Naunyn-Schmiedeberg's Arch Pharmacol (2003) 368 : 421–431
DOI 10.1007/s00210-003-0801-9

ORIGINAL ARTICLE

**M. Julia García-Fuster · Marcel Ferrer-Alcón
Antonio Miralles · Jesús A. García-Sevilla**

Modulation of Fas receptor proteins and dynamin during opiate addiction and induction of opiate withdrawal in rat brain

Received: 25 April 2003 / Accepted: 19 August 2003 / Published online: 3 October 2003

Abstract The Fas receptor is involved in the regulation of apoptosis but also can function as a non-apoptotic signal transducer. This study was mainly designed to quantitate Fas proteins in rat brain during heroin addiction and opiate withdrawal. In rat, mouse and human brains, and in SH-SY5Y cells, similar forms of Fas were immunodetected with different antibodies (i.e., 35 kDa native Fas and 48- and 51-kDa glycosylated Fas). Acute (2 h) treatments with the μ-opioid receptor agonists heroin (10 mg/kg) and morphine (30 mg/kg) increased the immunodensity of native Fas (124% and 36%) but not that of glycosylated Fas in the cerebral cortex. Chronic (5 days) heroin (5–30 mg/kg) and morphine (10–100 mg/kg) were also associated with increased native Fas (76% and 45%) and with different expressions of glycosylated Fas. In heroin-dependent rats, opiate withdrawal (48 h) resulted in a sustained increase in native Fas (107%) and in up-regulation of 51 kDa glycosylated Fas (51%). Acute treatments with selective δ-receptor (SNC-80, 10 mg/kg) or κ-receptor (U 50488-H, 10 mg/kg) agonists did not alter the content of native or glycosylated Fas. Chronic pentazocine (10–80 mg/kg, 5 days), a mixed opiate drug and σ_1 receptor agonist, decreased native (48%) and glycosylated (38–82%) Fas proteins. Similarly, the selective σ_1 agonist (+)-SKF 10047 also decreased native Fas (37%) and the effect was blocked by the σ_1 antagonist BD 1063. Brain dynamin was up-regulated by acute and/or chronic heroin (30–39%), morphine (47–85%), pentazocine (51%) and heroin withdrawal (74%). The main results indicate that chronic heroin/morphine treatment and heroin withdrawal are associated with up-regulation of 35 kDa native Fas (and with different expressions of glycosylated Fas), and also with concomitant increases of dynamin in rat brain.

Keywords Fas receptor proteins · Dynamin · Opiate drugs · σ Ligands · Naloxone · Opiate addiction · Opiate withdrawal · Rat brain

M. J. García-Fuster · A. Miralles · J. A. García-Sevilla (✉)
Laboratory of Neuropharmacology,
Associate Unit of the Institute of Neurobiology
"Ramón y Cajal" (CSIC), Department of Biology,
University of the Balearic Islands,
Cra. Valldemossa Km 7.5, 07122 Palma de Mallorca, Spain
Fax: +41-22-3055799,
e-mail: jesus.a.garcia-sevilla@hcuge.ch

M. Ferrer-Alcón · J. A. García-Sevilla
Clinical Research Unit, Department of Psychiatry,
University of Geneva,
HUG Belle-Idée, 2 Chemin du Petit-Bel-Air,
1225 Chêne-Bourg/GE, Switzerland

Introduction

The Fas/Fas-L and other signaling systems are well-known cellular pathways associated with the physiological regulation of programmed cell death (apoptosis; MacEwan 2002). The Fas receptor (also known as CD95 or APO-1) is a prototype member of the tumor necrosis factor/nerve growth factor superfamily of death receptors (Itoh et al. 1991; Watanabe-Fukunaga et al. 1992; Nagata and Golstein 1995; Nagata 1999; Orlinick et al. 1999), which is widely expressed in normal tissues (Watanabe-Fukunaga et al. 1992) including the brain (Bechmann et al. 1999; Choi et al. 1999; Boronat et al. 2001). The Fas receptor has an extracellular domain with two putative N-linked glycosylation sites and a cysteine-rich region for ligand binding (Fas-L), and an intracellular death domain near de C-terminal region of the molecule (Watanabe-Fukunaga et al. 1992; Orlinick et al. 1999). Native Fas receptor has a relative molecular mass ≈35 kDa, but after post-translational modifications mature Fas is mostly expressed as glycosylated proteins of ≈45–52 kDa (Itoh et al. 1991; Oehm et al. 1992; Watanabe-Fukunaga et al. 1992; Kamitami et al. 1997).

Upon receptor activation by Fas-L, the intracellular death domain of Fas rapidly recruits the adaptor molecule FADD (Fas-associated death domain protein), which is followed by activation of various caspase cascades (caspase-8 proenzyme) and specific effector caspases, such as caspase-3, leading to apoptosis (Nagata 1999; Krammer 2000; MacEwan 2002). The induction of apoptosis in neu-

rons has been demonstrated to share the same basic mechanisms with all other cell types (Sastry and Rao 2000; Yuan and Yankner 2000). Besides the primary role of Fas/Fas-L in apoptosis, increasing evidence also indicates the importance of this system as a non-apoptotic signal transducer (Wajant 2002). Among these non-apoptotic functions, the Fas receptor has been shown to transduce proliferative signals in normal human diploid fibroblasts and T cells (Siegel et al. 2000), to mediate cardiomyocyte hypertrophy in vitro and in vivo (Badorff et al. 2002), to stimulate axonal growth in primary neurons (Desbarats et al. 2003), and Fas-L to facilitate antigen acquisition by dendritic cells in melanomas (Tada et al. 2002). In contrast to Fas-mediated apoptosis events, the apoptosis-unrelated biological effects of Fas are still poorly understood.

In a recent study (Boronat et al. 2001), chronic treatment of rats with morphine was shown to be associated, through a naloxone-sensitive mechanism, with an opposite modulation of two key proteins involved in the regulation of the programmed cell death in the brain: up-regulation of pro-apoptotic Fas receptor and down-regulation of anti-apoptotic Bcl-2 oncoprotein. Similarly, morphine also was shown to increase the expression of Fas receptor mRNA in heart, lung, spleen and lymphocytes of mice as well as in human lymphocytes (Yin et al. 1999). Moreover, Fas mRNA expression was found enhanced in splenic lymphocytes of stressed mice (i.e., with abnormal high contents of endogenous opioids), an effect that was antagonized by naloxone, indicating that Fas-mediated lymphocyte apoptosis is dependent on endogenous opioids (Yin et al. 2000). Recently, Fas overexpression and Fas-mediated lymphocyte apoptosis in stressed mice was found abolished in μ-opioid receptor deficient mice (Wang et al. 2002). Together these findings indicated that morphine, through the activation of μ-opioid receptors, could promote abnormal programmed cell death in specific types of neurons (but see Raoul et al. 2002) and other cells. Alternatively, the modulation of Fas receptor by morphine in the brain could be related to the recent discovery that this system (Fas/Fas-L) can also function as a non-apoptotic signal transducer in various cells including neurons (Wajant 2002; Desbarats et al. 2003).

Because of the potential importance of this topic, the present study was mainly designed to assess the modulation of Fas-related proteins (native and glycosylated Fas) during heroin addiction (acute and chronic effects) and the induction of opiate withdrawal in rat brain. For comparison, the acute effects of other opiate and non-opiate drugs were also quantitated. Moreover, the effects of some opiate drugs (heroin, morphine, pentazocine) on dynamin, a recently discovered target of chronic morphine action (Noble et al. 2000) that plays an essential role in receptor endocytosis, were also investigated. A preliminary report of a portion of this work was given at the XXIV congress of the Spanish Society of Pharmacology (García-Fuster et al. 2002).

Materials and methods

Animals and treatments. Adult male Sprague-Dawley rats (250–300 g) were used. The rats were housed under controlled environmental conditions (22°C, 70% humidity, and 12-h light/dark cycle) with free access to a standard diet and tap water. For the acute drug treatments, the rats received a single intraperitoneal (i.p.) injection of heroin (10 mg/kg), morphine (30 mg/kg), SNC-80 (10 mg/kg, a selective δ-opioid receptor agonist), U 50488H (10 mg/kg, a selective κ-opioid receptor agonist), pentazocine (15 mg/kg, an opiate drug and also a potent σ_1 receptor agonist; see Mei and Pasternak 2001, 2002), (+)-SKF 10047 (5 mg/kg, a selective σ_1 receptor agonist; see Matsuno et al. 1995) and BD 1063 (10 mg/kg, a selective σ_1 receptor antagonist; see McCracken et al. 1999). For the chronic treatment with heroin, the rats were injected i.p. three times daily (at 08:00, 14:00 and 20:00 h) during 5 consecutive days with increasing doses of the opiate as follows: day 1: 5, 10, and 10 mg/kg; day 2: 10, 15, and 15 mg/kg; day 3: 15, 20, and 20 mg/kg; day 4: 20, 25, and 25 mg/kg; day 5: 25 and 30 mg/kg (Ventayol et al. 1997). After this chronic heroin treatment, naloxone (2 mg/kg i.p., 2 h)-precipitated withdrawal or spontaneous (48 h) opiate withdrawal was induced which resulted in various withdrawal reactions (data not shown; Gabilondo and García-Sevilla 1995). In another series of chronic experiments, the animals were similarly injected with morphine (10 to 100 mg/kg) or pentazocine (10 to 80 mg/kg) during 5 days (Ventayol et al. 1997). Other rats were chronically treated with (+)-SKF 10047 (3–10 mg/kg for 3 days). In all series of experiments, control rats received 0.9% saline vehicle or DMSO (in the case of SNC-80) i.p. (1 ml/kg) at the indicated treatment times. The animals were killed by decapitation 2 h after the last dose in the acute and chronic drug treatments or at the times indicated after heroin withdrawal. The brains were rapidly removed and specimens of the cerebral cortex were dissected on ice and stored at –80°C until assay. This study was approved by the research and ethical review board of the Dirección General de Investigación (MCT, Madrid), and the experiments in rats followed the "Principles of laboratory animal care" (NIH publication No. 85–23, revised 1985) and were performed according to the guidelines of the University of the Balearic Islands.

Brain sample preparations and immunoblotting of Fas-related proteins and dynamin. The preparation of rat brain samples and the immunodetection of target proteins were performed as described previously (Ventayol et al. 1997; Boronat et al. 2001) with some modifications. Briefly, 150–200 mg of cerebral cortex was homogenized (1:20, wt/vol) in cold 40 mM Tris HCl buffer, pH 7.5, containing 1% Triton X-100, 1 mM EDTA, 1 mM $MgCl_2$, 5 mM NaCl, and the protease inhibitors phenylmethylsulfonyl fluoride (1 mM) and leupeptin (40 μg/ml). The samples were centrifuged at 40,000 × *g* for 45 min, and then 200 μl of the resulting supernatant (total Fas and dynamin) was mixed with an equal volume of electrophoresis loading buffer (Ventayol et al. 1997), which was then boiled (denatured) and stored at –20°C until use. Protein concentrations were determined by the method of Bradford (Bradford 1976). In addition to rat brain, mouse (cerebral cortex) and human (prefrontal cortex) brain samples (Ferrer-Alcón et al. 2003) and human SH-SY5Y neuroblastoma cells (a clone from SK-N-SH cells which expresses high densities of μ- and δ-opioid receptors, proportion 4:1; Yu et al. 1986; López and Ferrer 2000) were also used for the immunodetection and characterization of Fas-related proteins with different anti-Fas antibodies (see Fig. 1). For these preliminary experiments, total membranes were prepared similarly as above except that the detergent Triton X-100 was not included in the homogenization buffer and the samples were not centrifuged (total homogenates). In some experiments, rat cortical membranes were incubated in the absence or presence of the enzyme N-glycosidase F (15 units for 3 h at 37°C) (Ozaita et al. 1999) to further ascertain the nature of the various Fas receptor proteins (anti-Fas M-20 antibody). In routine experiments, 40 μg protein of each rat (and mouse or human) brain sample or SH-SY5Y cells (20 μg protein) was subjected to SDS-PAGE on 15-well (6×8-cm gels, 1 mm thickness, Bio-Rad Laboratories, Hercules, CA, USA) 10% polyacrylamide

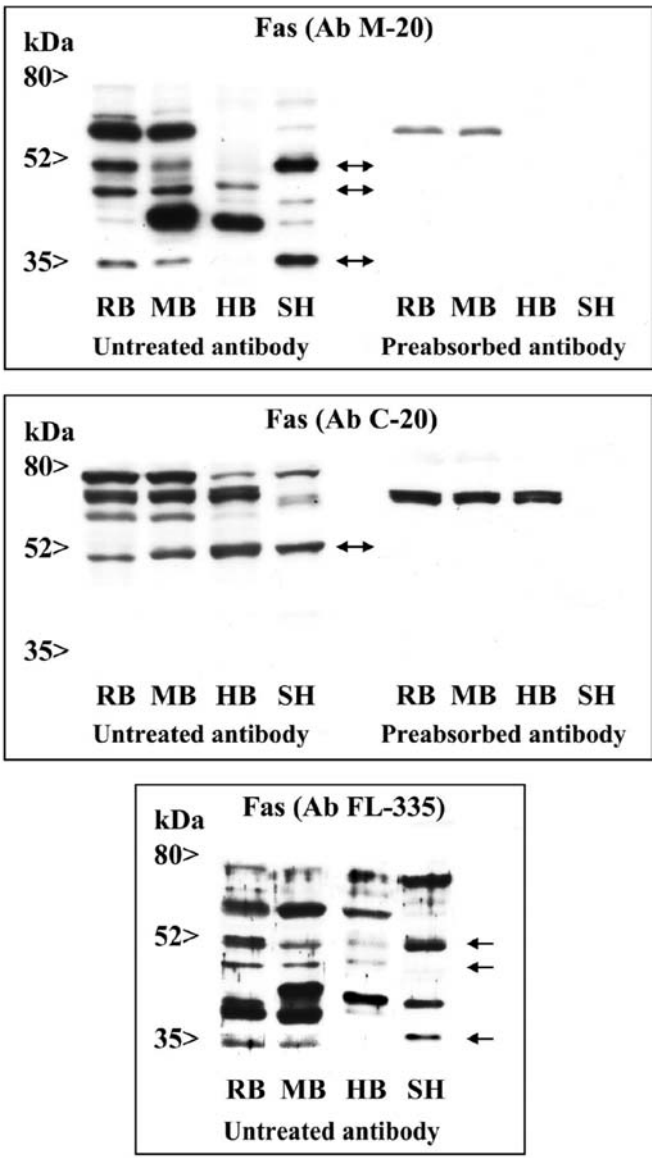

Fig. 1 Representative autoradiographs of Western blots depicting labeling of immunodetectable Fas-related proteins (≈35 kDa native Fas and ≈48 and 51 kDa glycosylated Fas, *arrows*) with various antibodies (M-20, C-20 and FL-335) in the rat brain (*RB*, cerebral cortex), mouse brain (*MB*, cerebral cortex), human brain (*HB*, prefrontal cortex) and human SH-SY5Y neuroblastoma cells (*SH*). The samples (40 µg protein for brain tissue and 20 µg protein for SH cells) were subjected to SDS-PAGE, transferred to nitrocellulose membranes (immunoblotting), incubated with the specific primary and secondary antibodies, and visualized by the Enhanced Chemiluminiscence method. The specificity of the antibodies anti-Fas M-20 and C-20 were assessed by preincubating the corresponding antibody with its antigenic peptide (preabsorbed antibody), which resulted in the blockade of the immunoreaction for the specific proteins (*double arrows*). The apparent molecular masses of Fas-related proteins were determined by calibrating the blots with prestained molecular weight markers as shown on the left hand side. For a given antibody all samples were run in the same gel

minigels. Proteins were electrophoretically transferred (110 V for 2–3 h) to nitrocellulose membranes (western blotting) that were incubated for 1 h in a phosphate-buffered saline containing 5% non-fat dry milk, 0.2% Tween 20 and 0.5% bovine serum albumin (blocking solution) (Harlow and Lane 1999). Then, the membranes were incubated overnight at 4°C in blocking solution containing the appropriate primary antibody: anti-Fas M-20 (rabbit polyclonal antibody raised against a peptide mapping the carboxyl terminus of Fas of mouse origin; dilution 1:2,000; sc-716, batches D219 and F251, Santa Cruz Biotechnology, CA, USA), anti-Fas C-20 (rabbit polyclonal antibody raised against a peptide mapping the carboxyl terminus of Fas of human origin [amino acids 300–319, cf. Kamitani et al. 1997]; dilution 1:2,000; sc-715, batch D172 Santa Cruz Biotechnology), anti-Fas FL-335 (rabbit polyclonal antibody raised against a recombinant protein corresponding to the full length Fas of human origin; dilution 1:1,000; sc-7886, batch C010 Santa Cruz Biotechnology) or anti-dynamin (mouse monoclonal antibody raised against a peptide mapping the carboxyl terminus of dynamin I of rat origin; dilution 1:2,000; clone 41, batch 4, Transduction Laboratories, Lexington, KY, USA). The anti-Fas antibodies used do not cross-react with other TNF superfamily transmembrane receptors. In order to test the selectivity of anti-Fas antibodies (M-20 and C-20) with specific proteins, the antigenic peptides (sc-716P and sc-715P, Santa Cruz Biotechnology) were preincubated in excess with the antisera to block the binding of the antibody to the specific protein species tested (Fig. 1). The secondary antibody, horseradish peroxidase-linked anti-rabbit or anti-mouse IgG, was incubated at 1:5,000 dilution in blocking solution at room temperature for 2 h. Immunoreactivity of target proteins was detected with the Enhanced Chemiluminiscence (ECL) Western Blot Detection system (Amersham International, Buckinghamshire, UK) and visualized by exposure to Hyperfilm ECL film (Amersham) for 30 s to 2 min (autoradiograms).

Quantitation of immunodensities of Fas-related proteins and dynamin. The autoradiograms were quantitated by densitometric scanning (GS-700 Imaging Densitometer, resolution: 42 µm, Bio-Rad, Hercules, CA, USA). The amount of target proteins (Fas and dynamin) in the cerebral cortex of rats treated with opiate or other drugs was compared in the same gel with that of control rats which received saline or DMSO solution. Prior to analyses, the linearity of protein concentration for Western blotting was ascertained by resolution of selected concentrations of protein (i.e., total protein loaded versus integrated optical density, IOD, units, consisting of 5 points of different protein content, usually 10 to 80 µg, resulting in good linear relations; data not shown, see Boronat et al. 2001). Experiments were performed by using 40 µg protein known to be within the linear range for immunolabeling of Fas-related proteins and dynamin. The quantification procedure was assessed 2–4 times in different gels (each gel with different brain 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. Since opiate drugs induce alterations in the content of cytoskeletal proteins such as neurofilaments (García-Sevilla et al. 1997; Boronat et al. 2001; Ferrer-Alcón et al. 2003), actin (Papakonstanti et al. 1998) and dynamin (Noble et al. 2000), a specific housekeeping cytoskeletal protein was not used as a negative control. Instead, unidentified peptides, not related to Fas, were also quantitated with anti-Fas antibodies (≈65 kDa band for M-20 and ≈70 kDa band for C-20, see Fig. 1) and the various opiate treatments did not alter significantly the immunodensity of these unknown peptides (e.g., mean ± SEM IOD values for the unidentified 65 kDa band, saline: 14.4±0.6, n=3; acute heroin: 14.1±0.3, n=3; chronic heroin: 14.0±0.3, n=3; chronic heroin plus naloxone: 14.6±0.4, n=3; chronic pentazocine: 12.8±0.6, n=3).

Data analyses and statistics. All series of data were analyzed with the program GraphPad Prism, version 3.0. Results are expressed as mean ± SEM values. One-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test, and Student's two-tailed t-test were used for the statistical evaluations. The level of significance was chosen as p=0.05.

Drugs and chemicals. Opiate drugs (and their sources) included heroin HCl and morphine HCl (Unión Químico-Farmacéutica S.A.E. (Madrid, Spain), (±) pentazocine HCl (Laboratorios Fides, Barce-

lona, Spain), SNC-80 ((+)-4-[(αR)-α-(2S,5R)-4-allyl-2,5-dimethyl-1-piperazinyl)-3-methoxybenzyl]-N-N,diethylbenzamide) (Tocris Cookson Ltd., Avonmouth, UK), U-50488-H HCl (1S-trans)-3,4-dichloro-N-methyl-N-[2-(1-pyrrolidinyl)cyclohexyl]-benzeneacetamide) (Sigma/RBI, St Louis, Mo, USA) and naloxone HCl (Endo Laboratories, Garden City, NY, USA). (+)-SKF 10047 HCl and BD 1063 HCl (1-[2-(3,4-dichlorophenyl)ethyl]-4-methylpiperazine) were purchased from Tocris. N-glycosidase F was from Sigma. Acrylamide (Protogel) was from BDH Brunschwig (Dorset, UK). Other materials such as the secondary antibodies, ECL reagents and autoradiography films were purchased from Amersham International (UK) or Santa Cruz Biotechnology (USA). All other chemicals were from Sigma Chemical.

Results

Immunodetection of Fas-related proteins in brain tissue and in SH-SY5Y cells

Fas is a widely expressed cell-surface receptor of relative molecular mass ≈35 kDa for native Fas and ≈45–52 kDa for their glycosylated forms (Itoh et al. 1991; Watanabe-Fukunaga et al. 1992). The antibodies used (M-20 and C-20) for the immunodetection of these Fas-related proteins (antisera directed against the cytoplasmic domain of Fas) were first tested for their specificity on Western blots of rat, mouse and human brain membranes as well as in human SH-SY5Y neuroblastoma cell membranes (no detergent added). In rat brain, the antiserum anti-Fas M-20 labeled specific proteins of Mr ≈35 kDa (native Fas) and ≈48 and 51 kDa (glycosylated Fas) (Fig. 1, top). Thus, previous preincubation of Fas M-20 antibody with the antigenic peptide (preabsorbed antibody) resulted in the blockade of the immunoreaction for the specific proteins (35-, 48- and 51-kDa), but not for other unknown unrelated peptide of higher molecular mass (≈65 kDa, Fig. 1, top). Similar results were obtained in mouse and human brains (including two prominent peptides of ≈45 kDa probably related to glycosylated Fas) and in SH-SY5Y cells (Fig. 1, top). In rat, mouse and human brain tissues as well as in SH-SY5Y cells, the antiserum anti-Fas C-20 labeled a specific protein of ≈51 kDa (glycosylated Fas) which was not immunodetected with the preabsorbed antibody (Fig. 1, middle). In these tissue and cell preparations, anti-Fas C-20 also immunodetected a specific band of ≈97 kDa (not shown) which probably represents a processed form of Fas aggregates (≈110–200 kDa; Kamitani et al. 1997). In rat, mouse and human brains and in SH-SY5Y cells, the pattern of Fas-related proteins immunodetected with the antiserum anti-Fas FL-335 (directed against the full length protein; antigenic peptide not available) was similar to that of anti-Fas M-20, although the immunoblots showed a higher background (Fig. 1, bottom). Therefore, with the use of various antibodies raised to independent epitopes of Fas it was possible to identify the same specific Fas-related proteins in rat brain (i.e., ≈35 kDa native Fas and ≈48 and 51 kDa glycosylated Fas).

In rat brain membranes extracted with 1% Triton X-100 and probed with anti-Fas M-20, the immunodensity of glycosylated 48 kDa Fas was greatly increased and that of 51 kDa Fas blunted (Fig. 2A). Protein extraction with this nonionic detergent also improved the immunodetection of native 35 kDa Fas (antibody M-20), glycosylated 51 kDa Fas (antibody C-20) and glycosylated 48- and 51-kDa Fas (antibody FL-335; see Figs. 1, 3). N-deglycosylation of Fas receptor induced a marked increase in the expression of native 35 kDa Fas and related peptides (≈43/45 kDa; Fig. 2B). Therefore, the effects of opiate and other drugs on Fas-related proteins were assessed in brain membranes extracted with Triton, and in which native 35 kDa Fas and glycosylated 48 kDa Fas were quantitated with antibody M-20, glycosylated 51 kDa Fas with antibody C-20, and both glycosylated forms of Fas also with antibody FL-335 (Fig. 3).

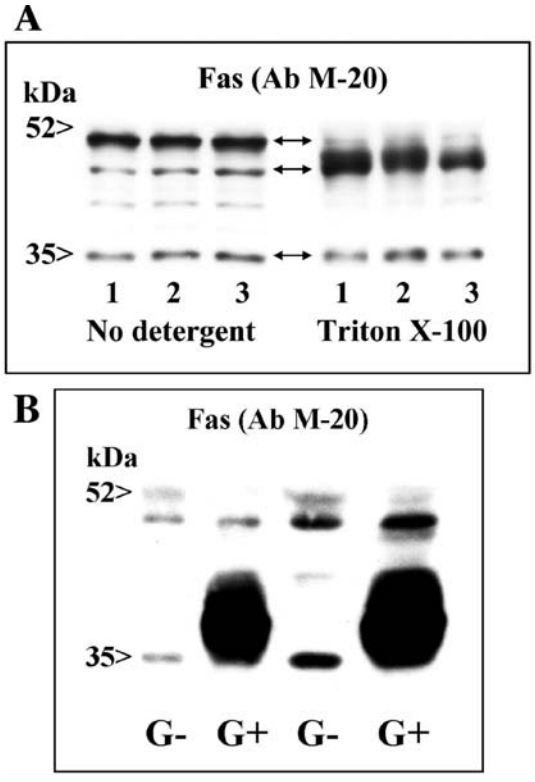

Fig. 2 **A** Immunodetection of Fas-related proteins (≈35 kDa native Fas and ≈48 and 51 kDa glycosylated Fas, *double arrows*) with antibody M-20 in the rat brain (cerebral cortex). The samples (40 µg protein, three animals in each group) were prepared in the absence or presence of a nonionic detergent (Triton X-100). Note that protein extraction with Triton markedly improved the immunodetection of 48 kDa glycosylated Fas and blunted that of 51 kDa glycosylated Fas. **B** Immunodetection of Fas-related proteins with antibody M-20 in the rat cerebral cortex after receptor deglycosylation. The samples (18 and 36 µg protein, prepared with Triton X-100) were incubated in the absence (G–) or presence (G+) of the enzyme N-glycosidase F (15 units for 3 h at 37°C). Note that N-deglycosylation of Fas receptor induced a marked increase in the expression of native 35 kDa Fas and related peptides (≈43/45 kDa). The apparent molecular masses of Fas-related proteins were determined by calibrating the blots with prestained molecular weight markers as shown on the left hand side

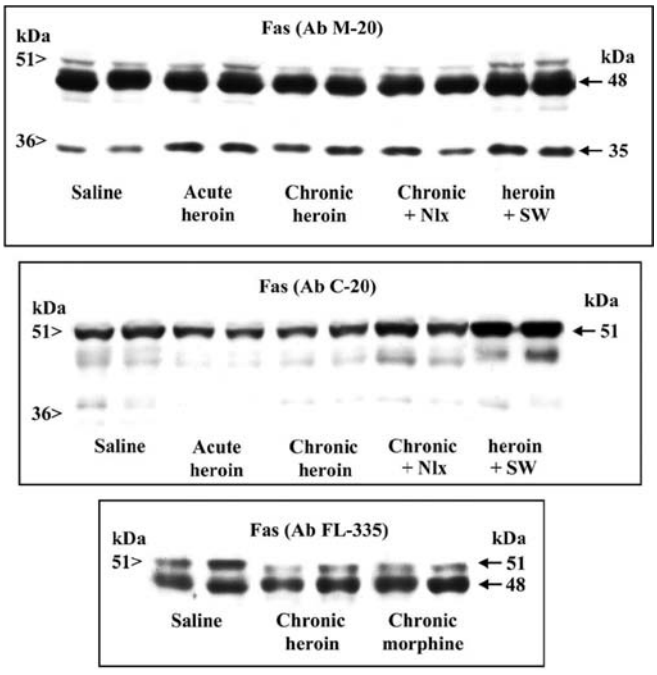

Fig. 3 Representative immunoblots of Fas-related proteins (≈35 kDa native Fas and ≈48 and 51 kDa glycosylated Fas, *arrows*) detected with different antibodies (M-20, C-20 and FL-335) in the rat cerebral cortex (40 µg protein extracted with Triton) after various heroin and morphine treatments. Groups of treatments (two animals for each group): saline, acute heroin (10 mg/kg, i.p., 2 h), chronic heroin (10–30 mg/kg i.p. for 5 days), chronic heroin plus naloxone (*Nlx*, 2 mg/kg i.p. for 2 h), chronic heroin plus spontaneous withdrawal (*SW*, 48 h) and chronic morphine (10–100 mg/kg for 5 days; only for antibody FL-335). The apparent molecular masses of Fas-related proteins were determined by calibrating the blots with prestained molecular weight markers as shown on the left hand side. For a given antibody all samples were run in the same gel

Effects of heroin and other opiate drugs on Fas-related proteins in rat brain

The acute treatment with the µ-opioid receptor agonist heroin (10 mg/kg, i.p. for 2 h), compared with saline solu-

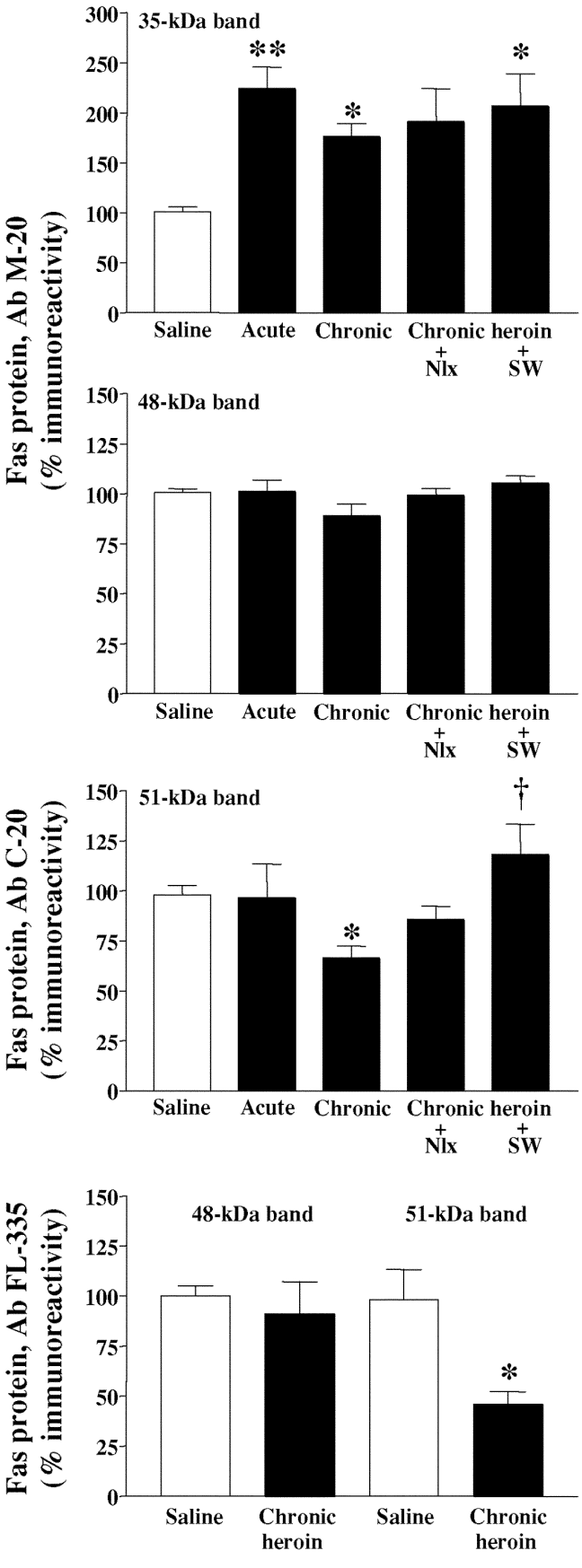

Fig. 4 Effects of acute and chronic treatments with heroin and of opiate withdrawal after the chronic treatment on the immunodensities of Fas-related proteins in the rat brain (cerebral cortex). Groups of treatments: saline, acute heroin (10 mg/kg, i.p., 2 h), chronic heroin (10–30 mg/kg i.p. for 5 days), chronic heroin plus naloxone (*Nlx*, 2 mg/kg i.p. for 2 h), chronic heroin plus spontaneous withdrawal (*SW*, 48 h). Quantitation of Fas-related proteins: 35 kDa native Fas and 48 kDa glycosylated Fas (antibody M-20); 51 kDa glycosylated Fas (antibody C-20), and 48 kDa and 51 kDa glycosylated Fas (antibody FL-335). Columns are means ± SEM of five experiments per group with an animal per experiment, and expressed as percentage of saline-treated rats. One-way ANOVA detected significant differences between groups with respect to 35 kDa native Fas (antibody M-20) [$F(4,21)=5.77$, $p=0.005$] and 51 kDa glycosylated Fas (antibody C-20) [$F(4,21)=3.38$, $p=0.028$]. *$p<0.05$; **$p<0.01$ when compared with the corresponding saline group, and †$p<0.05$ when compared with the chronic heroin group (ANOVA followed by Bonferroni's test). Chronic heroin treatment also reduced significantly the immunodensity of 51 kDa glycosylated Fas (antibody FL-335) *$p<0.05$ when compared with the saline group (Student's two-tailed *t*-test)

Table 1 Changes in Fas receptor protein immunoreactivities induced by various opiate drugs (heroin, morphine, μ-agonists; SNC-80, δ-agonist; U50488-H, κ-agonist) and σ_1 ligands (pentazocine, SKF10047, BD1063) in the rat cerebral cortex. Each value represents the mean ± SEM (expressed as percentage of the corresponding saline group [100%]) of *n* experiments per group with one animal per experiment. See Materials and methods for experimental details. *SW* spontaneous withdrawal, *NT* not tested

Treatment (time)	Dose (mg/kg)	Antibody M-20			Antibody 51 kDa	C-20 *n*
		35 kDa	48 kDa	*n*		
Acute heroin (2 h)	10	224±22**	101±6	5	97±16	5
Chronic heroin (5 days)	5–30	176±13*	89±6	5	67±6*	5
+ Naloxone (2 h)	2	191±33	99±3	5	86±6	5
+ SW (48 h)	–	207±32*	105±4	5	118±15†	5
Acute morphine (2 h)	30	136±8*	117±5	4	NT	
Chronic morphine (5 days)	10–100	145±7**	142±5**	4	NT	
Acute SNC-80 (2 h)	10	90±7	97±7	5	97±4	5
Acute U 50488-H (2 h)	10	119±9	102±7	4	95±2	4
Acute pentazocine (2 h)	15	95±10	118±9	3	97±5	3
Chronic pentazocine (5 days)	10–80	52±6**	62±9**	5	53±5***	5
Acute (+) SKF 10047 (2 h)	5	74±6*	93±6	4	93±9	4
Acute BD 1063 (2.5 h)	10	101±11	99±9	3	102±10	3
Acute BD + SKF	10+5	96±6	95±5	4	89±9	4
Chronic SKF 10047 (3 days)	3–10	61±6**	106±5	5	100±2	5

*$p<0.05$, **$p<0.01$, ***$p<0.0001$ when compared with its saline group; †$p<0.05$ when compared with the chronic heroin group (ANOVA followed by Bonferroni's test or Student's *t*-test)

tion administration, induced a marked increase in the immunodensity of 35 kDa native Fas in the rat cerebral cortex (124%, $p<0.01$) (Figs. 3 top, and 4). In contrast, acute heroin treatment did not alter significantly the immunodensities of 48- and 51-kDa glycosylated Fas in rat brain (Figs. 3, top and middle, 4; Table 1). Chronic treatment with heroin (5–30 mg/kg for 5 days) also was associated with an increase in the immunodensity of 35 kDa native Fas in rat the cerebral cortex (76%, $p<0.05$) (Figs. 3 top, 4), but this effect was less pronounced than that induced by the acute treatment, which suggested the induction of tolerance. Chronic heroin treatment did not modify significantly the immunodensity of 48 kDa glycosylated Fas (Figs. 3, top and bottom, 4; antibodies M-20 and FL-335) but decreased that of 51 kDa glycosylated Fas (33%, $p<0.05$ with antibody C-20; and 54%, $p<0.05$ with antibody FL-335; Figs. 3, middle and bottom, 4; Table 1).

Acute (30 mg/kg, i.p. for 2 h) and chronic treatment with morphine (10–100 mg/kg for 5 days) also increased the content of 35 kDa native Fas in the rat cerebral cortex (36% and 45%, $p<0.05$, respectively) (Table 1). Moreover, chronic morphine induced an increase in the immunodensity of 48 kDa glycosylated Fas (42%, $p<0.01$, Table 1, antibody M-20, and 43%, $p<0.01$, Fig. 3 bottom, antibody FL-335) and a decrease in that of 51 kDa glycosylated Fas (45%, $p<0.05$, Fig. 3 bottom, antibody FL-335).

Acute treatments with the opiate drugs SNC-80 (a selective δ-receptor agonist, 10 mg/kg i.p. for 2 h) or U 50488-H (a selective κ-receptor agonist, 10 mg/kg i.p. for 2 h) did not modify significantly the immunodensity of native or glycosylated Fas in the rat cerebral cortex (Table 1), which indicated that the rapid modulation of Fas proteins in the brain is related to the activation of μ-opioid receptors (heroin and morphine).

Effects of heroin withdrawal after chronic opiate treatment on Fas-related proteins in rat brain

In heroin-dependent rats (5–30 mg/kg for 5 days), naloxone (2 mg/kg)-precipitated withdrawal (2 h) or spontaneous opiate withdrawal (48 h) did not modify significantly the already increased immunodensities of 35 kDa native Fas (91%, $p=0.06$, and 107%, $p<0.05$, when compared to saline). Opiate withdrawal, like chronic heroin, did not induce significant changes in the content of 48-kDa glycosylated Fas (Figs. 3, top and middle, 4; Table 1). In these heroin-dependent rats, however, spontaneous opiate withdrawal (48 h) resulted in up-regulation in the density of 51-kDa glycosylated Fas (51% when compared to chronic heroin; Figs. 3 middle, 4; Table 1).

Effects of pentazocine and other σ ligands on Fas-related proteins in rat brain

The acute treatment with pentazocine (15 mg/kg, i.p. for 2 h), a σ_1 receptor agonist and a mixed nonselective δ/κ-opioid receptor agonist and μ-antagonist, did not modify significantly the immunodensity of native or glycosylated Fas in the rat cerebral cortex (Table 1). In contrast, chronic treatment with pentazocine (10–80 mg/kg for 5 days) resulted in marked decreases in the immunodensities of 35 kDa native Fas (48%, $p<0.01$), 48 kDa glycosylated Fas (38%, $p<0.01$ with antibody M-20, and 46%, $p<0.01$ with antibody FL-335), and 51 kDa glycosylated Fas (47%, $p<0.0001$ with antibody C-20, and 82%, $p<0.0001$ with antibody FL-335; Figs. 5, 6) in the brain (Table 1).

The acute treatment with the selective σ_1 receptor agonist (+) SKF 10047 (5 mg/kg i.p. 2 h) decreased the con-

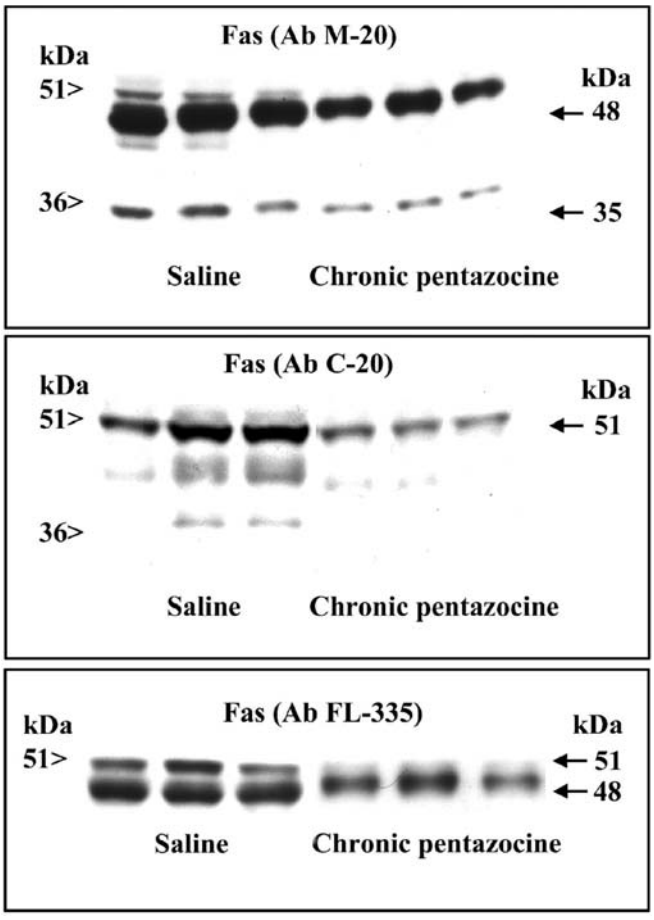

Fig. 5 Representative immunoblots of Fas-related proteins (≈35 kDa native Fas and ≈48 and 51 kDa glycosylated Fas, arrows) detected with different antibodies (M-20, C-20 and FL-335) in the rat cerebral cortex (40 µg protein extracted with Triton) after chronic pentazocine administration. Groups of treatments (three animals for each group): saline and chronic pentazocine (10–80 mg/kg i.p. for 5 days). The apparent molecular masses of Fas-related proteins were determined by calibrating the blots with prestained molecular weight markers as shown on the left hand side. For a given antibody all samples were run in the same gel

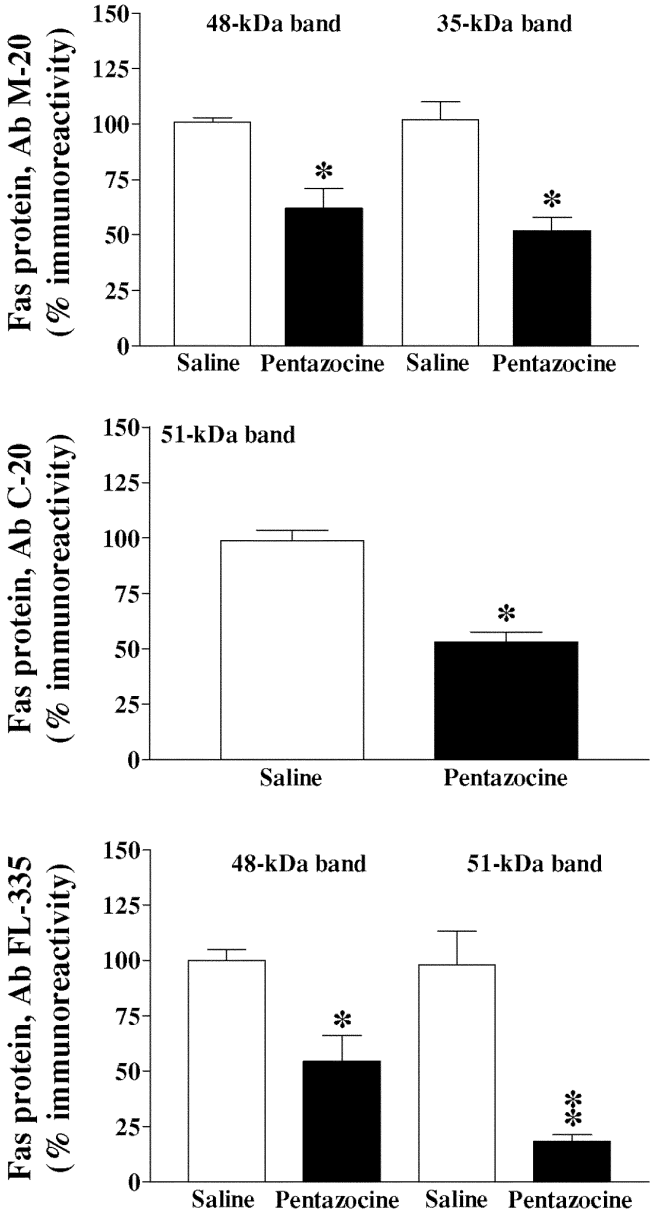

Fig. 6 Effect of chronic treatment with pentazocine on the immunodensities of Fas-related proteins in the rat brain (cerebral cortex). Groups of treatments: saline and chronic pentazocine (10–80 mg/kg i.p. for 5 days). Quantitation of Fas-related proteins: 48 kDa glycosylated Fas and 35 kDa native Fas (antibody M-20); 51 kDa glycosylated Fas (antibody C-20), and 48 kDa and 51 kDa glycosylated Fas (antibody FL-335). Columns are means ± SEM of five experiments per group with an animal per experiment, and expressed as percentage of saline-treated rats. *p<0.01; **p<0.0001 when compared with the corresponding saline group (Student's two-tailed t-test)

tent of 35 kDa native Fas (26%, p<0.05), but not that of glycosylated Fas, in the rat cerebral cortex (Table 1). This inhibitory effect on native Fas was antagonized by BD 1063, a selective σ_1 receptor antagonist (Table 1). Chronic treatment of rats with (+)-SKF 10047 (3–10 mg/kg for 3 days) also resulted in a sustained decrease in 35 kDa native Fas in the brain (37%, p<0.01) (Fig. 7, Table 1).

Effects of heroin, morphine and pentazocine, and of naloxone-precipitated heroin withdrawal on dynamin content in rat brain

The acute treatments with heroin (10 mg/kg, i.p. for 2 h) and morphine (30 mg/kg i.p. for 2 h), compared with saline solution administration, induced marked increases in the immunodensity of dynamin in the rat cerebral cortex (heroine: 39%, p<0.01; morphine: 85%, p<0.001; Fig. 8). Chronic treatments (5 days) with heroin (5–30 mg/kg) and morphine (10–100 mg/kg) were also associated with up-regulations in the content of dynamin in the brain (heroin: 30%, p<0.05; morphine: 47%, p<0.05), but these effects were less pronounced, although statistically not significant, than those induced by the acute treatments (especially in the case of morphine), which might suggest the

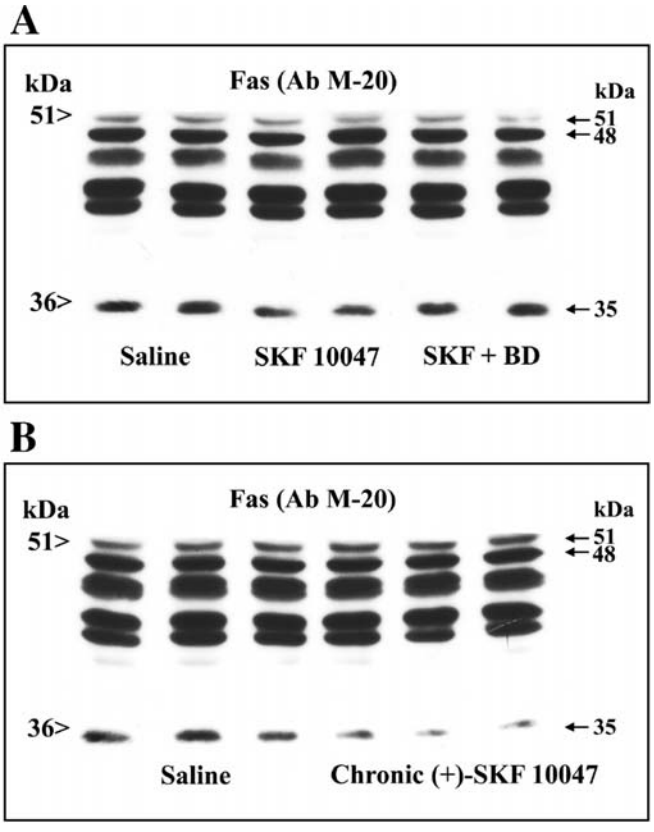

Fig. 7 Representative immunoblots of Fas-related proteins (≈35 kDa native Fas and ≈48 and 51 kDa glycosylated Fas, arrows) detected with antibody M-20 (batch F251) in the rat cerebral cortex (40 µg protein extracted with Triton) after **A** acute treatment with the selective σ_1 receptor agonist (+)-SKF 10047 and its interaction with the σ_1 antagonist BD 1063, and **B** chronic (+)-SKF 10047 administration. Groups of treatments (two or three animals for each group): saline, acute (+)-SKF 10047 (5 mg/kg i.p. for 2 h), acute BD 1063 (10 mg/kg i.p. for 2.5 h) + SKF 10047, and chronic SKF 10047 (3–10 mg/kg i.p. for 3 days). The apparent molecular masses of Fas-related proteins were determined by calibrating the blots with prestained molecular weight markers as shown on the left hand side. For **A** and **B** all samples were run in the same gel. Note that batch F251 for antibody M-20 (compared with batch D219 in Figs. 2, 3, 5) detected Fas related peptides of ≈44/46 kDa that were also immunodetected in mouse and human brains (see Fig. 1, top; batch D219)

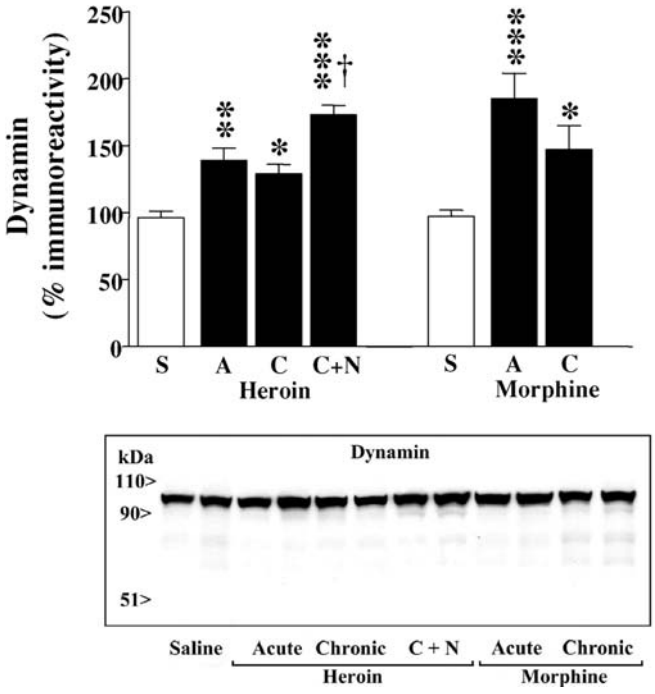

Fig. 8 Effects of acute and chronic treatments with heroin or morphine and of heroin withdrawal after the chronic treatment on the immunodensity of dynamin in the rat brain (cerebral cortex). Groups of treatments: saline (*S*), acute heroin (*A*, 10 mg/kg, i.p., 2 h), chronic heroin (*C*, 10–30 mg/kg i.p. for 5 days), chronic heroin plus naloxone (*C+N*, 2 mg/kg i.p. for 2 h), acute morphine (30 mg/kg i.p., 2 h) and chronic morphine (10–100 mg/kg i.p. for 5 days). Columns are means ± SEM of 4–8 (heroin) or 6–8 (morphine) experiments per group with an animal per experiment, and expressed as percentage of saline-treated rats. One-way ANOVA detected significant differences between groups with respect to dynamin immunodensity after heroin [$F(3,16)=22.7$, $p<0.0001$] and morphine [$F(2,17)=10.61$, $p=0.001$] treatments. *$p<0.05$; **$p<0.01$; ***$p<0.001$ when compared with the corresponding saline group, and †$p<0.01$ when compared with the chronic heroin group (ANOVA followed by Bonferroni's test). *Bottom:* Representative immunoblots for the effects of the various heroin and morphine treatments (two animals for each group, 40 µg protein extracted with Triton) on the immunodensity of dynamin in the rat cerebral cortex (all samples were run in the same gel). The apparent molecular mass of dynamin was determined by calibrating the blots with prestained molecular weight markers as shown on the left hand side

induction of tolerance (Fig. 8). Chronic pentazocine treatment (10–80 mg/kg for 5 days) also induced an increase in the immunodensity of dynamin in the rat cerebral cortex (51%, $p<0.05$) (data not shown). In heroin-dependent rats (5–30 mg/kg for 5 days), naloxone (2 mg/kg)-precipitated withdrawal (2 h) induced a marked up-regulation in the immunodensity of dynamin in the brain (74%, $p<0.001$), which was significantly greater (44%, $p<0.01$) than that observed after chronic heroin administration (Fig. 8).

Discussion

The immunodetection of Fas-related proteins (≈35 kDa native Fas and ≈48 and 51 kDa glycosylated Fas) in rat, mouse and human brains, as well as in SH-SY5Y neuroblastoma cells, was in good agreement with previous studies in various human cell lines, which used similar antibodies directed against the cytoplasmic domain of Fas (Kamitani et al. 1997). In Fas-deficient mice (disruption of the Fas gene by deletion of exon 9 coding for the receptor cytoplasmic region), normal size Fas mRNA and Fas-related proteins (≈40–45 kDa) were not expressed in thymocytes (Adachi et al. 1995). The heterogeneity of Fas expression in the mammal brain is most likely due to differential glycosylation of the two N-linked glycosylation sites in the extracellular domain of Fas (Itoh et al. 1991; Watanabe-Fukunaga et al. 1992; Kamitani et al. 1997; Krammer 2000), the expression of which apparently depends on sample preparation (e.g., protein extraction with the

nonionic detergent Triton X-100 improved the immunodetection of glycosylated Fas).

The major findings of the current study are that chronic heroin treatment (tolerant state) modulated differentially Fas receptor proteins (up-regulation of 35 kDa native Fas and down-regulation of 51 kDa glycosylated Fas) and that spontaneous heroin withdrawal (dependent state) was associated with increases of these forms of Fas in the brain (i.e. a sustained increase for native Fas and the induction of up-regulation for glycosylated Fas). Moreover, chronic morphine also induced up-regulation of 35 kDa native Fas. Interestingly, acute treatments with the μ-agonists heroin and morphine, but not with selective δ- and κ-agonists, increased the content of native Fas, which clearly indicated the involvement of μ-opioid receptors in the rapid modulation of Fas in the brain. The results extend previous findings on the modulation of Fas receptor (48 kDa glycosylated protein) in morphine-dependent rats, which was mediated through a naloxone-sensitive mechanism (Boronat et al. 2001). The results also confirm the general observation that activation of opioid receptors (mainly the μ-type) induces an increase in the expression of Fas receptor mRNA and protein in various tissues and cells (Yin et al. 1999, 2000; Chatzaki et al. 2001; Singhal et al. 2002; Wang et al. 2002). In this context, it is of interest to note that the basal immunodensity of 35 kDa native Fas was found decreased (30%, n=5, p<0.05) in μ-opioid receptor deficient mice (García-Fuster et al., unpublished results), suggesting that μ-receptors tonically stimulate, through endogenous opioid peptides, the activation of Fas in the brain. The content of glycosylated Fas proteins (48- and 51-kDa peptides) was not changed in μ-deficient mice (unpublished results; see also Wang et al. 2002). Moreover, the basal expression of 35 kDa native Fas was not modified in brains of δ- or κ-opioid receptor deficient mice (unpublished results). These data agree with the observed stimulating effects of acute heroin and morphine on 35 kDa Fas in rat brain and indicate the involvement of μ-opioid receptors in mediating the effects of these opiate agonists.

In a previous study, chronic morphine was associated with an increased content of 48 kDa Fas in the brain (Boronat et al. 2001). In the present study, chronic morphine also increased this glycosylated Fas form but decreased other (51 kDa protein). However, chronic heroin (and heroin withdrawal) did no alter 48 kDa glycosylated Fas in rat brain. The reason for this discrepancy is not known. Although morphine and heroin (diacetyl-morphine) may differ in some effects (Schuller et al. 1999), the in vivo efficacies of heroin, 6-acetylmorphine and morphine were shown to be mediated by pharmacologically similar populations of μ-opioid receptors (Negus et al. 2003). In any case, the current results indicate that heroin and morphine addiction in rats (tolerant and/or dependent states) is associated with up-regulation of 35 kDa native Fas (and with different expressions of glycosylated Fas) in the brain. At present, the molecular mechanisms by which heroin and other opiate drugs modulate the Fas receptor in the brain are not known. It is unclear whether the opioid receptors and the Fas receptor are physically associated or influence each other at the level of their signaling pathways. In a recent in vitro study (Singhal et al. 2002), morphine was shown to enhance the expression of FasL/Fas in macrophages which led to cell apoptosis, and this effect was mediated by opioid receptors via p38 mitogen-activated protein kinase (MAPK) phosphorylation. The MAPK and/or the modulation of other signaling pathways (one possibility would be through the interaction of inhibitory Gi/o proteins activated by opiate drugs and the Fas adaptor molecule FADD recruited after receptor stimulation) could also be involved in mediating the effects of opiate drugs on Fas in the brain.

In contrast to the acute and chronic effects of heroin and morphine on 35 kDa native Fas (up-regulation), chronic (±)-pentazocine induced down-regulation of native Fas in rat brain. Chronic pentazocine, but not chronic heroin, also reduced 48 kDa glycosylated Fas, and both treatments decreased 51 kDa glycosylated Fas in the brain. Pentazocine, a former "agonist-antagonist" opiate analgesic agent, is also a potent σ_1 receptor agonist (Mei and Pasternak 2001 and other references therein). Both enantiomers of pentazocine have good affinities for the cloned σ_1 receptor (Ki: 2.5–18 nM; Mei and Pasternak 2001), but (–)-pentazocine also has good affinity for the cloned μ- (Ki: 5.7 nM, antagonist), κ- (Ki: 7.2 nM, agonist) and δ- (Ki: 31 nM, agonist) opioid receptors (Raynor et al. 1994; Craft and McNiel 2003). Among other features, the receptors defined as σ are not longer opioid receptors and therefore they are naloxone-inaccessible (Quirion et al. 1992). Since chronic treatment with a high dose of naloxone (10 mg/kg for 13 days) did not alter the expression of Fas in the rat brain (Boronat et al. 2001), the inhibitory effects of chronic pentazocine on Fas cannot be related to its μ-antagonist properties. Similarly, acute treatments with selective δ- (SNC-80) and κ- (U 50488-H) agonists did not decrease Fas in the brain. In contrast, acute and chronic treatments with the selective σ_1 receptor agonist (+)-SKF 10047 decreased, through a σ_1 receptor-specific mechanism, the content of 35 kDa native Fas in the brain. It is of interest to note that the density of glycosylated Fas was also reduced by chronic pentazocine but not by the σ_1 agonist, suggesting that the former drug is also able to impair the proper post-translational modification of Fas receptor. At the functional level the σ_1 receptors comprise a potent anti-opioid system, the activation of which by (+)-pentazocine markedly reduces opiate analgesia in mice (Mei and Pasternak 2002). In line with this concept (anti-opioid effect), the down-regulation induced by chronic pentazocine on native Fas is also opposite to the up-regulation induced by heroin and morphine, and both effects are mediated trough different receptor mechanisms.

Dynamin is a neuronal phosphoprotein and a GTPase enzyme that plays an essential role in receptor-mediated endocytosis via clathrin-coated pits and caveoli (McClure and Robinson 1996). In the current study, chronic opiate drug treatments (heroin, morphine) and opiate withdrawal (heroin) resulted in marked up-regulations in the content of dynamin in the brain, indicating the importance of this

molecular target in opiate addiction. These results confirm and extend previous observations on the modulation of dynamin in morphine addiction (Noble et al. 2000). Conversely, chronic treatment with naltrexone, an opioid receptor antagonist, was associated with down-regulation (30%) of dynamin in mouse spinal cord (Patel et al. 2002). Moreover, the present data also indicate that acute heroin (10 mg/kg, 2 h) and morphine (30 mg/kg, 2 h) treatments resulted in up-regulation of dynamin in the rat cerebral cortex, which indicates its rapid modulation by opiate drugs. Furthermore, chronic pentazocine also up-regulated dynamin content in rat brain, which also suggests the involvement of σ_1 receptors in its regulation. The up-regulation of dynamin by opiate drugs could contribute to the plasticity of the endogenous opioid system, which is relevant in the development of tolerance and dependence to opiate drugs (see Noble et al. 2000). In this context, the up-regulation of dynamin in specific brain regions could regulate the level of expression of opioid receptors through modulation of receptor internalization induced by opiate drugs (Murray et al. 1998; Whistler and Von Zastrow 1999).

In conclusion, chronic heroin and morphine treatments and heroin withdrawal in rats are associated with up-regulation of 35 kDa native Fas (and with different expressions of glycosylated Fas) in the brain. At present, the functional consequences of the up-regulation of Fas receptor in opiate addiction are not known. Opiate drugs can promote, through the activation of Fas, abnormal cell death (Yin et al. 1999, 2000; Chatzaki et al. 2001; Singhal et al. 2002; Wang et al. 2002), but most neurons are resistant to Fas-induced apoptosis (Raoul et al. 2002). Alternatively, Fas could function as a non-apoptotic signal transducer (Wajant 2002; Desbarats et al. 2003), and in this case it would represent a new component in the opioid receptor signaling cascade potently modulated by opiate drugs.

Acknowledgements This study was supported by grant BFI2000–0306 from MCT (Madrid, Spain) and by grant 32.57066.99 from FNSRS (Bern, Switzerland). M.J.G.-F. was supported by a predoctoral fellowship from CSIC/MECD-Associated Units. J.A. García-Sevilla is a member of the Institut d'Estudis Catalans (Barcelona, Spain).

References

Adachi M, Suematsu S, Kondo T, Ogasawara T, Tanaka T, Yoshida N, Nagata S (1995) Targeted mutation in the Fas gene causes hyperplasia in peripheral lymphoid organs and liver. Nat Gen 11:294–300

Badorff C, Ruetten H, Mueller S, Stahmer M, Gehring D, Jung F, Ihling C, Zeiher AM, Dimmeler S (2002) Fas receptor signaling inhibits glycogen synthase kinase 3β and induces cardiac hypertrophy following pressure overload. J Clin Invest 109: 373–381

Bechmann I, Mor G, Nilsen J, Eliza M, Nitsch R, Naftolin F (1999) FasL (CD95L, Apo1L) is expressed in the normal rat and human brain. Glia 27:62–74

Boronat MA, García-Fuster MJ, García-Sevilla JA (2001) Chronic morphine induces up-regulation of the pro-apoptotic Fas receptor and down-regulation of the anti-apoptotic Bcl-2 oncoprotein in rat brain. Br J Pharmacol 134:1263–1270

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254

Chatzaki E, Makrigiannakis A, Margioris AN, Kouimtzoglou E, Gravanis A (2001) The Fas/FasL apoptotic pathway is involved in κ-opioid-induced apoptosis of human endometrial stromal cells. Mol Human Reprod 7:867–874

Choi C, Park JY, Lee J, Lim J-H, Shin E-C, Ahn Y, Kim C-H, Kim S-J, Kim J-D, Choi IS, Choi I-H (1999) Fas ligand and Fas are expressed constitutively in human astrocytes and the expression increases with IL-1, IL-6, TNF-α, or IFN-γ. J Immunol 162:1889–1895

Craft RM, McNeil DM (2003) Agonist/antagonist properties of nalbuphine, butorphanol and (–)-pentazocine in male vs. female rats. Pharmacol Biochem Behav 75:235–245

Desbarats J, Birge RB, Minouni-Rongy M, Weistein DE, Palerme J-S, Newell MK (2003) Fas engagement induces neurite growth through ERK activation and p35 upregulation. Nat Cell Biol 5:118–125

Ferrer-Alcón M, La Harpe R, Guimón J, García-Sevilla JA (2003) Down-regulation of neuronal cdk5/p35 in opioid addicts and opiate-treated rats: relation to neurofilament phosphorylation. Neuropsychopharmacology 28:947–955

Gabilondo AM, García-Sevilla JA (1995) Spontaneous withdrawal from long-term treatment with morphine accelerates the turnover of α_2-adrenoceptors in rat brain: up-regulation of receptors associated with increased receptor appearance. J Neurochem 64:2590–2597

García-Fuster MJ, Ferrer-Alcón M, Miralles A, García-Sevilla JA (2002) Modulation of pro-apoptotic Fas receptor proteins during heroin addiction in rat brain. Methods Find Exp Clin Pharmacol 24 [Suppl A]:161

García-Sevilla JA, Ventayol P, Busquets X, La Harpe R, Walzer C, Guimón J (1997) Marked decrease of immunolabelled 68 kDa neurofilament (NF-L) proteins in brains of opiate addicts. Neuroreport 8:1561–1565

Harlow E, Lane D (1999) Using antibodies. A laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor

Itoh N, Yonehara S, Ishii A, Yohenara M, Mizushima S-I, Sameshima M, Hase A, Seto Y, Nagata S (1991) The polypeptide encoded by the cDNA for human cell surface antigen Fas can mediate apoptosis. Cell 66:233–243

Kamitani T, Nguyen HP, Yeh ETH (1997) Activation-induced aggregation and processing of the human fas antigen. Detection with cytoplasmic domain-specific antibodies. J Biol Chem 272: 22307–22314

Krammer PH (2000) CD95's deadly mission in the immune system. Nature 407:789–795

López E, Ferrer I (2000) Staurosporine- and H-7-induced cell death in SH-SY5Y neuroblastoma cells is associated with caspase-2 and caspase-3 activation, but not with activation of the FAS/FAS-L-caspase-8 signaling pathway. Mol Brain Res 85:61–67

MacEwan DJ (2002) TNF ligands and receptors – a matter of life and death. Br J Pharmacol 135:855–875

Matsuno K, Senda T, Kobayashi T, Mita S (1995) Involvement of σ_1 receptor in (+)-N-allylnormetazocine-stimulated hippocampal cholinergic functions in rats. Brain Res 690:200–206

McClure SJ, Robinson PJ (1996) Dynamin, endocytosis and intracellular signalling. Mol Membr Biol 13:189–215

McCracken KA, Bowen WD, Matsumoto RR (1999) Novel σ receptor ligands attenuate the locomotor stimulatory effects of cocaine. Eur J Pharmacol 365:35–38

Mei J, Pasternak W (2001) Molecular cloning and pharmacological characterization of the rat sigma$_1$ receptor. Biochem Pharmacol 62:349–355

Mei J, Pasternak W (2002) σ_1 Receptor modulation of opioid analgesia in the mouse. J Pharmacol Exp Ther 300:1070–1074

Murray SR, Evans CJ, Von Zastrow M (1998) Phosphorylation is not required for dynamin-dependent endocytosis of a truncated mutant opioid receptor. J Biol Chem 273:24987–24991

Nagata S (1999) Fas ligand-induced apoptosis. Ann Rev Gen 33: 29–55

Nagata S, Golstein P (1995) The Fas death receptor. Science 267: 1449–1456

Negus SS, Brandt MR, Gatch MB, Mello NK (2003) Effects of heroin and its metabolites on schedule-controlled responding and thermal nociception in rhesus monkeys: sensitivity to antagonism by quadazocine, naltrindole and β-funaltrexamine. Drug Alcohol Depend 70:17–27

Noble F, Szücs M, Kieffer B, Roques BP (2000) Overexpression of dynamin is induced by chronic stimulation of μ- but not δ-opioid receptors: relationship with μ-related morphine dependence. Mol Pharmacol 58:159–166

Oehm A, Behrmann I, Falk W, Pawlita M, Maier G, Klas C, Li-Weber M, Richards S, Dhein J, Trauth B, Ponstingl H, Krammer P (1992) Purification and molecular cloning of the APO-1 cell surface antigen, a member of the tumor necrosis factor/nerve growth factor receptor family. J Biol Chem 267:10709–10715

Orlinick JR, Vaishnaw AK, Elkon KB (1999) Structure and function of Fas/Fas ligand. Int Rev Immunol 18:293–308

Ozaita A, Escribá PV, García-Sevilla JA (1999) The alkylating agent EEDQ facilitates protease-mediated degradation of the human brain α_{2A}-adrenoceptor as revealed by a sequence-specific antibody. Neurosci Lett 263:105–108

Papakonstanti EA, Bakogeorgou E, Castanas E, Emmanouel DS, Hartig R, Stournaras C (1998) Early alterations of actin cytoskeleton in OK cells by opioids. J Cell Biochem 70:60–69

Patel M, Gomes B, Patel V, Yoburn BC (2002) Antagonist-induced μ-opioid receptor up-regulation decreases G-protein receptor kinase-2 and dynamin-2 abundance in mouse spinal cord. Eur J Pharmacol 446:37–42

Quirion R, Bowen WD, Itzhak Y, Junien JL, Musacchio JM, Rothman RB, Su T-P, Tam SW, Taylor DP (1992) A proposal for the classification of sigma binding sites. Trends Pharmacol Sci 13:85–86

Raoul C, Estévez AG, Nishimune H, Cleveland DW, deLapeyrière O, Henderson CE, Haase G, Pettmann B (2002) Motoneuron death triggered by a specific pathway downstream of Fas: potentiation by ALS-linked SOD1 mutations. Neuron 35:1067–1083

Raynor K, Kong H, Chen Y, Yasuda K, Yu L, Bell GI, Reisine T (1994) Pharmacological characterization of the cloned kappa-, delta-, and mu-opioid receptors. Mol Pharmacol 45:330–334

Sastry PS, Rao KS (2000) Apoptosis and the nervous system. J Neurochem 74:1–20

Schuller AGP, King MA, Zhang J, Bolan E, Pan Y-X, Morgan DJ, Chang A, Czick ME, Unterwald EM, Pasternak GW, Pintar JE (1999) Retention of heroin and morphine-6β-glucoronide analgesia in a new line of mice lacking exon 1 of MOR-1. Nat Neurosci 2:151–156

Siegel RM, Ka-Ming Chang F, Chun HJ, Lenardo MJ (2000) The multifaceted role of Fas signaling in immune cell homeostasis and autoimmunity. Nat Immunol 1:469–474

Singhal PC, Bhaskaran M, Patel J, Patel K, Kasinath BS, Duraisamy S, Franki N, Reddy K, Kapasi AA (2002) Role of P38 mitogen-activated protein kinase phosphorylation and Fas-Fas ligand interaction in morphine-induced macrophage apoptosis. J Immunol 168:4025–4033

Tada Y, O-Wang J, Takiguchi Y, Tatsumi K, Kuriyama T, Okada S, Tokuhisa T, Sakiyama S, Tagawa M (2002) A novel role for Fas ligand in facilitating antigen acquisition by dendritic cells. J Immunol 169:2241–2245

Ventayol P, Busquets X, García-Sevilla JA (1997) Modulation of immunoreactive protein kinase C-α and β isoforms and G proteins by acute and chronic treatments with morphine and other opiate drugs in rat brain. Naunyn-Schmiedebergs Arch Pharmacol 355:491–500

Wajant H (2002) The Fas signalling pathway: more than a paradigm. Science 296:1635–1636

Wang J, Charboneau R, Barke RA, Loh HH, Roy S (2002) μ-Opioid receptor mediates chronic restraint stress-induced lymphocyte apoptosis. J Immunol 169:3630–3636

Watanabe-Fukunaga R, Brannan CI, Itoh N, Yonehara S, Copeland NG, Jenkins NA, Nagata S (1992) The cDNA structure, expression, and chromosomal assignment of the mouse Fas antigen. J Immunol 148:1274–1279

Whistler JL, Von Zastrow M (1999) Dissociation of functional roles of dynamin in receptor-mediated endocytosis and mitogenic signal transduction. J Biol Chem 274:24575–24578

Yin D, Mufson RA, Wang R, Shi Y (1999) Fas-mediated cell death promoted by opioids. Nature 397:218

Yin D, Tuthill D, Mufson RA, Shi Y (2000) Chronic restraint stress promotes lymphocyte apoptosis by modulating CD95 expression. J Exp Med 191:1423–1428

Yu VC, Richards ML, Sadée W (1986) A human neuroblastoma cell line expresses μ and δ opioid receptor sites. J Biol Chem 261:1065–1070

Yuan J, Yankner BA (2000) Apoptosis in the nervous system. Nature 407:802–809