

Effect of Cocaine on Fas-Associated Protein with Death Domain in the Rat Brain: Individual Differences in a Model of Differential Vulnerability to Drug Abuse

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This study was designed to (1) assess the effects of cocaine on Fas-associated protein with death domain (FADD) system and its role in the activation of apoptotic vs nonapoptotic events and (2) ascertain whether animals selectively bred for their differential propensity to drug-seeking show differences in FADD levels or response to cocaine. Acute cocaine, through D₂ dopamine receptors, induced a dose-response increase in FADD protein in the cortex, with opposite effects over pFADD (Ser191/194), and no induction of apoptotic cell death (poly-(ADP-ribose) polymerase cleavage). FADD was increased by cocaine in cytosol (~142%), membranes (~23%) and nucleus (~54%). The modulation of the FADD system showed tolerance of the acute effect over time, as well as a compensatory response on withdrawal that mirrored the acute effect—ie a transient FADD decrease on day 3 of withdrawal, both at mRNA and protein levels. In a second experiment, possible FADD differences were investigated in rats selectively bred for differential responsiveness to novelty, propensity for drug-seeking and cocaine sensitization. High-responders (HR), who were more prone to drug abuse, exhibited higher FADD and lower pFADD levels than low-responder (LR) rats. However, HR and LR rats showed similar rates of cocaine-induced apoptosis, and exhibited a parallel impact of cocaine over FADD within each phenotype. Thus, FADD is a signaling protein modulated by cocaine, regulating apoptosis/proliferative mechanisms in relation to its FADD/pFADD content. Interestingly, animals selectively bred for differential propensity to substance abuse show basal differences in the expression of this protein, suggesting FADD may also be a molecular correlate for the HR/LR phenotype.

Neuropsychopharmacology (2009) **34**, 1123–1134; doi:10.1038/npp.2008.88; published online 25 June 2008

Keywords: cocaine; FADD; apoptosis; proliferation; HR/LR phenotype; rat brain

INTRODUCTION

Cocaine is a highly abused psychostimulant that poses an enormous health, social, and economic burden on modern society. Cocaine addiction is both mental and physical, and largely depends on drug-induced neurochemical, structural, and behavioral brain alterations (Nestler, 2005). Indeed, a major focus in the field of addiction research has been to understand the early neural changes set in motion soon after the exposure to cocaine, because it is believed that such alterations can set the stage for long-lasting modifications of brain structure and function. Although different drugs of abuse have unique and specific mechanisms of action, similar molecular pathways likely mediate common functional effects (Nestler, 2005). Accordingly, different drugs of abuse share the property of increasing dopamine (DA) in limbic and motor brain areas, which has been

related to their addictive properties (Wise and Bozarth, 1985; Di Chiara and Imperato, 1988; Beitner-Johnson and Nestler, 1991).

Although effects on neurotransmission are clearly relevant, the impact of drugs of abuse on processes that affect neural plasticity are important to understand both short- and long-term consequences of drug exposure (Robinson and Kolb, 2004; Kalivas, 2007). In mammals, the regulation of cell fate to either proliferate, differentiate, arrest cell growth, or initiate cell death is the most fundamental mechanism for maintaining cellular function and homeostasis. In the developing and adult nervous system, these processes are regulated by complex interactions between several growth factors, neurotransmitters, and neuropeptides (Cameron *et al*, 1998; Sommer and Rao, 2002). Abundant evidence suggests that either necrotic or apoptotic neuronal death may underlie drug abuse-related behavioral changes (Cunha-Oliveira *et al*, 2006), although the literature concerning cocaine neurotoxicity in adult brain remains inconclusive (Dietrich *et al*, 2005).

In various cell systems, Fas-associated protein with death domain (FADD) stimulation, an adaptor protein that senses a death receptor signal and nucleates the death-inducing

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Received 8 February 2008; revised 2 May 2008; accepted 15 May 2008

signaling complex (DISC), is associated either with the activation of effector caspases leading to cell apoptosis or with survival/proliferation signals (Park *et al*, 2005; Peter *et al*, 2007). FADD also contributes to a number of nonapoptotic processes, including T-cell proliferation (Zhang *et al*, 1998), T-cell proliferation and/or survival (Beisner *et al*, 2003), and homeostatic proliferation (Zhang *et al*, 2005). In the central nervous system (CNS), Fas/FADD stimulation promotes neurite outgrowth (Desbarats *et al*, 2003) and neuronal branching (Zuliani *et al*, 2006) through ERK activation. Therefore, FADD is instrumental in controlling a variety of intracellular processes that regulate cell growth/survival/apoptosis in a complex dance of changing partners and overlapping steps. Indeed, other drugs of abuse (ie opiates) have recently been shown to promote either survival or proliferating neural signals (Tegeder and Geisslinger, 2004; Barry and Zuo, 2005) perhaps through inhibition of FADD by a mechanism dependent on the activation of the antiapoptotic ERK1/2 signaling pathway (Garcia-Fuster *et al*, 2007).

The present study assessed whether (1) FADD modulation is drug specific (opiate drugs; Garcia-Fuster *et al*, 2007) or is associated with common neuroplastic mechanisms induced by multiple drugs of abuse (cocaine in this study); (2) cocaine's impact on FADD shifts cell fate toward apoptotic mechanisms (drug-induced neurotoxicity measured by poly-(ADP-ribose) polymerase (PARP) enzyme cleavage, a molecular cell death marker involved in DNA damage following caspase-3 activation (Cagnol *et al*, 2006)) or nonapoptotic mechanisms; and (3) the activation and/or imbalance of these pathways could underlie a new potential mechanism relevant to the early neural changes instigated by different drugs of abuse.

To address the functional relevance of FADD modulation by cocaine, the second phase of this study assessed whether animals with differential propensity to drug-seeking show a differential FADD response to cocaine. We used a model which shows that rats' locomotor activity in the mild stress of a novel environment predicts subsequent behavioral responses to psychostimulants (Piazza *et al*, 1989; Piazza and Le Moal, 1996). High-responders rats (HR), exhibit high rates of novelty-induced locomotor activity, display exaggerated behavioral sensitization to psychostimulants, and will self-administer these drugs more readily than low-responders rats (LR) (Piazza *et al*, 1989; Pierre and Vezina, 1997; Marinelli and White, 2000; Kabbaj and Akil, 2001). Some of the known HR/LR neurochemical differences (ie dopaminergic) (Piazza *et al*, 1991; Dietz *et al*, 2005) that may contribute to their drug-taking behavior lead us to hypothesize certain HR/LR differences in FADD. As the HR/LR model is one of differential vulnerability to drug-seeking behavior, it was of particular interest to ascertain whether these animals would exhibit basal differences in FADD content, and whether they would show differential reactivity post-cocaine exposure. Stated differently, would measures of FADD signaling reflect a vulnerability trait, drug responsiveness, or an interaction between them?

MATERIALS AND METHODS

Animals

Commercially purchased rats. Male Sprague–Dawley rats (Charles River, Wilmington, MA), weighing 225–250 g on

arrival were used. Animals were housed three per cage, kept on a 12-h light/dark cycle (lights on at 0700 hours) with controlled temperature and humidity and maintained in accordance with the University of Michigan Committee on the Use and Care of Animals. Food and water were available *ad libitum*. Rats were allowed to acclimatize to housing conditions for 1 week before drug treatment or testing.

Selectively bred HR/LR rats. Our laboratory recently began to selectively breed HR and LR lines of Sprague–Dawley rats that show reliable differences across multiple behavioral and neurobiological dimensions. Selectively bred HR and LR male rats (fifteenth generation) were used ($n=4-5$ per group). A description of the breeding strategy and initial behavioral characterization of the HR and LR lines has been published (Stead *et al*, 2006).

Adult selectively bred HR and LR male rats weighing 225–250 g were first screened to determine their locomotor response to novelty. Horizontal and rearing activity was monitored by computer in 5 min intervals over 60 min by placing animals into clear acrylic $43 \times 21.5 \times 25.5$ cm (high) cages equipped with infrared photocell emitters mounted 2.3 and 6.5 cm above the grid floor. All testing was performed between 0800 and 1130 am. Total locomotion scores for each rat were calculated by adding the total number of horizontal and rearing movements. HR animals whose scores fell one standard deviation below the HR group average and LR animals whose scores fell one standard deviation above the group average were not used for the subsequent cocaine-treatment study.

Drug Treatment

Single injection. The dose–response profile was analyzed, with purchased rats receiving a single injection of cocaine at different doses (3, 7.5, 15, 30 mg/kg, i.p.); animals were killed by decapitation 1 h after the single administration. To evaluate the role of dopaminergic receptors in the acute cocaine-induced FADD modulation, selective blockers of D₁ (SCH 23390; 0.5 mg/kg, i.p.) and D₂ (raclopride; 0.5 mg/kg, i.p.) DA receptors were administered 30 min before treatment with cocaine (7.5 mg/kg, 1 h, i.p.), alone, or concomitantly. In all treatments control rats received saline (0.9% NaCl, 1 ml/kg, i.p.).

Repeated injections. The chronic cocaine study used a treatment regimen known to induce behavioral sensitization (Gosnell, 2005). Thus, one group of commercially purchased animals received injections of 15 mg/kg cocaine (i.p.), whereas the control group of animals received saline for 7 consecutive days. The acute cocaine group received 6 days of saline and an acute cocaine injection (15 mg/kg, i.p.) on day 7. All animals were transferred to an adjacent room to receive their daily injections (around 1000 hours) and were then returned to their colony room. On day 7, animals were killed 45 min following the last injection between 0800 and 1300 hours. After this chronic treatment, the impact of cocaine during the early days of withdrawal was assessed at three time points: days 1, 3, and 7.

Cocaine treatments with selectively bred HR/LR animals. Selectively bred HR and LR rats (fifteenth generation) were

used to determine basal HR/LR differences in FADD, pFADD, and PARP protein contents. As previous studies demonstrated that HR/LR rats exhibit distinct behavioral responses to cocaine, the effects of acute (15 mg/kg, i.p.), chronic (see paradigm above), and cocaine withdrawal (day 3) on FADD content were also studied to ascertain whether differing degrees of cocaine sensitization could be associated with differences in FADD levels.

Tissue Collection

Animals were killed at the indicated times and the brains removed. The right-half brain was freshly dissected (parieto-occipital cortex), fast frozen, and then stored at -80°C until use for western blot experiments (protein content), and the left-half brain was fast frozen and stored at -80°C for *in situ* hybridization experiments (ISH, mRNA levels).

Cocaine Effect on the Subcellular Distribution of FADD

The subcellular localization (cerebral cortex) and the effect of cocaine (7.5 mg/kg, i.p., 1 h) on the content of FADD in the different compartments was monitored by use of the Subcellular Proteome Extraction Kit (ProteoExtract; Calbiochem, Darmstadt, Germany) as per the manufacturer's instructions (García-Fuster *et al*, 2007). This sequential extraction method relies on the different solubility of proteins in certain subcellular compartments to yield four subproteomes enriched in cytosolic (F1), membrane and membrane organelle-localized (F2), soluble and DNA-associated nuclear (F3), and cytoskeletal (F4) proteins. The efficiency and selectivity of this subcellular extraction procedure have been reported (Abdolzade-Bavil *et al*, 2004), and in the current study, the assay's selectivity was assessed by the immunodetection of neurofilament (NF-L) protein, a specific cytoskeletal marker of mature neurons, which was only identified in F4 (García-Fuster *et al*, 2007).

Sample Preparations, Immunoblot Assays, and Quantitation of Target Proteins

Brain tissue samples (right-half cerebral cortex) were prepared in the presence of various protease inhibitors as previously reported (García-Fuster *et al*, 2007). Aliquots of total homogenate were mixed with equal volumes of electrophoresis loading buffer, denatured, and stored at -20°C until use. Protein concentrations were determined by the BCA Protein Assay (Pierce, IL). In routine experiments, brain proteins (40 μg) were separated under nonreducing conditions on 10% SDS-PAGE minigels (Bio-Rad Laboratories, Hercules, CA), which was followed by standard immunoblotting procedures (García-Fuster *et al*, 2003).

The primary antibodies used (overnight incubation at 4°C) were: anti-FADD (H-181) (affinity-purified rabbit polyclonal antibody raised against human FADD C-terminal 28–208 residues; dilution 1:5000; sc-5559, batch J-2004; Santa Cruz Biotechnology, USA); anti-p-Ser191 FADD (affinity purified rabbit polyclonal antibody raised against residues surrounding p-Ser191 of mouse FADD; oligomeric forms; dilution 1:750; Lot No 1; Cell Signaling Technology, Beverly, MA); anti-PARP (rabbit polyclonal antibody raised against a peptide corresponding to amino acids 215–228 of

human PARP; dilution 1:800; bacth B76146; Calbiochem); anti-NF-L (mouse monoclonal antibody; dilution 1:500; clone NR4, batch 094K4815; Sigma Chemical Co., St Louis, MO); and anti- β -actin (mouse monoclonal antibody; dilution 1:10 000; clone AC-15, batch 045K4831; Sigma). The secondary antibody, horseradish peroxidase-linked anti-rabbit or anti-mouse IgG, was incubated at dilution 1:5000 in blocking solution at room temperature for 1 h.

Immunoreactivity of target proteins was detected with an ECL Western Blot Detection system (Amersham International, Buckinghamshire, UK) and visualized by exposure to Hyperfilm ECL film (Amersham) for 60 s to 60 min (autoradiograms). The autoradiograms were quantified by densitometric scanning (IOD) using NIH Image software. The amount of target proteins in brain samples of treated rats was compared in the same gel with that of control rats, which received saline. This procedure was assessed 3–6 times in different gels (each gel with different samples from saline- 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. The content of β -actin (a cytoskeletal protein not altered in brain by cocaine treatments; Imam *et al*, 2005; Fumagalli *et al*, 2006; Zhang *et al*, 2007) was quantified as a loading control.

In Situ Hybridization Histochemistry

The ISH method used in this study is described in detail by Isgor *et al*, 2003. Cocaine-induced changes in mRNA levels were detected in the brain cortex (S1Tr, bregma -3.60 mm). Briefly, tissue (left-half brain) was cryostat-sectioned (at -20°C , 10 μm) and mounted onto poly(L-lysine)-coated slides, and stored at -80°C until use. Before probe hybridization, tissue was fixed in 4% paraformaldehyde at room temperature, rinsed with aqueous buffers, and dehydrated with graded alcohols. After air-drying, the sections were hybridized with an ^{35}S -labeled cRNA probe. The following probes were cloned from cDNA fragments with specific primers using standard *in vitro* transcription methodology: FADD (542 nucleotide fragment directed against the rat FADD mRNA coding region, nucleotides 355–898); β -actin (633 nucleotide fragment directed against the rat β -actin mRNA coding region, nucleotides 211–844). The probes were labeled with incorporation of ^{35}S -UTP and ^{35}S -CTP and hybridized to tissue overnight at 55°C . The next day, sections were washed with increasing stringency, dehydrated with graded alcohols, air-dried, and exposed to film. Film exposure time was chosen to maximize signal. Digital images of the brain sections were scanned and integrated optical density was measured using an image analysis system (Scion Image). The specificity of the hybridization signal was confirmed with sense probe controls for both probes (data not shown).

Data and Statistical Analysis

All series of data were analyzed with the program GraphPad Prism, version 3.0. Results are expressed as mean values \pm standard error of the mean (SEM). One- or two-way analysis of variance (ANOVA) followed by Bonferroni's multiple

comparison tests and Student's one- or two-tailed *t*-test were used for the statistical evaluations. The level of significance was chosen as $p \leq 0.05$.

Planned comparisons of main effects were employed and justified given the *a priori* hypothesized greater vulnerability to cocaine (degree of cocaine sensitization) of HR relative to LR phenotype (Kabbaj, 2006) regardless of the presence of significant two-way interaction terms.

Drugs and Chemicals

Cocaine-HCl was obtained from Mallinckrodt Inc. (St Louis, MO) and R(+)-SCH-23390 HCl and S(-)-Raclopride(+)-tartrate salt were purchased from Sigma. PARP Cleavage Detection Kit was obtained from Calbiochem. Other materials were purchased from Amersham, Santa Cruz Biotechnology and Sigma-Aldrich.

RESULTS

Studies with Commercially Purchased Outbred Rats

Acute effects of cocaine on FADD and pFADD protein content in rat brain cortex: lack of apoptosis measured by PARP cleavage enzyme. There was a main effect of cocaine treatment on FADD content ($F(4, 22) = 11.39$, $p < 0.0001$). Acute cocaine treatment (3–30 mg/kg, i.p., 1 h) induced complex effects on FADD content with an inverted U-shape dose-response curve (3 mg/kg, $14 \pm 5\%$ decrease, n.s.; 7.5 mg/kg, $46 \pm 5\%$ increase, $p < 0.001$; 15 mg/kg, $35 \pm 10\%$ increase, $p < 0.05$; and 30 mg/kg, $19 \pm 4\%$ increase, n.s.) in the cerebral cortex (total homogenate) compared to vehicle-treated animals (Figure 1a). FADD phosphorylation at Ser 191/194 is the principal mechanism by which this multifunctional adaptor protein regulates its nonapoptotic activities (Alappat *et al*, 2005). There was also a main effect of cocaine on pFADD protein content ($F(3, 20) = 5.347$, $p = 0.0072$). Acute cocaine treatment (7.5–30 mg/kg, i.p., 1 h) decreased the immunodensity of pFADD (Ser 191) at all doses tested: 7.5 mg/kg, $19 \pm 5\%$ ($p < 0.001$); 15 mg/kg, $14 \pm 3\%$ ($p < 0.05$); and 30 mg/kg, $6 \pm 2\%$ (n.s.) (Figure 1b). None of the cocaine treatments significantly altered β -actin content (Figures 1a and b).

As cocaine treatment increased FADD content (proapoptotic), the effect of cocaine exposure on PARP enzyme that is involved in DNA damage following DNA nicks was also investigated as a molecular marker of cell death (Cagnol *et al*, 2006). Acute cocaine treatment (7.5, 15, and 30 mg/kg, i.p., 1 h) did not alter the pattern of PARP cleavage (~85 kDa fragment) observed in the cerebral cortex of vehicle-treated rats ($F(7, 40) = 0.4964$, $p = 0.8315$, n.s.; Figure 1c), which indicated that a differential rate of cell death mediated by PARP cleavage was not activated by cocaine under the reported experimental conditions.

Effect of acute cocaine on the subcellular localization of FADD in rat brain cortex. Given that FADD was found to be induced by the cocaine treatment, its precise cellular localization was next examined. FADD in the cerebral cortex and at the subcellular level showed a similar pattern of subcellular distribution as previously reported (see García-Fuster *et al*, 2007). Thus, FADD was abundantly

localized in association with membranes (F2) and in the nucleus (F3), to a lesser extent in cytosol (F1), with no detectable levels in the cytoskeletal compartment (F4) (Figure 2). Acute cocaine (7.5 mg/kg, 1 h, i.p.) increased FADD protein content in all the compartments where it was expressed (given the expected increase in FADD by cocaine a one-tailed *t*-test was performed for each fraction): by $142 \pm 60\%$ in the cytosol ($F(2, 4) = 8.190$; $p < 0.05$); by $23 \pm 5\%$ in membranes ($F(2, 4) = 2.803$; $p < 0.05$); and by $54 \pm 30\%$ ($F(2, 4) = 2.536$; $p = 0.1$, n.s., all three animals increased FADD) in the nuclear fraction (Figure 2), indicating that FADD changes in total cerebral cortex homogenate reflects its regulation in key cellular compartments. As expected, NF-L was only expressed in the cytoskeletal compartment (F4). β -actin showed different relative protein contents per subcellular fraction, but its expression levels were not affected in any fraction following cocaine exposure. The graph in Figure 2 shows the ratio of FADD/ β -actin (IOD) for each fraction.

Effects of chronic cocaine and cocaine withdrawal on FADD content in rat brain cortex. In contrast to the acute effects of cocaine on FADD ($49 \pm 14\%$ increase, $p < 0.001$), there were no significant changes after chronic cocaine treatment (15 mg/kg, i.p., 7 days) on FADD protein content ($7 \pm 4\%$ increase, n.s.). There was a main effect of cocaine withdrawal on FADD ($F(5, 46) = 10.83$, $p < 0.0001$), which led to the inverse effect previously seen with acute cocaine administration, with a progressive decrease in FADD reaching a nadir at 3 days post-cocaine and reversing thereafter (day 1, $18 \pm 7\%$ decrease, n.s.; day 3, $27 \pm 6\%$ decrease, $p < 0.05$; and day 7, $9 \pm 3\%$ decrease, n.s.; Figure 3a). None of these treatments significantly altered β -actin content (Figure 3a).

At the mRNA level, parallel changes were observed after chronic cocaine treatment ($8 \pm 8\%$ decrease, n.s.) and cocaine withdrawal leading to a decrease in gene expression most notable after 3 days of withdrawal (day 1, $24 \pm 3\%$ decrease, $p < 0.05$; day 3, $30 \pm 3\%$ decrease, $p < 0.01$ and day 7, $17 \pm 5\%$ decrease, n.s.) ($F(4, 25) = 6.198$, $p = 0.0013$; Figure 3b). Indeed, there was a positive and significant correlation between the protein and mRNA FADD levels ($r = 0.43$; $n = 29$; $p < 0.02$) in the same cerebral cortices (Figure 3c), indicating that spontaneous withdrawal from chronic cocaine remarkably decreased FADD both at the transcriptional and post-translational level. The mRNA expression of β -actin was not altered with any of these treatments (data not shown), which is consistent with previous results (Yamaguchi *et al*, 2005).

D₂ DA receptors influence FADD activation by cocaine in rat brain cortex. Pretreatment of rats with raclopride (a D₂-type receptor antagonist; 0.5 mg/kg, i.p.) 30 min before cocaine fully prevented the acute cocaine-induced increase of FADD ($F(3, 27) = 7.522$, $p = 0.0008$; Figure 4a). However, pretreatment of rats with SCH-23390 (a D₁-type receptor antagonist; 0.5 mg/kg, i.p.) 30 min before cocaine did not block the acute cocaine-induced increase of FADD (Figure 4b). Indeed, SCH-23390 alone (0.5 mg/kg, i.p., 1 h 30 min) induced an increase ($40 \pm 9\%$, $p < 0.05$; $F(3, 14) = 7.887$) in FADD content in the cerebral cortex. None of these treatments significantly altered β -actin content (Figure 4).

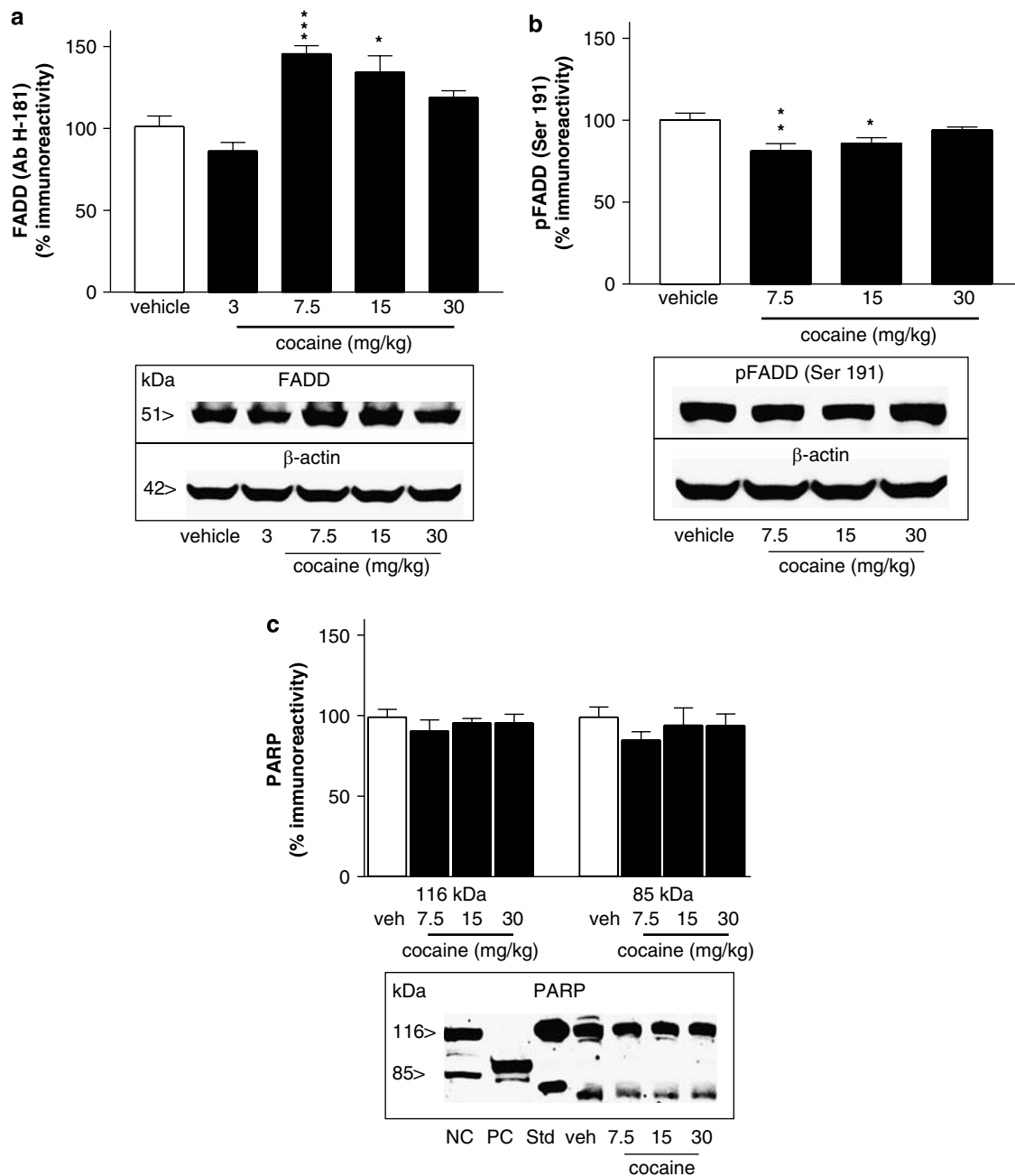


Figure 1 Acute effects of cocaine on Fas-associated protein with death domain (FADD) and pFADD in rat brain cortex: lack of apoptosis measured by poly-(ADP-ribose) polymerase (PARP) cleavage. (a) FADD protein content. This experiment was composed of two sets of experiments: (1) vehicle ($n = 6$), cocaine (7.5, 15, and 30 mg/kg, i.p., 60 min; $n = 6$ each) and (2) vehicle ($n = 3$), cocaine (3 mg/kg, i.p., 60 min; $n = 3$). Both sets of experiments were combined for the statistical analysis of FADD protein content. Columns are means \pm standard error of the mean (SEM) per group and expressed as percentage of vehicle-treated rats. One-way analysis of variance (ANOVA) detected a significant difference between the groups of treatments ($F(4, 22) = 11.39$, $p < 0.0001$). $*p < 0.05$; $***p < 0.001$ vs control (ANOVA followed by Bonferroni's test). (b) pFADD protein content. Groups of treatments: vehicle ($n = 6$), cocaine (7.5, 15, and 30 mg/kg, i.p., 60 min, $n = 6$ each). ANOVA ($F(3, 20) = 5.347$, $p = 0.0072$): $**p < 0.01$; $*p < 0.05$ vs control. (a–b, bottom) Representative immunoblots of FADD and pFADD and the corresponding for β -actin as a loading control (sample: 40 μ g protein). (c) PARP cleavage. Groups of treatments: vehicle ($n = 6$), cocaine (7.5, 15, and 30 mg/kg, i.p., 60 min, $n = 6$ each). One-way ANOVA did not detect a significant difference between the groups of treatments ($F(7, 40) = 0.4964$, $p = 0.8315$, n.s.). Bottom: representative immunoblot of PARP (sample: 40 μ g protein). NC, negative control (whole extract of human HL60 leukemia cells, basal rates of apoptosis); PC, positive control (etoposide-induced apoptosis in HL60 cells, PARP molecules fragmented); Std, PARP protein standard. The apparent molecular masses were determined by calibrating the blots with prestained molecular weight markers as shown on the left-hand side.

Studies with Selectively Bred HR/LR Rats

Basal HR/LR differences in FADD/pFADD and PARP.
HR/LR rats showed differences in basal FADD and pFADD

(Ser 191) protein content with HR exhibiting higher FADD levels ($45 \pm 8\%$ change, $p < 0.01$; $F(1, 8) = 1.593$; Figure 5a) and lower levels of pFADD (Ser 191) ($17 \pm 6\%$ change, $p < 0.05$; $F(1, 6) = 2.586$; Figure 5b) compared to LR rats.

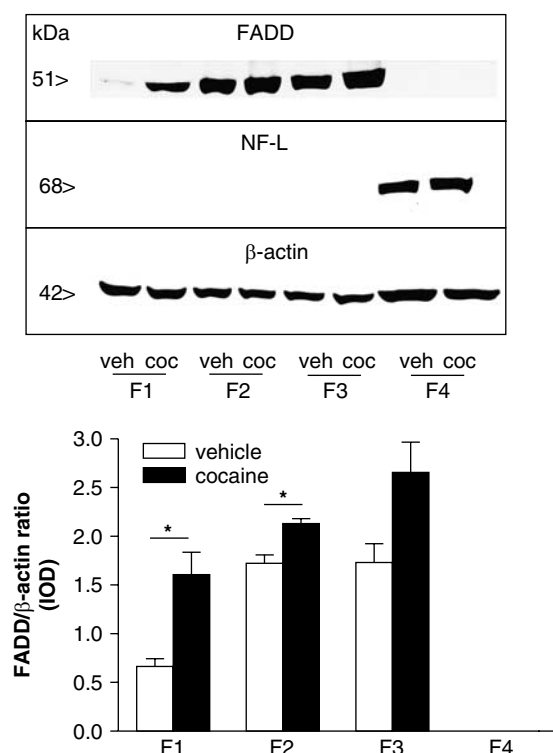


Figure 2 Representative immunoblot depicting the subcellular localization of Fas-associated protein with death domain (FADD) and the acute effect of cocaine (7.5 mg/kg, i.p., 60 min) on FADD content in various subcellular compartments of the rat cerebral cortex (F1, cytosol; F2, membrane/organelle; F3, nucleus; F4, cytoskeletal proteins; 40 μ g protein for F1–F3 fractions and 20 μ g protein for F4; see Materials and Methods for further details). Given the expected increase in FADD by cocaine a one-tailed *t*-test was performed for each fraction. Note that cocaine tends to increase FADD content in the cytosolic (F1 by $142 \pm 60\%$; $F(2, 4) = 8.190$; $*p < 0.05$), membranous (F2 by $23 \pm 5\%$; $F(2, 4) = 2.803$; $*p < 0.05$), and nuclear (F3 by $54 \pm 30\%$; $F(2, 4) = 2.536$; $p = 0.1$, n.s.) fractions. In the cytoskeletal fraction (F4) FADD was not immunodetected. Immunoblots of neurofilament (NF-L) proteins and β -actin were immunodetected in F1–F4. Note that NF-L was immunodetected in F4 but not in the other subcellular fractions. β -actin was not significantly changed in each fraction even though, as expected, the protein showed different relative protein contents per fraction. In fact, the ratio FADD/ β -actin (IOD) \pm standard error of the mean (SEM) was calculated for each fraction and a bar graph was represented with the results (see bar graph). This experiment was repeated 2–3 times with similar results. The apparent molecular masses were determined as indicated in Figure 1.

There were no significant differences in the basal pattern of PARP (116 kDa) cleavage (~ 85 kDa fragment) for the HR/LR phenotype ($F(3, 15) = 0.7523$, $p = 0.5379$; Figure 5c), indicating no different rates of basal induction of apoptotic cell death in the HR/LR phenotypes. There were no differences in the content of β -actin that was used as a loading control (Figure 5).

To further examine HR/LR differences in basal FADD and pFADD protein content, a correlation analysis of rats' novelty-induced locomotion scores and FADD (Figure 5d) or pFADD (Figure 5e) protein levels was performed. This analysis revealed a significant positive correlation between locomotion scores and FADD protein ($r = 0.84$; $n = 10$; $p < 0.003$) and a significant negative correlation with pFADD ($r = -0.76$; $n = 10$; $p = 0.01$) in the same cerebral cortices (HR/LR vehicle-treated rats) (Figures 5d and e).

FADD is not differentially regulated in HR/LR rats following repeated cocaine and cocaine withdrawal. Similar to findings in HR/LR control animals, cocaine-treated HR/LR rats also showed baseline differences in FADD protein content, with HR rats exhibiting higher FADD levels than LR rats (39% change, $p = 0.0002$, two-way ANOVA followed by Bonferroni's *post hoc* test; Figure 6). There was also a main effect for cocaine over FADD within each phenotype (LR rats: $F(3, 15) = 12.77$, $p < 0.001$; HR rats: $F(3, 14) = 8.745$, $p < 0.01$), which paralleled the findings previously observed with commercially purchased rats—ie acute cocaine treatment increased FADD (LR rats: $35 \pm 9\%$ increase, $p < 0.05$; HR rats: $29 \pm 8\%$ increase, $p < 0.05$) with a reversal following 3 days of withdrawal (LR rats: $25 \pm 8\%$ decrease, $p < 0.05$; HR rats: $33 \pm 6\%$ decrease, $p < 0.05$). However, there was no significant drug treatment phenotype interaction ($p = 0.8567$, n.s.; Figure 6).

DISCUSSION

The findings reported here characterize the effects of cocaine on the FADD system, and then ask whether inborn differences in propensity to substance abuse may relate to innate differences in the FADD system. These two series of studies are discussed in sequence.

Effects of Cocaine on FADD in the Rat Brain Cortex

The present results demonstrated that cocaine modulates FADD protein content in the brain as a part of a complex series of neuroplastic molecular mechanisms likely involved in the early stages of the addictive process. Specifically, we showed that (1) acute cocaine modulated FADD forms, increasing overall FADD, decreasing pFADD, with no activation of apoptosis; (2) cocaine treatment increased FADD protein in all subcellular compartments where it was expressed, but shifted FADD distribution, making it more cytosolic. We also observed that (3) repeated cocaine administration induced tolerance to its acute modulatory effect on FADD; (4) cocaine withdrawal was associated with a transient reduction in FADD density (main effect at day 3); (5) there was a positive correlation of FADD mRNA-protein levels; and (6) D₂ DA receptors were involved in FADD activation by cocaine. Taken together these data suggest that FADD is modulated by cocaine, that the acute effect is subject to tolerance but also shows an impact of withdrawal in a direction that mirrors the original effect. Thus, this modulation of FADD by cocaine not only has short-term consequences but long-term impact, particularly during the first days of withdrawal.

Concerning the acute modulation of FADD by cocaine, and in regard to its regulation of apoptotic vs nonapoptotic events, we examined the impact of cocaine exposure on the phosphorylated form of FADD, recently described as a nonapoptotic marker (Alappat *et al*, 2005; Park *et al*, 2005, 2007; Chen *et al*, 2005; Bhojani *et al*, 2005), and PARP, an enzyme cleaved by the protease caspase-3, as an apoptotic marker (Cagnol *et al*, 2006). Acute cocaine treatment induced significant decreases in pFADD protein content, and as judged from levels of PARP protein activation, did not appear to induce apoptosis in the brain cortex, at least

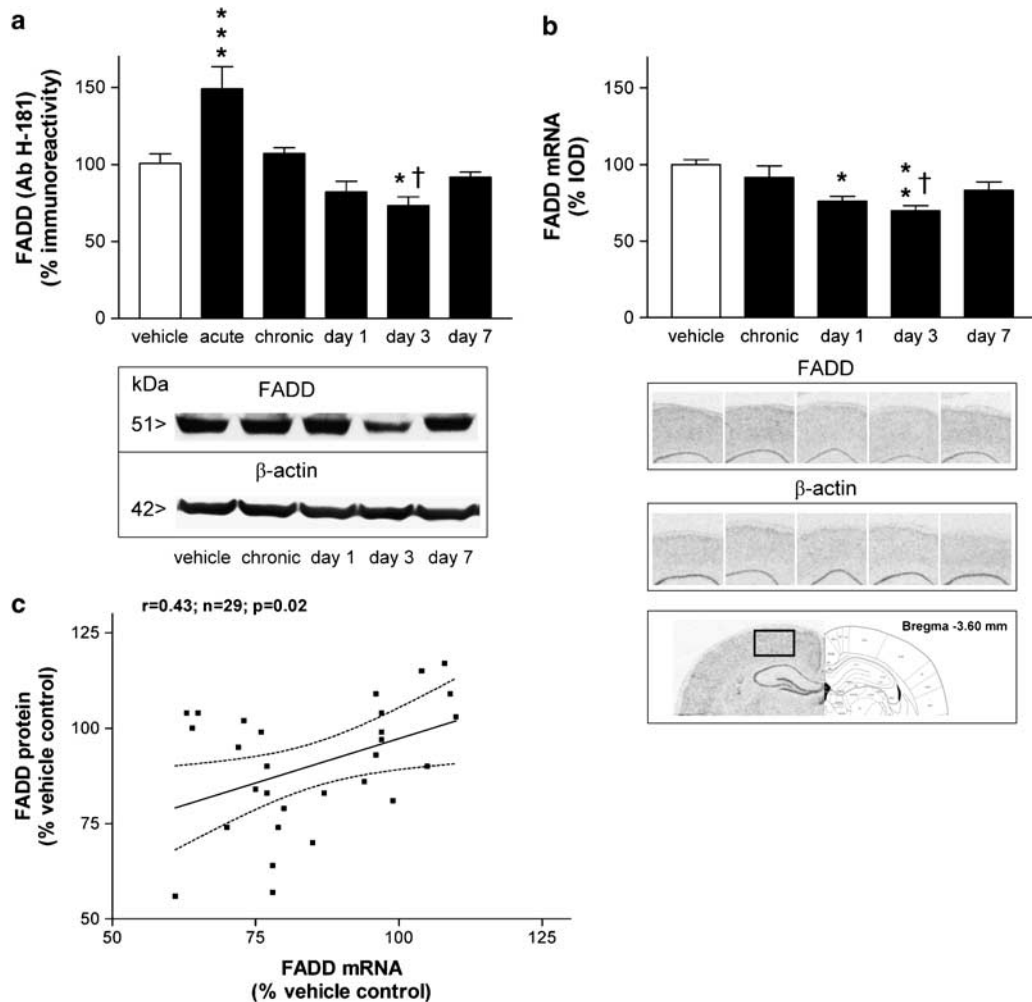


Figure 3 Effects of chronic cocaine and cocaine withdrawal on Fas-associated protein with death domain (FADD) content (protein and mRNA levels) in rat brain cortex. (a) FADD protein content. This experiment was composed of two sets of experiments: (1) vehicle ($n=8$), acute cocaine (acute, 6 days of saline and on day 7, 15 mg/kg of cocaine; $n=6$), and chronic cocaine (chronic, 15 mg/kg for 7 days; $n=8$) and (2) vehicle ($n=6$), chronic cocaine (chronic, 15 mg/kg for 7 days; $n=6$), and chronic cocaine followed by spontaneous withdrawal (day 1, 3, or 7; $n=6$ each). Both sets of experiments were combined for the statistical analysis at the protein level. Columns are means \pm standard error of the mean (SEM) per group and expressed as percentage of vehicle-treated rats. One-way analysis of variance (ANOVA) detected a significant difference between the groups of treatments ($F(5, 46) = 10.83$, $p < 0.0001$). *** $p < 0.001$; * $p < 0.05$ vs control; † $p < 0.05$ vs chronic cocaine (ANOVA followed by Bonferroni's test). Bottom: representative immunoblots of FADD (second experimental set) and the corresponding for β -actin as a loading control (sample: 40 μ g protein). The apparent molecular masses were determined as indicated in Figure 1. (b) FADD mRNA. Groups of treatments: animals of the second set of the above experiments. ANOVA ($F(4, 25) = 6.198$, $p = 0.0013$). * $p < 0.05$; ** $p < 0.01$ vs control; † $p < 0.05$ vs chronic cocaine. Bottom: representative X-ray images of FADD and β -actin mRNA for each treatment group. An image showing the representative region where the changes were measured is also shown (brain cortex, SI Tr, bregma -3.60 mm). (c) Scatter plot depicting a significant positive correlation between the immunodensity of FADD protein and FADD mRNA in the rat cerebral cortex (same samples, second experimental set) after chronic cocaine (chronic, 15 mg/kg for 7 days; $n=6$) and time course withdrawal (days 1, 3, and 7; $n=6$ each) treatments, and expressed as percentage of the corresponding vehicle-treated rats (controls). Each circle represents a different treated rat. The solid line ($y = 0.34x - 180.3$) is the best fit of the correlation ($r = 0.43$, $F = 6.02$, $n = 29$, $p = 0.02$). The dotted curves indicate the 95% confidence interval for the regression line.

at the 1 h time point chosen for this study (see García-Fuster *et al*, 2007 for FADD time course fluctuation). A previous study showed that opiate drugs also modulate FADD in cortex, but in the opposite direction, leading to decreased FADD content (García-Fuster *et al*, 2007). These disparate findings may be related to the fact that these drugs, a psychostimulant vs a psychodepressant, exert opposing effects on the CNS. In fact, opiate drugs (and specifically the δ -agonists) could promote survival signals in the brain through inhibition of FADD, which in turn is dependent on the activation of the antiapoptotic ERK1/2 signaling pathway (García-Fuster *et al*, 2007), crucial for protection

against Fas/FADD-mediated apoptosis (Holmstrom *et al*, 1999, 2000). Other experimental evidence has shown that cocaine alters proliferation, migration, and differentiation of human fetal brain-derived neural precursor cells (Hu *et al*, 2006). However, the potential effect of cocaine in inducing apoptosis of mature neurons is controversial. Several *in vitro* studies suggest that cocaine instigates cell death (reviewed in Cunha-Oliveira *et al*, 2006; Dey and Snow, 2007), but the majority of *in vivo* studies focus on prenatal cocaine exposure (Nassogne *et al*, 1997). Studies in the adult brain have found that cocaine is less neurotoxic compared to other drugs of abuse (eg D-amphetamine,

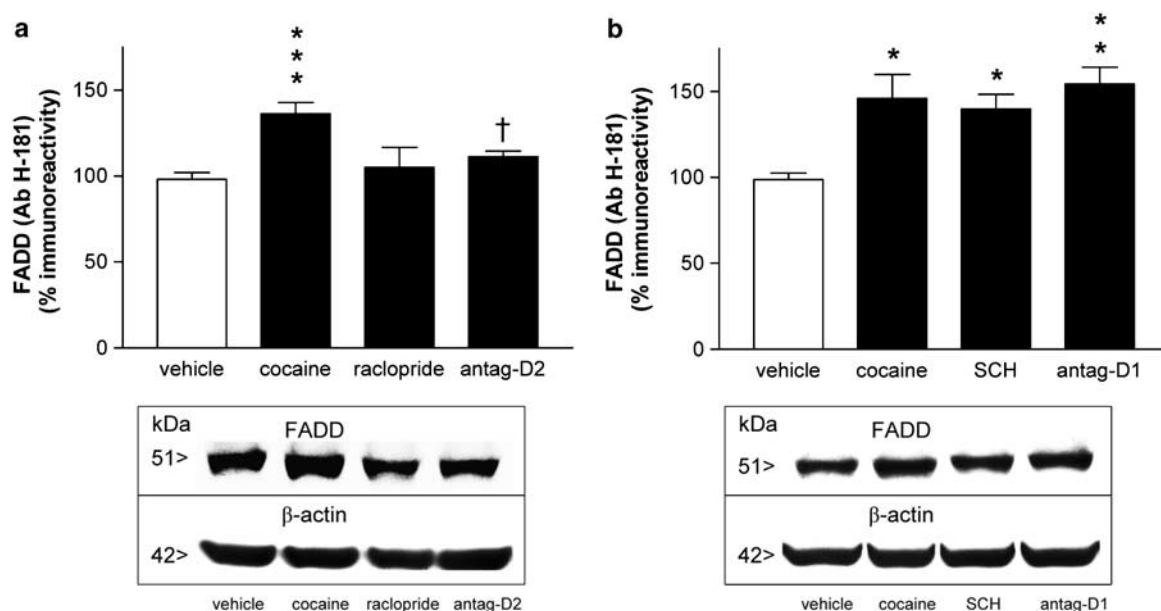


Figure 4 D₂ dopamine (DA)-receptors influence Fas-associated protein with death domain (FADD) activation by cocaine in rat brain cortex (a) D₂ antagonism by raclopride. Groups of treatments: vehicle ($n = 11$), cocaine (7.5 mg/kg, i.p., 60 min; $n = 9$), raclopride (0.5 mg/kg, 90 min; $n = 6$), and raclopride + cocaine (antag- D₂, $n = 5$). Columns are means \pm standard error of the mean (SEM) per group and expressed as percentage of vehicle-treated rats. One-way analysis of variance (ANOVA) detected a significant difference between the groups of treatments ($F(3,27) = 7.522$, $p = 0.0008$): *** $p < 0.001$ vs control; $^{\dagger}p < 0.05$ vs cocaine. (b) D₁ antagonism by SCH-23390. Groups of treatments: vehicle ($n = 5$), cocaine (7.5 mg/kg, i.p., 60 min; $n = 4$), SCH-23390 (SCH, 0.5 mg/kg, 90 min; $n = 4$), and SCH + cocaine (antag- D₁, $n = 5$). Other details are as above. ANOVA ($F(3,14) = 7.887$, $p = 0.0025$): * $p < 0.05$; ** $p < 0.01$ vs control. (a–b, bottom) Representative immunoblots of FADD for each set of experiments, and the corresponding for β -actin as a loading control (sample: 40 μ g protein). The apparent molecular masses were determined as indicated in Figure 1.

METH, and heroin), and does not appear to mediate any apoptotic events (Dietrich *et al*, 2005). Thus, based on these facts and on the results of the present study (increase FADD, decrease pFADD, but no apoptotic activation measured by PARP), it is feasible to suggest that cocaine might impair mechanisms controlling nonapoptotic functions (eg cell proliferation and survival), which still suggests a possible neurotoxic effect of the drug.

Several studies in a variety of cell types have reported that FADD is exclusively localized in the cytoplasm (O'Reilly *et al*, 2004), in the cytoplasm and nucleus (Gomez-Angelats and Cidlowski, 2003; Alappat *et al*, 2005; Lee *et al*, 2006; Yoo *et al*, 2007), or solely within the nucleus (Screaton *et al*, 2003; Bhojani *et al*, 2005). However, it is known that upon Fas ligation, cytoplasmic FADD is rapidly recruited to the plasma membrane where it forms, together with Fas and procaspase-8, the so-called DISC (Kischkel *et al*, 1995; Algeciras-Schimmich *et al*, 2002). In line with this fact and with a previous study (García-Fuster *et al*, 2007), the present results showed that FADD protein is mainly expressed in membrane (F2) and nucleus (F3), and to a lesser extent in cytoplasm (F1). Furthermore, acute cocaine exposure not only augmented the content of FADD overall, but also increased the proportion in the cytosolic fraction, indicating an alteration of distribution across cellular compartments.

After chronic cocaine treatment, the overall extent of FADD modulation in cortex was decreased compared with the single injection, presumably reflecting adaptative mechanisms set in motion by the chronic cocaine exposure. In fact, numerous studies have demonstrated that chronic cocaine administration reduces some of the acute drug

effects (ie tolerance) and, at the same time, enhances other neuronal responses (ie sensitization; for review see Berke and Hyman, 2000; Nestler, 2004). However, cocaine withdrawal (after 1, 3, and 7 days) following chronic cocaine exposure induced a new time course of downregulation of FADD protein content, with a peak decrease 3 days after the last cocaine dose and a reversal to normal levels after 7 days. Moreover, as previously described for this molecule in rat brain cortex (Bi *et al*, 2008), there was a positive correlation between FADD mRNA and protein levels after these treatments, suggesting that after spontaneous withdrawal from chronic cocaine, the entire machinery controlling the synthesis of FADD, including both the transcriptional and post-transcriptional level, was down-regulated. Thus, there might be multiple time domains of change, some transient and some sustained, some leading to tolerance and some to sensitization, which may led us to associate the rapid modulation of FADD by cocaine with the immediate changes that may show tolerance but are uncovered during the early days of withdrawal. Similarly, many of the known acute and chronic cocaine-induced alterations in gene expression and neurotransmitter systems are transient (Kreek, 1996; Nestler, 2001, 2004), and are hypothesized to represent compensatory adaptations to maintain homeostasis by reducing drug-induced effects.

The current study demonstrated that acute cocaine treatment increased FADD content in the cortex through a DA D₂-dependent mechanism, but not through D₁ DA receptors. Similarly, the stimulation of D₂, but not D₁, DA receptors induced a marked increase in FGF-2 expression in the striatum and cortex (Roceri *et al*, 2001; Fumagalli *et al*, 2003). The present study also showed that D₁ DA

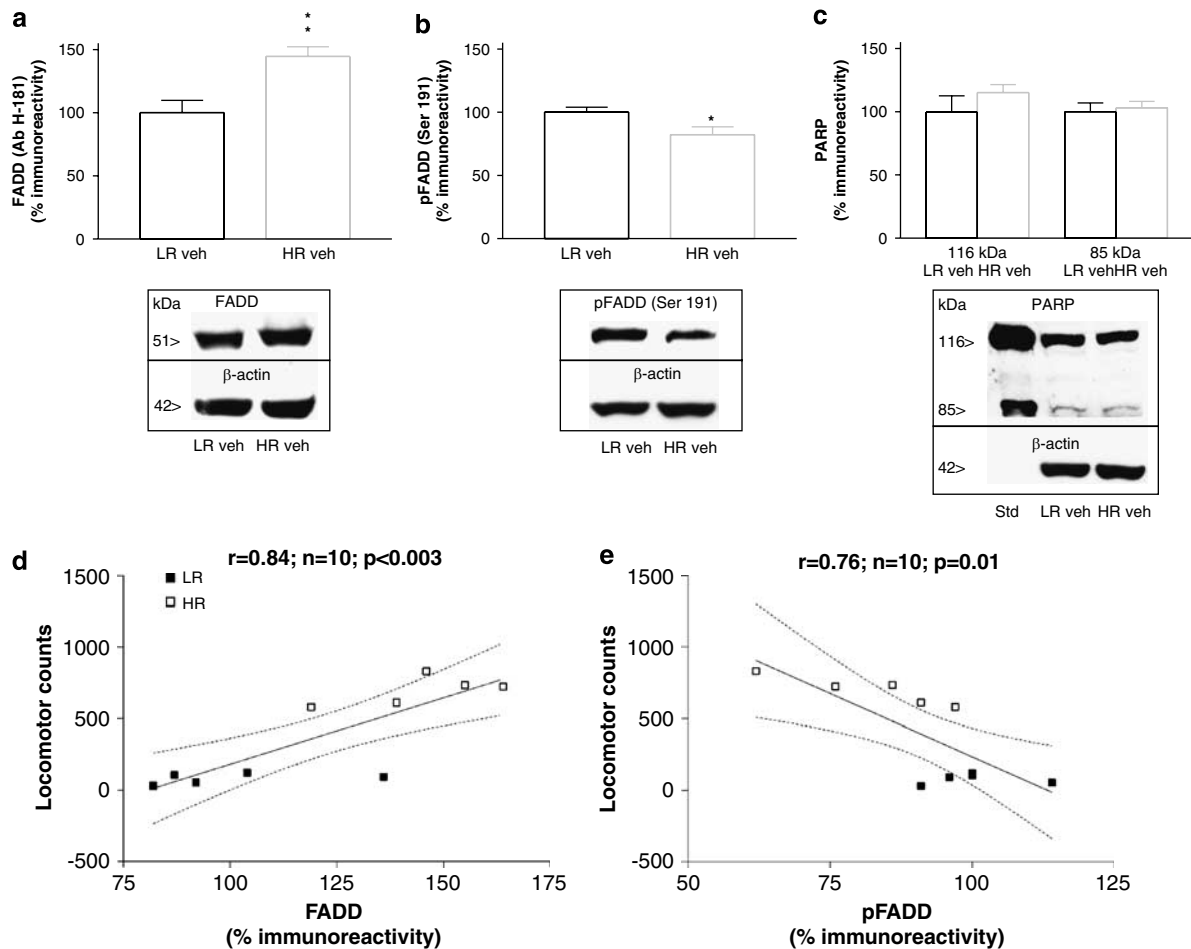


Figure 5 Basal molecular differences in high-responder (HR)/low-responder (LR) rats. (a) Fas-associated protein with death domain (FADD) protein content. Groups of treatments: LR vehicle ($n=5$), HR vehicle ($n=5$). Columns are means \pm standard error of the mean (SEM) per group and expressed as percentage of LR vehicle-treated rats. Student's two-tailed t -test detected a significant difference between the groups of treatments: $**p=0.0070$. (b) pFADD protein content. Student's two-tailed t -test: $*p=0.0395$. (c) Poly-(ADP-ribose) polymerase (PARP) cleavage. Columns are means \pm SEM per group and expressed as percentage of 116 kDa-band LR vehicle-treated rats. One-way analysis of variance (ANOVA) did not detect a significant difference between the groups of treatments ($F(3, 15)=0.7523$, $p=0.5379$). Bottom (a–c): representative immunoblots of FADD, pFADD, and PARP for each set of experiments, and the corresponding for β -actin as a loading control (sample: 40 μ g protein). Std, PARP protein standard. The apparent molecular masses were determined as indicated in Figure 1. (d) Positive correlation of FADD protein content and locomotion. Scatter plot depicting a significant positive correlation between locomotion scores and the immunodensity of FADD protein in the rat cerebral cortex (same samples) of basal HR and LR rats. Each circle represents a different rat. The solid line ($y=80.81x+9.33$) is the best fit of the correlation ($r=0.84$, $F=18.44$, $n=10$, $p<0.003$). The dotted curves indicate the 95% confidence interval for the regression line. (e) Negative correlation of pFADD protein content and locomotion. Other details are as above. The solid line ($y=113.2x-17.69$) is the best fit of the correlation ($r=-0.76$, $F=11.04$, $n=10$, $p=0.01$).

receptors blockade with a selective antagonist increased FADD content in the cortex. This then either suggests a possible serotonergic effect on FADD related to the agonistic properties of SCH-23390 on 5-HT_{1c/2c} receptors or the existence of an endogenous dopaminergic tone activating FADD through D₂ receptors when D₁ receptors are blocked. Recent studies (Kita *et al*, 2007) demonstrate that D₂ receptors provide a presynaptic component that can not only modulate autoinhibition, but also short-term facilitation of DA release, therefore impacting both D₁- and D₂-mediated postsynaptic plasticity. As for how D₂ signal transduction mechanisms modulate FADD activation, it has been previously described that activation of the D₂ receptors can activate ERK (Luo *et al*, 1998; Yan *et al*, 1999) and also that FADD modulation by opiate drugs was dependent on the activation of the antiapoptotic ERK1/2 signaling pathway (García-Fuster *et al*, 2007), which

suggests ERK as a possible mediator of FADD modulation after D₂ receptor activation by cocaine.

Functional Relevance of FADD System Modulation: Individual Differences in a Model of Differential Vulnerability to Drug Abuse

The second phase of this study utilized selectively bred animals that exhibit marked behavioral differences, including differential propensity to drug-seeking (Piazza *et al*, 1989; Piazza and Le Moal, 1996). The present study demonstrated that (1) individual differences in novelty-seeking behavior correlated with basal levels of FADD/pFADD proteins; and that (2) FADD was not differentially regulated in HR/LR rats following repeated cocaine and withdrawal. These results mainly suggest that FADD

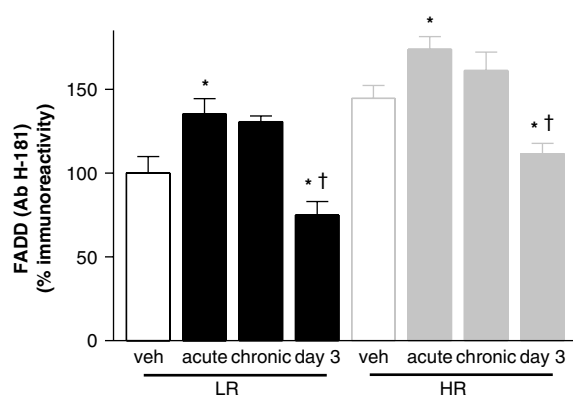


Figure 6 Fas-associated protein with death domain (FADD) is not differentially regulated in high-responder (HR)/low-responder (LR) rats following repeated cocaine and cocaine withdrawal. Groups of treatments: LR/HR vehicle ($n=5$), LR/HR acute cocaine (15 mg/kg, i.p., 45 min; $n=4$), LR/HR chronic cocaine (15 mg/kg for 7 days; $n=5$), and LR/HR chronic cocaine followed by spontaneous withdrawal (day 3; $n=4-5$). Columns are means \pm standard error of the mean (SEM) per group and expressed as percentage of LR vehicle-treated rats. Two-way analysis of variance (ANOVA) detected a significant difference in basal FADD protein content for HR–LR phenotype in all treatment groups, with HR rats exhibiting higher FADD levels than LR rats (39% change, $p=0.0002$, two-way ANOVA followed by Bonferroni's *post hoc* test). There was also a main effect for cocaine over FADD within each phenotype, which paralleled the findings previously observed with commercially purchased rats—ie acute cocaine treatment increased FADD (LR rats: $33 \pm 6\%$ increase, $p<0.05$; HR rats: $29 \pm 8\%$ increase, $p<0.05$) with a reversal following 3 days of withdrawal (LR rats: $25 \pm 8\%$ decrease, $p<0.05$; HR rats: $23 \pm 4\%$ decrease, $p<0.05$) (see Figure 3). One-way ANOVA detected significant differences between the groups of treatments if split by group: * $p<0.05$ vs LR/HR vehicle; † $p<0.05$ vs LR/HR cocaine (ANOVA followed by Bonferroni's test). However, no drug \times phenotype interaction was found ($p=0.8567$, n.s.).

signaling could reflect a molecular correlate for the HR/LR phenotype. The basal differences are maintained post-cocaine, as cocaine produced an increase in FADD both in HR and LR animals. As we have argued about, a consistent elevation on FADD is likely to promote, even if it does not cause apoptosis (eg impairing mechanisms controlling nonapoptotic functions), potential mechanisms for enhanced neurotoxicity. Therefore, we would hypothesize that HR may be more prone to neurotoxicity to repeated cocaine.

In the present study we show that selectively bred HR/LR rats display baseline differences in the FADD system, with HRs exhibiting higher FADD and lower pFADD levels than LR rats. Notably, HR/LR rats showed similar levels of the basal 116 kDa PARP cleavage (~ 85 kDa fragment) indicating similar rates of basal induction of apoptotic cell death in the cortex. This finding reinforces the notion that the FADD/pFADD system regulates nonapoptotic mechanisms in the brain. Interestingly, the basal changes in FADD/pFADD protein content were positively and negatively correlated, respectively, with the rats' rates of locomotion activity in a novel environment, suggesting the FADD system as a possible molecular correlate for the HR/LR phenotype. In addition to differences in sensitivity to psychostimulants (Piazza *et al*, 1989; Piazza and Le Moal, 1996; reviewed in Kabbaj, 2006), HR/LR animals display a variety of other behavioral differences including differences in anxiety behavior (Stead *et al*, 2006). Thus, future studies

will focus on evaluating other behavioral or neuroendocrine measures to determine how they may relate to FADD levels. In the same vein, several prior studies revealed other neurochemical and neural gene expression differences that contribute, at least in part, to the observed HR/LR behavioral phenotypes (Piazza *et al*, 1991; Hooks *et al*, 1994a,b; Kabbaj, 2004; Kabbaj *et al*, 2000; Ballaz *et al*, 2007a,b). The present observations, together with the D_2 mechanism involved in FADD modulation and the importance of the dopaminergic system in the regulation of locomotion, point to the possibility that basal HR/LR differences in FADD and pFADD may be driven by differences in dopaminergic circuits (Piazza *et al*, 1991; Hooks *et al*, 1994a,b; Dietz *et al*, 2005). In fact, Fligel *et al*, 2007, recently showed basal differences in D_2 DA-receptor mRNA expression in striatum of selectively bred HR/LR rats. The present study also demonstrated a main effect of cocaine on FADD levels in both HR and LR animals, a finding that paralleled our results in commercially purchased animals—ie increased FADD after acute cocaine with a reversal following 3 days of withdrawal. However, there was no drug \times phenotype interaction, at least with the dosage and method of cocaine administration we used and in our analysis of the response in cortex.

We have focused this work on the cortex as this brain region has been implicated in the cognitive and affective consequences of cocaine, and particularly in the consolidation of the addictive processes (Kalivas and Volkow, 2005). Moreover, the cortex appears critical in the translation of environmental and pharmacological stimuli into adaptive motor responses, including those seen during behavioral sensitization (Pierce and Kalivas, 1997). Of direct relevance to our findings, the cortex exhibits alterations in neural plasticity following cocaine administration (Ballesteros-Yanez *et al*, 2007), and these plasticity changes, including the novel mechanisms described in our studies, likely mediate critical behavioral sequelae of exposure to cocaine.

Nevertheless, equally relevant changes in the FADD system are likely taking place in subcortical regions and are worthy of further investigation. In particular, HR–LR differences in cocaine's impact on the FADD system may emerge in other brain regions. For instance, previous studies have shown HR animals had an increased DAT function in the NAcc and a decreased DAT function in cortex with no differences on striatum, suggesting a region-specific role for DAT in mediating information transfer within the corticolimbic circuitry involved in response to novelty (Chefer *et al*, 2003; Zhu *et al*, 2007). Therefore, future work should extend the present findings by examining the impact of cocaine on other brain regions in HR and LR rats. Moreover, given the differential propensity of HR vs LR rats for self-administration and our observation of preexisting differences in the FADD system, it will be of great interest to ascertain how well the FADD basal differences correlate with drug-seeking behavior and with the development of long-term addiction.

ACKNOWLEDGEMENTS

This study was supported by NIH fundings RO1DA13386, 5P01MH42251, 5P01DA021633-02, Conte Center grant no. L99MH60398, and Office of Naval Research (ONR) N00014-

02-1-0879 to HA and SJW. We thank Dr Jesús A García-Sevilla and Dr Shelly B Flagel for useful comments and Sharon Burke and Jennifer Fitzpatrick for excellent technical assistance.

DISCLOSURE/CONFLICT OF INTEREST

The authors state no conflict of interest.

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