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Stress induces altered CRE/CREB pathway activity and BDNF expression in the hippocampus of glucocorticoid receptor-impaired mice

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ABSTRACT

The gene coding for the neurotrophin Brain-Derived Neurotrophic Factor (BDNF) is a stress-responsive gene. Changes in its expression may underlie some of the pathological effects of stress-related disorders like depression. Data on the stress-induced regulation of the expression of BDNF in pathological conditions are rare because often research is conducted using healthy animals. In our experiments, we used transgenic mice with glucocorticoid receptor impaired (GR-i) expression in the hypothalamus created as a tool to study the neuroendocrine changes occurring in stress-related disorders.

First, under basal condition, GR-i mice displayed lower levels of BDNF exons IX and IV and decreased CRE(BDNF) binding activity with respect to wild-type (WT) mice in the hippocampus. Then, we exposed GR-i and WT mice to an acute restraint stress (ARS) to test the hypothesis that GR-i mice display: 1] different ARS induced expression of BDNF, and 2] altered activation of signaling pathways implicated in regulating BDNF gene expression in the hippocampus with respect to WT mice.

Results indicate that ARS enhanced BDNF mRNA expression mainly in the CA3 hippocampal sub-region of GR-i mice in the presence of enhanced levels of pro-BDNF protein, while no effect was observed in WT mice. Moreover, ARS reduced CREB signaling and binding to the BDNF promoter in GR-i mice but enhanced signaling and binding, possibly through ERK1/2 activation, in WT mice.

Thus, life-long central GR dysfunction resulted in an altered sensitivity at the transcriptional level that may underlie an impaired response to an acute psycho-physical stress.

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1. Introduction

In the brain, particularly in the hippocampus, stress evokes a large array of molecular effects, which produce structural, functional, molecular and behavioural changes (Sapolsky, 2003; McEwen, 2001). It was demonstrated that hippocampal Brain Derived Neurotrophic Factor (BDNF) mRNA was reduced by different types of stress, supporting the hypothesis that low BDNF levels may contribute to atrophy and neuronal cell loss observed in subjects suffering from stress-related disorders (Duman et al., 1997; Duman and Monteggia, 2006; Marmigere et al., 2003; Smith et al., 1995; Ueyama et al., 1997). On the other hand, BDNF gene transcriptional activity has a direct impact on neuronal growth and plasticity, affects neurogenesis and contributes to the therapeutic effects of antidepressants (Duman and Monteggia, 2006).

In humans and rodents, the BDNF gene presents a coding region that is under transcriptional control of at least seven non coding exons (Aid et al., 2007; Liu et al., 2006). The use of distinct promoters provides a diverse alternative regulatory mechanism that permits to modulate BDNF expression, localization and synaptic delivery (Greenberg et al., 2009). Importantly, activity-dependent transcription of BDNF in hippocampal neurons, starts from exon IV (Tao et al., 1998). The genomic analyses of the

Abbreviations: ARS, Acute Restraint Stress; BDNF, Brain Derived Neurotrophic Factor; CA3, Ammon's Horn 3; CRE, cAMP Response Element; CREB, cAMP Response Element Binding Protein; DG, Dentate Gyrus; ERK, Extracellular Signal-Regulated Kinase; GR-i, Glucocorticoid Receptor- impaired; HPA, Hypothalamic-Pituitary-Adrenal; IEG, Immediate Early Gene; MAPK, Mitogen-Activated Protein Kinase; WT, Wild-Type.

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structure of this promoter indicates the presence of several cAMP responsive element (CRE) sites that binds the transcription factor cAMP Responsive Element-Binding protein (CREB) when phosphorylated at Ser 133 (Blandy, 2006; Tardito et al., 2006). CREB is involved both in the adaptative response to stress, as well as, in the physiological and pharmacological regulation of the expression of BDNF (Alfonso et al., 2006; Bilang-Bleuel et al., 2002; Nair et al., 2007; Nibuya et al., 1996; Tao et al., 1998).

Even though the vast majority of studies use healthy animals to investigate the consequences of stress on the expression of BDNF and the intracellular regulation of the CRE/CREB pathway, it is very likely that stress induces different molecular mechanisms in animal models of those pathologies characterized by a different vulnerability to stressful events, like depression (Molteni et al., 2009; Ridder at al., 2005).

Given this, the general aim of our study was to analyze the molecular effects of acute psycho-physiological stress on the expression of BDNF and on the CREB/CRE pathway in the presence of a genetic-induced neuroendocrine state similar to those observed in major depression. Depressed patients exhibit disturbance in the hypothalamic-pituitary-adrenal (HPA) axis functionality, a possible marker of decreased GR functionality that has been described both *in vivo* and *in vitro* (Holsboer, 2001; Holsboer and Barden, 1996; Pariante, 2003, 2004, 2006). In the present study we employed a validated model of stress-related pathologies characterized by impaired glucocorticoid receptor (GR-i) expression (Barden et al., 1997; Froger et al., 2004; Montkowski et al., 1995; Pepin et al., 1992). In these GR-i mice, reduced GR mRNA expression and binding, specifically in the hypothalamus, ultimately lead to impaired HPA axis feedback control with a low sensitivity to dexametasone and HPA axis hyperactivity (Barden, 2004). Several of the HPA dysfunctions and cognitive impairments described in these GR-i mice are normalized specifically by antidepressant treatments (Barden, 2004). Moreover, previous studies demonstrated that GR-i mice have abnormal responses both to environmental stimuli and pharmacological treatments (Blom et al., 2002; Froger et al., 2004; Linthorst et al., 2000; Païzanis et al., 2010; Vinet et al., 2004).

Therefore, we used an acute restraint stress (ARS) of 30 min period to test the first wave of stress-induced effects on the expression of BDNF and the activation of the CRE/CREB pathway. We hypothesized that these early effects initiate a cascade that, through modifications in synaptic plasticity shapes the behavioural outcome of mice when confronted with a specific environmental stimuli.

2. Materials and methods

2.1. Animals

Adult male transgenic (line 5.4) mice, with partial knockout of the glucocorticoid receptor (GR-i), and wild-type (WT) mice (B6C3F1) (Charles River Laboratories, Lecco, Italy) were used in this study ($n = 8–10$), as previously reported (Blom et al., 2002; Barden, 2004; Vinet et al., 2004). GR-i mice were generated using a B6C3F1 background by insertion into the genome of a transgene expressing antisense RNA complementary to a fragment of the glucocorticoid receptor (GR) cDNA (Pepin et al., 1992). GR-i and WT animals were housed in polycarbonate cages (28 × 17 × 12 cm) with ad *libitum* access to food and tap water throughout the study, and maintained under a 12:12 light–dark cycle in an ambient temperature of 21 ± 3 °C with relative humidity controlled.

Mice were restrained individually for 30 min (ARS) in Plexiglas cages (5 cm length, 2.5 cm width, 3.5 cm height) between 9:00 am to 11:00 pm. We chose restraint as stress procedure because it is a physical stress with an important emotional component but without pain. Animals were checked for signs of discomfort as indicated by animal care and use guidelines (National Academy of Sciences. Guide for the care and use of laboratory animals, 1998, "Guidelines for the Care and Use of Mammals in Neuroscience and Behavioural Research" (National Research Council, 2003)). EC guidelines (EEC Council Directive 86/609 1987), and the Italian legislation on animal experimentation (Decreto Legislativo 116/92) were followed throughout the whole experiment. All efforts were made to minimise animal suffering and to reduce the number of animals used in this study.

Mice were sacrificed, immediately (t_0) or 140 min (t_{140}) after the ARS ended. Control, unstressed animals were similarly handled and were killed at the same time intervals as the stressed mice. Hippocampi were rapidly removed and stored at −80 °C until RNA and protein extraction.

2.2. Protein extraction

For protein extraction, hippocampi were homogenized by potter (12 strokes at 600 rpm) in lysis buffer containing Hepes 10 mM, EGTA 0.1 mM, Sucrose 0.28 M, $Na_4P_2O_7$ 5 mM, in the presence of a protease inhibitor (Phenylmethanesulfonyl Fluoride 0.1 mg/mL) and two phosphatase inhibitors (NaF 20 mM, Na_3VO_4 1 mM), (buffer A). A fraction of the lysate was collected (total extract) and the remaining fraction was centrifuged at 1000 × *g* for 5 min at 4 °C. The pellets were resuspended in a buffer containing NaCl 120 mM, Hepes 20 mM, EGTA 0.1 mM, Dithiothreitol 0.1 mM, Sodium Pyrophosphate 5 mM, NaF 20 mM, Na_3VO_4 1 mM, Phenylmethanesulfonyl Fluoride 0.1 mg/mL (nuclear enriched extract). Protein concentration was determined with a standard protocol using Coomassie® reagent (Pierce).

2.3. Western blot

Western blots were carried out on 6 μL of total extract, for the detection of BDNF, ERK1/2 and p-ERK1/2, or nuclear enriched extracts for p-CREB and CREB, and electrophoretically separated on a 14% polyacrylamide gel. Proteins were transferred to PVDF membranes (Amersham®). The membranes were blocked for 1 h with 5% non-fat dry milk in TBS-Tween 20 followed by overnight incubation at 4 °C with primary antibodies: anti-BDNF dil 1:1000 (Santa Cruz N-20, sc-545), anti-phospho-ERK1/2 dil 1:1000 (Cell Signaling, #9106), anti-ERK1/2 dil 1:1000 (Cell Signaling, #9102), anti-phospho-CREB dil 1:1000 (Cell Signaling, #9191), anti-CREB dil 1:1000 (Cell Signaling #9192) and anti-β–tubulin dil 1:1000 Santa Cruz D-10, sc-5274). Membranes were then washed three times and incubated with the respective appropriate secondary antibodies (Anti-rabbit IgG-HRP-linked Cell Signaling, #7071 or goat anti-mouse IgG-HRP-linked sc-2005) and then washed again three times. Bands were detected using ECL + Plus chemiluminescence reagent (Amersham®). Proteins levels were calculated by measuring the peak densitometric area of the autoradiography analyzed with an image analyzer (GS-690 BIORAD). The signals were normalized according to the optical density of β-tubulin, or to the relative unphosphorylated protein. To ensure that the autoradiographic bands were in the linear range of intensity, different exposure times were used. Ratios were expressed as percentage of control ±SEM.

2.4. Preparation of nuclear extracts

Nuclear extracts were prepared from hippocampi by homogenization in buffer A using a Teflon-glass homogenizer and then centrifuged at 1500 × *g* for 5 min at 4 °C. The nuclear pellet obtained was resuspended in a low salt solution (Hepes 20 mM, glycerol 25%, $MgCl_2$ 2 mM, KCl 0.1 M, EDTA 1 mM, DTT 5 mM), with the addition of protease and phosphatase inhibitors and then centrifuged at 1500 × *g* for 5 min at 4 °C after which the pellet was resuspended in a high salt solution (Hepes 20 mM, glycerol 25%, $MgCl_2$ 2 mM, KCl 1 M, EDTA 1 mM, DTT 5 mM), with protease and phosphatase inhibitors added. The suspension was incubated on ice for 40 min, and then centrifuged at 16,000 × *g* for 30 min at 4 °C. Supernatant protein concentration was determined as previously described.

2.5. Electrophoretic mobility shift assay (EMSA)

For EMSA assay 1 μg of nuclear extract protein was incubated in a buffer containing 20 mM Tris–HCl (pH 7.5), 5 mM $MgCl_2$, 40 mM KCl, 5 mM DTT, 10% glycerol, 1.5 μg poly dI:dC, 3 μg Bovine Serum Albumine (BSA), for 20 min at room temperature. For the binding of CREB to the labeled *BDNF* CRE probe, 75 fmol [^{32}P]-labeled probe was added and incubated at room temperature for an additional 15 min. The DNA-protein complexes were electrophoretically separated on a 6% polyacrylamide nondenaturing gel. After electrophoresis, gels were dried and the complexes visualized by autoradiography. The following oligonucleotide sequences were used for EMSA: CRE(BDNF) sense; 5′-TGACAGCTCACGTCAAGGCAGC-3′; CRE(BDNF) antisense; 5′-GCTGCCTTGACGTG AGCTGTCA-3′. The probes were labeled with [^{32}P]-ATP using T4 polynucleotide kinase (Roche). For the competition assay of CRE binding, a 100 times excess unlabeled probe or CREm probe, was incubated with 1 μg of nuclear extract 15 min prior to the addition of a fixed amount of [^{32}P]-labeled CRE probe. CREm probe was synthesized as complementary oligodeoxyribonucleotide strands: 5′-GACAGCCAGCTGCAAGGCAGC-3′, 5′-GCTGCCTTGCAGCTGGCTGTC-3′. For the supershift assay, 1 μg of nuclear extract was preincubated with 3 μg of CREB-1 mouse monoclonal antibody (Santa Cruz 24H4B, sc-271×) at room temperature for 30 min and then the gelshift buffer and the labeled probe were added. Optical densities of gelshift bands were quantified using an image analyzer (GS-690 BIO-RAD). Values were expressed as percentage of control ±SEM.

2.6. Real time RT-PCR

Total RNA, extracted using TRIzol® (Sigma Aldrich) was treated with a TURBO DNA-free™ kit (Ambion®) to remove genomic DNA contamination.