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Acute Stress Responsiveness of the Neurotrophin BDNF in the Rat Hippocampus is Modulated by Chronic Treatment with the Antidepressant Duloxetine

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Compelling evidence suggests that mood disorders are characterized by reduced neuronal plasticity that might be normalized by pharmacological intervention. Our study aimed to establish whether chronic antidepressant treatment could alter the modulation of the neurotrophin brain-derived neurotrophic factor (BDNF) under a stressful condition. Therefore, adult male Sprague–Dawley rats were treated for 21 days with vehicle or with the SNRI duloxetine and, 24 h after the last injection, exposed to an acute swim stress (5 min) before being killed 15 min later. We found that chronic duloxetine treatment was able to modulate the rapid transcriptional changes of BDNF isoforms produced by an acute swim stress. Indeed whereas the mRNA levels of BDNF exon IV were upregulated by stress in vehicle as well as in duloxetine-treated rats, a significant increase of exon VI and exon IX was only found in rats that were pretreated with the antidepressant. These differential effects are in part because of selective changes in signaling pathways involved in the control of BDNF transcription. Moreover, the acute stressful episode significantly increased the levels of mature BDNF protein in the synaptosomal compartment in rats that were pretreated with the antidepressant, but not in control animals. Our results suggest that chronic antidepressant treatment might affect the responsiveness of BDNF under stressful conditions, suggesting that pharmacological intervention could 'prime' neuroprotective pathways and render them more responsive to preserve cell function and viability.
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INTRODUCTION

Depression is a complex disorder also characterized by a reduced ability of the brain to cope and adapt under challenging conditions (Duman and Monteggia, 2006). At a molecular level, this compromised neuroplasticity is associated with altered expression and function of neuroplastic molecules (McClung and Nestler, 2008). Accordingly, the potential of antidepressant drugs to 'correct' these abnormalities may be linked to the regulation of specific proteins, which are critical for these mechanisms. One of these molecules is the neurotrophin brain-derived neurotrophic factor (BDNF), whose modulation represents a key step in long-term adaptive changes brought about by antidepressant drugs (Berton and Nestler, 2006; Groves, 2007; Martinowich *et al*, 2007; Tardito *et al*, 2006). The effects on BDNF are complex and may occur at several levels including transcription (Calabrese *et al*, 2007; Coppell *et al*, 2003; Kozisek *et al*, 2008; Molteni *et al*, 2006; Nair *et al*, 2007; Nibuya *et al*, 1995), translation and trafficking in specific subcellular compartments (Calabrese *et al*, 2007) as well as receptor activation and signaling (Duman *et al*, 2007; Fumagalli *et al*, 2005; Saarelainen *et al*, 2003; Wyneken *et al*, 2006).

However, because the concept of neuronal plasticity implies that adaptive changes are set in motion in response to 'external' stimuli, it is expected that antidepressants should not only improve compromised neuronal plasticity by affecting the expression of key proteins, but also modulate the responsiveness of these systems under challenging conditions (Martinowich *et al*, 2007). These effects could be particularly relevant for BDNF that responds to rapid or more protracted manipulations and represents a key mediator for neuronal plasticity (Bramham and Messaoudi, 2005). Stress can be particularly relevant in

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this context because it represents a challenge for the individual, who can, in turn, be resistant or vulnerable to the development of a psychopathology according to his ability of coping with the adverse situation (McEwen, 2007; Pariante, 2003). This individual difference is probably because of the activation of neuronal pathways and mechanisms that will eventually affect cellular responsiveness and contribute to resilience or vulnerability.

Along this line of thinking the aim of this study has been to investigate the ability of chronic antidepressant treatment to modulate the responsiveness of BDNF under a challenging condition. Accordingly, we investigated if rapid transcriptional and translational changes of the neurotrophin in response to an acute stress could be affected by chronic treatment with duloxetine (DLX), a serotonin-noradrenaline reuptake blocker (SNRI) antidepressant. We focused our analysis on the hippocampus, a brain structure that is altered in depressed subjects (Sheline *et al*, 2002; Stockmeier *et al*, 2004) and that is highly responsive to stress (Joels *et al*, 2008). Thus we investigated, the changes in the transcription of different 5′ BDNF isoforms in this brain region, the levels and activation of some of the proteins controlling their expression (Aid *et al*, 2007; Liu *et al*, 2006), as well as modifications of BDNF protein in specific subcellular compartments.

MATERIALS AND METHODS

Materials

General reagents were purchased from Sigma-Aldrich (Milan, Italy), and molecular biology reagents were obtained from Ambion (Austin, TX, USA), New England Biolabs (Beverly, MA, USA), Promega (Milan, Italy), and Bioline (London, UK). DLX was obtained from Eli Lilly & Co. (Indianapolis, IN, USA).

Animals

Adult (2- to 3-month old) male Sprague–Dawley rats (Charles River, Calco, Italy) weighing 225–250 g were used throughout the experiments. Rats were housed in groups of 4 per cage under standard conditions (12-h light/dark cycle with food and water available *ad libitum*) and were exposed to daily handling for 2 weeks before any treatment. All animal handling and experimental procedures were performed in accordance with the EC (EEC Council Directive 86/609, 1987), the Italian legislation on animal experimentation (Decreto Legislativo 116/92), and the *National Institutes of Health Guide for the Care and Use of Laboratory Animals*. All efforts were made to minimize animal suffering and to reduce the number of animals used.

Pharmacological Treatments and Stress Paradigm

Rats were treated with vehicle (VEH; water) or DLX (10 mg/kg per day, by gavage) for 21 days and then randomly subdivided in two groups: half of the animals were maintained undisturbed in their home cages whereas the others were exposed 24 h after the last drug treatment to an acute stress episode. For this purpose, animals were individually placed in a bucket filled with water at room temperature (25°C) that was deep enough so that the animals' paws did not touch the bottom. After 5 min, they were dried with a towel and returned to their home cage. Rats were killed by decapitation 15 min after the end of the stressful episode and the hippocampi were rapidly dissected, frozen on dry ice and stored at −80°C for molecular analysis. One hippocampus was used for mRNA analysis and one was used for protein expression studies.

Plasma Corticosterone Assay

Samples of blood from each rat were collected in heparinized tubes. Plasma was separated by centrifugation (5000 rpm for 10 min) and corticosterone was determined by an enzyme-linked immunosorbent assay using a commercial kit according to the manufacturers' instructions (IBL, Hamburg, Germany).

RNA Preparation

The hippocampus was homogenized in 4 M guanidinium isothiocyanate (containing 25 mM sodium citrate pH 7.5, 0.5% sarcosyl, and 0.1% 2-mercaptoethanol). Total RNA was isolated by phenol/chloroform extraction and quantification was carried out by spectrophotometric analysis. RNA quality controls were performed using Agilent Bioanalyzer 2100 Lab-on-a-Chip technologies.

Quantification of BDNF mRNA Expression by Real-Time Quantitative PCR

Total RNA (2 μg) were mixed with 1 μl of 0.5 μg/μl random hexamer (Invitrogen, Carlsbad, CA, USA), 10 μl of 5 × buffer (Invitrogen), 1 μl of 20 mM dNTPs, 1 μl of 20 mM DTT (Invitrogen), 0.4 μl of 40 U/μl RNaseOUT (Invitrogen), 2 μl SuperScript II Transcriptase (Invitrogen), in a final volume of 50 μl. The reaction mix was incubated at 42°C for 55 min, at 70°C for 15 min, and then at 95°C for 10 min. Real-time PCR was performed using the 7700 (Applera, Norwalk, CT, USA) sequence detection system.

The mRNA levels of the target genes (ID total BDNF TaqMan probe: Rn02531967_s1; ID BDNF transcript I TaqMan Probe: Rn01484924_m1; ID BDNF transcript IIa TaqMan Probe: Rn00560868_m1; ID BDNF transcript IIb TaqMan Probe: Rn01484926_m1; ID BDNF transcript IIc TaqMan Probe: Rn01484925_m1; BDNF transcript III forward primer: ATGCTTCATTGAGCCCAGTT and reverse primer: GTGGACGTTTGCTTCTTTCA; ID BDNF transcript IV TaqMan Probe: Rn01484927_m1; ID BDNF transcript VI TaqMan Probe: Rn01484928_m1, transcript IXa forward primer: TGGTGTCCCCAAGAAAGTAA and reverse primer: CACGTGCTCAAAAGTGTCAG) and the housekeeping gene β-actin (ID TaqMan β-actin probe: Rn00667869_m1) were determined by real-time PCR (7700 Applied Biosystem, Foster City, CA, USA). PCR reactions were carried out with TaqMan Universal PCR Master Mix (Applera), which contained AmpliTaq Gold DNA polymerase, AmpErase UNG, dNTPs with dUTP, passive reference, and optimized buffer components. AmpErase UNG treatment was used to prevent the possible reamplification of carryover PCR products. Thermal cycling was initiated with an incubation at 50°C for 2 min and then at 95°C for 10 min for optimal

AmpErase UNG activity and activation of AmpliTaq Gold DNA polymerase, respectively. After this initial step, 40 cycles of PCR were performed. Each PCR cycle consisted of heating the samples at 95°C for 15 s to enable the melting process and then for 1 min at 60°C for the annealing and extension reaction. Each sample was assayed in duplicate and using two independent retrotranscription products. Data analyses have been performed according to the comparative C_t method by the use of Applied Biosystem Real-Time software, which automatically determines, using the auto C_t determination feature, the optimal baseline and threshold settings. Data have been expressed as percentage calculated from the expression of the target genes normalized on β-actin mRNA levels.

Preparation of Protein Extract

Different subcellular fractions were prepared from the hippocampus. Tissues were manually homogenized using a dounce homogenizer in 600 µl of a pH 7.4 cold buffer (solution A) containing 0.32 M sucrose, 10 mM Tris–HCl, pH 7.4, and a commercial cocktail of protease (Roche, Monza, Italy) and phosphatase (Sigma-Aldrich) inhibitors. The total homogenate (H) was centrifuged at 2000 *g* for 1 min at 4°C, thus obtaining a pellet (P1) corresponding to the nuclear fraction, which was resuspended in solution A and centrifuged again (2000 *g* for 2 min at 4°C). The supernatant obtained from each centrifugation step was collected and centrifuged at 23 000 *g* for 5 min at 4°C to obtain a pellet (P2) corresponding to the crude synaptosomal fraction, which was resuspended in solution A. Discontinuous Ficoll 400 (Sigma-Aldrich) gradients were prepared by layering 2 ml of 5% Ficoll 400 on top of 2 ml of 13% Ficoll 400 into 5 ml polycarbonate centrifuge tubes. The Ficoll 400 was dissolved in solution A. The resuspended P2 pellet was then layered on top of the gradient and centrifuged at 45 000 *g* for 45 min at 4°C. After centrifugation, synaptosomes were collected (interphase between 13 and 5% Ficoll), diluted 1–10 with solution A and centrifuged at 23 000 *g* for 20 min at 4°C. The supernatant was removed and the pellet (synaptosomes) was resuspended in 70 µl of solution A. Total protein content was measured according to the Bradford protein assay procedure (Bio-Rad, Milan, Italy), using bovine serum albumin as calibration standard.

Western Blot Analysis

Protein analysis was performed on total homogenate, nuclear (P1), cytosolic (S2), and synaptosomal fractions. Equal amounts of protein were run under reducing conditions on SDS–polyacrylamide gels (14% SDS–PAGE for BDNF and 8% SDS–PAGE for glucocorticoid receptor (GR), α-calcium/calmodulin-dependent protein kinase II (α-CaMKII), CREB, methyl CpG-binding protein 2 (MeCP2), mitogen-activated protein kinase/extracellular signal-regulated kinase kinase (MEK)) and then electrophoretically transferred onto PVDF (for BDNF) or nitrocellulose (for the other proteins) membranes (Bio-Rad). The blots were blocked with 10% nonfat dry milk and then incubated with the following primary antibodies: anti-BDNF polyclonal antibody (1 : 1000, 4°C, overnight; Santa Cruz Biotechnology, Santa Cruz, CA, USA) able to recognize both the mature form of the neurotrophin (mBDNF; 14 kDa) and its precursor (proBDNF; 32 kDa); monoclonal anti-GR (Affinity Bioreagent, Golden, CO, USA; dilution 1 : 2500); monoclonal anti-Thr^{286}-phospho-α-CaMKII (Affinity Bioreagent; dilution 1 : 2500); monoclonal anti-α-CaMKII (Chemicon International Inc., Temecula, CA, USA; dilution 1 : 2500); monoclonal anti-Ser^{133}-phospho-CREB (Cell Signaling Technology, Danvers, MA, USA; dilution 1 : 1000); monoclonal anti-CREB (Cell Signaling Technology; dilution 1 : 1000); monoclonal anti-$Ser^{217/221}$ phospho-MEK (Santa Cruz Biotechnology; dilution 1 : 500); monoclonal anti-MEK (Santa Cruz Biotechnology; dilution 1 : 5000); monoclonal anti-Ser^{421}-phospho-MeCP2 and monoclonal anti-MeCP2 (both generous gifts of Professor ME Greenberg; dilution 1 : 4000 and 1 : 2000, respectively). Membranes were then incubated for 1 h at room temperature with a peroxidase-conjugated antirabbit IgG (1 : 5000 for BDNF and 1 : 2000 for the other proteins) and immunocomplexes were visualized by chemiluminescence using the ECL Western Blotting kit (Amersham Life Science, Milan, Italy). Results were standardized using β-actin as the control protein, which was detected by evaluating the band density at 43 kDa after probing the membranes with a polyclonal antibody (1 : 10 000, Sigma) followed by a 1 : 10 000 dilution of peroxidase-conjugated antimouse IgG (Sigma).

Densitometric and Statistical Analyses

Protein levels were calculated by measuring the optical density of the autoradiographic bands using Quantity One software (Bio-Rad). To ensure that autoradiographic bands were in the linear range of intensity, different exposure times were used. β-Actin was employed as an internal standard because its expression is not regulated by the experimental paradigm used.

Data from gene expression and protein analyses were analyzed with two-way analysis of variance (ANOVA), in which drug (VEH *vs* DLX) and treatment (no stress *vs* stress) were considered as independent factors, although the examined molecules as dependent variables. When appropriate, further differences were analyzed by single contrast *post hoc* test (SCPHT). Data are presented as mean ± standard error (SEM), with each individual group comprising at least six independent samples. For graphic clarity, optical densities of experimental groups were expressed and presented as mean percent of the control group, namely the nonstressed VEH-treated rats. Significance for all tests was assumed at least at $p < 0.05$.

RESULTS

Analysis of Stress Responsiveness

In this study, we investigated the influence of chronic DLX treatment on stress-induced modulation of hippocampal BDNF expression, both at a transcriptional and translational level.

To assess the effectiveness of the stress paradigm adopted, we first measured plasma corticosterone levels, which were significantly modulated by the forced swim stress ($F_{1,26} = 48.286$, $p < 0.001$). As shown in Figure 1, corticosterone was increased by stress both in VEH

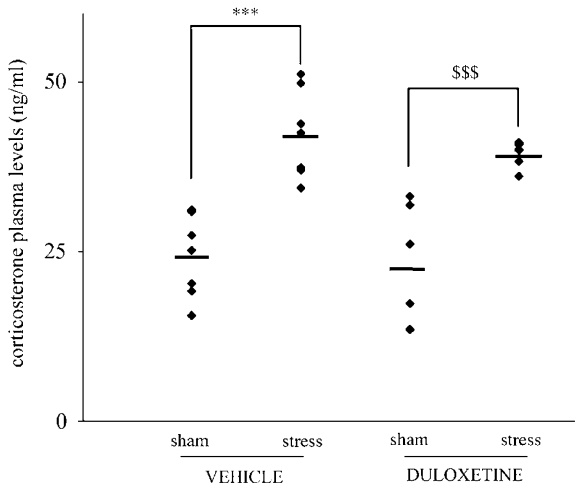

Figure 1 Analysis of corticosterone plasma levels in rats treated for 21 days with vehicle (VEH) or duloxetine (DLX) either in basal condition (sham) or exposed to a forced swim 24 h after the last drug administration (stress) and killed 15 min after the end of the stress session. Values are the mean ± SEM from at least six independent determinations. $^{***}p<0.001$ *vs* VEH- and $^{\$\$\$}p<0.001$ *vs* DLX-treated rats (two-way ANOVA with SCPHT).

($p<0.001$ with SCPHT) and in DLX-treated rats ($p<0.001$ with SCPHT), without any differences between the two experimental groups.

Analysis of BDNF Transcription Following Stress

It is well known that the rat *BDNF* gene has a complex organization consisting of eight 5′ untranslated exons and one protein coding exon (Aid *et al*, 2007). As separate promoters drive the transcription of each exon, the rat *BDNF* gene produces 11 different transcripts consisting of the 5′ exons spliced with the 3′ coding exon (Aid *et al*, 2007). We used real-time RT-PCR to investigate the modulation of total BDNF mRNA (exon IX) as compared to eight major splice variants of the neurotrophin that are expressed in the brain, namely transcript I, IIa, IIb, IIc (as a result of using alternative splice-donor sites within exon II), III, IV, VI, and IXa.

Total (exon IX) BDNF mRNA levels were significantly modulated by DLX ($F_{1,27}=45.408$, $p<0.001$) as well as by stress ($F_{1,27}=4.796$, $p<0.05$), with a significant drug × stress interaction ($F_{3,27}=5.256$, $p<0.05$). In fact, as shown in Figure 2g, DLX treatment by itself upregulated exon IX (+27% *vs* VEH, $p<0.01$ with SCPHT). Exposure to swim stress increased the expression of BDNF coding exon in DLX-treated rats (+29% *vs* DLX, $p<0.01$ with SCPHT), but not in control animals (+2% *vs* VEH, $p>0.05$ with SCPHT).

To establish, which BDNF exon may contribute to the observed changes, we have analyzed eight isoforms that are highly expressed in the brain. The mRNA levels for exons I and II (all splice variants) were not altered by antidepressant treatment or stress (Figure 2a and b). Among the other isoforms, the expression of exons III and IXa (Figure 2c and f) was slightly, but significantly, increased following chronic DLX treatment ($F_{1,27}=15\,543$, $p<0.005$ and $F_{1,27}=9819$, $p<0.01$, respectively), although they were not modulated by the acute stress ($F_{1,27}=4762$, $p>0.05$ and $F_{1,27}=3667$, $p>0.05$, respectively). With regard to BDNF exon IV mRNA levels (Figure 2d), we found a significant effect for DLX ($F_{1,25}=5.159$, $p<0.05$) and stress ($F_{1,25}=25.660$, $p<0.05$) but no drug × stress interaction ($F_{3,25}=0.101$, $p>0.05$). Even if DLX slightly reduced basal BDNF exon levels (−15% *vs* VEH, $p>0.05$ with SCPHT), stress upregulated its mRNA levels in VEH (+27% *vs* VEH, $p<0.01$ with SCPHT) as well as in DLX-treated rats (+64% *vs* DLX, $p<0.001$ with SCPHT). Conversely, chronic DLX treatment was able to influence stress-induced changes of BDNF exon VI (Figure 2e), as indicated by the significant drug × stress interaction ($F_{3,25}=18.333$, $p<0.05$). In fact, the expression of exon VI was significantly increased by swim stress in DLX-treated rats (+42% *vs* DLX, $p<0.001$ with SCPHT), but not in control animals (+2% *vs* VEH, $p>0.05$ with SCPHT).

Analysis of Proteins Involved in BDNF Transcription

To address the potential mechanisms underling transcriptional changes of BDNF, we investigated the expression of specific proteins that are involved in the regulation of BDNF exons IV and VI promoters, which have been studied in detail (Tabuchi *et al*, 2000, 2002; Takeuchi *et al*, 2000, 2002). Figure 3 displays prototypical western blot analyses of these proteins, as measured in nuclear (panel a) and cytosolic (panel b) fractions.

It has been reported that the regulation of BDNF exon IV depends primarily on the activity of Ca^{2+} and CREB (Tabuchi *et al*, 2000, 2002; Tao *et al*, 2002), which stimulate exon IV transcription, as well as on MeCP2 that represses it (Chen *et al*, 2003; Zhou *et al*, 2006). Hence, we investigated their expression (total levels) and activation (phosphorylated levels) in response to DLX treatment as well as to acute stress. As shown in Figure 4a, neither DLX nor stress produced any change in total CREB protein ($F_{1,20}=1.976$, $p>0.05$; $F_{1,20}=0.527$, $p>0.05$, respectively) and its phosphorylated form ($F_{1,18}=0.904$, $p>0.05$; $F_{1,18}=1.878$, $p>0.05$, respectively). Conversely, although total MeCP2 levels were not affected by stress or antidepressant treatment ($F_{1,21}=1.209$, $p>0.05$; $F_{1,21}=0.063$, $p>0.05$, respectively), we found a significant effect of stress ($F_{1,18}=8.185$, $p<0.05$) on P-MeCP2. Indeed the acute swim stress significantly increased P-MeCP2 levels in VEH- (+60% *vs* VEH, $p<0.05$ with SCPHT) and DLX- (+54% *vs* DLX, $p<0.05$ with SCPHT) treated rats. As the phosphorylation of MeCP2 triggers its release from the BDNF promoter IV and allows gene transcription (Zhou *et al*, 2006), the increased P-MeCP2 may contribute to the upregulation of BDNF exon IV in response to stress.

BDNF exon VI promoter contains glucocorticoid-responsive elements and its activity is influenced by corticosterone (Hansson *et al*, 2006; Schaaf *et al*, 2000). We investigated GR levels in the cytosol as well as in the nuclear fraction where they translocate upon activation. As shown in Figure 5a, cytosolic GR levels were significantly increased by chronic DLX treatment ($F_{1,20}=18.268$, $p<0.05$), but they were not modulated by stress ($F_{1,20}=1.138$, $p>0.05$). Conversely we found a significant effect of DLX ($F_{1,20}=7.088$, $p<0.05$) as well as of acute stress ($F_{1,20}=18.275$, $p<0.05$) on nuclear GRs. In fact receptor levels were significantly decreased following chronic DLX

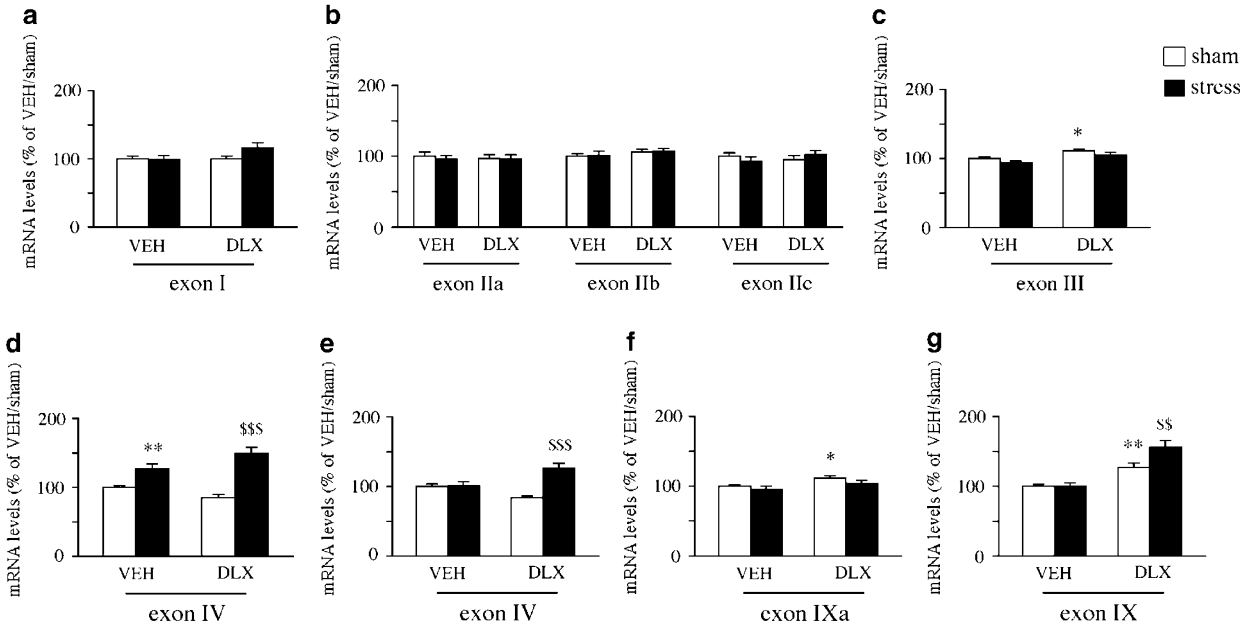

Figure 2 Effect of acute swim stress on *BDNF* gene expression in the hippocampus of rats chronically treated with duloxetine (DLX; 10 mg/kg) or vehicle (VEH) and killed 15 min after the end of the stress session. The data represent the mRNA levels for exon I (a), exon II a/b/c (b), exon III (c), exon IV (d), exon VI (e), exon IX a (f), and coding exon IX (g) expressed as a percentage of control values (VEH/sham: unstressed animals treated with VEH, set at 100%). Bar graphs are the mean ± SEM from at least eight independent determinations. $^{*}p<0.05$, $^{**}p<0.01$ *vs* VEH- and $^{\$\$}p<0.01$, $^{\$\$\$}p<0.001$ *vs* DLX-treated rats (two-way ANOVA with SCPHT).

treatment (−40% *vs* VEH, $p<0.01$ with SCPHT; Figure 5), whereas stress exposure increased nuclear GR levels in VEH- (+41% *vs* VEH, $p>0.05$ with SCPHT) and DLX-treated rats (+59% *vs* DLX, $p<0.05$ with SCPHT), although only in the latter group the effect was statistically significant. When stress-induced changes of GR levels within the two compartments are expressed as percent of sham animals (Figure 5c), a modest but not significant elevation was found in the nucleus of VEH-injected animals (+41%, $p>0.05$), whereas a marked increase of nuclear GR levels was detected in rats chronically treated with DLX (+110%, $p<0.05$), an effect paralleled by a slight reduction of its cytosolic expression (−17%, $p>0.05$).

Mitogen-activated protein kinase (MAPK) and α-CaMKII pathways can also contribute to the regulation of BDNF exon VI promoter (Takeuchi *et al*, 2000, 2002). To assess the function and responsiveness of the MAPK pathway, we measured MEK (total and phosphorylated) in the cytosolic fraction (Figure 6a). Although total protein levels were not affected by chronic DLX ($F_{1,24}=0.426$, $p>0.05$) or stress ($F_{1,24}=0.002$, $p>0.05$), a significant effect of antidepressant treatment was found on P-MEK ($F_{1,21}=5.449$, $p<0.05$). In fact, the levels of P-MEK were increased in DLX-treated rats (+53% *vs* VEH, $p<0.05$ with SCPHT). Stress slightly increased P-MEK in control rats (+33%, *vs* VEH, $p>0.05$ with SCPHT), but did not affect its levels in DLX-treated rats (−11% *vs* DLX, $p>0.05$ with SCPHT).

As shown in Figure 6b, nuclear α-CaMKII levels were not affected by antidepressant treatment ($F_{1,18}=0.841$, $p>0.05$) or stress ($F_{1,18}=0.045$, $p>0.05$), whereas DLX influenced the stress-dependent phosphorylation of this signaling protein, as indicated by the significant drug × stress interaction ($F_{3,18}=5.143$, $p<0.05$). Chronic DLX reduced P-α-CaMKII levels (−41% *vs* VEH, $p<0.05$ with SCPHT) in the nucleus. Upon exposure to stress, a reduction of P-α-CaMKII was found only in VEH-treated animals (−43% *vs* VEH, $p<0.05$ with SCPHT), without any significant change in DLX-pretreated rats (+12% *vs* DLX, $p>0.05$ with SCPHT).

Analysis of BDNF Protein

As we have previously demonstrated that chronic DLX treatment can alter the subcellular localization of BDNF protein in rat frontal cortex (Calabrese *et al*, 2007), we decided to investigate possible changes in the subcellular redistribution of BDNF in response to acute stress. As shown in Figure 7b, the levels of proBDNF and mBDNF in the whole homogenate were not affected either by DLX treatment ($F_{1,24}=0.001$, $p>0.05$; $F_{1,22}=0.044$, $p>0.05$, respectively) or by stress ($F_{1,24}=0.012$, $p>0.05$; $F_{1,24}=0.103$, $p>0.05$, respectively). However at synaptosomal level (Figure 7c), although proBDNF levels were not changed, mBDNF was significantly modulated by DLX ($F_{1,30}=14.822$, $p<0.001$) and stress ($F_{1,30}=9.262$, $p<0.01$). Basal levels of mBDNF were significantly upregulated by DLX treatment in the synaptic compartment (+51% *vs* VEH, $p<0.05$ with SCPHT). Upon exposure to stress a further significant increase of mBDNF levels was found in DLX-treated rats (+42% *vs* DLX, $p<0.05$ with SCPHT), but not in control animals (+30% *vs* VEH, $p>0.05$ with SCPHT).

DISCUSSION

It is widely accepted that antidepressant drugs produce relevant neuroplastic changes, when chronically administered (Berton and Nestler, 2006; Schloss and Henn, 2004).

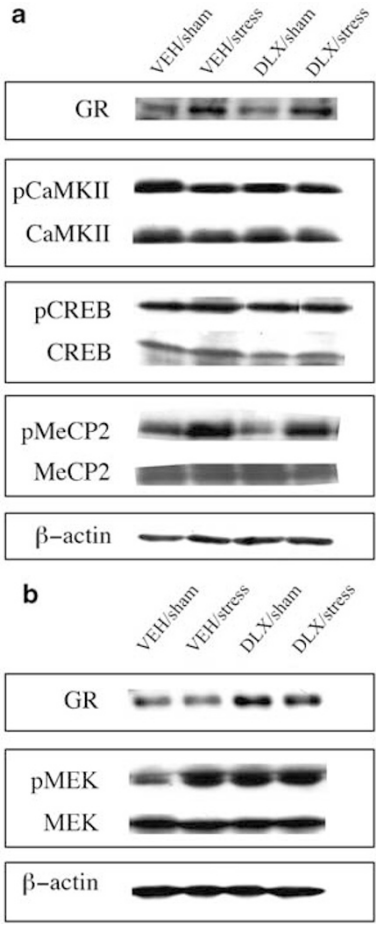

Figure 3 Representative western blot analysis of protein that might contribute to the changes in the levels of BDNF transcripts observed in vehicle (VEH)- or duloxetine (DLX)-treated rats exposed to an acute stress. Proteins were analyzed in the nuclear (panel a: GR, α-CamKII; CREB, MeCP2) or cytosolic fraction (panel b: GR, MEK) in their total or phosphorylated forms. Experimental conditions are described in Materials and methods section.

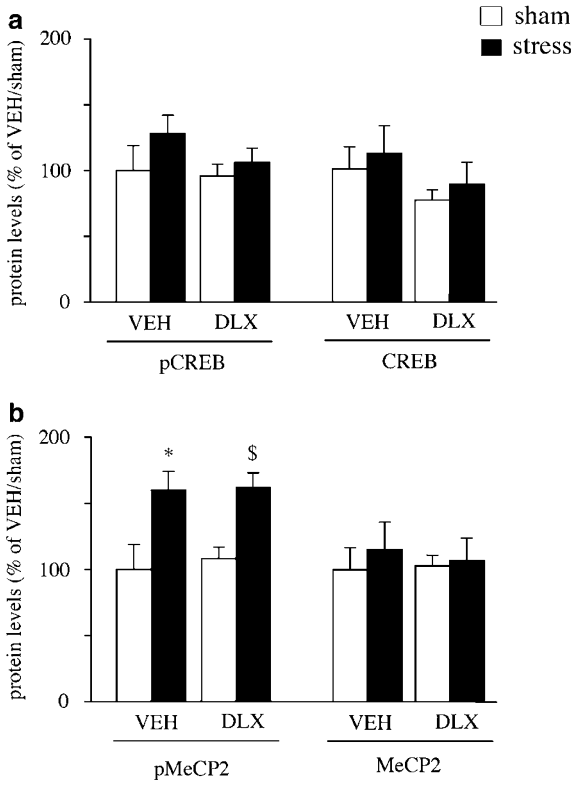

Figure 4 Effect of acute swim stress on CREB (a) and MeCP2 (b) protein levels in the nuclear fraction obtained from the hippocampus of rats chronically treated with duloxetine (DLX; 10 mg/kg) or vehicle (VEH) and killed 15 min after the end of the stress session. β-Actin was used as an internal standard. Quantitative data represent the levels of the phosphorylated (P-CREB and P-MeCP2, respectively) and the native (CREB and MeCP2, respectively) forms expressed as a percentage of control values (VEH/sham: unstressed animals treated with VEH, set at 100%). Bar graphs are the mean ± SEM from at least four independent determinations. $^{*}p < 0.05$ *vs* VEH-treated rats, $^{\$}p < 0.05$ *vs* DLX-treated rats (two-way ANOVA with SCPHT).

Along this line of thinking, it has been demonstrated that different classes of antidepressants upregulate the expression of key neuroplastic molecules, such as the neurotrophin BDNF, in selected brain regions (Duman, 2002; Kozisek *et al*, 2008; Tardito *et al*, 2006). These alterations, which affects systems and pathways defective in mood disorders, are thought to contribute to their therapeutic activity (Duman, 2002; Pittenger and Duman, 2008).

In this paper, we show that the ability of antidepressant drugs to modulate neuronal plasticity is not limited to basal changes in the expression of proteins such as BDNF, but can also lead to altered responsiveness of these proteins under challenging conditions. This suggests that the neuroadaptive changes that take place in response to chronic antidepressant treatment can affect cellular resiliency not only by increasing the expression of 'protective' proteins, but also by altering the coping ability under potential 'threatening' situations.

Using the SNRI DLX, we demonstrate that changes in gene transcription or protein expression/activation may not be evident under 'resting' conditions but become manifest when the system is challenged. Indeed, the transcriptional and translational changes of the neurotrophin BDNF, which occur following an acute swim stress, are significantly affected by prior antidepressant treatment. We found a small but significant increase of the mRNA levels for BDNF coding exon (IX) in the hippocampus 24 h after the last drug injection. Although this effect is in line with published work (Duman and Monteggia, 2006; Dwivedi *et al*, 2006; Kozisek *et al*, 2008), we have previously reported that chronic DLX treatment has a major impact on BDNF mRNA levels in frontal cortex without affecting the neurotrophin transcription in the hippocampus (Calabrese *et al*, 2007), suggesting that even under the same experimental conditions slight differences in these regulatory mechanisms might be observed. The increased levels of total BDNF mRNA is probably because of the slight upregulation of isoforms III and IXa, because no major changes were detected in all the others. Interestingly, the levels of total BDNF mRNA in DLX-treated rats were significantly upregulated by an acute stress episode, which was not effective in VEH-injected animals. This suggests that chronic antidepressant treatment might determine an upregulation of the neurotrophin shortly after the end of a stressful experience. This mechanism represents a rapid response of the cell to a

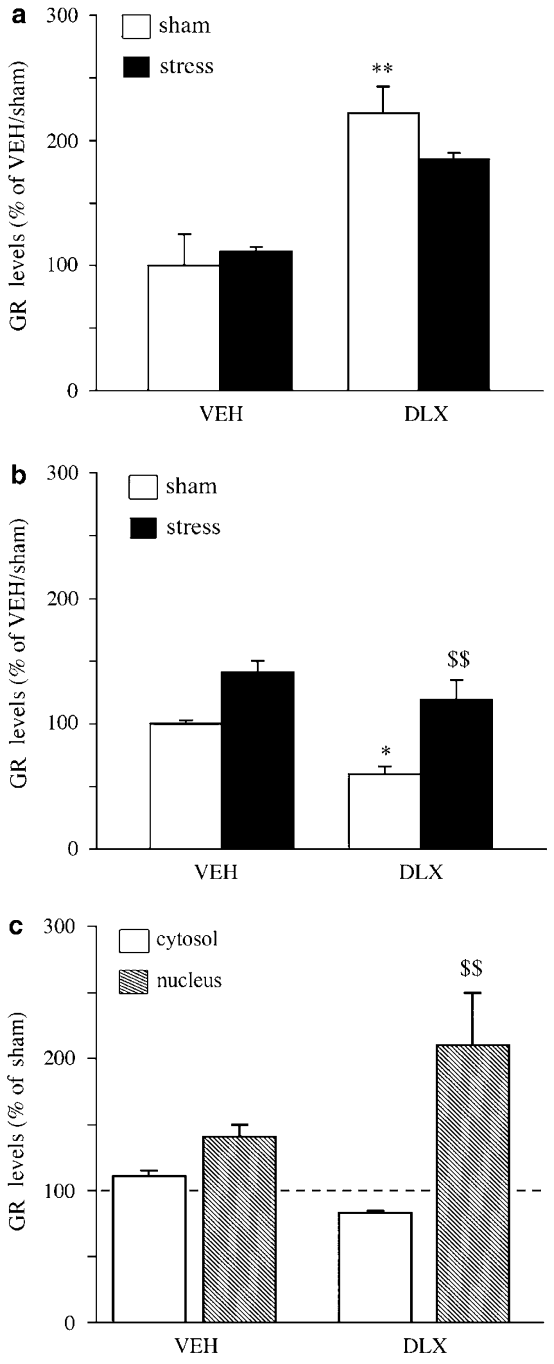

Figure 5 Effect of acute swim stress on glucocorticoid receptor protein levels in the cytosolic (a) and nuclear (b) compartments obtained from the hippocampus of rats chronically treated with duloxetine (DLX; 10 mg/kg) or vehicle (VEH) and killed 15 min after the end of the stress session. β-Actin was used as an internal standard. Data are expressed as a percentage of control values (VEH/sham: unstressed animals treated with VEH, set at 100%). Stress-induced changes in the nuclear or cytosolic compartments, expressed as percent of sham animals, are also presented (panel c). Bar graphs are the mean ± SEM from at least five independent determinations. $^{*}p<0.05$ and $^{**}p<0.01$ *vs* VEH-treated rats and $^{\$\$}p<0.001$ *vs* DLX-treated rats (two-way ANOVA with SCPHT).

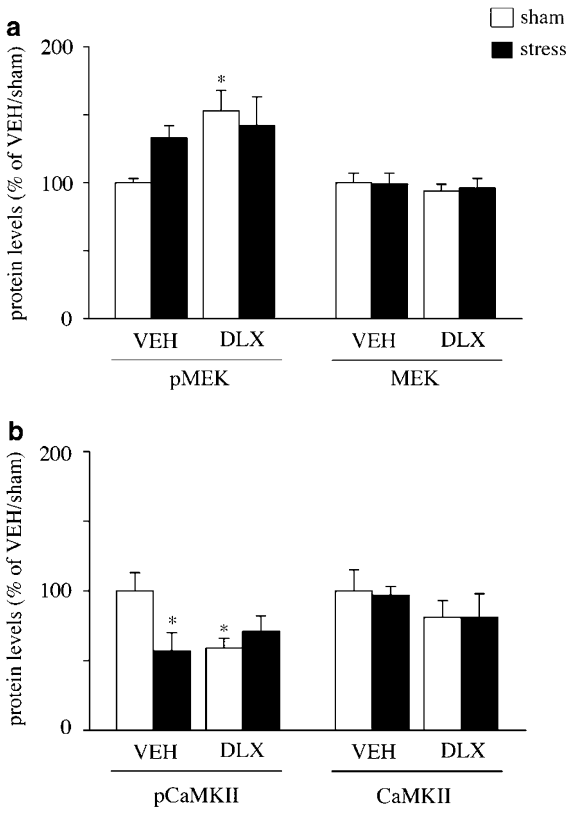

Figure 6 Effect of acute swim stress on cytosolic MEK (a) and nuclear α-CamKII (b) protein levels in their total or phosphorylated forms measured in the hippocampus of rats chronically treated with duloxetine (DLX; 10 mg/kg) or vehicle (VEH) and killed 15 min after the end of the stress session. β-Actin was used as an internal standard. Data are expressed as a percentage of control values (VEH/sham: unstressed animals treated with VEH, set at 100%). Bar graphs are the mean ± SEM from at least four independent determinations. $^{*}p<0.05$ *vs* VEH-treated rats (two-way ANOVA with SCPHT).

short-lasting threatening situation, an effect that might be different if compared to longer stressors that are known to decrease the neurotrophin expression (Roceri *et al*, 2004; Smith *et al*, 1995). We hypothesize that such an effect could result from the activation of different intracellular pathways that regulate *BDNF* gene promoters. Among the major BDNF isoforms expressed in the brain and investigated in this study, we found that exons IV and VI are regulated by acute stress, although their responsiveness is differently affected by chronic antidepressant treatment. The rapid modulation of exons IV and VI is in agreement with the possibility that they might be induced as 'immediate early genes,' without intervening protein synthesis (Lauterborn *et al*, 1996). Levels of exon IV mRNA were significantly upregulated by stress independently from DLX administration, although the magnitude of this effect is larger in antidepressant-treated rats. This effect is in agreement with changes reported following immobilization stress at a similar time point (Marmigere *et al*, 2003), although longer stressors appear to be ineffective or even inhibitory on its transcription (Marmigere *et al*, 2003; Nair *et al*, 2007). BDNF exon IV is highly responsive to neuronal activation and its expression is upregulated under different experimental conditions, through the activation of calcium and cAMP responsive element (Tabuchi *et al*, 2000). CREB does not appear to contribute to this effect although, as our analysis was limited to a single time point, the possibility exits that changes in CREB activation may occur earlier

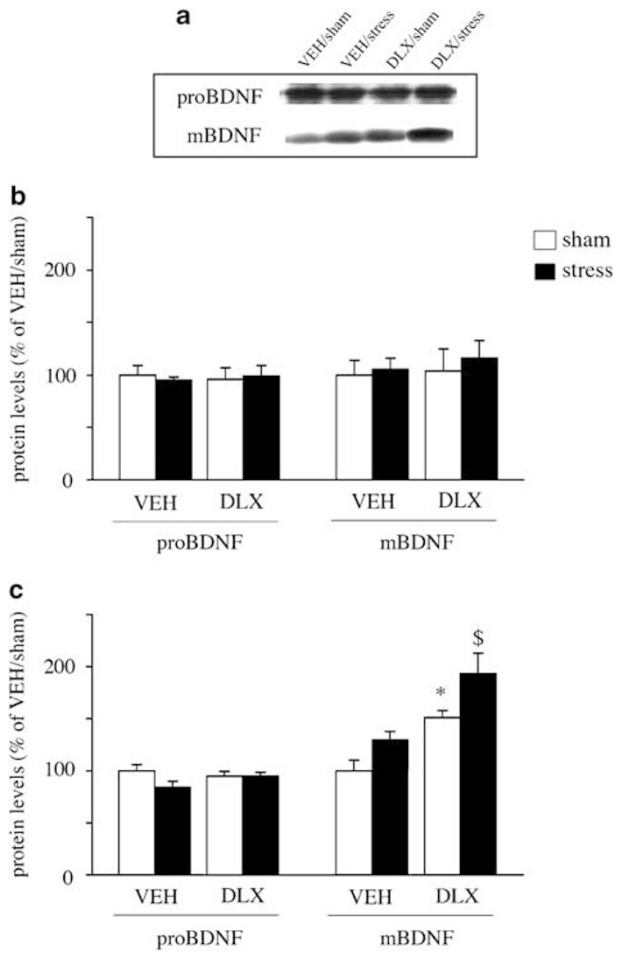

Figure 7 Effect of acute swim stress on BDNF protein levels in the hippocampus of rats chronically treated with duloxetine (DLX; 10 mg/kg) or vehicle (VEH) and killed 15 min after the end of the stress session. (a) Representative immunoreactive bands of the precursor form (proBDNF) and the mature form (mBDNF) of the neurotrophin resulting from western blot analysis performed on the synaptosomal fraction. Quantitative analyses of the observed effects are reported in panel b (the whole homogenate) and in panel c (synaptosomal fraction). The data represent protein levels of proBDNF and mBDNF, which are expressed as percentage of control values (VEH/sham: unstressed animals treated with VEH, set at 100%). Bar graphs are the mean ± SEM from at least five independent determinations. $^{*}p<0.05$ *vs* VEH- and $^{\$}p<0.05$ *vs* DLX-treated rats (two-way ANOVA with SCPHT).

(or later). We believe that stress-induced changes of exon IV are because of the modulation of MeCP2, which acts as a global repressor of transcription. It is known that MeCP2 binds to the methylated CpG island on BDNF exon IV and that, upon phosphorylation, it is released, allowing gene activation (Zhou *et al*, 2006). Our data suggest that this mechanism is rapidly activated in response to a brief stressful episode and might determine a rapid chromatin remodeling that contributes to the fast transcription of this BDNF exon (Chen *et al*, 2003), and possibly of other genes that are regulated in a similar manner. It will be interesting to examine, in future experiments, whether the phosphorylation of MeCP2 can be differentially modulated by a longer stressor, which has a negative impact on BDNF transcription (Roceri *et al*, 2004). The stress-induced increase of P-MeCP2 does not appear to be related to CaMKII, whose activation is actually reduced in response to stress (see below), suggesting that *in vivo* modulation of MeCP2 might be more complex than observed in neuronal cultures (Zhou *et al*, 2006).

Isoform VI mRNA levels were significantly upregulated by stress only in animals previously treated with DLX, suggesting that chronic antidepressant treatment may render the system more responsive under a challenging condition. It has been demonstrated that GRs regulate the transcription of BDNF exon VI (Hansson *et al*, 2006; Schaaf *et al*, 2000). Circulating corticosterone levels were similarly increased after stress in VEH- and DLX-treated rats, suggesting that the HPA axis responsiveness is not affected by chronic antidepressant treatment. Although at clinical level there is consistent evidence that antidepressant treatment can normalize HPA axis alterations, animal studies do not unequivocally demonstrate reduced basal or poststress HPA axis function (Pariante and Miller, 2001). With regard to our results, we must bear in mind that poststress corticosterone levels were only investigated at one time point. Therefore, we cannot rule out the possibility that time-dependent changes of HPA axis responsiveness can be determined by chronic DLX treatment.

On the other hand, hippocampal GR levels were increased in the cytosolic compartment after chronic DLX treatment, in agreement with the results obtained with other antidepressants (Budziszewska, 2002; Pariante and Miller, 2001). The concomitant decrease of GR levels in the nuclear compartment of DLX-treated rats suggests that the receptors may be retained in the cytosol, possibly through the interaction with chaperone proteins, inferring that under basal conditions GR-dependent transcription may be reduced following chronic antidepressant treatment. Conversely, we observed a significant increase of nuclear GR levels in response to stress, which is more pronounced in antidepressant-treated animals, suggesting that their activation can be facilitated under these conditions. This might occur through changes in glucocorticoid accessibility to the brain (Pariante *et al*, 2003), as well as changes in signaling mechanisms that regulate GR function (Rangarajan *et al*, 1992). These data are in line with a previous study showing that antidepressants can increase GR translocation and facilitate dexamethasone effects (Pariante *et al*, 1997), and suggest that the neuronal encoding of stress response could be significantly affected by the pharmacological treatment. The changes in nuclear GR trafficking observed in DLX-treated animals after stress might have an impact on the regulation of several different genes and possibly on the rapid coping responses to a challenging experience.

At first sight it may be difficult to reconcile the increased nuclear translocation of GR with the enhanced transcription of BDNF exon VI in animals treated with DLX and challenged with an acute stress episode, because it is generally believed that glucocorticoids have a direct inhibitory role on this isoform (Hansson *et al*, 2006). However, alternative mechanisms, which, for example, imply an epigenetic control of neurotrophin transcription, could be considered. In fact it has been shown that forced swim stress increases the phosphorylation of histone-3 through a GR-dependent mechanism (Bilang-Bleuel *et al*, 2005) and posttranslational modifications of histones

(phosphorylation and/or acetylation) appear to be relevant for neurotrophin transcription, particularly for exons IV and VI (Tsankova *et al*, 2006).

Although it has been shown that MEK and α-CaMKII influence the transcription of BDNF isoform VI (Takeuchi *et al*, 2002), our data do not provide a clear-cut response on the potential contribution of these signaling pathways to BDNF exon VI modulation. Chronic DLX treatment decreases phospho-α-CaMKII levels whereas increasing MEK activation and these changes are not affected by the acute stress episode. We may therefore speculate that the enhanced levels of BDNF exon VI after stress in DLX-treated rats may involve the contribution and cooperation of multiple pathways that are differentially modulated by antidepressant treatment. The reduction of −α-CaMKII Thr^{286} phosphorylation after chronic treatment with DLX is in line with the effect of other antidepressant, such as fluoxetine (L Musazzi and M Popoli, unpublished observations). Moreover a reduction of α-CaMKII at synaptic level might contribute to the functional changes produced by antidepressant drugs, such as the inhibition of glutamate release (Bonanno *et al*, 2005).

Interestingly, the modulation of exon VI by stress has potential implications for the subcellular distribution of the neurotrophin. Although exons I and IV transcripts display a somatic localization, exon VI (as well as exon II) can also be found in dendrites (Chiaruttini *et al*, 2008; Pattabiraman *et al*, 2005). On the basis of our data, it can be inferred that the somatic BDNF pool can be enhanced by stress despite ongoing antidepressant treatment. Conversely, DLX facilitates activity-dependent transcription of a BDNF transcript that might undergo dendritic targeting, which could lead to an increase in neurotrophin availability at synaptic level, a possibility supported by our analysis of BDNF protein. Indeed, the levels of mBDNF were significantly increased upon stress exposure in the synaptosomal compartment of animals that had been previously treated with DLX, whereas a more limited effect was seen in VEH-treated stressed animals. The increased synaptic levels of mBDNF could be the result of local translation of BDNF transcripts (Tongiorgi *et al*, 1997) or of trafficking of BDNF protein from other compartments. We believe that the synaptic delivery of mBDNF after stress is somewhat related to the activation of GRs, because this mechanism is impaired in animals with a partial deletion of type 2 *GR* gene (Molteni *et al*, 2008), and might contribute to rapid synaptic events taking place at this level in response to stress.

Our results provide novel evidence for a link between BDNF and antidepressant treatment suggesting that, in agreement with recent review/perspective articles (Groves, 2007; Martinowich *et al*, 2007), a better understanding of this neurotrophin's function in depression can be achieved only by a thorough investigation of its components and of how they might be differentially affected under pathologic conditions or pharmacological treatments.

To summarize, our data suggest that antidepressants may have a potential impact on activity-dependent plasticity within regions involved in emotional processing, the integrity of which is compromised in depression (Castren, 2005). This leads to the hypothesis that pharmacological intervention could 'prime' neuroprotective pathways and render them more responsive to preserve cell function and viability.

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DISCLOSURE/CONFLICT OF INTEREST

All authors have no financial interests or potential conflicts of interest to declare. Dr Michele Mancini is an employee of Eli Lilly Italia holding Eli Lilly nonqualified stock option shares.

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