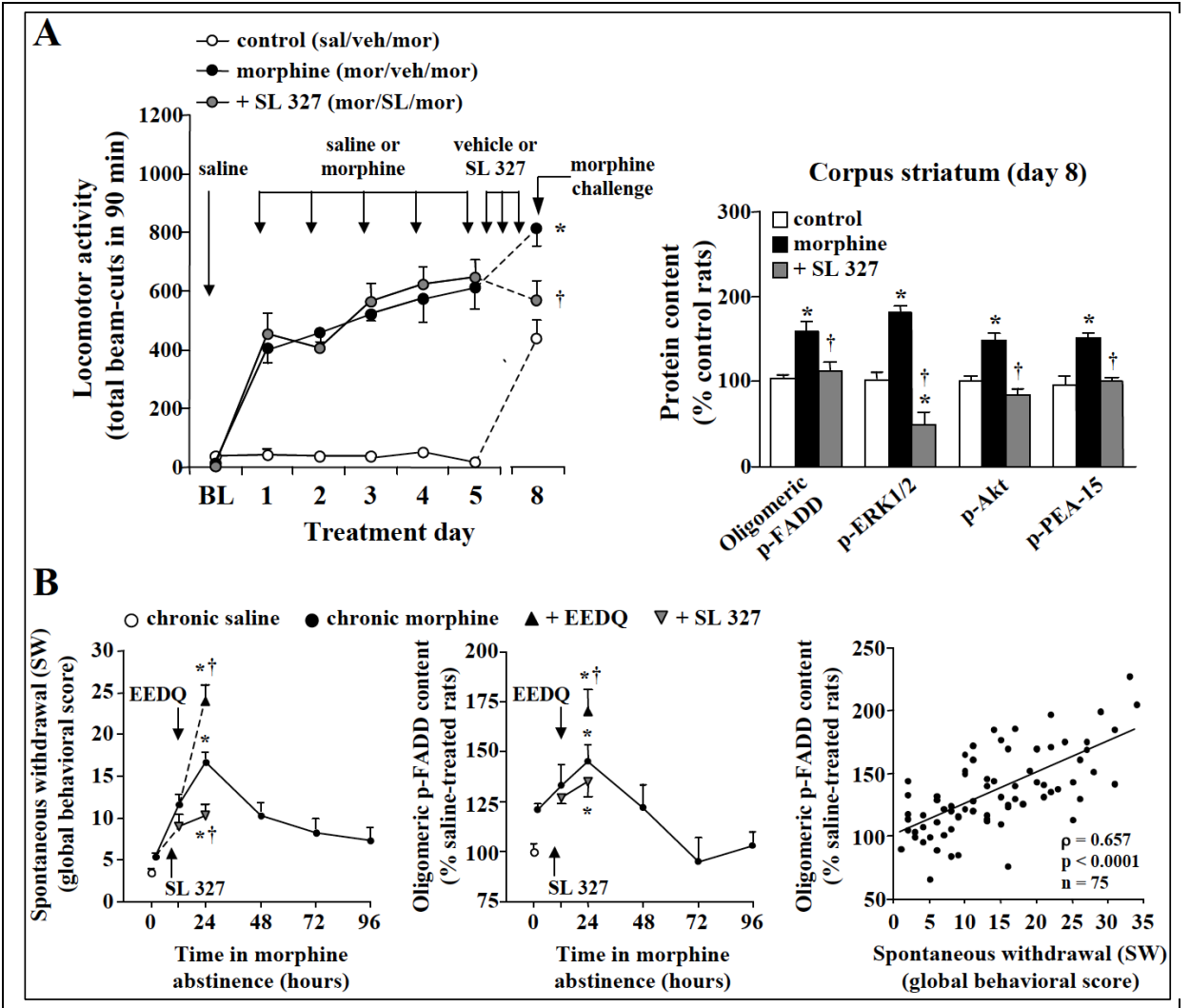


Fig. 7. (A) FADD phosphorylation and expression of unconditioned morphine-induced psychomotor sensitization. Note that day 8 of the treatment corresponds to SW3 (see text). (B) FADD phosphorylation and intensity of spontaneous morphine abstinence syndrome. BL, baseline; SL327, an inhibitor of MEK in vivo; EEDQ, an alkylating of α_2 -adrenoceptors. *At least $p < 0.05$ versus controls, †at least $p < 0.05$ versus morphine-treated rats. (Modified from Ramos-Miguel et al., 2010, 2011).

To explore the role of FADD in the mechanisms of morphine-induced physical dependence, the regulation of cortical p-FADD was investigated during the development of spontaneous opiate withdrawal (SW) in morphine-dependent rats (10-100 mg/kg for 6 days) (Ramos-Miguel et al., 2011). Notably, cortical p-FADD mirrored the time course of morphine SW (12-96 h; peak at 24 h) (Fig. 7B, left), which resulted in a striking correlation between p-FADD and the intensity of morphine abstinence (Fig. 7B, right). On the other hand, the involvement of α_2 -adrenoceptors in opiate addiction is well-known, and the stimulation of these inhibitory receptors induces anti-withdrawal effects in morphine-dependent animals and in human addicts. Interestingly, the inactivation of brain α_2 -adrenoceptors (EEDQ at SW12) (Fig. 7B, left) further enhanced morphine abstinence intensity and cortical p-FADD content at SW24 (Fig. 7B, right and middle panels). The disruption of ERK signalling (SL 327

at SW4 and SW8) did not alter morphine abstinence at SW12, but did attenuate the behavioral syndrome at SW24 (Fig. 7B, left). ERK inhibition, however, did not prevent the up-regulation of p-FADD at SW12 and SW24 (Fig. 7B, middle panel). Taken together, these findings reveal that cortical p-FADD, mainly through an interaction with α_2 -adrenoceptors, plays a functional role in the behavioral expression of morphine abstinence in rats.

Together, these studies indicate that relevant behavioral adaptations induced by repeated morphine exposure in rats correlate with an increased p-FADD/FADD ratio in the cerebral cortex, which strongly suggests that multifunctional FADD is involved in the complex molecular mechanisms of opiate-induced neuroplasticity.

3.4 Regulation of apoptotic pathways and associated signalling in brains of opiate addicts: p-FADD and neuroplasticity

Recent studies have investigated the role of Fas receptor, FADD adaptor and its phosphorylation, other pro- and anti-apoptotic proteins, and FADD-associated signalling pathways, in postmortem brains of long-term opiate addicts (García-Fuster et al., 2008b; Ramos-Miguel et al., 2009). The prefrontal cortex (Brodmann’s area 9, middle frontal gyrus; PFC/BA9) was the region selected for examination because it is directly related with the mesocorticolimbic dopaminergic system and the rewarding and addictive properties of opiates and other drugs of abuse.

First, the hypothesis was tested that human opiate addiction is associated with an increased cell death in the brain (García-Fuster et al., 2008b). In a well-characterized cohort (n=48) of heroin or methadone abusers (including the assessment of opiates and metabolites in blood, urine, and hair samples), the content of Fas receptor in PFC/BA9 did not differ from that in age-, gender-, and postmortem delay-matched controls (Fig. 8A). In contrast, FADD adaptor was down-regulated in the same brain samples of short- and long-term opiate addicts (Fig. 8A). Furthermore, initiator caspase-8 was not altered, but FLIP_L content, a dominant inhibitor of caspase-8, was increased in long-term opiate addicts.

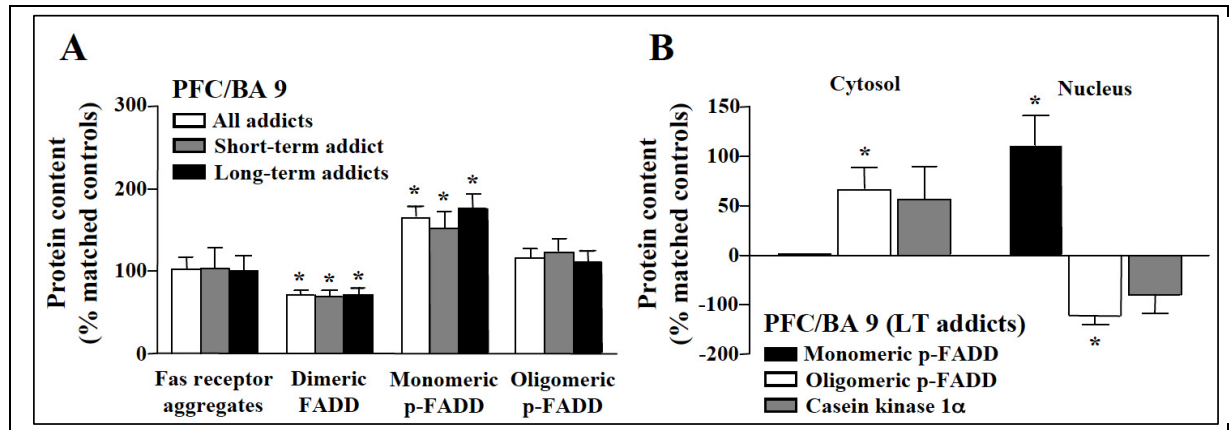


Fig. 8. (A) Contents of Fas receptor aggregates, FADD adaptor, and p-FADD in the prefrontal cortex/Brodmann’s area 9 (PFC/BA 9; total homogenate samples) of short- and long-term opiate abusers. (B) Subcellular content (increases or decreases in cytosol and nucleus) of p-FADD and CK1 α in the PFC/BA9 of long-term (LT) opiate addicts. *At least $p<0.05$ versus matched controls. (Modified from García-Fuster et al., 2008b; Ramos-Miguel et al., 2009).

In the intrinsic mitochondrial pathway, pro-apoptotic Bax and AIF (apoptosis-inducing factor) were unchanged, cytochrome c (a potent caspase-3 activator) was reduced, and anti-apoptotic Bcl-2 augmented in long-term opiate addicts. Importantly, the content of executioner caspase-3/active fragments and the pattern of cleavage of nuclear PARP-1 (poly-(ADP-ribose)-polymerase-1), a hallmark of apoptosis, were very similar in opiate addicts and control subjects.

Taken together, these findings indicate that the molecular machineries of canonical apoptotic pathways are not abnormally activated enough in the PFC/BA9 of opiate abusers to suggest higher rates of cell death in this brain region. Instead, the long-term adaptations of FADD and cytochrome c (down-regulation) and those of FLIP_L and Bcl-2 (up-regulation) could be related to the induction of non-apoptotic actions including phenomena of neuroplasticity in brains of opiate addicts.

Therefore, the role of p-FADD and FADD-associated signalling pathways involved in neuroplasticity was investigated (Ramos-Miguel et al., 2009) in the same cohort and brain region of opiate abusers (García-Fuster et al., 2008b). In these subjects, the content of monomeric, but not oligomeric, p-FADD was markedly increased in the PFC/BA9 of short- and long-term opiate abusers (Fig. 8A, total homogenate samples). At the subcellular level (PFC/BA9), long-term opiate addiction was associated with up-regulation of monomeric p-FADD and down-regulation of oligomeric p-FADD in the nucleus (Fig. 8B). In the cytosol, in contrast, oligomeric p-FADD was increased (Fig. 8B). Along this line, CK1 α , the enzyme that mediates p-FADD, was found co-localized with FADD in cytosol and nucleus (Fig. 8B). These findings appear to indicate that FADD is phosphorylated (and oligomerized) in the cytosol of cortical cells (PFC/BA9), and translocates to the nucleus, where it is disaggregated to monomers to develop its nuclear functions (see Fig. 3).

In long-term opiate addicts, on the other hand, marked down-regulation of ERK1/2, JNK1/2 (c-Jun N-terminal Kinase), PEA-15 and Akt1 signalling were observed in the PFC/BA9 (total homogenate and subcellular compartments) (Ferrer-Alcón et al., 2004; Ramos-Miguel et al., 2009). Remarkably, down-regulation of ERK1/2 and Akt1 in the PFC of chronic opiate addicts could also play a major role in the induction of tolerance to opiate reward (Ramos-Miguel et al., 2009). A complex cross-talk between FADD/p-FADD and Akt1/PEA-15 and ERK1/2 signalling would take place in the brain to finally result in the induction of neuroplasticity without an abnormal rate of cell death in the PFC/BA9 of chronic opiate addicts (see Fig. 3).

Taken together, the results of these studies (García-Fuster et al., 2008b; Ramos-Miguel et al., 2009) clearly indicate that opiate addiction in humans is associated with an altered balance between p-FADD (content increased) and FADD (content decreased) in brain, which may favor the neuroplastic actions of FADD adaptor (ratio p-FADD/FADD: a 3.3-fold increase over matched controls). In fact, relevant roles of p-FADD in modulating morphine-induced behavioral plasticity have been demonstrated in the rat brain (see subheading 3.3.).

4. Role of FADD adaptor in cocaine addiction

Cocaine and/or its oxidative metabolites (e.g. norcocaine) can induce various forms of neurotoxicity (Büttner, 2011), including apoptotic effects in both cultured cells (Xiao et al.,

2000; Cunha-Oliveira et al., 2008) and the developing brain (Novikova et al., 2005). However, the aberrant activation of several cell death mechanisms by cocaine, including those mediated by the Fas/FADD complex, in the adult rat brain remains inconclusive (Dietrich et al., 2005; García-Fuster et al., 2009). Nevertheless, self-exposure to cocaine in humans was recently shown to enhance the degradation of a DNA-repairing enzyme in the PFC/BA9 of long-term addicts, which is compatible with the induction of aberrant cell death by the psychostimulant (Álvaro-Bartolomé et al., 2011).

4.1 Acute, chronic and withdrawal effects of cocaine on FADD and associated signalling in the brain

Acute treatments of rats with cocaine (7.5-30 mg/kg) modulated FADD protein forms in brain cortex, increasing the content of FADD and moderately decreasing that of p-FADD with the lower doses (Fig. 9A) (García-Fuster et al., 2009; Álvaro-Bartolomé et al., 2011). In contrast to opiate and cannabinoid drugs, cortical FADD and p-FADD do not correlate after acute cocaine (Fig. 6C), suggesting that psychostimulants favours the expression of pro-apoptotic FADD form (increased). Acute cocaine increased FADD in all subcellular compartments where it was expressed, with the greater effects in the cytosol and nucleus (García-Fuster et al., 2009). Dopamine D₂ receptors were involved in FADD activation by cocaine as pretreatment with raclopride, a D₂-type receptor antagonist, fully prevented the acute cocaine-induced increase of FADD in rat brain cortex (Fig. 9B). Pretreatment with a D₁-type receptor antagonist (SCH-23390) did not block the acute effect of cocaine on FADD (Fig. 9B). In fact, SCH-23390 by itself increased cortical FADD (Fig. 9B), an effect possibly mediated by its agonistic properties at 5-HT_{1c/2c} receptors (García-Fuster et al., 2009).

A non-contingent experimenter-administered regimen of chronic cocaine in rats (15 or 40 mg/kg, for 6-7 days), known to induce behavioral sensitization, induced tachyphylaxis to the acute modulatory effect of the psychostimulant on cortical FADD (Fig. 9C). Cocaine withdrawal (1-7 days) was associated with a transient reduction in cortical FADD, which was significant 3 days after discontinuation of the chronic treatment (Fig. 9C) (García-Fuster et al., 2009; Álvaro-Bartolomé et al., 2011). It is worth noting that there was a positive correlation between FADD protein and the levels of FADD mRNA in rat brain cortex ($r=0.43$; $n=29$; $p<0.05$, see García-Fuster et al., 2009).

Acute cocaine (20 mg/kg) stimulated p-Thr34 DARPP-32 (dopamine- and cAMP-regulated phosphoprotein of 32 kDa) in rat brain cortex, consistent with the engagement of dopamine signalling. Chronic cocaine (40 mg/kg for 6 days) and cocaine withdrawal (3 days), however, were not associated with activation of cortical p-DARPP-32 (tachyphylaxis after the repeated treatment). Interestingly, chronic cocaine and abstinence, but not acute cocaine, increased the content of t-DARPP (a truncated 30 kDa isoform of DARPP-32 with striking anti-apoptotic actions; El-Rifai et al., 2002) in rat brain cortex. Moreover, acute cocaine, but not the chronic/abstinence treatments, stimulated Akt1 in rat brain cortex. Neither treatment with cocaine (acute, chronic, and abstinence) altered the basal stimulation of anti-apoptotic PEA-15 and pro-apoptotic JNK1/2 signaling (Álvaro-Bartolomé et al., 2011).

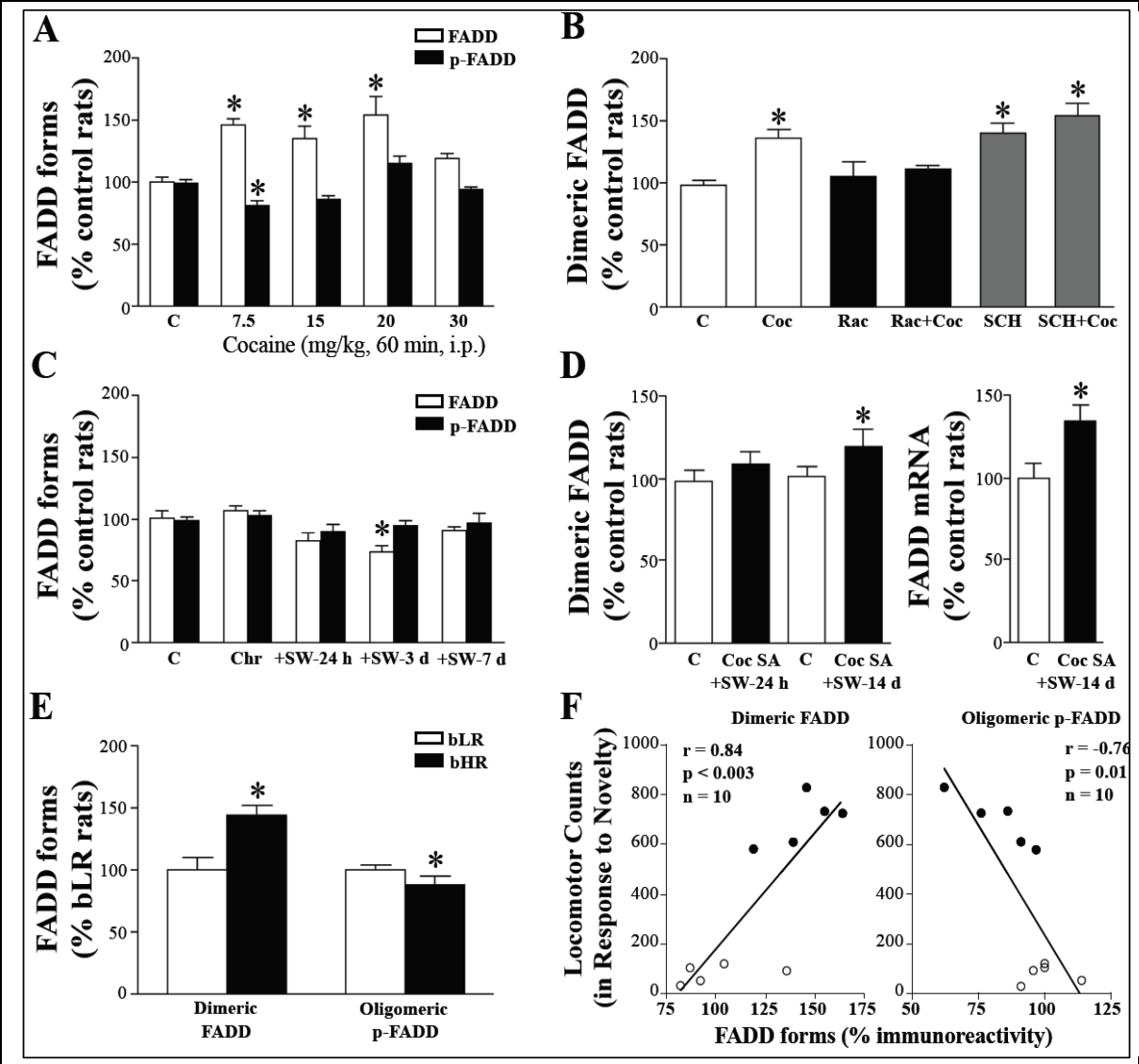


Fig. 9. (A) Acute dose-response effects of cocaine (7.5-30 mg/kg) on FADD and p-FADD in rat brain cortex. (B) FADD is modulated by acute cocaine (Coc, 7.5 mg/kg) through the activation of dopamine D₂ receptors in rat cortex. Rac: raclopride (0.5 mg/kg), SCH: SCH-23390 (0.5 mg/kg). (C) Non-contingent chronic cocaine (Chr, 15 mg/kg for 7 days) and spontaneous withdrawal (+SW) time course effects on cortical FADD and p-FADD. (D) Effects of spontaneous withdrawal (+SW) following contingent cocaine self-administration (Coc SA) on hippocampal FADD protein and mRNA. (E) Basal cortical differences in FADD and p-FADD in bred low-responder (bLR) and high-responder (bHR) rats. (F) Individual differences in locomotor response to novelty correlated (Pearson's r) with basal contents of FADD and p-FADD in rat cortex; non-parametric analysis (Spearman's ρ) also resulted in significant correlations for FADD ($\rho = 0.85$; $p < 0.003$; $n = 10$) and p-FADD ($\rho = -0.70$; $p < 0.05$; $n = 10$) (open circle: bLR rats; closed circles: bHR rats). *At least $p < 0.05$ versus control (C) or bLR rats. (Modified from García-Fuster et al., 2009, 2011; Álvaro-Bartolomé et al., 2011).

It is unlikely that cocaine-induced up-regulation of pro-apoptotic FADD in rat brain (Fig. 9A) could result in the induction of aberrant cell death. In fact, neither acute and chronic cocaine treatments nor cocaine spontaneous withdrawal altered the content of Fas receptor

forms or mitochondrial cytochrome c (a potent caspase-3 activator) and AIF (a mitochondrial mediator of caspase-independent apoptosis) in rat cortex (Álvarez-Bartolomé et al., 2011). Moreover, none of these cocaine treatments altered the pattern of cleavage of nuclear PARP-1 in rat brain cortex (García-Fuster et al., 2009; Álvarez-Bartolomé et al., 2011).

A recent study has examined how a contingent extended daily access to cocaine self-administration impacts the hippocampus at the cellular and molecular levels, and how these alterations can change over the course of cocaine withdrawal (García-Fuster et al., 2011). This animal model has good validity in that it results in the escalation of drug intake (as controlled by the animal, see Ahmed and Koob, 1998) and in cognitive deficits (Briand et al., 2008) similar to those seen in human addicts. Moreover, hippocampal plasticity likely plays an important role in addiction-related behaviors. For example, suppression of hippocampal neurogenesis enhanced resistance to extinction of drug-seeking behavior (Noonan et al., 2010). The results of this study indicated that 5-hour of extended daily access to cocaine for 14 days elicits a profound increase in drug intake from the first self-administration session to the last (García-Fuster et al., 2011), providing a model to study the hippocampal adaptations associated with cocaine withdrawal after abuse of the psychostimulant. This cocaine paradigm led to alterations of hippocampal cell fate regulation (in various hippocampal subregions) during the course of withdrawal (1, 14 and 28 days) with significant changes observed at 14 days (García-Fuster et al., 2011). Notably, FADD adaptor (protein and mRNA; Fig. 9D) was increased in the hippocampus of rats with impaired cell proliferation rates (Ki-67+ mitotic progenitor cells and NeuroD+ neural progenitor cells). The increase in hippocampal FADD (14 days of cocaine withdrawal) did not parallel changes in apoptotic cell death, as measured by cleavage of nuclear PARP-1 (García-Fuster et al., 2011). These data suggest that FADD adaptor is an important hippocampal cell fate regulator during cocaine withdrawal in rats.

4.2 Relevance of FADD in novelty-seeking behaviour and cocaine abuse

Selectively breeding for divergence in locomotor reactivity to a novel environment (bred high-responder (bHR) and low-responder (bLR) lines of Sprague-Dawley rats) has been shown to display reliable differences across multiple behavioural and neurochemical dimensions (Stead et al., 2006). For example, bHR compared to bLR rats have shown an increased behavioural sensitization to cocaine (García-Fuster et al., 2010) and a greater initial propensity to self-administer cocaine (Davis et al., 2008). Interestingly, bHR and bLR rats showed significant basal differences in cortical FADD (higher content in bHR) and p-FADD (lower content in bHR) (Fig. 9E) (García-Fuster et al., 2009). However, bHR/bLR rats showed similar levels of basal nuclear PARP-1 cleavage, indicating similar rates of basal induction of cell death in the cortex (García-Fuster et al., 2009). Moreover, locomotion in a novel environment (bLR *versus* bHR) correlated with the basal content of cortical FADD (positive relation) and p-FADD (inverse relation) (Fig. 9F, $n=10$). Similarly to the acute, chronic and withdrawal cocaine effects observed in commercially purchased Sprague-Dawley rats (see Fig. 9A/C), the basal differences observed between bHR and bLR rats were maintained post-cocaine (i.e., increased FADD after acute cocaine with a reversal following 3 days of withdrawal) for both phenotypes (García-Fuster et al., 2009). These results suggest that FADD signalling could represent a molecular correlate for the bHR and/or bLR phenotype and therefore the initial propensity to initiate cocaine use (Belin et al., 2008).

4.3 Regulation of apoptotic pathways and associated signalling in brains of cocaine addicts: Increased degradation of nuclear PARP-1

In a recent study (Álvaro-Bartolomé et al., 2011), the hypothesis was tested that cocaine addiction in humans results in abnormal activation of canonical (extrinsic and intrinsic) apoptotic pathways leading to increased cell death in the brain (Fig. 10).

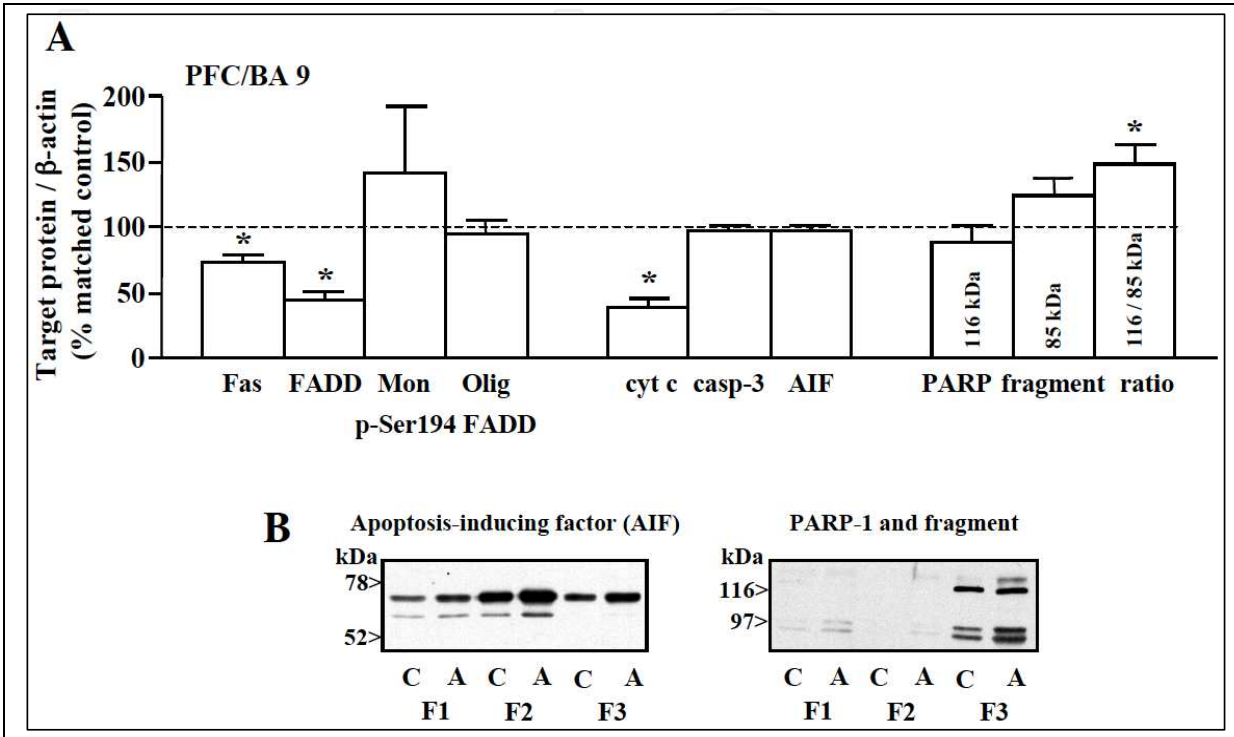


Fig. 10. (A) Contents of apoptotic proteins of the intrinsic (Fas receptor, FADD adaptor and p-FADD) and intrinsic (cytochrome c, caspase-3 and AIF) pathways, as well as the cleavage of nuclear PARP-1 in the prefrontal cortex/Brodmann’s area 9 (PFC/BA9; total homogenate samples) of cocaine abusers. (B) Subcellular content of AIF and PARP-1 (F1: cytosol; F2: membranes; F3: nucleus) in the PFC/BA9 of a representative chronic cocaine addict. *At least $p<0.05$ versus matched controls. (Modified from Álvaro-Bartolomé et al., 2011).

In a small ($n=10$) and well-characterized cohort of “pure” cocaine abusers (including the assessment of cocaine and metabolites in blood, urine, and hair samples), Fas aggregates and FADD adaptor were down-regulated in the PFC/BA9 (Fig. 10A), which was associated with a modest increase in p-FADD/FADD ratio. Moreover, mitochondrial cytochrome c was also reduced, but not caspase-3 or AIF (Fig. 10A) (AIF, however, was increased in the nuclear fraction, Fig. 10B). Importantly, the proteolytic cleavage of nuclear PARP-1 (ratio of 85 kDa fragment to 116 kDa PARP-1) was augmented in the same brain samples of cocaine addicts (Fig. 10A), including an increase in the cortical nuclear fraction (Fig. 10B). In chronic cocaine abusers (PFC/BA9), several signalling molecules associated with cocaine/dopamine and/or apoptotic pathways (Akt1, PEA-15, JNK1/2) were found unaltered, with the exception of DARPP-32 and anti-apoptotic t-DARPP whose contents were decreased.

These findings indicate that cocaine addiction in humans is not associated with abnormal upregulation of major components of the extrinsic and intrinsic apoptotic machineries in the

PFC/BA9. On the contrary, the downregulation of Fas-FADD receptor complex and cytochrome c could reflect the induction of contraregulatory adaptations or non-apoptotic (neuroplastic) actions induced by the repeated abuse of the psychostimulant. In any case, the enhanced degradation of nuclear PARP-1 (Fig. 10B), a hallmark of apoptosis, clearly indicates the possibility of aberrant cell death in brains of chronic cocaine addicts. The molecular mechanism appears to involve the induction of oxidative stress by cocaine metabolites (norcocaine and derivatives) and the activation of the mitochondrial death effector AIF after its translocation to the nucleus (Fig. 10B) (Álvaro-Bartolomé et al., 2011), where it interact with PARP-1 and induces chromatin condensation and large-scale DNA fragmentation (Strosznajder et al., 2010). This particular (caspase-independent) cell death subroutine, involving the nuclear interaction of AIF and PARP-1, has been named *parthanatos* and has a role in multiple pathophysiological conditions (Galluzzi et al., 2011), which could include the induction of neurotoxic effects in the brain of human cocaine addicts (see Fig. 3).

5. Role of FADD adaptor in the neurobiology of the cannabinoid system

Among the many effects induced by natural and synthetic cannabinoids (Pertwee, 1997), their beneficial or deleterious actions on neuronal survival remain a controversial topic (Guzmán et al., 2002; Álvaro-Bartolomé et al., 2010). Although cannabinoids can induce pro-apoptotic activity in several cellular models (Maccarrone and Finazzi-Agró, 2003), recent evidence also demonstrates that these compounds, acting through cannabinoid CB₁ (Aguado et al., 2007) or CB₂ receptors (Viscomi et al., 2009) can also protect neurons from death. It is conceivable therefore that the neuroprotection induced by some cannabinoids *in vivo* could be the result of a favorable balance between the relative activation of anti- and pro-apoptotic signalling pathways in the brain.

5.1 Regulation of basal Fas/FADD complex by cannabinoid receptors: Pro-apoptotic CB₁ receptor tone

CB₁ receptors are highly expressed in the CNS (Howlett et al., 2002) and display a high level of constitutive activity (Gifford and Ashby, 1996). This contrasts with brain CB₂ receptors, which pharmacological activation has been questioned in conscious rats (Chin et al., 2008) and, therefore, the presence of any receptor constitutive activity is uncertain. Similarly to δ -opioid receptors (see Fig. 4; García-Fuster et al., 2007b), the remarkable constitutive activity of CB₁ receptors was also postulated to be involved in the tonic control of pro-apoptotic Fas/FADD complex. This possibility was investigated using gene-targeted mice lacking CB₁ or CB₂ receptors (Álvaro-Bartolomé et al., 2010).

In brain regions of CB₁-KO mice (cerebral cortex, corpus striatum and cerebellum), the content of Fas receptor and/or FADD was reduced (Fig. 11A), suggesting that endocannabinoids acting on CB₁ receptors stimulate the expression of pro-apoptotic Fas/FADD complex. In these mice, non-apoptotic p-FADD and p-FADD/FADD ratio are increased (Fig. 11A), indicating that CB₁ receptors tonically inhibit the phosphorylation of brain FADD, which could also favour the induction of pro-apoptotic actions. In brain regions of CB₂-KO mice, in contrast, the changes of Fas receptor, FADD and p-FADD (somehow opposite to those observed in CB₁-KO mice) did not indicate that CB₂ receptors

are involved in the tonic regulation of Fas/FADD complex. The alterations of Fas/FADD in brains of CB₁ and CB₂ receptors KO mice did not appear to result in an increased cell death because the pattern of cleavage of nuclear PARP-1 was very similar to that measured in WT mice (Álvaro-Bartolomé et al., 2010).

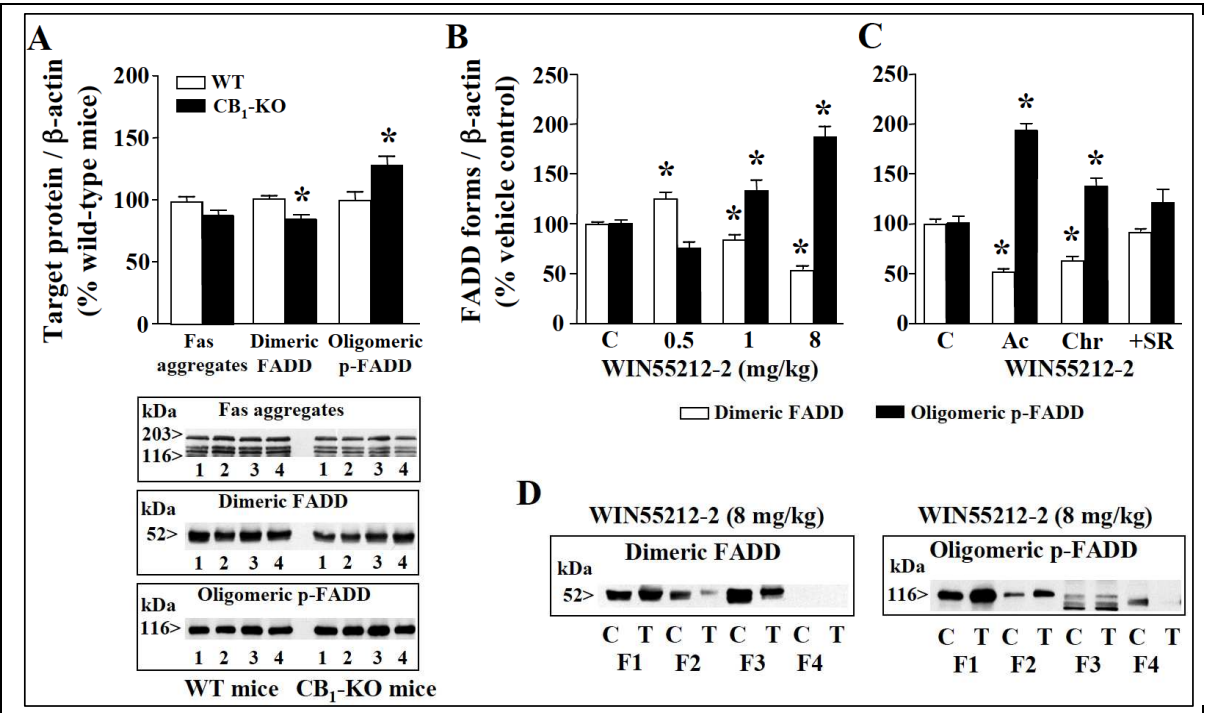


Fig. 11. (A) Fas receptor aggregates, FADD adaptor, and p-FADD in the cerebral cortex of WT mice and cannabinoid CB₁ receptor KO mice. (B) Acute (dose-response) effects of the CB₁ receptor agonist WIN55212-2 on the content of FADD and p-FADD in mouse brain cortex. (C) Acute (Ac, 8 mg/kg), chronic (Chr, 1-8 mg/kg, increasing doses for 5 days) and withdrawal effects (SR, 10 mg/kg; precipitated with the antagonist SR141716A or rimonabant) of WIN55212-2 on FADD and p-FADD content in mouse brain cortex. (D) Effects of WIN55212-2 (8 mg/kg) on the content of FADD and p-FADD at the subcellular level (F1: cytosol; F2: membranes; F3: nucleus; F4: cytoskeleton). *At least $p < 0.05$ versus WT or control (C). (Modified from Álvaro-Bartolomé et al., 2010).

Therefore, CB₁ receptors appear to exert a tonic activation of Fas/FADD complex in brain (Fig. 11A) that is opposite to that induced by δ -opioid receptors (inhibitory tonic control; Fig. 4). Given the interactions between cannabinoids and opiates (Bushlin et al., 2010), and particularly between CB₁ receptors and δ -opioid receptors (Urigüen et al., 2005), the opposite tonic control of these inhibitory receptors on pro-apoptotic Fas/FADD complex could be of relevance in drug mechanisms leading to neuronal cell death or neuroprotection.

5.2 Acute, chronic and withdrawal effects of cannabinoid drugs on FADD and associated signalling in the brain

Acute treatment of mice with the CB₁ receptor agonist WIN55212-2 (0.5, 1 and 8 mg/kg) did not alter the content of Fas receptor forms in the cerebral cortex. However, a low dose

of WIN55212-2 (0.5 mg/kg) increased FADD, whereas higher doses of the agonist (1 and 8 mg/kg) decreased FADD content in mouse brain cortex (Fig. 11B). WIN55212-2 also induced bell-shaped dose effects on p-FADD, but in the opposite direction (Fig. 11B). Pretreatment of mice with the antagonist rimonabant prevented the opposite effects of WIN55212-2 on FADD and p-FADD, indicating a CB₁ receptor-related mechanism. At the subcellular level, WIN55212-2 increased p-FADD in the cytosol and membranes, and to a lesser extent in the nucleus (Fig. 11D right). In contrast, WIN55212-2 decreased FADD in membranes and nucleus, and increased its content in cytosol (Fig. 11D left). WIN55212-2 also increased CK1 α in cytosol, which was coincident with the marked enhancement of p-FADD in this compartment (Fig. 11D right). In marked contrast to the activation CB₁ receptors, high doses of the CB₂ receptor agonist JWH133 were not associated with significant changes of Fas receptor forms, FADD or p-FADD in mouse brain cortex (Álvaro-Bartolomé et al., 2010).

These data indicate that the activation of CB₁ receptors decreases (lower dose) or increases (higher doses) the ratio of cortical p-FADD/FADD (an index of non-apoptotic activity). Interestingly, and as observed for opiate drugs, acute WIN55212-2 treatment induced opposite changes on p-FADD and FADD (Fig. 6B), and this interconversion of FADD forms associated with the activation of CB₁ receptors could be important in the actions of cannabinoids in the brain. For example and consistent with the findings observed in the cerebral cortex of CB₁ receptor KO mice (Fig. 11A: decreased FADD and increased p-FADD), a low dose of WIN55212-2 (0.5 mg/kg) increased FADD and decreased p-FADD in mouse brain cortex (Fig. 11B). This opposite regulation of FADD forms is also consistent with the existence of a pro-apoptotic CB₁ receptor tone. However, the selective CB₁ receptor antagonist/inverse agonist rimonabant (10 mg/kg) did not alter FADD or p-FADD in brains of mice, suggesting that the receptor tonic control on this system is moderate (Álvaro-Bartolomé et al., 2010).

It is noteworthy that chronic WIN55212-2 administration (1-8 mg/kg for 5 days) also resulted in down-regulation of FADD and up-regulation of p-FADD in mouse brain cortex (Fig. 11C), which indicates a sustained attenuation of apoptotic signalling in spite of the induction of some tolerance (tachyphylaxis) upon the repeated stimulation of CB₁ receptors. Rimonabant-precipitated WIN55212-2 withdrawal did not cause a rebound of FADD or p-FADD over control values (Fig. 11C). Along this line, the acute and chronic treatments of mice with WIN55212-2, as well as rimonabant-precipitated withdrawal, did not alter the contents of mitochondrial cytochrome c, AIF, or the cleavage of nuclear PARP-1 in the cerebral cortex. These negative findings further discount the induction of cell death after the activation of CB₁ receptors in the mouse brain.

On the other hand, acute, but not chronic, treatment with WIN55212-2 markedly stimulated the activation of anti-apoptotic ERK1/2 and Akt1/PEA-15, as well as pro-apoptotic JNK1/2 and p38 MAPK in the mouse cerebral cortex. This suggests that the acute neuroprotection *in vivo* induced by some cannabinoids could be the result of a favorable balance between the relative activation of anti- and pro-apoptotic signalling pathways. In contrast to FADD and p-FADD, the lack of a sustained stimulation of anti- and pro-apoptotic cascades upon chronic WIN55212-2 treatment probably reflects the rapid induction of CB₁ receptor desensitization in the regulation of these systems (Álvaro-Bartolomé et al., 2010).

The current findings indicate that the chronic stimulation of CB₁ receptors is associated with a marked downregulation 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 chronic stimulation of CB₁ receptors is also associated with up-regulation of p-FADD, the protein form that mediates non-apoptotic actions including brain plasticity (see Fig. 3). The link between CB₁ receptors and the multifunctional FADD adaptor provides new insights into the complex neurobiology of the cannabinoid system.

6. General conclusions

The modulation of FADD adaptor by drugs of abuse is a new and relevant molecular process in the complex neurobiology of addictions. The regulation of FADD and associated signalling by opiate drugs (heroin/methadone) and the psychostimulant cocaine can lead to neurotoxicity and/or neuroplasticity in brains of human addicts. The ratio of p-Ser194 FADD (anti-apoptotic form) to FADD (pro-apoptotic form) appears to represent a novel marker of cortical plasticity.

In the prefrontal cortex of long-term opiate addicts, the observed down-regulation of FADD (i.e. attenuation of Fas signals), the up-regulation of FLIP_L and Bcl-2 (greater anti-apoptotic effects), the increased Bcl-2/Bax ratio (positive balance for cell survival), the reduction of cytochrome c (lesser activation of other pro-apoptotic factors), the lack of abnormal caspase-3 activation, and the normal pattern of nuclear PARP-1 cleavage (Fig. 3) clearly indicate the absence of aberrant cell death. In contrast, p-FADD and p-FADD/FADD ratio are increased in brains of opiate addicts, which suggests the induction of neuroplastic actions. In fact, other studies in laboratory rats have shown that the behavioural response to morphine-induced psychomotor sensitization, as well as the severity of opiate abstinence syndrome (two well-known neuroplastic responses) correlated with increased p-FADD and reduced FADD in the brain, which further supports the role of p-FADD/FADD ratio as a marker of neuronal plasticity.

In the prefrontal cortex of long-term cocaine addicts, Fas/FADD receptor complex and mitochondrial cytochrome c are down-regulated, suggesting contraregulatory adaptations or non-apoptotic actions (Fig. 3). Importantly, however, the degradation of nuclear PARP-1 is increased in the absence of caspase-3 activation. This type of caspase-independent cell death (named *parthanatos*) involves the induction of oxidative stress by cocaine metabolites (norcocaine and derivatives) and the nuclear translocation of the mitochondrial death effector AIF (Fig. 3). Therefore, cocaine addiction in humans appears to be associated with aberrant cell death in the brain. However, p-FADD/FADD ratio is also increased which also suggest the induction of neuroplastic changes in brains of cocaine addicts. In fact, other studies in laboratory rats have shown that p-FADD and FADD in the cortex represent a molecular correlate of the initial brain plasticity that might predispose to some facets of addictive-like behaviours such as locomotor response to novelty.

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