

# Repeated stress prevents cocaine-induced activation of BDNF signaling in rat prefrontal cortex

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## Abstract

In this report we provide evidence that repeated stress prevents cocaine-induced activation of BDNF expression and signaling in rat prefrontal cortex. A single injection of cocaine up-regulates BDNF expression in sham (i.e. unstressed) rats but not in repeatedly stressed rats. Similarly, the expression as well as trafficking of the high affinity BDNF receptor *trkB* promoted by the psychostimulant is impaired in chronically-stressed rats challenged with cocaine. Moreover, among the different intracellular signaling pathways that can be activated by the neurotrophin, i.e. ERK1/2-, Akt- and PLC- $\gamma$ -pathway, we found that cocaine is able to selectively activate the ERK1/2 pathway in sham animals, but not in rats exposed to repeated stress. Notably, such changes take place in chronically-stressed animals although they still retain the ability to increase neuronal activity as measured by the enhancement of Arc gene expression. In summary, we have demonstrated that stress globally interferes with BDNF-mediated signaling responses to cocaine challenge, providing key insights into the molecular basis of stress-cocaine interaction and indicating the critical role of the prefrontal cortex in mediating such interaction. © 2009 Elsevier B.V. and ECNP. All rights reserved.

## 1. Introduction

Preclinical and clinical work indicate that stress is a strong trigger for cocaine abuse. Evidence exists that stress causes craving for cocaine (Sinha et al., 2006), sensitizes behavioral and neurochemical responses to cocaine (Piazza and Le Moal, 1998; Rouge-Pont et al., 1995) and facilitates the reinstatement of drug-taking behavior after extinction periods (Brown and Erb, 2007). In line with these observations, exposure to corticoster-

one facilitates, whereas adrenalectomy attenuates, cocaine self-administration (Erb et al., 1996; Goeders and Guerin, 1996). Taken together, these findings reveal that stress influences the action of cocaine. Moreover, stress and drug abuse are critical elements for the vulnerability of mental illnesses (Brady and Sinha, 2005), suggesting that this interaction may represent an important determinant of these pathological states.

Among the various factors that could be affected by the combined action of stress and cocaine, a plausible candidate may be the neurotrophin Brain Derived Neurotrophic Factor (BDNF) since previous work from different laboratories, including our own, has shown that chronic stress alters BDNF expression (Fumagalli et al., 2003; Nibuya et al., 1995; Roceri

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et al., 2004) and cocaine administration evokes regional- and temporal-specific changes in the expression of BDNF (Filip et al., 2006; Fumagalli et al., 2007; Le Foll et al., 2005). In concert with other proteins, BDNF and its high affinity receptor *trkB* are indeed critical regulators of neuronal plasticity and behavioral coping in adult rat brain (Castren, 2004; Nagappan and Lu, 2005). Activation of BDNF and its receptor may activate multiple signaling cascades involved in the regulation of different cellular processes spanning from neuroprotection and cognition to behavioral and molecular responses to psychoactive drugs (Reichardt, 2006; Russo et al., 2009). To this end, the major aim of our work was to address the question of whether exposure to repeated stress could influence the expression of BDNF and the activation of its intracellular signaling promoted by cocaine in the rat brain. In order to achieve this goal, we exposed chronically-stressed adult male rats to a single challenge with cocaine, focusing our analysis on prefrontal cortex, which is crucial for the addictive properties of cocaine (Steketee, 2005) and hippocampus, the brain region with the highest expression of corticosteroid receptors (Joels, 2007).

## 2. Material and methods

### 2.1. Materials

General reagents were purchased from Sigma (Milan, Italy) and molecular biology reagents were obtained from Celbio (Pero, Milan, Italy) and Promega (Milan, Italy). Cocaine hydrochloride was purchased from MacFarlan-Smith, Edinburgh, UK.

### 2.2. Stress procedure and treatment with cocaine

Male Sprague Dawley rats (275–300 g) (Charles River, Calco, Lecco) were maintained under a 12-h light/dark cycle with food and water available *ad libitum*. Animals were allowed to adapt to laboratory conditions for 1 week and handled 5 min a day during this period. We have recently performed a dose–response experiment of cocaine and have determined that the dose of 10 mg/kg induces a stable and reliable enhancement of BDNF gene expression in prefrontal cortex, frontal cortex and hippocampus (Fumagalli et al., 2007). For this reason, we decided to administer, via intraperitoneal injection, such a dose of psychostimulant to challenge both, stressed or unstressed animals. From our previous experiments we also mutated the time of sacrifice, i.e. 2 h after the single injection, (Fumagalli et al., 2007).

Chronic stress was employed to evaluate the effects of stress on cocaine-induced BDNF elevation [for a detailed explanation of the stress paradigm, please see (Fumagalli et al., 2008)]. Our experiment consisted of 4 experimental groups: 1) unstressed animals treated with saline; 2) unstressed animals treated with cocaine; 3) chronically stressed animals treated with saline and 4) chronically stressed animals treated with cocaine. Animals from groups 3 and 4 were subjected to repeated stress (14 days), mainly consisting of restraint and swim stress administered at a random time interval between treatments in order to avoid habituation. On the day of sacrifice, rats from groups 3 and 4 were exposed to restraint stress for 30 min, received a single injection of cocaine (10 mg/kg) immediately after the end of the stress session and decapitated 2 h later. Brain regions were immediately dissected according to the atlas of Paxinos and Watson (Watson, 1996), frozen on dry ice and stored at  $-80^{\circ}\text{C}$ . The prefrontal cortex (defined as Cg1, PL, and IL subregions corresponding to the plates 6–10) was dissected from 2-mm thick slices, whereas the hippocampus (including both ventral and dorsal parts) was dissected from the whole brain.

All animal handling and experimental procedures were performed in accordance with the EC guidelines (EEC Council Directive 86/609 1987), the Italian legislation on animal experimentation (Decreto Legislativo 116/92) and the National Institute of Health Guide for the Care and Use of Laboratory Animals.

### 2.3. RNA preparation

Different brain structures were homogenized in 4 M guanidinium isothiocyanate (containing 25 mM sodium citrate pH 7.5, 0.5% sarcosyl and 0.1% 2-mercaptoethanol) and total RNA was isolated by phenol-chloroform extraction. Quantification was carried out by spectrophotometric analysis and RNA aliquots were re-precipitated in ethanol for RNase protection assays. Quantification was performed by spectrophotometric analysis (absorption at 260 nm) and RNA re-precipitated in ethanol for RNase protection assay. To verify the quality of RNA, parallel samples were loaded onto an agarose/formaldehyde gel, run (35 V for 16 h) and stained with ethidium bromide.

### 2.4. cRNA probes and RNase protection assay

A transcription kit (MAXI script, Ambion) was used to generate cRNA probes and  $^{32}\text{P}$ -CTP was used as a radiolabelled nucleotide. The following plasmids were employed in the RNase protection assay: plasmid pSK-rB(C1) containing rat BDNF coding region, which is common to all the BDNF transcripts produced by different 5' exons (Aid et al., 2007) (generous gift of Dr. George Yancopoulos, Regeneron, Tarrytown, NY) and pTRI- $\beta$ -Actin-Rat (Ambion) containing a portion of rat  $\beta$ -Actin. Arc plasmid containing a portion of rat 5' coding region (a generous gift of Dr. Worley) and plasmid pSK-rTRKB(A1) (a generous gift of Dr. Yancopoulos). The cRNA probes and the relative protected fragment (p.f.) were the following: BDNF=800; p.f.=740; *trkB*=366; p.f.=336; Arc=630, p.f.=620;  $\beta$ -actin=280, p.f.=126.

The RNase protection assay was performed on a 10  $\mu\text{g}$  sample of total RNA as described previously (Riva et al., 1996). Briefly, after ethanol precipitation, total RNA was dissolved in 20  $\mu\text{l}$  of hybridization solution containing 150,000 cpm of  $^{32}\text{P}$ -labelled BDNF, *trkB* or Arc and 50,000 cpm of  $^{32}\text{P}$ -labelled  $\beta$ -actin cRNA probe. After being heated at  $85^{\circ}\text{C}$  for 10 min, the cRNA probes were allowed to hybridize the endogenous RNAs at  $45^{\circ}\text{C}$  overnight. At the end of hybridization, the solution was diluted with 200  $\mu\text{l}$  of RNase digestion buffer containing a 1/400 dilution of an RNase cocktail (RNase A and RNase T1) and incubated for 30 min at  $30^{\circ}\text{C}$ . Proteinase K and SDS were then added to the sample and the mixture was incubated at  $37^{\circ}\text{C}$  for an additional 15 min. At the end of incubation, the sample was extracted with phenol/chloroform and ethanol-precipitated. The pellet, containing the RNA:RNA hybrids, was dried and resuspended in loading buffer, boiled at  $95^{\circ}\text{C}$  for 5 min and separated on 5% polyacrylamide gel under denaturing conditions.

### 2.5. RNA calculation

The levels of mRNA for BDNF or  $\beta$ -actin were calculated using the Quantity One software from Biorad. In order to ensure that the autoradiographic bands were in the linear range of intensity, different exposure times were used.  $\beta$ -actin was employed as internal standard for RNase protection assay as its expression was not regulated by single or repeated cocaine treatment. Results were compiled as the unitless ratio of BDNF/ $\beta$ -actin mRNA, Arc/ $\beta$ -actin mRNA and *trkB*/ $\beta$ -actin mRNA.

### 2.6. Preparation of protein extracts and Western Blot Analysis

Prefrontal cortex and hippocampus were homogenized in a glass-glass potter in cold 0.32 M sucrose containing 1 mM Hepes solution, 0.1 mM EGTA, 0.1 mM PMSE, pH=7.4, in presence of a complete set of

protease inhibitors and a phosphatase inhibitor cocktail. Different subcellular fractions were prepared as previously described (Fumagalli et al., 2006). The homogenized tissues were centrifuged at 1000  $\times$ g for 10 min. The resulting pellet (P1), corresponding to the nuclear fraction, was resuspended in a buffer (20 mM Hepes, 0.1 mM DTT, 0.1 mM EGTA) with protease and phosphatase inhibitors; the supernatant (S1) was centrifuged at 9000  $\times$ g for 15 min to obtain a clarified fraction of cytosolic proteins (S2) and the pellet, corresponding to the crude synaptosomal fraction (membrane fraction, P2), was resuspended in the same buffer used for P1. Total protein content was measured in the homogenate and P2 fraction by the Bio-Rad Protein Assay (Bio-Rad Laboratories, Segrate, Milan, Italy).

Total protein concentrations were adjusted to the same amount for all samples (10  $\mu$ g per lane). All the samples were run on a sodium dodecyl sulfate (SDS)-10% polyacrilamide gel under reducing conditions and proteins were then electrophoretically transferred onto nitrocellulose membranes (Bio-Rad Laboratories, Segrate, Milan, Italy). Blots were blocked with 10% nonfat dry milk in TBS/0.1% Tween-20 buffer and then incubated with primary antibody. The blots were first probed with antibodies against the phosphorylated forms of the protein and then stripped with SDS 2%, 100 mM  $\beta$ -mercaptoethanol and 62.5 mM Tris-HCl pH 6.7 at 50  $^{\circ}$ C for 30 min and re-probed with antibodies against total proteins of same type. The conditions of the primary antibodies are the following: polyclonal anti-TrkB full length (145 kDa) (Santa Cruz Biotechnology, 1:750 in 5% nonfat dry milk, overnight, 4  $^{\circ}$ C), monoclonal anti-phospho-ERK1/2 (44-42 kDa) (Santa Cruz Biotechnology, 1:10,000 in 1% nonfat dry milk, overnight, 4  $^{\circ}$ C), polyclonal anti-ERK1/2 (Santa Cruz Biotechnology, 1:5000 in 1% nonfat dry milk, 2 h, room temperature), polyclonal anti-phospho-PLC $\gamma$  Tyr783 (155 kDa) (Cell Signaling Technology, 1:1000, in 3% nonfat dry milk, 4  $^{\circ}$ C, O/N), polyclonal anti-PLC (Cell Signaling Technology, 1:1000 in 5% nonfat dry milk, overnight, 4  $^{\circ}$ C), polyclonal anti-phospho-AKT Ser473 (60 kDa) (Cell Signaling Technology, 1:1000 in 5% nonfat dry milk, overnight, 4  $^{\circ}$ C), polyclonal anti-AKT (Cell Signaling Technology, 1:1000 in 10% nonfat dry milk, 2 h, room temperature). Blots were then incubated 1 h at room temperature with horseradish peroxidase-conjugated secondary antibody (anti-rabbit or anti-mouse IgG, by Sigma-Aldrich) and immunocomplexes were visualized by chemiluminescence utilizing the ECL Western Blotting kit (Amersham Life Science, Milano, Italy) according to the manufacturer's instructions. Only for the detection of ERK1/2 phosphorylated forms, immunocomplexes were visualized by chemiluminescence utilizing the SuperSignal West Femto (Pierce). Results were standardized by using  $\beta$ -actin as a housekeeping protein which was detected by evaluating the band density at 43 kDa after probing with a polyclonal antibody with a 1:20,000 dilution in 3% non fat dry milk (1 h, room temperature, Sigma-Aldrich).

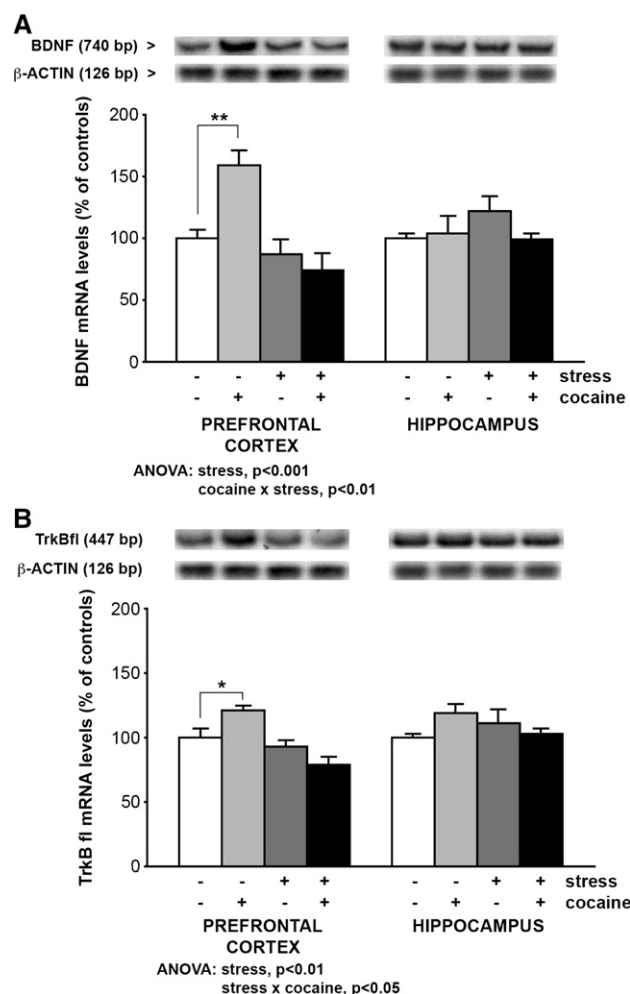
## 2.7. Statistical analysis

Data are presented as means and standard errors, with each individual group comprising at least 5 samples. Changes between the different experimental groups were analyzed by two-way analysis of variance (ANOVA), with status (unstressed- and chronically-stressed animals) and treatment (cocaine injection) as independent variables and have been converted in percent in the Figures. When appropriate, further differences were analyzed by single contrast post hoc test (SCPHT). Significance for all tests was assumed at  $P < 0.05$ .

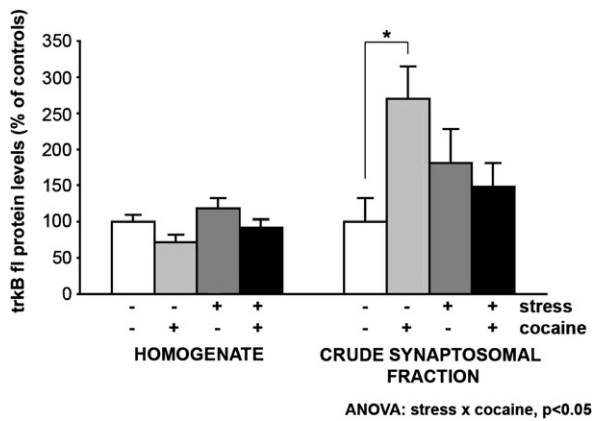
## 3. Results

The rationale for the present manuscript stems from our recent findings showing that administration of cocaine evokes changes in BDNF expression primarily in rat prefrontal cortex (Fumagalli et al., 2007). Since acute stress exposure did not alter the

expression of BDNF following an acute challenge with cocaine (controls:  $100 \pm 8$ ; acute cocaine:  $150 \pm 7$ ; acute stress:  $134 \pm 8$ ; acute stress+acute cocaine:  $176 \pm 9$ ; Fumagalli and Riva,



**Figure 1** Effect of a single injection of cocaine on BDNF and trkB expression: modulation by chronic stress. (A) The upper panel shows a representative RNase protection assay of BDNF and  $\beta$ -actin (employed as an internal standard) mRNA levels in response to a single dose of cocaine administered to unstressed and to chronically stressed animals immediately after the end of the last stress session. Animals were sacrificed 2 h after the single drug administration. The lower panel shows the quantitative analysis of the effects of a single injection of cocaine on BDNF mRNA levels in the prefrontal cortex and the hippocampus. The results, expressed as % of control (unstressed) rats, represent the mean  $\pm$  S.E.M. of at least 5 independent determinations. \*\* $P < 0.01$  vs. controls (two way ANOVA with SCPHT). (B) The upper panel shows a representative RNase protection assay of trkB full length (fl) mRNA levels in response to a single dose of cocaine administered to unstressed and to chronically stressed animals immediately after the end of the last stress session. The lower panel shows the quantitative analysis of the effects of a single injection of cocaine on trkB mRNA levels in the prefrontal cortex and the hippocampus. The results, expressed as % of control (unstressed) rats, represent the mean  $\pm$  S.E.M. of at least 5 independent determinations. \* $P < 0.05$  vs. controls (two way ANOVA with SCPHT).



**Figure 2** Effect of a single injection of cocaine on trkB full length (fl) protein levels: modulation by chronic stress. The analysis was carried out in the whole homogenate or crude synaptosomal fraction of rat prefrontal cortex. Cocaine was administered immediately after the end of the last stress session. Animals were sacrificed 2 h after the single drug administration. The results, expressed as % of control (unstressed) rats, represent the mean  $\pm$  S.E.M. of at least 5 independent determinations. \* $P < 0.05$  vs. controls (two way ANOVA with SCPHT).

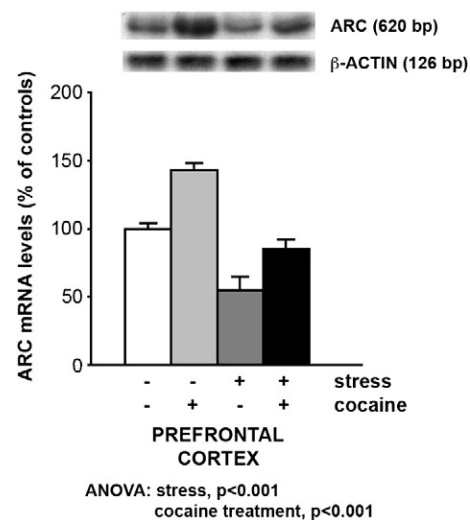
unpublished observation), the main aim of our investigation was to evaluate whether chronic exposure to unpredictable stress could interfere with the response to the psychostimulant, and the consequent activation of BDNF-dependent intracellular pathways. We focused our analysis on the rat prefrontal cortex, the terminal region of the mesocortical dopaminergic network, which has been involved in cocaine seeking and withdrawal (Steketee, 2005), although some measurements were undertaken in the hippocampus as well, since this brain region contains the highest density of corticosteroid receptors which mediate the response to stress (Joels, 2007).

To address this issue, we first examined the effect of cocaine and stress, alone or in combination, on BDNF mRNA levels. Two-way ANOVA indicated a significant effect of repeated stress ( $F_{3,21} = 17.6355$ ,  $P = 0.0006$ , two-way ANOVA) and only a marginal effect of cocaine treatment ( $F_{3,21} = 3.9487$ ,  $P = 0.063$ , two-way ANOVA), whereas the response to a single injection of cocaine depended upon whether the animals were exposed to chronic stress (cocaine  $\times$  stress interaction,  $F_{3,21} = 9.2935$ ,  $P = 0.007$ , two-way ANOVA) (Fig. 1A). In light of the significant interaction between the two treatments we made intergroup comparisons, which showed that cocaine elicited an up-regulation of BDNF mRNA levels in sham animals ( $F_{1,11} = 13.2553$ ,  $P = 0.004$ , two-way ANOVA with SCPHT) but not in chronically-stressed rats ( $F_{1,10} = 0.53981$ ,  $P = 0.94504$ , two-way ANOVA with SCPHT) (Fig. 1A). No significant changes were instead observed in the hippocampus under these different experimental conditions (Fig. 1A). A similar increase was observed for the high affinity BDNF receptor, i.e. trkB. Two-way ANOVA indicated a significant effect of repeated stress ( $F_{3,24} = 14.2836$ ,  $P = 0.001$ , two-way ANOVA), no effect of cocaine treatment ( $F_{3,24} = 0.2964$ ,  $P = 0.592$ , two-way ANOVA) and a significant cocaine treatment  $\times$  repeated stress interaction ( $F_{3,24} = 7.4045$ ,  $P = 0.013$ , two-way ANOVA) (Fig. 1B). Similar to the effects on BDNF gene expression, cocaine produced a significant elevation of trkB mRNA levels in unstressed animals ( $F_{1,14} = 6.3223$ ,  $P = 0.04$ , two-

way ANOVA with SCPHT) which was prevented in chronically-stressed animals ( $F_{1,10} = 2.0481$ ,  $P = 0.3357$ , two-way ANOVA with SCPHT).

We also analyzed the trafficking of trkB toward the membrane compartment by comparing its levels in the whole homogenate with those in the crude synaptosomal fraction (Fig. 2). Whereas no changes were observed in the whole homogenate under any of the experimental conditions, significant changes were found in the synaptosomal compartment as indicated by a significant cocaine  $\times$  repeated stress interaction ( $F_{3,19} = 6.62$ ,  $P = 0.02$ , two-way ANOVA) (two-way ANOVA). Subdividing the data for individual intergroup comparison, it was evident that cocaine evoked a strong increase of trkB total levels in the crude synaptosomal fraction of sham animals ( $F_{1,10} = 9.86$ ,  $P = 0.01$ , two-way ANOVA with SCPHT) but not in chronically-stressed rats ( $F_{1,9} = 0.33$ ,  $P = 0.57$ , two-way ANOVA with SCPHT) (Fig. 2).

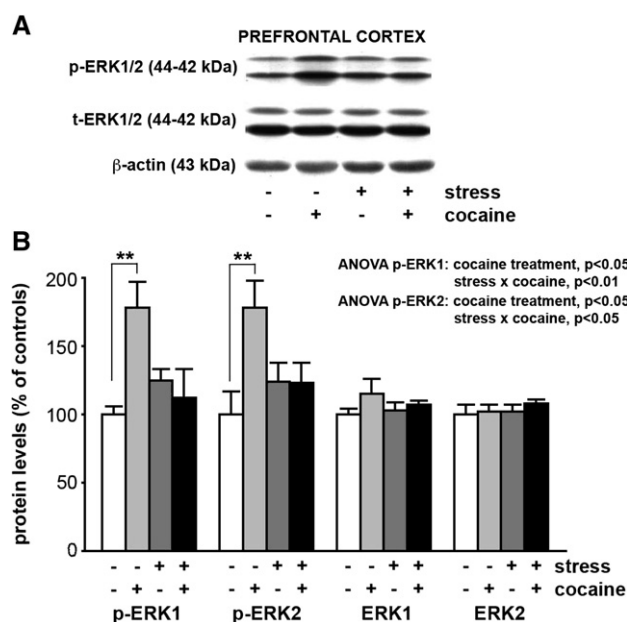
The differential modulation of BDNF by cocaine in sham versus stressed animals was not due to altered neuronal responsiveness. In fact the expression of Arc (Activity Regulated Cytoskeletal Associated protein), widely considered a marker of neuronal activation (Lyford et al., 1995), was significantly affected by cocaine treatment ( $F_{3,25} = 22.60$ ,  $P = 0.000001$ , two-way ANOVA) and prolonged stress exposure ( $F_{3,25} = 45.28$ ,  $P = 0.0001$ , two-way ANOVA). However, no cocaine treatment  $\times$  stress interaction ( $F_{3,25} = 0.787$ ,  $P = 0.385$ , two-way ANOVA) was found suggesting that chronically stressed animals still retain the ability to respond to challenging stimuli (Fig. 3).



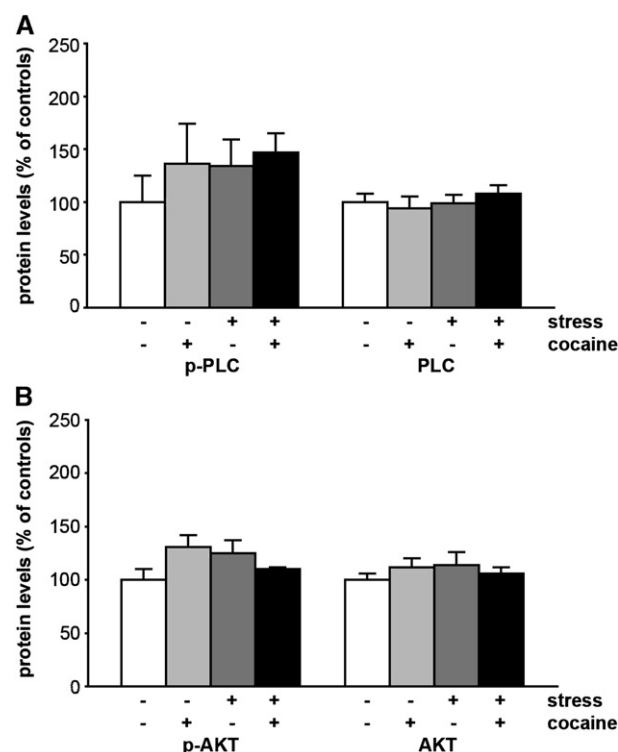
**Figure 3** Effect of a single cocaine injection on Arc mRNA levels in rat prefrontal cortex: modulation by chronic stress. The upper panel shows a representative RNase protection assay of Arc and  $\beta$ -actin (employed as an internal standard) mRNA levels in response to a single dose of cocaine administered to unstressed or chronically stressed animals immediately after the end of the last stress session. Animals were sacrificed 2 h after the single drug administration. The lower panel shows the effect of a chronic stress procedure on the modulation of Arc mRNA levels produced by a single injection of cocaine (10 mg/kg) in comparison to control (unstressed) animals in prefrontal cortex, measured 2 h after the injection. The results, expressed as % of control rats, represent the mean  $\pm$  S.E.M. of at least 5 independent determinations.



To further characterize if the effects of cocaine on BDNF-dependent signaling were affected by stress exposure, we investigated the modulation of MAP kinase (ERK1/2), Akt- or PLC $\gamma$ -dependent pathways. It is well known that cocaine is able to trigger ERK activation (Valjent et al., 2006, 2004) thereby suggesting that the psychostimulant might have a broad impact on intracellular signaling given the multiple roles exerted by ERK1/2. With respect to pERK1, two way ANOVA indicated a significant effect of cocaine treatment ( $F_{3,24}=4.832$ ,  $P=0.040$ , two-way ANOVA), no effect of repeated stress ( $F_{3,24}=1.926$ ,  $P=0.180$ , two-way ANOVA) and a significant cocaine treatment  $\times$  repeated stress interaction ( $F_{3,24}=9.466$ ,  $P=0.006$ , two-way ANOVA) (Fig. 4A). Examining the individual treatment effects, it was evident that cocaine significantly enhanced phosphorylation of pERK1 in unstressed animals ( $F_{1,12}=13.912$ ,  $P=0.002$ , two-way ANOVA with SCPHT) but not in rats exposed to prolonged stress ( $F_{1,12}=1.426$ ,  $P=0.492$ , two-way ANOVA with SCPHT). Analysis of pERK2 revealed a significant effect of cocaine treatment ( $F_{3,25}=5.112$ ,  $P=0.035$ , two-way ANOVA), no effect of repeated stress ( $F_{3,25}=0.764$ ,  $P=0.392$ , two-way ANOVA) and a significant cocaine treatment  $\times$  repeated stress interaction ( $F_{3,25}=0.392$ ,  $P=0.031$ , two-way ANOVA) (Fig. 4A). Given the interaction between the two treatment paradigms, we made all intergroup comparisons. As previously observed for pERK1, cocaine induced a significant elevation of pERK2 phosphorylation in unstressed animals ( $F_{1,13}=10.85$ ,  $P=0.006$ , two-way ANOVA with SCPHT) but not in repeatedly stressed rats



**Figure 4** Effect of a single cocaine injection on ERK1/2 phosphorylation and expression in rat prefrontal cortex: modulation by chronic stress. (A) The photographs are representative immunoblots of native (ERK) and phosphorylated (p-ERK) forms of ERK 1 and ERK 2 in the crude synaptosomal fraction. (B) Quantitative analysis of the effects of chronic stress, acute cocaine or their combination on ERK 1/2 expression and phosphorylation in rat prefrontal cortex 2 h after last drug injection. Cocaine was administered immediately after the end of the last stress session. Data are the mean  $\pm$  SEM at least 5 independent determinations.  $**P<0.01$  vs. saline-injected rats (two way ANOVA with SCPHT).



**Figure 5** Effect of a single cocaine injection on phospholipase C (PLC) and Akt phosphorylation and expression in rat prefrontal cortex: modulation by chronic stress. Quantitative analysis of the effects of cocaine challenge on Akt or PLC $\gamma$  expression and phosphorylation in crude synaptosomal fraction of rat prefrontal cortex 2 h after last drug injection in unstressed or chronically stressed animals. Cocaine was administered immediately after the end of the last stress session.

( $F_{1,12}=0.001$ ,  $P=1.946$ , two-way ANOVA with SCPHT) (Fig. 4A). No changes were observed in the total levels of ERK1 and ERK2 in rat prefrontal cortex.

Fig. 5 shows no effect of cocaine or prolonged stress when administered alone as well as no effect of their combination on Akt or PLC $\gamma$  phosphorylation and expression in the crude synaptosomal fraction of rat prefrontal cortex.

## 4. Discussion

Our findings show that a single injection of cocaine has a different effect on BDNF expression and signaling depending on whether or not the animal has been exposed to repeated stress. We found that chronic stress prevents cocaine-induced up-regulation of the neurotrophin BDNF and its high affinity receptor trkB, as well as trkB trafficking to the membrane compartment, which might be considered an index of activation upon neurotrophin release (Saarelainen et al., 2003). In addition, we demonstrate that stress prevents increased ERK1/2 phosphorylation promoted by cocaine, whereas no effect of both experimental conditions, alone or in combination, was observed on the activation of Akt- and PLC $\gamma$ -mediated signaling pathways. These results reveal a previously unappreciated, and highly dynamic, mechanism through which stress alters the response to this

psychostimulant. Note that chronically stressed animals still retain the ability to respond to the challenge produced by cocaine, as evidenced by increased Arc gene expression, a widely accepted measure of neuronal activity (Lyford et al., 1995), implicating that stress does not interfere with the degree of cortical activation thus adding specificity to the changes observed in BDNF signaling pathway.

The role of BDNF in the modulation of addictive behaviors is indeed very complex. Most of the studies primarily investigated the effect of cocaine on BDNF expression or, otherwise, how the exogenous administration of the neurotrophin could influence the propensity to develop cocaine seeking (Filip et al., 2006; Le Foll et al., 2005) (Berhow et al., 1996; Grimm et al., 2003; Hall et al., 2003; Lu et al., 2004; Pu et al., 2006); (Berglind et al., 2007; Graham et al., 2007). Conversely, no molecular evidence exists whether BDNF is implicated in cross-sensitization mechanisms between stress and cocaine, an issue of critical relevance since stress is known to increase cocaine craving (Sinha et al., 2006). The increased expression of the neurotrophin and the activation of its intracellular signaling pathway, that was observed in unstressed animals, might represent a defensive strategy but it could also contribute to the neuroplastic changes required for the rewarding properties of cocaine. Because of the intrinsic nature of the neurotrophin, it is tempting to speculate that, at variance from chronic cocaine treatment, which may cause adaptive responses presumably involved in drug addiction, enhancement of BDNF expression following acute cocaine may represent a homeostatic, neuroprotective response of the cell to the exaggerated neuronal activity caused by the psychostimulant, an option corroborated by evidence that inhibition of BDNF enhances cocaine's cytotoxicity in cultured cells (Yan et al., 2007). Although we focused our attention on BDNF-dependent intracellular signaling, we are aware that other factors may converge and cooperate to modulate ERKs, such as norepinephrine which is critical for stress responses (Pascoli et al., 2005). In chronically stressed animals, if BDNF cannot be recruited to attenuate or counteract the cellular demands caused by acute cocaine, then cellular resilience may be at risk: chronic stress might cause a tolerance-like state, which might increase the threshold for the activation of the trophic response thus rendering the animals more vulnerable to cocaine. Although the nature of such activation is still unclear, the infusion of BDNF into the medial prefrontal cortex attenuates cocaine seeking (Berglind et al., 2007). In view of these results, it is conceivable to hypothesize that stress-induced prevention of acute BDNF up-regulation promoted by cocaine might be deleterious for cortical neurons.

Although we are aware that changes in the expression of a single neurotrophic factor cannot fully explain the complex interactions occurring in a stressed brain exposed to cocaine, the evidence that stress and cocaine interact to modulate the expression of the trophic factor FGF-2 in rat brain (Fumagalli et al., 2008) further strengthens the possibility that modulation of trophic factors participates in determining the influence of stress on cocaine exposure, and presumably, the functional outcome.

In conclusion, our data indicate that BDNF-dependent signaling represents a specific vulnerable target of stress and cocaine interaction in rat prefrontal cortex, thus providing a

mechanism for higher vulnerability to cocaine in chronically stressed rats and highlighting the critical role of prefrontal cortex in mediating such interaction. The findings described herein further reinforce the opportunity to evaluate the response to drugs of abuse in relationship with the environmental context and in animals bearing molecular or behavioral alterations that might be found in humans.

## Role of the funding source

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## Contributors

Fabio Fumagalli, Giorgio Racagni and Marco A. Riva designed the study. Fabio Fumagalli and Marco A. Riva wrote the manuscript. Fabio Fumagalli coordinated the different phases of the experiments and managed the literature searches. Fabio Fumagalli and Lucia Caffino performed all in vivo animal experiments. Lucia Caffino performed the biochemical experiments and undertook the statistical analysis.

## Conflict of interest

None of the authors has any potential conflict of interest nor financial interests to disclose.

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