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Opioid receptor agonists enhance the phosphorylation state of Fas-associated death domain (FADD) protein in the rat brain: Functional interactions with casein kinase Iα, $G\alpha_i$ proteins, and ERK1/2 signaling

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ABSTRACT

Opioid drugs have been proposed to promote anti-apoptotic signals in brain through inhibition of FADD protein [García-Fuster et al., 2007. Effects of opiate drugs on Fas-associated protein with death domain (FADD) and effector caspases in the rat brain: Regulation by the ERK1/2 MAP kinase pathway. Neuropsychopharmacology 32, 399–411]. FADD phosphorylation by casein kinase Iα (CKIα) appears to regulate its non-apoptotic activity. This study investigated the effects of opioids on p-FADD in rat brain, as well as various mechanisms that could link opioid receptors with p-FADD, including the modulation of CKIα, $G\alpha_i$ proteins and ERK1/2 signaling. In rat, mouse and human brains, various anti-p-FADD antibodies immunodetected the monomeric and oligomeric forms of this protein, irrespective of the antibody origin and specific Ser191 or Ser194 phosphorylation site. Acute μ- and δ-agonists increased, through specific opioid receptor mechanisms, the content of oligomeric and monomeric p-FADD forms in rat cortical homogenates (25–61%) and subcellular compartments, with most relevant effects for sufentanil in membrane (239%) and nucleus (136%). p-FADD induction vanished with repeated (5 days) morphine but not SNC-80, and opioid withdrawal induced a new (morphine) or sustained (SNC-80) stimulatory effect (32–33%). The κ-agonist (−)-U-50488H failed to stimulate p-FADD. Sufentanil reduced CKI protein and kinase activity in the cytosol (30–37%). Morphine, but not SNC-80, augmented CKIα in cytosol, membrane and nucleus (36–104%). In contrast to FADD, the ability of SNC-80 to stimulate p-FADD was not sensitive to ERK1/2 blockade. Pertussis toxin did not prevent the opposite effects of SNC-80 on p-FADD and FADD because the toxin by itself markedly altered their basal contents, indicating that FADD could be a novel toxin target. The upregulation of p-FADD induced by μ/δ-agonists could play a relevant role in the anti-apoptotic and/or neuroplastic effects of opioids.

1. Introduction

Fas-associated death domain (FADD) protein was initially reported as an adaptor molecule that mediates the signaling of various death receptors belonging to the tumor necrosis factor receptor superfamily (Chinnaiyan et al., 1995; Kischkel et al., 1995). The function of FADD is essential in Fas-induced apoptosis, a fundamental process for homeostasis and development in all tissues (Sharma et al., 2000; Sastry and Rao, 2000). Upon Fas activation and receptor trimerization, a death-inducing signaling complex (DISC) is formed by recruitment of at least FADD and initiator pro-caspase-8, which provides a molecular scaffold to induce the death signal with the activation of effector caspases (Algeciras-Schimnich et al., 2002; Lee et al., 2006; Feig et al., 2007). Notably, 23 kDa monomeric FADD has the potential to be highly oligomeric through a self-association process that appears to increase the efficiency of Fas signaling (Sandu et al., 2006; Werner et al., 2006). FADD, a postulated cytoplasmic protein (see O'Reilly et al., 2004), whose structure possesses sequences for nuclear localization and export signals (Zhang et al., 2004), may also have a function in the nucleus most probably unrelated to its pro-apoptotic activity (Screaton et al., 2003).

In addition to its well-established function in mediating apoptotic signals, Fas (Siegel et al., 2000; Lambert et al., 2003; Peter et al., 2007) and FADD have additional roles in cell growth and differentiation (Park et al., 2005; Tourneur et al., 2005; Werner et al., 2006). In fact, FADD and caspase-8 appear to be instrumental in linking Fas to non-apoptotic pathways through the activation of

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JNK (Matsumoto et al., 2007). In the CNS, Fas/FADD stimulation has been demonstrated to promote neurite outgrowth (Desbarats et al., 2003) and neuronal branching (Zuliani et al., 2006) through ERK activation. Interestingly, FADD displays, outside the death domain, a single serine phosphorylation site (human: Ser194; mouse: Ser191), which is crucial for its non-apoptotic activity (Zhang et al., 2004). Casein kinase Iα (CKIα), a serine/threonine kinase of wide subcellular distribution (Zhang et al., 1996; Knippschild et al., 2005), is the main enzyme that mediates p-Ser FADD in various cells, and through this mechanism the multifunctional protein can regulate the cell cycle and the sensitivity to antineoplastic drugs (Alappat et al., 2005). Moreover, phosphorylation of FADD also appears to be essential for its accumulation in the nucleus and export to the cytoplasm (Screaton et al., 2003; Alappat et al., 2005).

Opioid drugs were shown to induce Fas upregulation and FADD downregulation in the rat brain (Boronat et al., 2001; García-Fuster et al., 2003, 2004, 2007a). This opposite modulation suggested that possible apoptotic signals engaged by opioids through Fas (Mao et al., 2002; Liao et al., 2005; Pérez-San Emeterio et al., 2006) would be offset through FADD inhibition, which in turn was dependent on the activation of anti-apoptotic ERK1/2 signaling (García-Fuster et al., 2007a). Notably, the basal contents of Fas aggregates and FADD are increased in the cerebral cortex of δ-opioid receptor-deficient mice, indicating that this receptor can tonically inhibit the expression of Fas/FADD complex (García-Fuster et al., 2007b). The opioid modulation of p-FADD might also be relevant in mediating some anti-apoptotic signals induced by opioid receptors (Tegeder and Geisslinger, 2004). Against this background, the aim of this study was to assess the effects of opioid drugs on brain p-FADD, and to investigate the roles of CKIα, $G\alpha_i$ proteins and ERK1/2 signaling in its regulation.

2. Materials and methods

2.1. Drug treatment of rats

Adult male Sprague–Dawley rats (200–250 g; housed at 22 °C, 70% humidity, and 12-h light/dark cycle) were used. The animals were treated in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and in agreement with the local ethical committee. All efforts were made to minimize the number of animals used and their suffering. The rats received fentanil (100 μg/kg, s.c., 15 min), sufentanil (2.5–30 μg/kg, s.c., 30 min), morphine (100 mg/kg, i.p., 2 h), SNC-80 (δ-agonist, 10 and 30 mg/kg, i.p., 30 min), or (−)-U50488H (κ-agonist, 30 mg/kg, i.p., 1 h). Other rats were injected with naloxone (10 mg/kg, i.p.) alone (90 min) or 60 min before sufentanil (15 μg/kg), or with naltrindole (δ-antagonist, 5 mg/kg, i.p.) alone (60 min) or 30 min before SNC-80 (10 mg/kg). Control rats received saline or dimethyl sulfoxide (DMSO, in the case of SNC-80). For the chronic treatment with morphine, the rats were injected (i.p.) three times daily during 5 days with increasing doses of the opioid (10–100 mg/kg) and killed 2 h after the last dose. After this treatment, naloxone (2 mg/kg, i.p.)-precipitated withdrawal (2 h) or spontaneous withdrawal (48 h) was induced, which resulted in the expected behavioral reaction (data not shown; Miralles et al., 2005). In other chronic experiments, the rats were treated with SNC-80 (10 mg/kg) or (−)-U50488H (10 mg/kg) once daily during 5 days. After these treatments, naltrindole (5 mg/kg, i.p., 2 h) or nor-binaltorphimine (κ-antagonist, 5 mg/kg, i.p., 2 h)-precipitated withdrawal was induced, which resulted in a mild behavioral (κ) reaction or the absence of behavioral (δ) signs (data not shown; Milanés and Laorden, 1998; Brandt et al., 2001). Control rats received chronic saline or DMSO in parallel. The animals were killed by decapitation at the indicated times, and specimens of the cerebral cortex and corpus striatum dissected on ice, frozen in liquid nitrogen, and then stored at −80 °C.

2.2. FADD dephosphorylation, dissociation of FADD oligomers, and monitoring of apoptotic cell death

In addition to rat brain, mouse (cerebral cortex) and human (prefrontal cortex) samples were also used for the immunodetection of p-FADD with different antibodies (Table 1). The specificity of anti-p-FADD antibodies was assessed by Western blot analysis using calf intestinal mucosa alkaline phosphatase (94–118 units, Product 79390, Sigma–Aldrich) (García-Fuster et al., 2007a). The dissociation of p-FADD oligomers (disulfide-cross linked forms) to monomeric forms was assessed as described (García-Fuster et al., 2004). Briefly, rat cortical membranes were incubated (1 h at 37 °C) in the absence or presence of the lipophilic sulfhydryl alkylating reagent dithiothreitol (DTT, 200 mM) or *N*-ethylmaleimide (NEM, 1 mM), followed by a freeze/thaw treatment to −70 °C, to block native cysteine residues (rat FADD contains three cysteine residues; see Zhang et al., 2004). The potential of morphine to induce apoptosis was assessed by measuring the cleavage of poly(ADP-ribose)-polymerase (Garnett et al., 2006), using the PARP Cleavage Detection Kit (Calbiochem, Darmstadt, Germany).

2.3. Subcellular distribution of p-FADD and CKIα, and effects of opioid agonists

The subcellular localization of p-FADD and CKIα in the cerebral cortex, and the effects of sufentanil (15 μg/kg, s.c., 30 min), morphine (100 mg/kg, i.p., 2 h) and SNC-80 (10 mg/kg, i.p., 30 min) in the different compartments were monitored by use of the Subcellular Proteome Extraction Kit (ProteoExtract™, Calbiochem) (see García-Fuster et al., 2007a). Protein concentrations were determined by the Non-Interfering Protein Assay Kit (Calbiochem). This sequential extraction method is based on the different solubility of proteins in certain subcellular compartments to yield four subproteomes enriched in cytosolic (F1, cytosolic fraction), membrane and membrane organelle-localized (F2, membrane fraction), soluble and DNA-associated nuclear (F3, nuclear fraction) and cytoskeletal (F4, cytoskeletal fraction) proteins. The efficiency and selectivity of this subcellular extraction procedure have been reported (Abdolzade-Bavil et al., 2004), and in the current rat brain study it was assessed by the immunodetection of stathmin in F1 (Di Paolo et al., 1997), Fas in F2 (a membrane receptor), nuclear protein prostate apoptosis response 4 (PAR-4) in F3 (Wang et al., 2006), and neurofilament (NF-L) in F4.

2.4. Determination of CKIα activity

The effect of sufentanil (30 μg/kg, s.c., 30 min) on CKI activity was measured in the rat cerebral cortex using a specific peptide substrate (RRKDLHDDEEDEAMSITA) and following standard procedures with some modifications (Murga et al., 1996; Izeradjene et al., 2004). Briefly, aliquots of cytosolic preparations (S2) from saline- and sufentanil-treated rats (22 and 44 μg protein) were incubated with the peptide (0.1 μM), in the absence or presence (10 μM) of the CKI inhibitor D4476 in a reaction buffer (25 mM Hepes, pH 7.5; 10 mM $MgCl_2$; 130 mM KCl; 0.2 mM Na_3VO_4; 1.5 mM NaF; 50 μM ATP; 4 μCi [γ-^{32}P]ATP) at 30 °C during 30 min. Reactions (50 μl) were stopped by addition of 30% $C_2HCl_3O_2$. Other procedures and incorporation of [^{32}P] into the substrate were assessed as described (Murga et al., 1996).

2.5. Effect of pertussis toxin on SNC-80-induced changes in FADD and p-FADD

Rats were pretreated with pertussis toxin (PT, i.c.v.) to ADP-ribosylate (cysteine 350) and inactivate $G\alpha_{i/o}$ proteins (Clark and Traynor, 2006). PT (Sigma–Aldrich, P7208, Batch 034K1409) was administered essentially as described

Table 1
Characteristics of the antibodies used for the detection of FADD and other proteins in rat brain tissue

Protein	Antibody	Antigen	Host	Catalog	Batch	Company
p-FADD	Ab I	Mouse p-FADD (short peptide containing p-Ser191)	Rabbit	2785	1	Cell Signaling, USA
p-FADD	Ab II	Human p-FADD (short peptide containing p-Ser194)	Rabbit	sc-12439	C072	Santa Cruz, USA
p-FADD	Ab III	Human p-FADD (short peptide containing p-Ser194)	Rabbit	2781	2	Cell Signaling, USA
FADD	H-181	Human FADD (28–208 residues)	Rabbit	sc-5559	J-2004	Santa Cruz, USA
FADD	Clone 28	Rat FADD (97–178 residues)	Mouse	558202	08738	BD Biosciences, USA
CKIα	C-19	Human casein kinase Iα (C-terminal residues)	Goat	sc-6477	K2305	Santa Cruz, USA
$G\alpha_{i2}$	Clone L5	Protein-purified whole molecule	Mouse	G111	086L200	Leinco, USA
PARP	512739	Human PARP (215–228 residues)	Rabbit	512729	D33259	Calbiochem, FRG
Stathmin	CS-3352	Human stathmin (residues surrounding Ser38)	Rabbit	3352	1	Cell Signaling, USA
Fas	M-20	Mouse Fas receptor (C-terminal residues)	Rabbit	sc-716	F1306	Santa Cruz, USA
PAR-4	A10	Rat nuclear protein PAR-4 (1–334 residues)	Mouse	sc-1666	J-305	Santa Cruz, USA
Neurofilament-L	Clone NR4	Pig spinal cord	Mouse	5139	086K4823	Sigma Chemical, USA
β-actin	Clone AC-15	Human β-actin (2–16 residues)	Mouse	A1978	016K4817	Sigma Chemical, USA

(García-Sevilla et al., 1978). The rats were anesthetized with isoflurane (700 cm^3/min, Abbott) and their skulls exposed by means of a midline incision. A hole was drilled on the right side 2 mm posterior to bregma and 2 mm lateral from the sagittal suture. Then PT (3 μg in 10 μl vehicle, pH 7.0) was slowly administered into the cavity of the lateral ventricle (4 mm below the surface of the skull) by means of a Hamilton syringe. The hole was closed with dental cement and the skin sutured with sterile staplers. Then the animals were housed in individual cages for recovery. Control rats received the vehicle (10 μl, i.c.v.) following the same procedure, which was verified by injection and localization of dye into the right lateral ventricle. The experiments (PT alone or followed by SNC-80) were performed 24 h after the administration of the toxin when the gross behavior of the animals was normal. The contents of FADD, p-FADD and $G\alpha_{i2}$ proteins were quantified in the right cerebral cortex.

2.6. Effect of MEK inhibition on the basal contents of FADD, p-FADD and CKIα, and on SNC-80-induced changes in p-FADD

The compound SL327 is a brain-penetrating and selective inhibitor of MAP/ERK kinases (MEK1/2): IC_{50} for MEK1/2, 0.18–0.22 μM; IC_{50} for ERK1/2, >50 μM (Scherle et al., 2000). Groups of rats were treated with SL327 (10, 20 and 30 mg/kg, i.p., 90 min) to assess the effects of an inhibition of ERK1/2 activity on the basal contents of FADD, p-FADD and CKIα in the cortex and striatum. Other rats were treated with SL327 (20 mg/kg, i.p.) alone (90 min) or 60 min before the δ-agonist SNC-80 (10 mg/kg, 30 min) to determine the influence of an impaired ERK1/2 signaling in the regulation of p-FADD by this agonist. Control rats received DMSO or SNC-80 (10 mg/kg, i.p., 30 min). The doses of SL327 were chosen from previous studies that demonstrated effective brain ERK inhibition (Valjent et al., 2006; García-Fuster et al., 2007a).

2.7. Sample preparations, immunoblot assays, and quantitation of target proteins

Brain samples (cortex and striatum) were prepared as described (García-Fuster et al., 2007a). Proteins were determined by the BCA Assay (Pierce Biotechnology, Rockford, IL, USA). In routine experiments, proteins (40 μg) were separated on 10% SDS–PAGE minigels (Bio-Rad Laboratories, Hercules, CA, USA), which was followed by immunoblotting standard procedures (García-Fuster et al., 2003, 2007a). The primary affinity-purified antibodies (overnight incubation at 4 °C) are listed in Table 1, and were used at the appropriate dilutions (1:500–1:10 000). The secondary antibody, horseradish peroxidase-linked anti-rabbit, anti-goat or anti-mouse IgG, was incubated at 1:5000 dilution in blocking solution at room temperature for 1 h. In order to test the selectivity of anti-p-FADD and anti-CKIα antibodies with specific proteins, the corresponding antigenic peptide (sc-12439P, batch C201, and sc-6477P, batch J-1802, Santa Cruz) was preincubated in excess with the antiserum to block the binding of the antibody.

Protein immunoreactivity was detected with the ECL system and visualized by exposure to Hyperfilm (Amersham, Buckinghamshire, UK) for 60 s to 60 min. The autoradiograms were quantified by densitometric scanning (IOD, GS-800 Calibrated Densitometer, Bio-Rad). Prestained markers were run on the gels to serve as internal markers for antigen molecular-weight determinations (Harlow and Lane, 1999), using the Bio-Rad software. The amount of target proteins in brain samples of rats treated with opioid drugs was compared in the same gel with that of control rats which received saline or DMSO solution. This procedure was assessed 3–6 times in different gels (each gel with different samples from saline/DMSO- and drug-treated rats). Finally, percent changes in immunoreactivity with respect to control samples (100%) were calculated for each rat treated with the specific drug in the various gels and the mean value used as a final estimate. As a control for sample loading and protein transfer, the blots were stripped (Boronat et al., 2001) and reprobed with anti-β-actin monoclonal antibody (negative control). The brain content of this cytoskeletal protein is not altered by opioids (Ammon et al., 2003; Marie-Claire et al., 2004). Very similar results were obtained when control and experimental immunoblots (IODs) were corrected by the corresponding β-actin content (data not shown).

2.8. Data and statistical analysis

Data were analyzed with GraphPad Prism™. Results are expressed as means ± S.E.M. One-way ANOVA followed by Bonferroni's test, and Student's *t*-test were used for the statistical evaluations. Correlation coefficients were calculated to test for possible association between variables. The level of significance was chosen as $p \leq 0.05$.

2.9. Drugs and chemicals

Fentanil citrate (Sigma–Aldrich Química, Madrid, Spain); sufentanil citrate (Janssen-Cylag, Madrid, Spain); morphine HCl (Unión Químico-Farmacéutica, Madrid, Spain); (+)-4-[(αR)-α-(2S,5R)-4-allyl-2,5-dimethyl-1-piperazinyl)-3-methoxybenzyl]-*N*,*N*-diethylbenzamide, SNC-80; (1*S*-*trans*)-3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl)cyclohexyl]-benzene-acetamide HCl, (−)-U50488H; naloxone HCl; naltrindole HCl; nor-binaltorphimine HCl; and α-amino-(4-amino-phenyl-thio)-methylene-2-(trifluoromethyl)-benzeneacetonitrile, SL 327 (Tocris Cookson Ltd., Avonmouth, UK). Recombinant FADD (DD: 109–208 residues) with glutathione-*S*-transferase (GST) tag (Abnova Corporation, Taipei, Taiwan). CKI substrate, lot B50445, and CKI inhibitor D4476, lot D22439 (Calbiochem, Darmstadt, Germany). Other materials were purchased from Cell Signaling, Amersham or Sigma.

3. Results

3.1. Immunodetection of p-FADD forms in brain tissue

Western blot analysis of rat brain tissue with anti-p-FADD antibodies revealed three major species of p-FADD: ≈116, 37–40, and 20–23 kDa (Fig. 1A–D; see Table 1 for antibodies I, II, and III). These putative p-FADD forms and others (≈204 and 92 kDa) were sensitive to enzymatic dephosphorylation (60–100%), demonstrating the specificity of p-FADD species detected in total homogenate and cytosolic brain preparations (Fig. 1A–D). Monomeric p-FADD (23 kDa) was immunodetected in rat cortex (weak signal) and striatum (strong signal), but only with Ab II (Fig. 1B,C). Preincubation of Ab II with its antigenic peptide resulted in the blockade of the various p-FADD immunoreactions (data not shown). Other bands recognized by these antibodies, but not sensitive to alkaline phosphatase or peptide competition, were considered nonspecific. Preincubation of rat cortical membranes with DTT (200 mM) or NEM (1 mM, not shown) reduced the immunodensity of high-molecular mass p-FADD forms (20–45%) and increased that of the monomer (120–150%) (Fig. 1E,F), suggesting that disulfide bonds could participate in FADD oligomerization.

Very similar patterns of p-FADD forms were immunodetected in mouse and human cerebral cortices with Abs I and II (Fig. 2A,B), which indicated that p-FADD can accurately be measured in rat brain tissue irrespectively of the antibody origin (Ab I: anti-mouse pS191; Ab II: anti-human pS194). On the other hand, rat and mouse p-FADD (i.e. ≈116, 37 and 23 kDa bands) were also immunodetected in brain with two antibodies recognizing total FADD protein regardless of its phosphorylation state (Fig. 2C; see also García-Fuster et al., 2007a). As expected, recombinant FADD was immunodetected with Ab H-181 (total FADD) (Fig. 2D), but not with Ab I (p-FADD) (not shown). Moreover, the μ-opioid receptor agonist fentanil (100 μg/kg, 15 min) significantly increased the content of ≈116 kDa p-FADD in the rat cerebral cortex independently of the anti-p-FADD antibody used (Ab I: 61%, Ab II: 36%, Ab III: 44%) (Fig. 3).

From these experiments, various p-FADD forms were selected to further assess the effects of opioid drugs in the rat cerebral cortex. Thus, oligomeric p-FADD was mainly immunodetected with Ab I, and monomeric p-FADD with Ab II after prolonged exposure times (weak signals).

3.2. Subcellular distribution of p-FADD in rat brain

In the cerebral cortex, p-FADD forms (116 and 37 kDa) were mainly expressed in the cytosolic (F1) and/or nuclear (F3) fractions (Fig. 4E,F), and monomeric p-FADD (23 kDa) was only detected in the nucleus (F3) (Fig. 4F). The contents of these p-FADD forms were lower in the membrane (F2) and cytoskeletal compartments (F4) (Fig. 4E,F, and G for specific subcellular markers). Other bands recognized by these anti-p-FADD antibodies were most probably nonspecific or degradation products (see above). A similar subcellular distribution for dimeric FADD has been reported (García-Fuster et al., 2007a).

3.3. Effects of μ-opioid receptor agonists on the content of p-FADD in rat brain

Similarly to fentanil (Fig. 3), acute sufentanil (15 μg/kg, 30 min) increased p-FADD content (116 kDa: 34%; 23 kDa: 24 ± 2%, $n = 3$, $p < 0.01$) in the cerebral cortex, and naloxone antagonized these

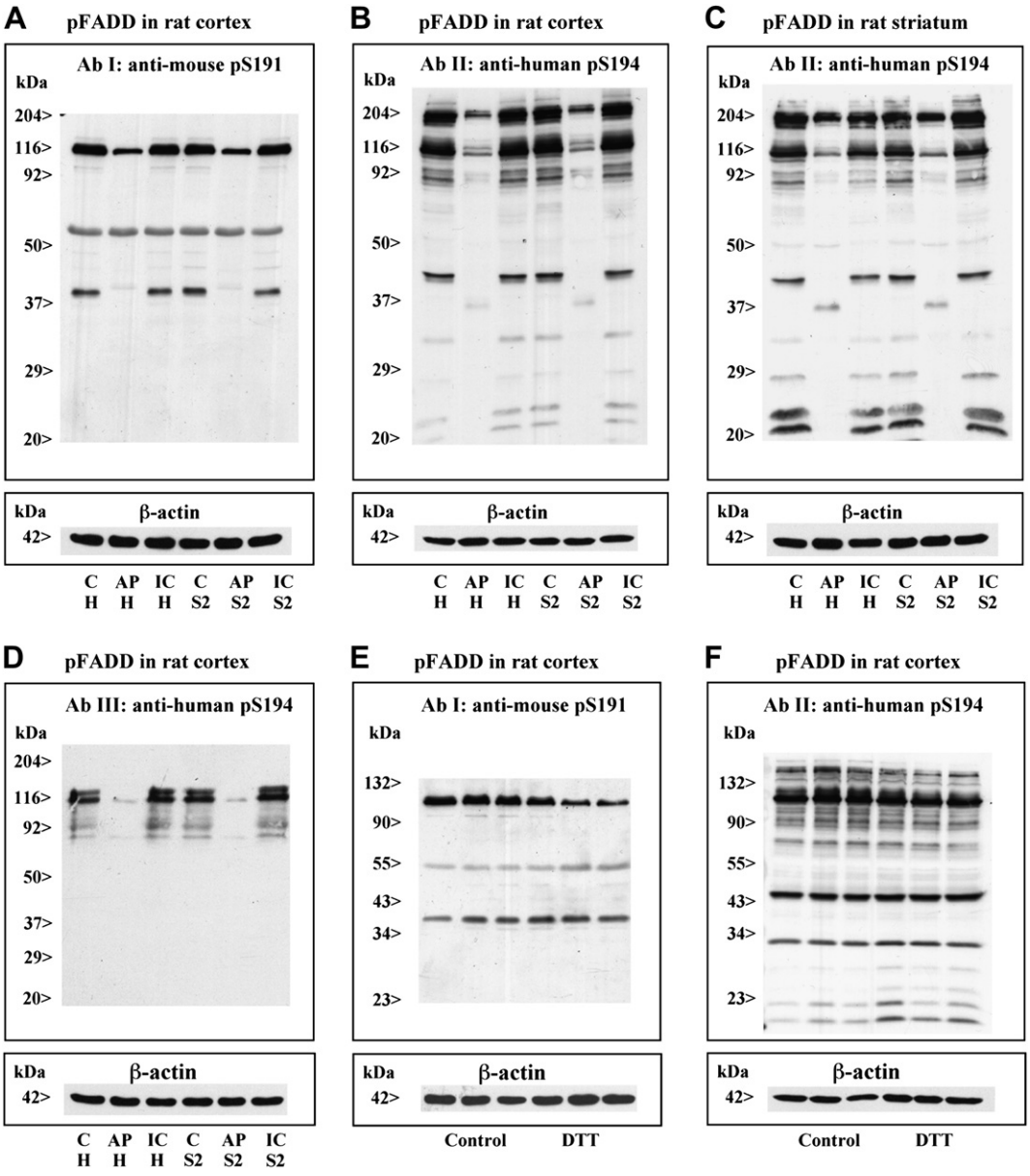


Fig. 1. (A–D) Representative autoradiograms of Western blots depicting labeling of immunodetectable p-FADD species (≈204–92 kDa, ≈37–40 kDa and ≈20–23 kDa forms) with various affinity-purified polyclonal antibody (A: Ab I; B-C: Ab II; D: Ab III; see Table 1), and the effect of protein enzymatic dephosphorylation in the rat cerebral cortex and corpus striatum. Total tissue homogenate (H) and the cytosolic fraction (S2) were incubated at 37 °C for 15 min in the absence (C, control samples) or presence of alkaline phosphatase (AP, 94–118 units). Samples containing the enzyme were also incubated with 100 mM sodium pyrophosphate (IC, inhibited controls). The amount of protein loaded on the gel was 40 μg for all samples. Note that appropriate exposure times allowed the simultaneous visualization of the various forms of p-FADD, including the ≈20–23 kDa monomeric form (Ab II). (E, F) Effect of the sulfhydryl alkylating reagent dithiothreitol (DTT, 200 mM, 1 h) on the immunodensities of p-FADD species (Ab I and II) in the rat cerebral cortex. The experiments, including β-actin immunoblots, were repeated 3–5 times with similar results. The apparent molecular masses of p-FADD forms are reported according to blot calibration with standard molecular weight markers (kDa).

effects (Fig. 4A–C). These treatments did not modify the 37 kDa p-FADD species (Fig. 4A,B) or β-actin (Fig. 4D). At the subcellular level, sufentanil increased 116 kDa p-FADD in the cytosol ($13 \pm 12\%$, $n = 4$, $p > 0.05$), membranes ($239 \pm 49\%$, $n = 4$, $p < 0.01$) and nucleus ($136 \pm 35\%$, $n = 4$, $p < 0.05$) (Fig. 4E,F), and monomeric p-FADD in the nucleus ($88 \pm 22\%$, $n = 3$, $p < 0.05$) (Fig. 4F).

Acute morphine (100 mg/kg, 2 h) also stimulated FADD phosphorylation in the cortex (116 kDa: 51%; 23 kDa: $98 \pm 11\%$, $n = 3$, $p < 0.02$) (Fig. 5A–C). In contrast, chronic morphine did not result in upregulation of these p-FADD forms (Fig. 5A–C), indicating the induction of tachyphylaxis to the repeated treatment. In these rats, naloxone-precipitated withdrawal (2 h) was associated with increased p-FADD (116 kDa: 40% vs. control; 32% vs. chronic morphine) (Fig. 5A,B). Spontaneous opioid withdrawal (48 h) did not induce significant increases of p-FADD forms (Fig. 5A–C). None of these treatments altered the content of 37 kDa p-FADD or that of β-actin (Fig. 5A,B).

The highest dose of morphine used (100 mg/kg) did not alter the pattern of nuclear PARP cleavage observed in the cortex of saline-treated rats (Fig. 5D), indicating that caspase-3 was not activated.

3.4. Effects of a δ-opioid receptor agonist on the contents of p-FADD and FADD in rat brain

SNC-80 (10 mg/kg, 30 min) increased p-FADD in the cerebral cortex (116 kDa: 37%; 23 kDa: $47 \pm 6\%$, $n = 4$, $p < 0.01$) (Fig. 6A, and Fig. 9B for the monomeric form), and this effect was blocked

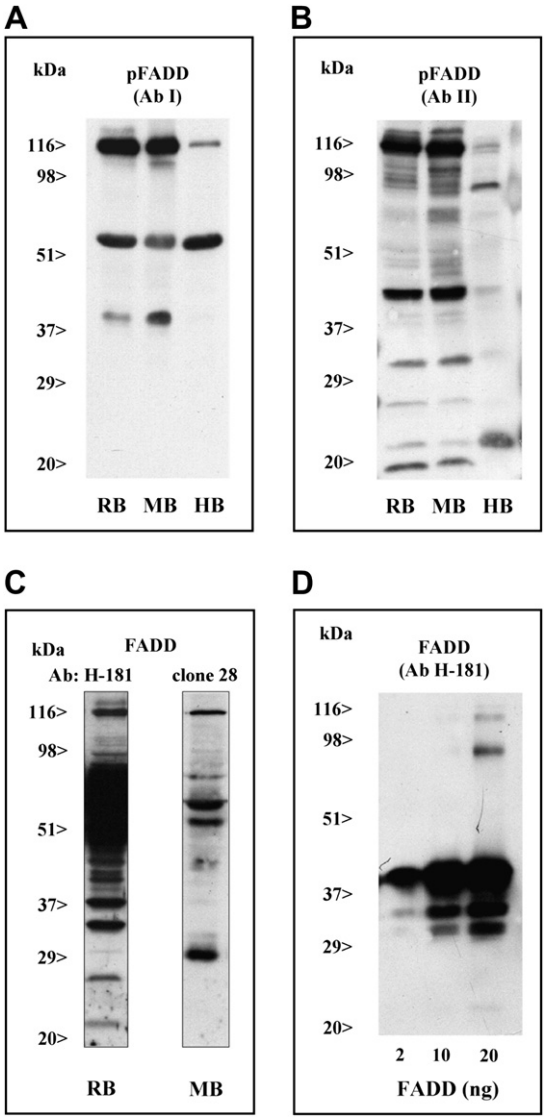


Fig. 2. (A,B) Representative immunoblots depicting labeling of p-FADD species in rat (RB), mouse (MB) and human (HB) cerebral cortex (Ab I and II). (C) Immunodetection of FADD with Ab H-181 and Ab clone 28 (total FADD protein regardless of phosphorylation state; see Table 1) in the rat (RB) and mouse (MB) cerebral cortex. Note that both antibodies revealed the presence of p-FADD species (e.g., ≈116 kDa band) after a prolonged exposure time (≈1 h). (D) Immunodetection of ≈37–40 kDa GST-tagged FADD protein (samples: 2–20 ng) with Ab H-181 (FADD) in the rat cerebral cortex.

by naltrindole (Fig. 6A). Treatment with a higher dose of SNC-80 (30 mg/kg) reduced FADD (30%) and augmented p-FADD (116 kDa: 45%; 23 kDa: 38 ± 18%, $n = 4$, $p < 0.05$) (Fig. 6C–E). At the subcellular level, SNC-80 (10 mg/kg) increased 116 kDa p-FADD in the cytosol (50 ± 7%, $n = 3$, $p < 0.02$), membranes (67 ± 12%, $n = 3$, $p < 0.05$) and to a lesser extent in the nucleus (13 ± 5%, $n = 3$, $p > 0.05$), and monomeric p-FADD in the nucleus (21 ± 2%, $n = 3$, $p < 0.005$) (Fig. 6F).

The repeated treatment with SNC-80 (10 mg/kg; 5 days) also induced an increase of 116 kDa p-FADD (34%) in the cortex (Fig. 6B). The sustained chronic effect of SNC-80 was in contrast with the development of tachyphylaxis associated with chronic morphine treatment (Fig. 5). In chronic SNC-80-naltrindole withdrawn rats, the content of 116 kDa p-FADD (33%) was much like that quantified after the chronic treatment (Fig. 6B).

As reported for sufentanil and morphine, SNC-80 did not modify the content of 37 kDa p-FADD or that of β-actin (Fig. 6A,B; and data not shown).

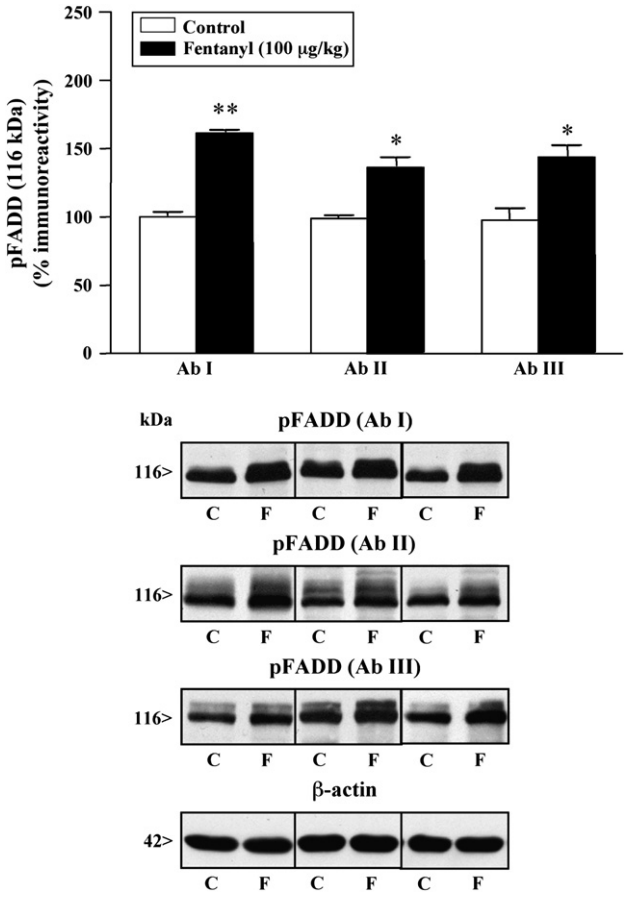


Fig. 3. Acute effect of fentanil on p-FADD content (≈116 kDa species) quantified with Ab I, Ab II and Ab III (see Table 1) in the rat cerebral cortex (same brain samples). Groups of treatments: saline (C, $n = 6$), fentanil (F, 100 μg/kg, s.c., 15 min; $n = 6$). Columns are means ± S.E.M. of n experiments per group with an animal per experiment, and expressed as percentage of saline-treated rats. $^{*}p < 0.01$, $^{**}p < 0.001$ vs. control (Student's t-test). Bottom: representative immunoblots of p-FADD and β-actin for each set of experiments.

3.5. Effects of a κ-opioid receptor agonist on the contents of p-FADD and FADD in rat brain

Treatment with a high dose of (−)-U50488H (30 mg/kg, 1 h) decreased the content of FADD (39%), but it failed to stimulate FADD phosphorylation (oligomeric and monomeric forms) in the cerebral cortex (Fig. 7A,B). Furthermore, repeated treatment with (−)-U50488H (10 mg/kg; 5 days) and κ-antagonist-precipitated withdrawal were not associated with alterations p-FADD forms in brain (Fig. 7C).

Similarly to sufentanil, morphine and SNC-80, (−)-U50488H did not alter the content of 37 kDa p-FADD or that of β-actin (Fig. 7B,C).

3.6. Effects of opioid receptor agonists on the content of casein kinase Iα in rat brain

In rat, mouse and human cerebral cortices, CKIα was immunodetected as a doublet of ≈37/40 kDa, sensitive to antigenic peptide competition (data not shown), which corresponded to CKIα and CKIαL isoforms (Fig. 8A) (see Zhang et al., 1996). CKIα was more abundantly expressed in the cytosol and nucleus (Fig. 8C).

The μ-agonist sufentanil (2.5–30 μg/kg, 30 min) induced modest but sustained decreases in CKIα content in the cerebral cortex (18% at the highest dose, $p < 0.05$) (Fig. 8A). In sufentanil-treated rats (30 μg/kg), the stimulation of cortical cytosolic CKI activity was reduced compared with that in controls (37%, $p < 0.001$) (Fig. 8B).

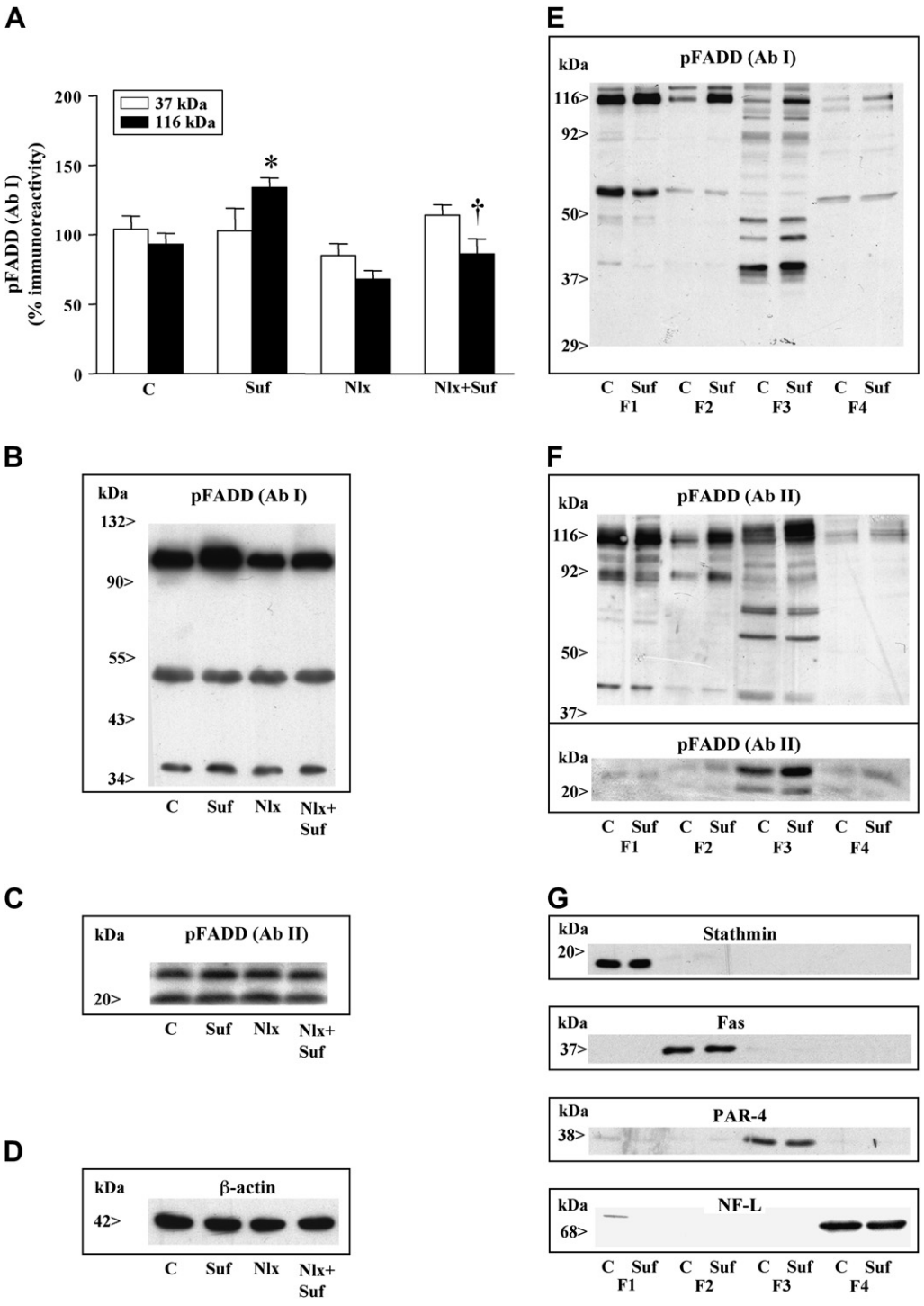
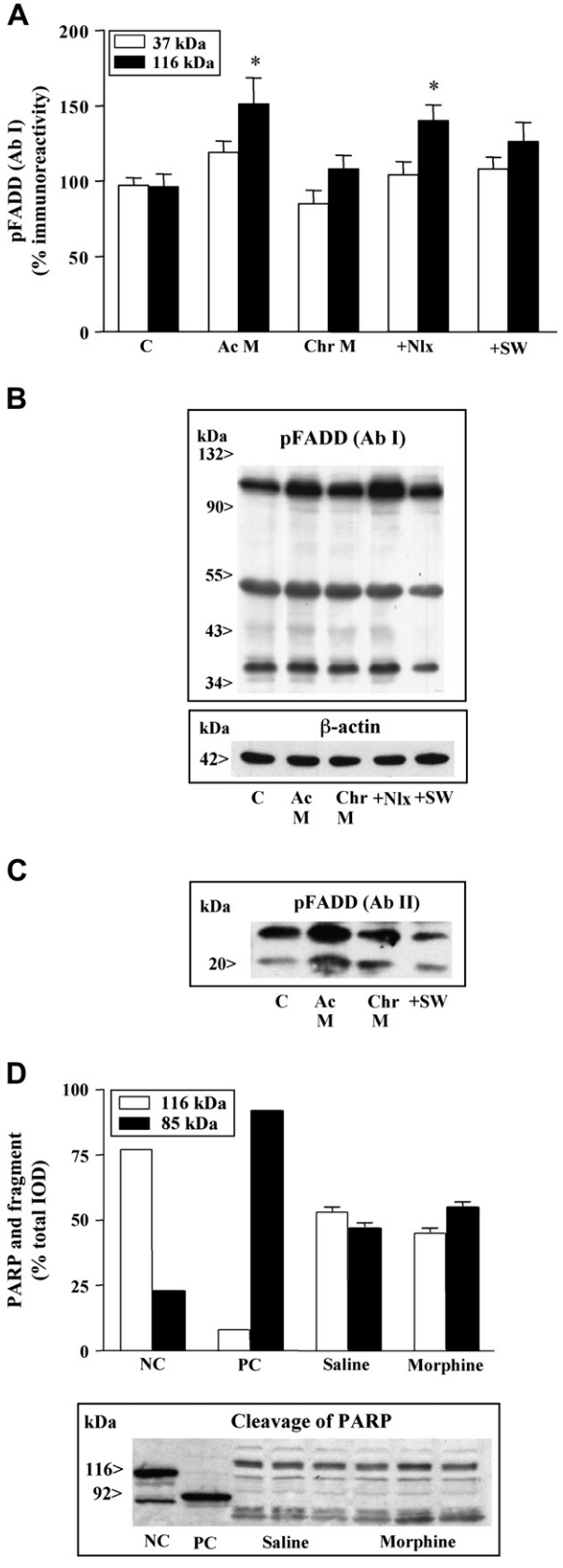


Fig. 4. (A–C) Acute effect of sufentanil on p-FADD content (Ab I) and antagonism by naloxone in rat cerebral cortex. (A) Groups of treatments: saline (C, $n = 6$), sufentanil (Suf, 15 μg/kg, s.c., 30 min; $n = 4$), naloxone (Nlx, 10 mg/kg, i.p., 60 min; $n = 4$) and Nlx + Suf ($n = 4$). Columns are means ± S.E.M. of n experiments per group with an animal per experiment, and expressed as percentage of saline-treated rats. For ≈116 kDa p-FADD, but not the ≈37 kDa band, ANOVA detected a significant difference between the groups of treatments [$F(3,14) = 8.80$, $p = 0.0019$]. $^{*}p < 0.05$ vs. control, and $^{\dagger}p < 0.05$ vs. sufentanil (ANOVA followed by Bonferroni's test). (B–D) Representative immunoblots of p-FADD species and β-actin. (E, F) Representative immunoblots depicting the subcellular localization of p-FADD (E: Ab I; F: Ab II) in the cerebral cortex of control rats (C), and the acute effects of sufentanil (Suf, 15 μg/kg, s.c., 30 min) on p-FADD content in the various compartments (F1: cytosolic fraction; F2: membrane/organelle fraction; F3: nuclear fraction; F4: cytoskeletal fraction). (G) Immunoblots of selective subcellular markers: stathmin (F1), Fas receptor (F2), PAR-4 (F3) and NF-L (F4).

In contrast, the δ-agonist SNC-80 (10 mg/kg, 30 min) and the κ-agonist (−)-U50488H (10 and 30 mg/kg, 30 min) did not alter the content of CKIα (data not shown). Similarly, chronic (5 days) treatments with morphine (10–100 mg/kg), SNC-80 (10 mg/kg) and (−)-U50488H (10 mg/kg) did not result in significant modulations of CKIα in the cortex (data not shown). At the subcellular level, sufentanil (15 μg/kg) reduced CKIα in the cytosol (30 ± 3%, $n = 3$, $p < 0.01$) with a concomitant increase in membranes (43 ± 11%, $n = 3$, $p < 0.05$) but not in nucleus (9 ± 11%, $n = 3$, $p > 0.05$) (Fig. 8C). A high dose of morphine (100 mg/kg) increased CKIα in the cytosol (104 ± 9%, $n = 3$, $p < 0.01$), membranes (62 ± 11%, $n = 3$, $p < 0.05$) and nucleus (36 ± 6%, $n = 3$, $p < 0.05$) (Fig. 8D).



SNC-80 (10 mg/kg) did not alter CKIα content in any subcellular compartment (Fig. 8E).

3.7. Effects of pertussis toxin on FADD basal contents and on δ-opioid receptor-induced changes in FADD and p-FADD in rat brain

PT was used as a tool to assess the involvement of inactivation of $G\alpha_{i/o}$ proteins in the in vivo modulation of FADD and p-FADD by SNC-80. Unexpectedly, PT (3 μg, 24 h) markedly reduced the basal content of FADD (63%) and augmented that of p-FADD (116 kDa: 41%; 23 kDa: 45 ± 6%, $n = 4$, $p < 0.05$) in the cerebral cortex (Fig. 9A,B). In the same brain samples, PT did not alter the expression of native, glycosylated and aggregated Fas receptor forms to which FADD is coupled (data not shown). PT decreased the amount of $G\alpha_{i2}$ proteins in the cortex (24%) (Fig. 9C).

As expected, SNC-80 (10 mg/kg) resulted in downregulation of FADD and upregulation of p-FADD forms in the cortex (Fig. 9A,B). In PT-pretreated rats, however, the effects of SNC-80 on FADD and p-FADD were not attenuated (Fig. 9A,B). None of these treatments (PT, SNC-80, PT + SNC) altered the content of 37 kDa p-FADD or that of β-actin in the cortex (Fig. 9A,B).

3.8. Effects of MEK inhibition on δ-opioid receptor-induced changes in p-FADD in rat brain

Brain MEK1/2 inhibition with SL327 was shown to prevent the downregulation of FADD induced by SNC-80, demonstrating the involvement of ERK1/2 activation in this regulation (García-Fuster et al., 2007a). Therefore, the influence of MEK-ERK signaling in the regulation of p-FADD by SNC-80 was investigated. MEK1/2 inhibition with SL327 (10, 20 and 30 mg/kg, 90 min) did not alter the basal contents of FADD, p-FADD (except a marginal increase with the highest dose) and CKIα in the cerebral cortex (Fig. 10A,B) or corpus striatum (data not shown).

As expected, SNC-80 (10 mg/kg) induced upregulation of oligomeric and monomeric p-FADD in the cortex (Fig. 10A,B). Pretreatment with SL327 (20 mg/kg), however, did not prevent the increases of p-FADD forms induced by SNC-80, but instead the effects were slightly potentiated (Fig. 10A,B). Similar results were obtained in the striatum (data not shown). None of these treatments (SL327, SNC-80, SL + SNC) altered the content of 37 kDa p-FADD or that of β-actin in the cortex (Fig. 10A,C).

4. Discussion

4.1. Immunodetection and subcellular localization of phosphorylated FADD in brain

Monomeric and oligomeric (≈116 kDa) p-FADD forms were immunodetected in rat, mouse and human brain tissue irrespective of the antibody origin, anti-mouse or anti-human, and the specific Ser191 or Ser194 phosphorylation site that lies outside the FADD apoptotic region. To our knowledge the equivalent p-Ser on rat

Fig. 5. (A) Acute, chronic, and withdrawal effects of morphine on p-FADD content (Ab I) in the rat cerebral cortex. Groups of treatments: saline (C, $n = 9$), acute morphine (Ac M, 100 mg/kg, i.p., 2 h; $n = 5$), and chronic morphine (Chr M, 10–100 mg/kg for 5 days; $n = 9$) followed by naloxone (+Nlx, 2 mg/kg; $n = 6$)-precipitated (2 h) or spontaneous (+SW, 48 h; $n = 4$) opioid withdrawal. Columns are means ± S.E.M. of n experiments per group with an animal per experiment, and expressed as percentage of saline-treated rats. For ≈116 kDa p-FADD, but not the ≈37 kDa band, ANOVA detected a significant difference between the groups of treatments [$F(4,28) = 4.10$, $p = 0.0094$]. $^*p < 0.05$ vs. control (ANOVA followed by Bonferroni's test). (B, C) Representative immunoblots of p-FADD species and β-actin. (D) Acute effects of saline and morphine (100 mg/kg, 2 h; $n = 3$) on PARP cleavage in the rat cerebral cortex. NC: negative control (whole cell extract of human HL60 leukemia cells). PC: positive control (etoposide-induced apoptosis in HL60 cells).

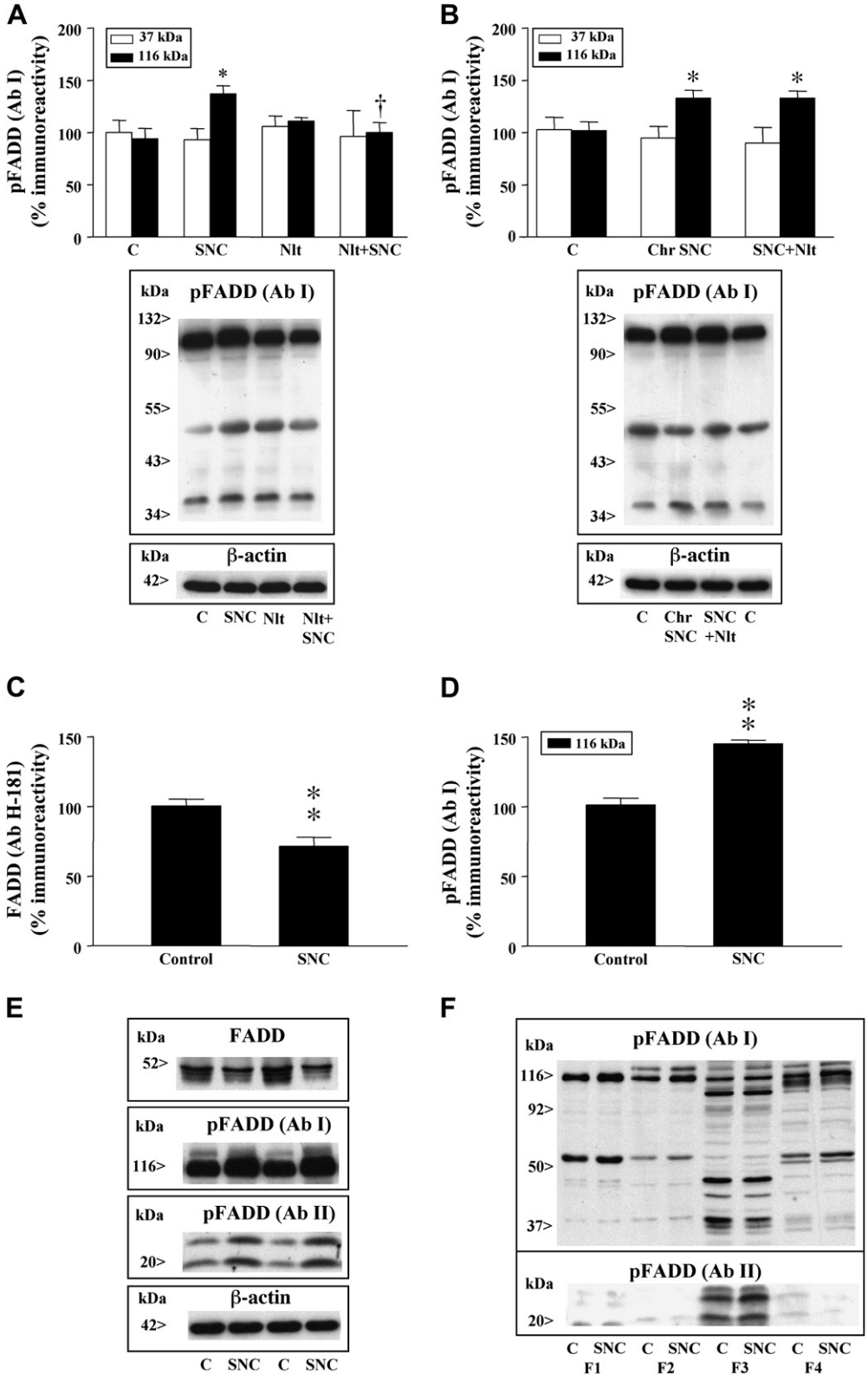


Fig. 6. Acute, chronic and withdrawal effects of SNC-80 on p-FADD content (Ab I) in the rat cerebral cortex. (A) Antagonism by naltrindole. Groups of treatments: vehicle (C, $n = 5$), SNC-80 (SNC, 10 mg/kg, i.p., 30 min; $n = 6$), naltrindole (Nlt, 5 mg/kg, 60 min; $n = 3$) and Nlt + SNC ($n = 5$). Columns are means ± S.E.M. of n experiments per group with an animal per experiment, and expressed as percentage of saline-treated rats. For ≈116 kDa p-FADD, but not the ≈37 kDa band, ANOVA detected a significant difference between the groups of treatments [$F(3,15) = 5.30$, $p = 0.010$]: $^{*}p < 0.05$ vs. control; $^{\dagger}p < 0.05$ vs. SNC-80 (ANOVA followed by Bonferroni's test). (B) Chronic effects of vehicle (C, $n = 8$) and SNC-80 (Chr, SNC 10 mg/kg for 5 days; $n = 4$) followed by naltrindole (SNC + Nlt, 5 mg/kg)-precipitated (2 h) opioid withdrawal ($n = 5$). Other details as above. ANOVA, [$F(2,14) = 5.40$, $p = 0.017$]: $^{*}p < 0.05$ vs. control. (C, D) Opposite acute effects of SNC-80 (30 mg/kg, 30 min; $n = 7$) on FADD (C, Ab H-181) and p-FADD (D, Ab I) in the rat cerebral cortex (C, control, $n = 6$). $^{**}p < 0.005$ vs. the corresponding control (Student's t-test). (E) Representative immunoblots of FADD, p-FADD species (Ab I and Ab II) and β-actin. (F) Representative immunoblots for the effect of SNC (10 mg/kg, 30 min) on the subcellular content of p-FADD (Ab I and Ab II) in the rat cerebral cortex (F1: cytosol; F2: membrane; F3: nucleus; F4: cytoskeleton). The corresponding immunoblots of selective subcellular markers are shown in Fig. 8E.

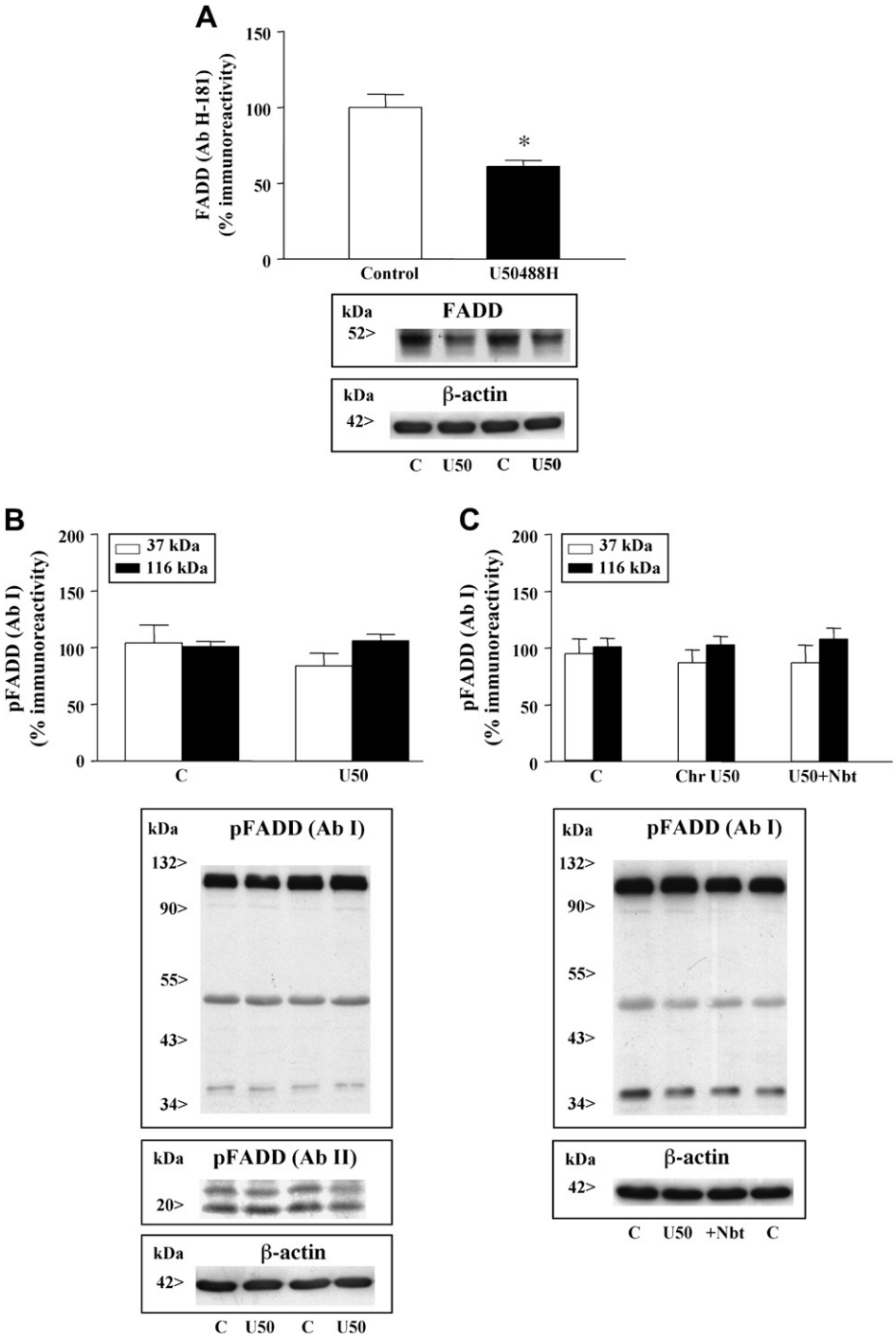


Fig. 7. Acute effects of (−)-U50488H on FADD (A, Ab H-181) and p-FADD (B, Ab I) contents in the rat cerebral cortex. Groups of treatments: saline (C, $n = 5$) and (−)-U50488H (U50, 30 mg/kg, i.p., 60 min, $n = 6$). Columns are means ± S.E.M. of n experiments per group with an animal per experiment, and expressed as percentage of saline-treated rats. $^*p < 0.005$ vs. the corresponding control (Student's t-test). (C) Chronic effects of saline (C, $n = 5$) and (−)-U50488H (Chr U50, 10 mg/kg for 5 days, $n = 5$) followed by nor-binaltorphimine (U50 + Nbt, 5 mg/kg)-precipitated (2 h) opioid withdrawal ($n = 5$) on p-FADD (Ab I) in the rat cerebral cortex. Other details as above. ANOVA did not detect significant differences between the groups of treatments for p-FADD forms [$F(2,12) = 0.08–0.20$, $p = 0.90$]. Bottom (A–C): representative immunoblots of FADD, p-FADD species (Ab I and Ab II) and β-actin.

FADD has not been mapped yet. However, there exists a good homology between rat and mouse FADD (87% sequence identity, 92% amino acid similarity) as well as rat and human FADD (68% identity, 82% similarity) (Zhang et al., 2004). This fact strongly suggests that one of the adjacent Ser194-Ser195 on rat FADD (Zhang et al., 2004) could be the specific phosphorylation site that, being recognized by the antibodies used, would mediate the anti-apoptotic function in this species. In a previous study, FADD (Ab H-181) was recognized as a homo-dimer/trimer in the mammalian brain (García-Fuster et al., 2007a). In the current study, ≈116 kDa oligomeric FADD was also identified, after prolonged film time exposures, in rat and mouse brain tissue with two antibodies recognizing total FADD protein (Ab H-181; Ab clone 28) regardless of its phosphorylation state. These data indicated that the adaptor FADD has the capacity to be oligomeric in brain, being expressed as self-associated high-order structures. It has been demonstrated

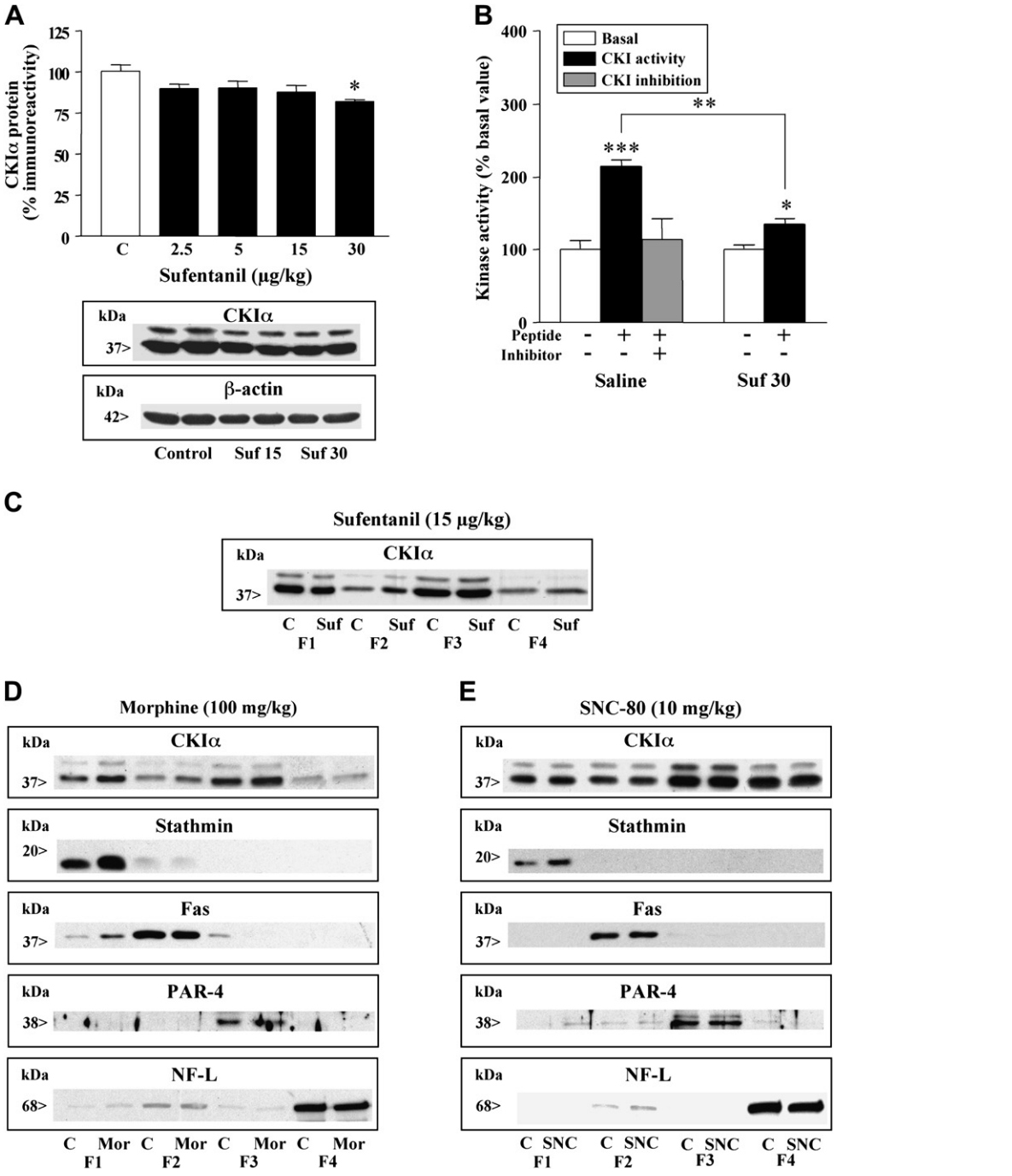


Fig. 8. (A) Dose-response for the acute effect of sufentanil on casein kinase Iα (CKIα) protein content in the rat cerebral cortex. Groups of treatments: saline (C, $n = 6$) and sufentanil (Suf, 2.5–30 μg/kg, s.c., 30 min, $n = 4$ for each group). Columns are means ± S.E.M. of n experiments per group with an animal per experiment, and expressed as percentage of saline-treated rats. ANOVA detected a significant difference between the groups of treatments [$F(4,17) = 3.30$, $p = 0.037$]. $^{*}p < 0.05$ vs. control (ANOVA followed by Bonferroni's test). A post-test for linear trend with increasing sufentanil doses was also significant ($p = 0.007$). Bottom: representative immunoblots of CKIα and β-actin (C, Suf 15 and suf 30 in duplicate). (B) Cortical cytosolic CKI activity in saline- and sufentanil (Suf, 30 μg/kg)-treated rats. Columns are means ± S.E.M. of three experiments (two concentrations of protein in duplicate), and expressed as percentage of the corresponding basal kinase activity (saline: 99 ± 12 cpm/μg protein; Suf: 192 ± 13 cpm/μg protein). Basal kinase activity for Suf reflects the activity of multiple kinases. CKI activity: specific peptide substrate phosphorylation. CKI inhibition: CKI activity in the presence of CKI inhibitor D4476. For other details see Section 2. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. corresponding basal value or when compared saline vs. sufentanil on CKI activity (Student's t-test). (C–E) Representative immunoblots for the effects of sufentanil (15 μg/kg, 30 min), morphine (100 mg/kg, 2 h) and SNC-80 (10 mg/kg, 30 min) on the subcellular content of CKIα protein in the rat cerebral cortex (F1: cytosol; F2: membrane; F3: nucleus; F4: cytoskeleton), with the corresponding blots of selective subcellular markers, except for sufentanil which are shown in Fig. 4G.

that FADD self-association, possibly dimers of receptor trimers, is functionally important for Fas signaling (Carrington et al., 2006; Muppidi et al., 2006; Sandu et al., 2006).

Since FADD couples Fas with initiator caspases in association with the plasma membrane, it was assumed that the adaptor was a cytoplasmic protein. Cell studies have shown that FADD or p-FADD is exclusively localized in the cytoplasm (O'Reilly et al., 2004), in cytoplasm and nucleus (Gómez-Angelats and Cidlowski, 2003; Alappat et al., 2005; Lee et al., 2006; Yoo et al., 2007) or solely within the nucleus (Screaton et al., 2003; Bhojani et al., 2005; Osborn et al., 2007), including that of neurons (Takagi et al., 2007). In rat brain, FADD was mainly localized in cytosol and nucleus

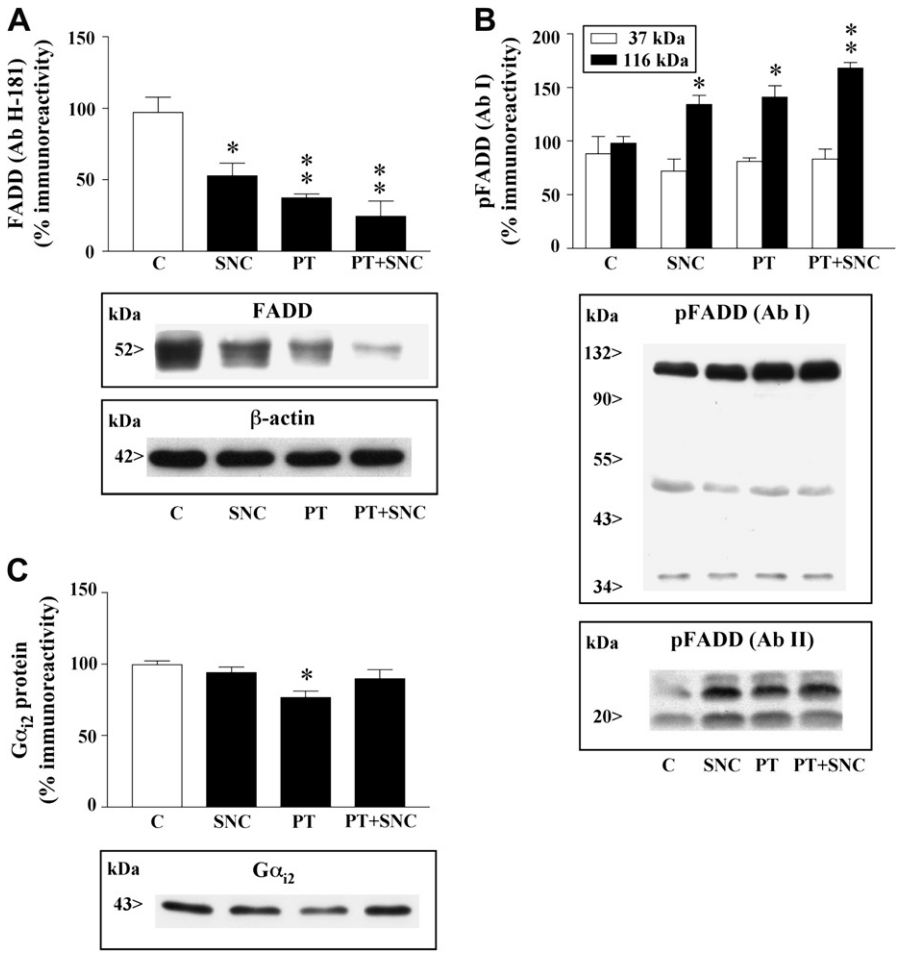


Fig. 9. (A, B) Effects of pertussis toxin (PT) on the contents of FADD (Ab H-181) and p-FADD (Ab I and Ab II), and lack of effects of the toxin on the downregulation of FADD and the upregulation of p-FADD induced by SNC-80 in the rat cerebral cortex. Groups of treatments: vehicle (C, $n = 5$), SNC-80 (SNC, 10 mg/kg, i.p., 30 min; $n = 5$), PT (3 μg, i.c.v, 24 h; $n = 5$) and PT + SNC ($n = 4$). Columns are means ± S.E.M. of n experiments per group with an animal per experiment, and expressed as percentage of vehicle-treated rats. ANOVA detected significant differences between the groups of treatments for FADD [$F(3,15) = 15.1$, $p = 0.0001$] and 116 kDa p-FADD [$F(3,15) = 11.2$, $p = 0.004$], but not for the 37 kDa p-FADD band. $^{*}p < 0.05$ and $^{**}p < 0.001$ when compared with the corresponding vehicle group (ANOVA followed by Bonferroni's test). (C) Effects of SNC-80 and PT (alone or in combination with the opioid) on $G\alpha_{i2}$ immunodensity in the rat cerebral cortex. Groups of treatments as above. ANOVA [$F(3,15) = 4.59$, $p = 0.012$]. $^{*}p < 0.05$ when compared with the control group. Bottom (A–C): representative immunoblots of FADD, p-FADD species, $G\alpha_{i2}$ proteins and β-actin.

(García-Fuster et al., 2007a). In the present study, oligomeric p-FADD followed a similar pattern and the monomeric p-form was detected in the nucleus, a localization associated with its non-apoptotic action (Screaton et al., 2003; Alappat et al., 2005; Werner et al., 2006).

4.2. μ/δ-Opioid receptor agonists enhance the phosphorylation state of FADD in brain

Acute treatments with the μ-agonists fentanil, sufentanil and morphine and the δ-agonist SNC-80 stimulated, through specific receptor mechanisms, the phosphorylation of oligomeric and monomeric FADD in cortical homogenates and, depending of the opioid, in the cytosolic, membrane and nuclear compartments. The induction of p-FADD vanished with repeated morphine, but not SNC-80, and opioid withdrawal induced a new (morphine) or sustained (SNC-80) stimulatory effect. In contrast, the κ-agonist (−)-U50488H failed to increase p-FADD in the cortex. These opioid treatments did not modulate the content of a 37 kDa p-FADD form, most probably a proteolytic product of oligomeric p-FADD.

Sufentanil, morphine and SNC-80 (acute, chronic and/or withdrawal effects) increased the content of oligomeric p-FADD (116 kDa; Ab I) with a concomitant decrease of FADD (Ab H-181) in the same cortical samples ($r = -0.42$; $n = 30$; $p = 0.018$; present data and García-Fuster et al., 2007a). This inverse relationship suggests that these opioids induced an increase in the ratio of p-FADD to FADD in rat brain. However, the κ-agonist (−)-U50488H failed to show a similar tendency (present data and García-Fuster et al., 2007a). The observed changes in the immunoreactivity of p-FADD and total FADD are likely to be due to changes in the phosphorylation status of the protein induced by the opioids or eventually to other changes of unknown nature. Recent in vitro studies with cancer cells have also reported the induction of p-FADD by paclitaxel, and that the balance between p-FADD and FADD can affect cell survival (Shimada et al., 2004, 2005). The current results support the concept of an interconversion between non-phosphorylated FADD and p-FADD after opioid treatment. In fact, FADD dephosphorylation resulted in an increased immunoreactivity of the non-phosphorylated form using Ab H-181 (García-Fuster et al., 2007a).

Sufentanil reduced total FADD in the cytosolic and nuclear compartments (García-Fuster et al., 2007a). In the present study, sufentanil markedly increased oligomeric p-FADD in membrane and nucleus, as well as nuclear monomeric p-FADD, suggesting a translocation of these p-forms to the nuclear compartment. FADD phosphorylation does not play a role in Fas signaling leading to

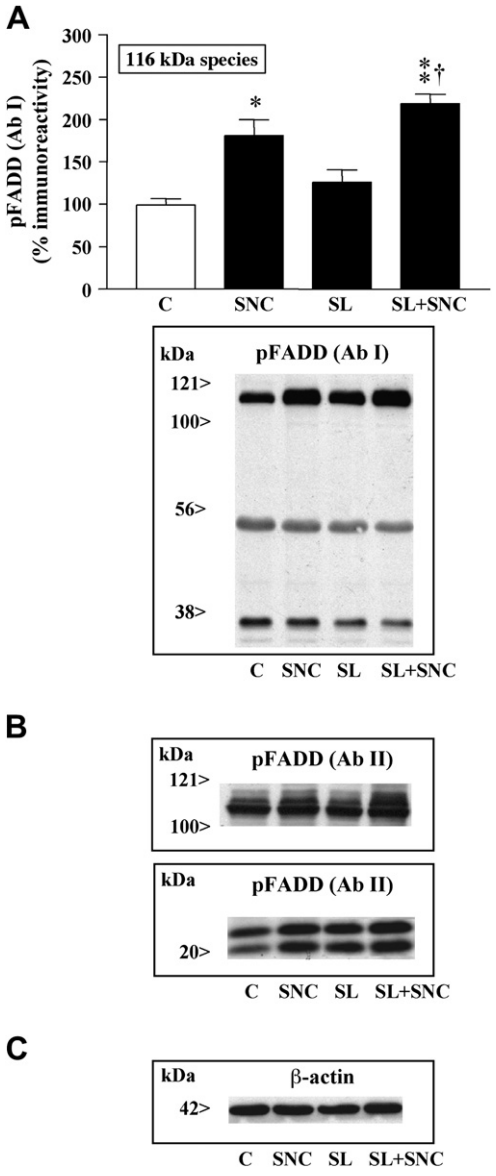


Fig. 10. (A) Effects of SL 327 (a selective MEK1/2 inhibitor) on the upregulation of 116 kDa p-FADD (Ab I and Ab II) content induced by SNC-80 in the rat cerebral cortex. Groups of treatments: vehicle (C, $n = 5$), SNC-80 (SNC, 10 mg/kg, i.p., 30 min; $n = 5$), SL 327 (SL, 20 mg/kg, i.p., 90 min; $n = 7$) and SL + SNC ($n = 5$). Columns are means ± S.E.M. of n experiments per group with an animal per experiment, and expressed as percentage of vehicle-treated rats. ANOVA detected a significant difference between the groups of treatments [$F(3,18) = 14.0$, $p < 0.001$]. $^{*}p < 0.01$; $^{**}p < 0.001$ when compared with the vehicle group; $^{\dagger}p < 0.001$ when compared with the SL 327 group (ANOVA followed by Bonferroni's test). Bottom (A–C): representative immunoblots of p-FADD (Ab I and Ab II) and β-actin.

apoptosis (Scaffidi et al., 2000), but it can regulate several non-apoptotic activities (Zhang et al., 2004; Alappat et al., 2005). In fact, a high dose of morphine that markedly increased p-FADD did not induce apoptosis, at least through the activation of caspase-3 and the subsequent PARP cleavage in rat cortex. This result was in line with previous negative findings dealing with the cleavage of pro-caspases-8/3 by various opioids and treatments (García-Fuster et al., 2007a). It has been suggested that the nuclear localization of p-FADD may be a regulatory mechanism where FADD is sequestered to abrogate Fas apoptotic signaling (Screaton et al., 2003; Bhojani et al., 2005). In contrast to sufentanil, SNC-80 did not increase nuclear p-FADD although the δ-opioid receptor could induce anti-apoptotic actions through its inhibitory endogenous tone on Fas/FADD complex (García-Fuster et al., 2007b).

These findings strongly suggest that the upregulation of p-FADD induced by μ- and especially δ-agonists might contribute to their anti-apoptotic (Tsao and Su, 2001; Narita et al., 2006) and neuroprotective effects (Zhang et al., 2002; Ma et al., 2005; Narita et al., 2006) (see Tegeder and Geisslinger, 2004; Barry and Zuo, 2005). Emerging evidence also suggests that apoptotic proteins can regulate brain synaptic plasticity (Gilman and Mattson, 2002). In this context, morphine-induced locomotor sensitization was associated with an increased p-FADD/FADD ratio in the rat corpus striatum and cerebral cortex (Ramos-Miguel et al., 2007), which supports the functional significance to the present findings.

4.3. Molecular interactions between opioid receptor signaling and FADD in brain

Because opioid receptors do not directly interact with FADD, various molecular mechanisms were explored in an attempt to clarify the positive signaling of opioids on FADD phosphorylation. CKIα is the main enzyme that phosphorylates specific Ser residues on FADD, and this interaction regulates FADD nuclear localization and non-apoptotic activities (Alappat et al., 2005). Therefore, the modulation of CKIα by opioids was investigated in rat cortex. The key results indicated that sufentanil induced a translocation of CKIα from the cytosol (protein and activity decreased) to the membrane (protein increased), and that morphine upregulated the content of this kinase in the cytosolic, membrane and nuclear compartments. In contrast, SNC-80, which also increased p-FADD in cytosol/membrane, did not alter CKIα content in any cellular compartment. Although CKIα could mediate the acute phosphorylation of FADD induced by μ-agonists, another kinase distinct from CKI (Scaffidi et al., 2000; Hua et al., 2003; Osborn et al., 2007) may be responsible for the observed effects of the δ-agonist SNC-80.

Opioid receptors transduce their signals via $G_{i/o}$ proteins (Law et al., 2000). SNC-80 was shown to stimulate ERK1/2 activation in vitro through a PT-sensitive mechanism (Audet et al., 2005). The inhibitory effect of SNC-80 on brain FADD was mediated through a mechanism fully dependent on ERK1/2 activation (García-Fuster et al., 2007a), which in turn is crucial in protecting against Fas/FADD-mediated apoptosis (Holmström et al., 1999, 2000). In the present study, the MEK inhibitor SL327 did not prevent the upregulation of p-FADD induced by SNC-80, indicating that ERK1/2 signaling is not required for this modulation. As mentioned, other kinases are responsible for the exclusive phosphorylation of FADD. On the other hand, it was anticipated that PT would block the opposite effects of SNC-80 on FADD and p-FADD in brain. Surprisingly, PT by itself markedly altered the basal levels of FADD (downregulation) and p-FADD (upregulation), precluding the net effects of its interaction with the δ-agonist which, contrary to the expectations, were not attenuated. To note that FADD does not contain any specific amino acid sequence with the cysteine residue that is required for ADP-ribosylation by PT (for G-proteins, see Fields and Casey, 1997). This finding revealed that brain FADD is a novel target of PT, whose mechanism of action remains uncertain. Interestingly, PT, presumably by blocking $G\alpha_{i2}$ signaling, was shown to impair neuroepithelial cell proliferation in the embryonic mouse brain, without altering cell death in the cortex (Shinohara et al., 2004), a finding that could be related, in part, to the toxin effects on FADD. Similarly, PT was reported to block etorphine-induced apoptosis in SK-N-SH cells (Yin et al., 1997), which could also be explained by the marked decrease in FADD induced by the toxin. It is worth noting that the opposite modulation of p-FADD and FADD induced by PT was similar to that reported for opioid drugs, which may indicate that a variety of agents shared common mechanisms for FADD regulation.

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