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Deglycosylation of Fas receptor and chronic morphine treatment up-regulate high molecular mass Fas aggregates in the rat brain

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Abstract

This study was designed to immunodetect and characterize Fas receptor aggregates (oligomerization) in the brain and to assess its possible modulation in opiate addiction. High molecular mass, sodium dodecyl sulfate (SDS)- and β-mercaptoethanol-resistant Fas aggregates (~ 110/120 and ~ 203 kDa specific peptides) were immunodetected with a cytoplasmic domain-specific antibody in brain tissue (rat, mouse and human) and SH-SY5Y cells by Western blot analysis. Preincubation of rat cortical membranes with *N*-ethylmaleimide (NEM; 1 mM for 1 h at 37 °C) reduced the immunodensity of ~ 203 kDa Fas aggregates (51%) and increased that of 35 kDa native Fas (172%) and 51/48 kDa glycosylated Fas (47%), indicating that disulfide bonds are involved in Fas dimerization. Enzymatic *N*-deglycosylation of Fas receptor increased the content of Fas aggregates (~ 110/120 kDa: five- to sixfold, and ~ 203 kDa: two- to threefold), suggesting that Fas glycosylation is involved in regulating receptor dimerization. Chronic (10–100 mg/kg for 5 days), but not acute (30 mg/kg for 2 h), treatment with morphine (a μ-opioid peptide receptor agonist) induced up-regulation of Fas aggregates in the brain (~ 110/120 kDa: 39%, and ~ 203 kDa: 89%). The acute and/or chronic treatments with δ- and κ-opioid peptide receptor agonists and with a σ_1-receptor agonist did not readily alter the content of Fas aggregates in the rat brain. The results indicate that Fas aggregates are natively expressed in the brain and that its density is regulated by the state of Fas glycosylation. These forms of Fas (receptor homodimerization) are functionally relevant because they were up-regulated in the brain of morphine-dependent rats.

Keywords: Fas receptor aggregate; Fas protein; Deglycosylation; Opiate drug; Morphine addiction; Brain, rat

1. Introduction

The Fas receptor [also known as cytotoxicity-dependent protein (CD95) or apoptosis-1 protein (APO-1)] is a well-known transmembrane protein critically involved in the regulation of apoptosis (Nagata, 1999; Krammer, 2000; Sastry and Rao, 2000; Yuan and Yankner, 2000) which can also function as a nonapoptotic signal transducer (Siegel et al., 2000b; Badorff et al., 2002; Tada et al., 2002; Wajant, 2002; Desbarats et al., 2003). Although initially restricted to the immune system, Fas protein is widely expressed in normal tissues (Watanabe-Fukunaga et al., 1992), including the brain of mammals (Bechmann et al., 1999; Choi et al., 1999; Boronat et al., 2001; García-Fuster et al., 2003). Native Fas receptor has a molecular mass of ~ 35 kDa, but after posttranslational modifications, mature Fas is mostly expressed as glycosylated proteins of ~ 45–52 kDa (the N-terminal domain of Fas contains two putative N-linked glycosylation sites at N22 and N93; Itoh et al., 1991; Oehm et al., 1992; Watanabe-Fukunaga et al., 1992; Kamitani et al., 1997). In addition, high molecular mass (~ 110 to >200 kDa), sodium dodecyl sulfate (SDS)- and β-mercaptoethanol-resistant Fas aggregates have been detected by Western blot analysis, immunoprecipitation and other techniques in various cell lines (Kischkel et al., 1995; Kamitani et al., 1997;

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Papoff et al., 1999; Siegel et al., 2000a; Algeciras-Schimnich et al., 2002). These Fas aggregates appears to be composed of a complex of intact Fas monomers (Kamitani et al., 1997), and this receptor homo-oligomerization appears to be the initial signaling form of Fas (Kischkel et al., 1995; Algeciras-Schimnich et al., 2002).

Recently, Fas receptor proteins (35-kDa native and 48-kDa glycosylated Fas) were shown to be modulated by acute and/or chronic treatments with various opiate drugs in the rat brain (Boronat et al., 2001; García-Fuster et al., 2003). Notably, the observed up-regulation of 35-kDa native Fas after chronic heroin/morphine treatment and heroin withdrawal (García-Fuster et al., 2003) clearly indicated a relevant role of Fas in opiate addiction either as promoter of abnormal cell death, but most neurones are resistant to Fas-induced apoptosis (Raoul et al., 2002), or as a new nonapoptotic signal transducer in the opioid receptor signaling pathways. It is not known, however, whether chronic opiate treatment can also modulate the formation of high molecular mass Fas aggregates, one of the earliest events in Fas signaling (Algeciras-Schimnich et al., 2002). On the other hand, recent findings in heterologous cellular systems indicated that heterodimerization of α_{2A}- and β_1-adrenoceptors is regulated by receptor glycosylation (Xu et al., 2003), and this post-translational receptor modification could be also relevant in the regulation of Fas aggregates (homodimerization) in brain tissue.

In this context, the present study was designed to immunodetect and characterize high molecular mass Fas aggregates in brain tissue, to investigate the relation between Fas receptor glycosylation and the expression of Fas aggregates, and to assess whether chronic morphine treatment modulate these Fas forms in the rat brain. A preliminary report of a portion of this work was given at the X Congress of the Spanish Society of Neuroscience (García-Fuster et al., 2003).

2. Materials and methods

2.1. Dissociation of Fas aggregates and enzymatic deglycosylation of Fas receptor

For the dissociation of Fas aggregates, rat cortical membranes were incubated in sample buffer (see below) in the absence or presence of the lipophilic sulfhydryl alkylating reagents *N*-ethylmaleimide (NEM) or *N*-methylmaleimide (NMM; 1 mM for 1 h at 37 °C) to block native cysteine residues (the extracellular domain of Fas contains 18 cysteine residues; Itoh et al., 1991; Oehm et al., 1992). For the enzymatic deglycosylation of Fas receptor, cortical membranes were incubated in the absence or presence of *N*-glycosidase F (15 units for 3 h at 37 °C) as described previously (Ozaita et al., 1999; García-Fuster et al., 2003). Control and pretreated (NEM or *N*-glycosidase F) brain membranes were then further prepared for the immunodetection of Fas receptor and Fas aggregates by Western blot analysis (all samples were denatured by incubation for 4 min at 95 °C in electrophoresis buffer prior to loading, see below).

2.2. Animals and treatments

Adult male Sprague–Dawley rats (200–250 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 morphine (30 mg/kg), (+)-4-[(αR)-α-(2S,5R)-4-allyl-2,5-dimethyl-1-piperazinyl)-3-methoxybenzyl]-*N*-*N*,diethylbenzamide) (SNC-80, 10 mg/kg), 1S-*trans*(-3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl)cyclohexyl]-benzeneacetamide) (U 50488 HCl, 10 mg/kg) and (+)-*N*-allyl-normetazocine (SKF 10047, 5 mg/kg) (see García-Fuster et al., 2003 for details of these drugs). For the chronic treatment with morphine, the rats were injected i.p. three times daily during five consecutive days with increasing doses of the opiate (10–100 mg/kg), which resulted in a marked degree of opiate tolerance and dependence (García-Fuster et al., 2003). Other rats were chronically treated with (+)-SKF10047 (3–10 mg/kg for 3 days; García-Fuster et al., 2003). In all series of experiments, control rats received 0.9% saline vehicle or dimethyl sulphoxide (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. 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.

2.3. Brain samples and immunoblotting of Fas-related proteins

The preparation of rat brain samples and the immunodetection of Fas-related proteins were performed as described in detail previously (García-Fuster et al., 2003). Briefly, 150–200 mg of cerebral cortex was homogenised in 40 mM Tris–HCl buffer, pH 7.5, containing 1% Triton X-100, 1 mM EDTA, 1 mM $MgCl_2$, 5 mM NaCl, and various protease inhibitors. After centrifugation (40,000 × *g*, 45 min), aliquots of the supernatant (total Fas content) were mixed with electrophoresis-loading buffer (50 mM Tris–HCl pH 6.8, 10% glycerol, 1.5% SDS, 2.5% β-mercaptoethanol), denatured and stored at −20 °C until use. In addition to rat brain, mouse (cerebral cortex)

and human (prefrontal cortex) brain samples and human SH-SY5Y neuroblastoma cells (a cell line that expresses MOP and DOP receptors; i.e. μ/δ-opioid peptide receptors; Yu et al., 1986) were also used for the immunodetection and characterization of Fas aggregates with the anti-Fas M-20 antibody (García-Fuster et al., 2003). Protein concentrations were determined by the biuret reaction using bicinchoninic acid for colorimetric detection of cuprous cation (BCA, Protein Assay Reagent, Pierce Chemical, Rockford, IL, USA). 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 sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 10% polyacrylamide minigels (Bio-Rad Laboratories, Hercules, CA, USA). Proteins were transferred to nitrocellulose membranes and incubated in a blocking solution containing the appropriate primary polyclonal antibody (affinity-purified): anti-Fas M-20 (rabbit polyclonal antibody raised against a peptide mapping the carboxyl terminus of Fas of mouse origin; dilution 1:2000; sc-716, batches D219 and F251, Santa Cruz Biotechnology, CA, USA) or anti-Fas C-20 (rabbit polyclonal antibody raised against a peptide mapping the carboxyl terminus of Fas of human origin [amino acids 300–319] (Kamitani et al., 1997); dilution 1:2000; sc-715, batch D172 Santa Cruz Biotechnology). The secondary antibody, horseradish peroxidase-linked anti-rabbit immunoglobulin G, was incubated at 1:5000 dilution in blocking solution at room temperature for 1 h. Immunoreactivity of Fas 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 2 to 15 min (autoradiograms).

2.4. Quantitation of immunodensities of Fas-related proteins

The autoradiograms were quantitated by densitometric scanning as described previously (García-Fuster et al., 2003). The amount of Fas in the cerebral cortex of rats treated with opiate drugs was compared in the same gel with that of control rats which received saline or DMSO solution. Experiments were performed by using 40 μg protein known to be within the linear range for immunolabeling of Fas-related proteins. The quantification procedure was assessed two to three 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. A similar procedure was used to quantitate the effects of NEM and enzymatic deglycosylation (control versus pretreated membranes) on Fas proteins.

2.5. Data analyses and statistics

Results are expressed as mean ± S.E.M. 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 $P = 0.05$.

2.6. Drugs and chemicals

Opiate drugs (and their sources) included morphine HCl (Unión Químico-Farmacéutica S.A.E., Madrid, Spain), SNC-80 (Tocris Cookson, Avonmouth, UK), U-50488-H HCl (Sigma/RBI, St. Louis, MO, USA) and (+)-SKF 10047 HCl (Tocris). The enzyme *N*-glycosidase F and the sulfhydryl alkylating reagents NEM and NMM were 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 or Santa Cruz Biotechnology. All other chemicals were from Sigma.

3. Results

3.1. Immunodetection of Fas aggregates in brain tissue and in SH-SY5Y cells

High molecular mass, SDS- and β-mercaptoethanol-resistant Fas aggregates (~ 110/120 and ~ 203 kDa) were readily detected in rat, mouse and human brain membranes as well as in human SH-SY5Y cells by Western blot analysis (Fig. 1). These peptides were specific Fas-related

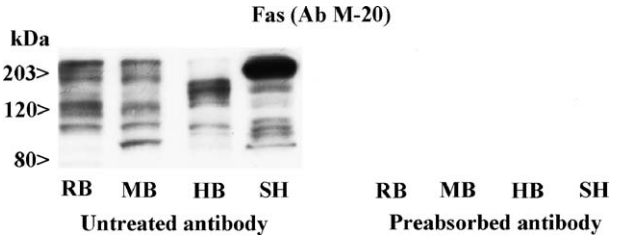

Fig. 1. Representative autoradiograph of Western blots depicting labelling of immunodetectable Fas receptor aggregates (~ 110/120- and ~ 203-kDa peptides) with anti-Fas M-20 antibody in the rat brain (RB, cerebral cortex), mouse brain (MB, cerebral cortex), human brain (HB, prefrontal cortex) and human 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 (under denaturing conditions), transferred to nitrocellulose membranes (immunoblotting), incubated with the specific primary and secondary antibodies, and visualized by the Enhanced Chemiluminiscence method. The specificity of anti-Fas M-20 antibody was assessed by preincubating the antiserum with its antigenic peptide (preabsorbed antibody), which resulted in the blockade of the immunoreaction for the specific Fas aggregates. The apparent molecular masses of Fas aggregates were determined by calibrating the blots with prestained molecular weight markers as shown on the left-hand side.

proteins because preincubation of anti-Fas M-20 antibody with the antigenic peptide (preabsorbed antibody) resulted in the blockade of the immunoreaction of these Fas forms (Fig. 1), as it was also the case for native (35 kDa) and glycosylated (45–52 kDa) Fas (see García-Fuster et al., 2003).

3.2. Effects of NEM on the immunodensities of Fas aggregates and native and glycosylated Fas

In order to determine whether the cysteine residues on Fas play a role in Fas aggregation and to assess the relation between high molecular mass Fas aggregates and other Fas-related proteins (native and glycosylated Fas), the sulfhydryl alkylating reagent NEM was used as a tool to dissociate Fas aggregates. Preincubation of rat cerebral cortical membranes with NEM (1 mM for 1 h at 37 °C) significantly reduced the immunodensity of ~ 203 kDa Fas aggregates (51 ± 4%, $n=6$, $P<0.001$) but not that of ~ 110/120-kDa aggregates (Fig. 2). Concomitantly, NEM markedly increased the densities of 35-kDa native Fas (172 ± 10%, $n=6$, $P<0.0001$) and 51/48-kDa glycosylated Fas (47 ± 6%, $n=6$, $P<0.0001$; Fig. 2). Similar effects were observed with the parent compound NMM (data not shown). These data indicated that disulfide bonds are involved in Fas dimerization in brain tissue.

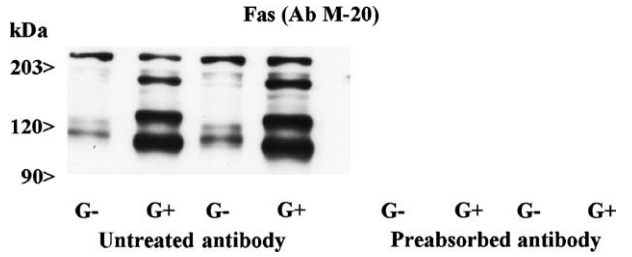

Fig. 3. Effect of enzymatic *N*-deglycosylation (*N*-glycosidase F for 3 h at 37 °C) on the immunodensities of Fas aggregates (~ 203- and ~ 110/120-kDa peptides) detected with anti-Fas M-20 antibody in rat cortical membranes. The samples [18 μg protein (lines 1–2 and 5–6) and 36 μg protein (lines 3–4 and 7–8)] were incubated in the absence (G–) or presence (G+) of the *N*-glycosidase F (15 units). Other details are the same as for Fig. 1 (preabsorbed antibody) and Fig. 2 (203- and 110/120-kDa peptides were quantitated as doublets). The apparent molecular masses of Fas aggregates were determined by calibrating the blots with prestained molecular weight markers as shown on the left-hand side. The experiment shown in this figure was repeated three times with very similar results.

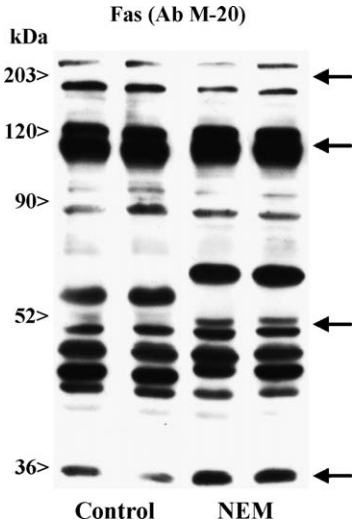

Fig. 2. Effect of *N*-ethylmaleimide (NEM, 1 mM for 1 h at 37 °C) on the immunodensities of Fas aggregates (~ 203- and ~ 110/120-kDa peptides), glycosylated Fas (48- and 51-kDa peptides) and native Fas (35-kDa peptide) detected with anti-Fas M-20 antibody (batch F251) in rat cortical membranes. The apparent molecular masses of Fas-related proteins (arrows) were determined by calibrating the blots with prestained molecular weight markers as shown on the left-hand side. An appropriate film time exposure (15 min) allowed the simultaneous visualization and quantitation of the various forms of Fas in the same blot (203-, 110/120-, 48/51-kDa peptides were quantitated as doublets, see arrow position). In less-exposed films (3 min), no modulation by NEM on ~ 110/120-kDa Fas aggregates was observed (see Results). Note that batch F251 for antibody M-20 detected Fas-related peptides of ~ 44/46 kDa that were not immunodetected with batch D219 (see Fig. 4; see also García-Fuster et al., 2003 for other details on these Fas-related proteins). The experiment shown in this figure was repeated three times with very similar results.

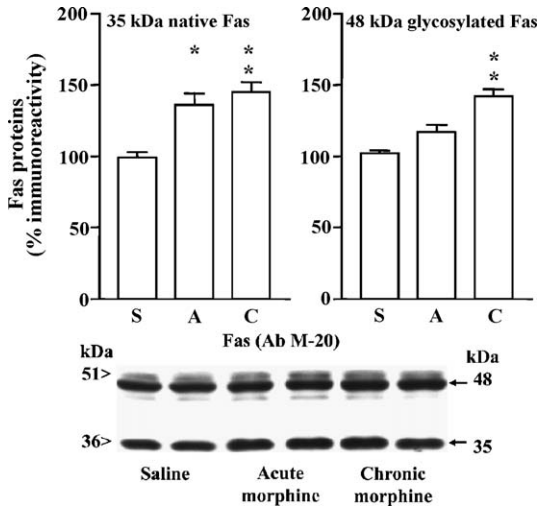

Fig. 4. Effects of acute and chronic treatments with morphine on the immunodensities of native Fas (35-kDa peptide) and glycosylated Fas (48-kDa peptide), detected with anti-Fas M-20 antibody, in the rat brain (cerebral cortex). Groups of treatments: saline (S), acute morphine (A, 30 mg/kg i.p., 2 h) and chronic morphine (C, 10–100 mg/kg i.p. for 5 days). Columns are means ± S.E.M. of four 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 native Fas [$F(2,9)=12.4$, $P=0.004$] and glycosylated Fas [$F(2,9)=24.5$, $P=0.0004$]. *$P<0.05$, **$P<0.01$ when compared with the corresponding saline group (ANOVA followed by Bonferroni's test). Bottom: representative immunoblot for the effects of morphine treatments (two animals for each group, 40 μg protein) on the immunodensity of native and glycosylated Fas (arrows) in the rat cerebral cortex (all samples were run in the same gel). The apparent molecular masses of Fas proteins were determined by calibrating the blots with prestained molecular weight markers as shown on the left-hand side.

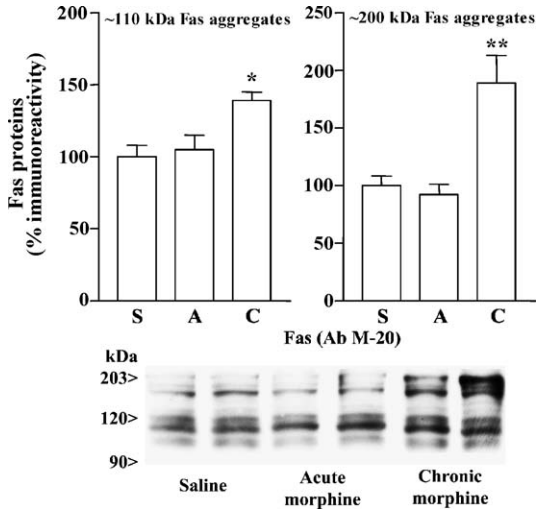

Fig. 5. Effects of acute and chronic treatments with morphine on the immunodensities of Fas aggregates (~ 110/120- and ~ 203-kDa peptides), detected with anti-Fas M-20 antibody, in the rat brain (cerebral cortex). Groups of treatments: saline (S), acute morphine (A, 30 mg/kg i.p., 2 h) and chronic morphine (C, 10–100 mg/kg i.p. for 5 days). Columns are means ± S.E.M. of four 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 ~ 110/120-kDa Fas [$F(2,9)=8.2$, $P=0.012$] and ~ 203-kDa Fas [$F(2,9)=11.8$, $P=0.0031$]. *$P<0.05$, **$P<0.01$ when compared with the corresponding saline group (ANOVA followed by Bonferroni's test). Bottom: representative immunoblot (upper part of the immunoblot shown in Fig. 4) for the effects of morphine treatments (two animals for each group, 40 μg protein) on the immunodensity of Fas aggregates in the rat cerebral cortex (all samples were run in the same gel). Other details are the same as for Fig. 3 (203- and 110/120-kDa peptides were quantitated as doublets). The apparent molecular masses of Fas aggregates were determined by calibrating the blots with prestained molecular weight markers as shown on the left-hand side.

3.3. Changes in the immunodensity of Fas aggregates after Fas receptor deglycosylation

In the rat cerebral cortex, enzymatic *N*-deglycosylation of Fas receptor was previously shown to induce a marked increase in the expression of 35-kDa native Fas and related peptides (~ 43/45 kDa), which were immunodetected with the anti-Fas M-20 antibody (García-Fuster et al., 2003). In rat brain tissue, enzymatic *N*-deglycosylation of Fas receptor (*N*-glycosidase F, 15 units for 3 h at 37 °C), followed by immunoblotting with anti-Fas C-20 antibody that only recognizes 51-kDa glycosylated Fas (García-Fuster et al., 2003), also resulted in the appearance of a related peptide of lower molecular mass (~ 45 kDa) which was not immunodetected with the preabsorbed antidody (data not shown).

In membranes from rat cerebral cortex, enzymatic *N*-deglycosylation of Fas receptor markedly increased the immunodensity of Fas aggregates (~ 110/120-kDa aggregates: five- to sixfold, $n=4$, $P<0.0001$, and ~ 203-kDa aggregates: two- to threefold, $n=4$, $P<0.0005$; Fig. 3). Again, these forms of high molecular mass Fas aggregates and their up-regulations following receptor deglycosylation were not visualized with the preabsorbed anti-Fas M-20 antibody (Fig. 3).

3.4. Effects of opiate drugs on Fas receptor and Fas aggregates in rat brain

The acute (30 mg/kg for 2 h) and chronic (10–100 mg/kg for 5 days) treatments with morphine, a μ-opioid peptide receptor agonist, increased the immunodensities of 35-kDa native Fas (36–45%, $n=4$, $P<0.01$) and 48-kDa glycosylated Fas (17–42%, $n=4$, $P<0.001$) in the rat cerebral cortex (Fig. 4). In the same rat brain samples (and same immunoblots), chronic morphine treatment was also associated with a significant up-regulation in the immunodensity of Fas aggregates (~ 110/120-kDa aggregates: 39%, $n=4$, $P<0.05$, and ~ 203-kDa aggregates: 89%, $n=4$, $P<0.01$; Fig. 5). In contrast, acute morphine treatment did not alter the content of high molecular mass Fas aggregates in the brain (Fig. 5).

The acute treatment (10 mg/kg for 2 h) with SNC-80 (a selective δ-opioid peptide receptor agonist) modestly, but significantly, decreased the immunodensity of Fas aggregates (15%, $P<0.02$) in the rat brain (Table 1). The acute treatment (10 mg/kg for 2 h) with U 50488-H (a selective κ-opioid peptide receptor agonist), and the acute (5 mg/kg for 2 h) and chronic (3–10 mg/kg for 3 days) treatments with (+)-SKF10047 (a potent σ_1-receptor agonist) did not modify significantly the immunodensity of Fas aggregates in the rat cerebral cortex (Table 1).

Table 1
Effects of opiate drugs on the immunodensity of Fas receptor aggregates in rat brain

Treatment (time)	Dose (mg/kg)	Fas aggregates % immunoreactivity	*n*
DMSO (2 h)	–	101 ± 3	6
Acute SNC-80 (2 h)	10	86 ± 4*	8
Saline (2 h)	–	99 ± 3	5
Acute U 50488-H (2 h)	10	100 ± 5	4
Saline (2 h)	–	100 ± 4	4
Acute (+)-SKF10047 (2 h)	5	98 ± 1	4
Saline (3 days)	–	98 ± 3	5
Chronic (+)-SKF10047 (3 days)	3–10	90 ± 5	5

Rats were treated with SNC-80 (a selective δ-opioid peptide receptor agonist), U 50488-H (a selective κ-opioid peptide receptor agonist) and (+)-SKF10047 (a potent σ_1-receptor agonist and a mixed δ/κ-opioid peptide receptor agonist and μ-opioid peptide receptor antagonist), and the immunodensity of Fas aggregates (~ 110/120 kDa) was quantitated (SDS-PAGE; anti-Fas M-20 antibody) in the cerebral cortex. See Materials and methods for further experimental details. Each value represents the mean ± S.E.M. (expressed as percentage of the corresponding control group) of *n* experiments per group with one animal per experiment.

*$P<0.02$ when compared with its control group (Student's *t*-test).

4. Discussion

The immunodetection of high molecular mass Fas receptor aggregates (~ 110/120- and ~ 203-kDa peptides) with anti-Fas M-20 antibody in rat, mouse and human brains, as well as in SH-SY5Y neuroblastoma cells, was in general agreement with previous findings revealing similar forms of Fas aggregates (stable in SDS and β-mercaptoethanol) in various cell lines (Kischkel et al., 1995; Kamitani et al., 1997; Papoff et al., 1999; Siegel et al., 2000a). These studies (Kamitani et al., 1997; Papoff et al., 1999) demonstrated that cross-linking of Fas with a chemical agent or an agonistic antibody induced the formation of Fas aggregates (i.e., complexes of intact Fas monomers of 120, 180 and/or ~ 206 kDa), which were immunodetected with antibodies also directed against the cytoplasmic domain of Fas. These Fas aggregates could correspond to dimeric or trimeric receptors, or even higher-order receptor aggregates (oligomerization forms of Fas; Papoff et al., 1999; Algeciras-Schimnich et al., 2002). Several studies have also reported the immunodetection of dimers and oligomers for other receptors, following receptor solubilization and resolution by denaturing SDS-PAGE, demonstrating that these receptor species are not the result of spurious disulfide bonding during tissue preparation (Ng et al., 1996; Cvejic and Devi, 1997; Lee et al., 2003; Salahpour et al., 2003). Moreover, the dissociation of oligomeric species of β_2-adrenoceptors was accompanied by an increase in the content of monomeric forms (Salahpour et al., 2003). In rat brain membranes, the current results demonstrate that NEM (a sulfhydryl alkylating reagent) reduced the immunodensity of ~ 203-kDa Fas aggregates with a concomitant marked increase in the densities of 35-kDa native Fas and 51/48-kDa glycosylated Fas, indicating a clear relation between Fas aggregates (disulfide bridges would be required for the stability of Fas oligomers) and Fas monomeric forms in brain tissue. Therefore, the current findings demonstrate that high molecular mass Fas aggregates are expressed natively in the brain of mammals and that these Fas forms (receptor oligomerization) can be detected by Western blot analysis under denaturing conditions. These Fas aggregates appear to be the initial signaling form of Fas (Algeciras-Schimnich et al., 2002).

Glycosylation is one of the most common posttranslational modifications of receptor proteins and these attached glycans are relevant for ligand receptor binding and cell signaling, as is the case for Fas that possesses two putative *N*-glycosylation sites at N22 and N93 (Itoh et al., 1991; Oehm et al., 1992; Watanabe-Fukunaga et al., 1992; Kamitani et al., 1997). Notably, the enzymatic *N*-deglycosylation of Fas receptor markedly increased the immunodensity of high molecular mass Fas aggregates (~ 110/120-kDa Fas: five- to sixfold, and ~ 203-kDa Fas: two- to threefold) in the rat cerebral cortex. In this context, α_{2A}/β_1-adrenoceptor heterodimerization in heterologous cellular systems was shown to be enhanced by point mutations in either receptor (N10A, N15A) that blocked N-linked glycosylation, indicating that the interaction between these two adrenoceptors is regulated by glycosylation (Xu et al., 2003). In this study, it was suggested that lack of glycosylation alters the conformations of both adrenoceptors such that the efficiency of their heterodimerization is increased (Xu et al., 2003). Moreover, the enhanced heterodimerization of the mutant adrenoceptors was functionally relevant, because it modulated receptor internalization and altered the pharmacological properties of one of the receptors (Xu et al., 2003). In a previous study by the same group (He et al., 2002), it was shown that blocking β_1-adrenoceptor N-linked glycosylation (N15A) reduced β_1-adrenoceptor homodimerization. It is known, however, that the functional effects of *N*-glycosylation on receptors are highly variable (see He et al., 2002 for discussion). The current results clearly indicate that the native expression of Fas receptor homodimerization in the brain was markedly increased after receptor deglycosylation, and, therefore, Fas glycosylation must play a role in regulating receptor dimerization. Because these Fas aggregates have been shown to be one of the earliest events in Fas signaling (Algeciras-Schimnich et al., 2002), drug modulation (e.g., opiate drugs) of these forms of Fas appeared of relevance.

Another major finding of the present study is that chronic, but not acute, morphine administration (opiate tolerance and dependence induced by the μ-opioid peptide receptor agonist; García-Fuster et al., 2003) was associated with up-regulation of high molecular mass Fas aggregates (~ 110/120 and ~ 203 kDa) in the rat cerebral cortex. As expected (García-Fuster et al., 2003), the immunodensity of native (35 kDa) and glycosylated (48 kDa) Fas were increased by acute and chronic morphine treatments. Moreover, the acute and/or chronic treatments with selective δ- (SNC-80) and κ- (U 50488-H) opioid peptide receptor agonists, and a σ_1-receptor agonist (SKF10047) did not readily alter the content of Fas aggregates in rat brain (SNC-80 induced a modest decrease). In a previous study (García-Fuster et al., 2003), these δ- and κ-opioid peptide receptor agonists did not modulate native or glycosylated Fas, but acute and chronic treatments with the σ_1-receptor agonist (SKF 10047 is also a mixed δ/κ-opioid peptide receptor agonist) induced a down-regulation of these Fas forms in the brain. Taken together, these results clearly indicate that opiate tolerance and dependence in rats, mediated through the sustained stimulation of μ-opioid peptide receptors, result in up-regulation of high molecular mass Fas aggregates in the brain. Therefore, these processes not only are associated with increased immunodensities of native Fas and glycosylated Fas (Boronat et al., 2001; García-Fuster et al., 2003) but also with an up-regulation of Fas aggregates (present results), which appear to trigger Fas signaling (Algeciras-Schimnich et al., 2002). At present, the functional implications of the up-regulation of Fas in morphine-treated rats (abnormal cell death and/or non-apoptotic cell signaling) are not precisely known (see

García-Fuster et al., 2003 for further discussion on this topic).

In summary, high molecular mass Fas aggregates are expressed natively in rat, mouse and human brain membranes, and its density is regulated by the state of Fas glycosylation. Moreover, these forms of Fas (receptor homodimerization) appear to be functionally relevant because they were up-regulated (together with native and glycosylated Fas) in the brain of morphine-dependent rats.

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References

Algeciras-Schimnich, A., Shen, L., Barnhart, B.C., Murmann, A.E., Burkhardt, J.K., Peter, M.E., 2002. Molecular ordering of the initial signaling events of CD95. Mol. Cell. Biol. 22, 207–220.

Badorff, C., Ruetten, H., Mueller, S., Stahmer, M., Gehring, D., Jung, F., Ihling, C., Zeiher, A.M., 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, M.A., García-Fuster, M.J., García-Sevilla, J.A., 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.

Choi, C., Park, J.Y., Lee, J., Lim, J.-H., Shin, E.-C., Ahn, Y., Kim, C.-H., Kim, S.-J., Kim, J.-D., Choi, I.S., 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.

Cvejic, S., Devi, L., 1997. Dimerization of the δ-opioid receptor: implication for a role in receptor internalization. J. Biol. Chem. 272, 26959–26964.

Desbarats, J., Birge, R.B., Minouni-Rongy, M., Weistein, D.E., Palerme, J.-S., Newell, M.K., 2003. Fas engagement induces neurite growth through ERK activation and p35 upregulation. Nature Cell Biology 5, 118–125.

García-Fuster, M.J., Ferrer-Alcón, M., Miralles, A., García-Sevilla, J.A., 2003. Modulation of Fas receptor proteins and dynamin during opiate addiction and induction of opiate withdrawal in rat brain. Naunyn-Schmiedeberg's Arch. Pharmacol. 368, 421–431.

He, J., Xu, J., Castleberry, A.M., Lau, A.G., Hall, R.A., 2002. Glycosylation of β_1-adrenergic receptors regulates receptor surface expression and dimerization. Biochem. Biophys. Res. Commun. 297, 565–572.

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, H.P., Yeh, E.T.H., 1997. Activation-induced aggregation and processing of the human fas antigen. Detection with cytoplasmic domain-specific antibodies. J. Biol. Chem. 272, 22307–22314.

Kischkel, F.C., Hellbardt, S., Behrmann, I., Germer, M., Pawlita, M., Krammer, P.H., Peter, M.E., 1995. Cytotoxicity-dependent APO-1 (Fas/CD95)-associated proteins form a death-inducing signaling complex (DISC) with the receptor. EMBO J. 14, 5579–5588.

Krammer, P.H., 2000. CD95's deadly mission in the immune system. Nature 407, 789–795.

Lee, S.P., O'Dowd, B.F., Rajaram, R.D., Nguyen, T., George, S.R., 2003. D2 Dopamine receptor homodimerization is mediated by multiple sites of interaction, including an intermolecular interaction involving transmembrane domain 4. Biochemistry 42, 11023–11031.

Nagata, S., 1999. Fas ligand-induced apoptosis. Annu. Rev. Genet. 33, 29–55.

Ng, G.Y.K., O'Dowd, B.F., Lee, S.P., Chung, H.T., Brann, M.R., Seeman, P., George, S.R., 1996. Dopamine D2 receptor dimers and receptor-blocking peptides. Biochem. Biophys. Res. Comm. 227, 200–204.

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.

Ozaita, A., Escribá, P.V., García-Sevilla, J.A., 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.

Papoff, G., Hausler, P., Eramo, A., Pagano, M.A., Di Leve, G., Signore, A., Ruberti, G., 1999. Identification and characterization of a ligand-independent oligomerization domain in the extracellular region of the CD95 death receptor. J. Biol. Chem. 274, 38241–38250.

Raoul, C., Estévez, A.G., Nishimune, H., Cleveland, D.W., deLapeyrière, O., Henderson, C.E., 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.

Salahpour, A., Bonin, H., Bhalla, S., Petäjä-Repo, U., Bouvier, M., 2003. Biochemical characterization of β_2-adrenergic receptor dimers and oligomers. Biol. Chem. 384, 117–123.

Sastry, P.S., Rao, K.S., 2000. Apoptosis and the nervous system. J. Neurochem. 74, 1–20.

Siegel, R.M., Frederiksen, J.K., Zacharias, D.A., Ka-Ming Chang, F., Johnson, M., Lynch, D., Tsien, R.Y., Lenardo, M.J., 2000a. Fas preassociation required for apoptosis signaling and dominant inhibition by pathogenic mutations. Science 288, 2354–2357.

Siegel, R.M., Ka-Ming Chang, F., Chun, H.J., Lenardo, M.J., 2000b. The multifaceted role of Fas signaling in immune cell homeostasis and autoimmunity. Nat. Immunol. 1, 469–474.

Tada, Y., 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.

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

Watanabe-Fukunaga, R., Brannan, C.I., Itoh, N., Yonehara, S., Copeland, N.G., Jenkins, N.A., Nagata, S., 1992. The cDNA structure, expression, and chromosomal assignment of the mouse Fas antigen. J. Immunol. 148, 1274–1279.

Xu, J., He, J., Castleberry, A.M., Balasubramanian, S., Lau, A.G., Hall, R.A., 2003. Heterodimerization of α_{2A}- and β_1-adrenergic receptors. J. Biol. Chem. 278, 10770–10777.

Yu, V.C., Richards, M.L., Sadée, W., 1986. A human neuroblastoma cell line expresses μ and δ opioid receptor sites. J. Biol. Chem. 261, 1065–1070.

Yuan, J., Yankner, B.A., 2000. Apoptosis in the nervous system. Nature 407, 802–809.