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BRIEF REPORT

THEMATIC SECTION
New Aspects in the Treatment of Affective Disorders

CINP

Reduced peripheral brain-derived neurotrophic factor mRNA levels are normalized by antidepressant treatment

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Abstract

Consistent data coming from biochemical studies have evidenced a brain-derived neurotrophic factor (BDNF) serum reduction in depressed patients compared to controls and a restoration following antidepressant treatment. However, to date, no study has evaluated whether BDNF synthesis in leukocytes could contribute to such modulation. Therefore, in this study, we analysed BDNF mRNA levels in leukocytes from 21 depressed patients prior to and during escitalopram treatment and from 23 control subjects showing that BDNF mRNA levels were decreased in drug-free depressed patients and that 12 wk escitalopram treatment was able to reverse this deficit. Interestingly, changes in BDNF mRNA levels paralleled BDNF serum increase during antidepressant treatment, and were correlated with symptoms improvement. Our results indicate that BDNF serum modulation observed in depressed patients is associated with BDNF synthesis alteration in leukocytes and suggest that these peripheral cells might play an active role in the mechanisms of action of antidepressants.

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Introduction

Major depression (MD) is a severe mental disease characterized by a reduced ability of the brain to respond to external stimuli. In particular, compelling evidence has demonstrated that stress-adverse lifetime events represent a challenge for individuals who, depending on the genetic background susceptibility, may be vulnerable to the development of a depressive symptomatology (Charney & Manji, 2004). Molecular correlations underlying the mechanisms of the stress response involve the modulation of various neurotrophic factors, including brain-derived neurotrophic factor (BDNF), which is known to play a pivotal role in the neurodevelopment and the maintenance of adult brain homeostasis through regulation of neurogenesis and neuronal plasticity (Duman & Monteggia, 2006).

Several preclinical studies have demonstrated that BDNF expression levels are significantly lower in the brains of rats exposed to different stress paradigms (Roceri *et al*. 2002), whereas chronic antidepressant treatments increase BDNF brain levels and enhance neurogenesis (Kozisek *et al*. 2008). The hypothesis suggesting an involvement of BDNF in the pathogenesis of MD has also been corroborated by meta-analyses demonstrating that BDNF serum, which is probably reflective of BDNF levels in the brain (Karege *et al*. 2002*a*), was significantly decreased in drug-free depressed patients (Sen *et al*. 2008), whereas pharmacological and non-pharmacological antidepressant treatments (Sen *et al*. 2008; Brunoni *et al*. 2008) induce a progressive normalization of BDNF blood deficit. However, to date, very little is known about the origin of BDNF blood fluctuations observed in patients with MD.

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Interestingly, blood BDNF may derive in part from brain production and subsequent egress from the blood–brain barrier (BBB) (Pan *et al*. 1998), and also from synthesis in different populations of peripheral cells such as vascular endothelial cells, smooth muscle cells, in addition to leukocytes (Gielen *et al*. 2003; Nakahashi *et al*. 2000). In this regard, leukocytes have been proposed as a useful peripheral model to study mental pathologies (Gladkevich *et al*. 2004; Iga *et al*. 2008; Rokutan *et al*. 2005), since their expression profiles have shown similarities to those observed for brain cells, especially for genes encoding neurotransmitter receptors and transporters, stress mediators, cytokines, hormones, and growth factors (Glatt *et al*. 2005; Sullivan *et al*. 2006). Interestingly, altered mRNA levels of genes encoding dopamine and glucocorticoid receptors, the serotonin transporter, the transcription factor cAMP response element-binding protein (CREB), and other genes involved in calcium signalling have been found in the peripheral leukocytes of MD patients (Iga *et al*. 2008). Furthermore, in a recent study, Pandey and colleagues observed a reduction of BDNF mRNA levels in leukocytes of bipolar paediatric patients that was able to be normalized by administration of mood-stabilizing drug therapies (Pandey *et al*. 2008). Finally, no difference in leukocyte mRNA levels was observed between controls and drug-treated MD patients (Otsuki *et al*. 2008).

Therefore, the aims of this study were: (1) to evaluate whether leukocyte BDNF gene expression was altered in drug-free MD patients compared to control subjects; (2) to observe putative longitudinal changes in BDNF gene expression during 12 wk treatment with a selective serotonin reuptake inhibitor (SSRI) escitalopram, and (3) to assess whether putative changes in BDNF mRNA levels could correlate with BDNF protein serum content.

Method

Subjects

Twenty-one outpatients [17 females, 4 males; mean ± standard deviation (S.D.) age 43.57 ± 9.19 yr; body mass index (BMI) 23.55 ± 3.14] subsequently admitted to the Psychiatry Rehabilitation Unit of the IRCCS San Giovanni di Dio FBF, Brescia, Italy were enrolled in the study. The inclusion criteria were: diagnosis of MD according to either ICD-10 and DSM- IV criteria, age 18–65 yr, parents of caucasoid European ethnicity. Exclusion criteria were: a personal history of bipolar affective disorder, schizophrenia, mood-incongruent psychotic symptoms, primary substance abuse or primary organic disease, current or actively seeking pregnancy, current treatment with antidepressants, antipsychotics or a mood stabilizers or any regular treatment for a medical condition. Moreover, no patient was on the oral contraceptive pill. Upon entering the study, the wash-out period (only low doses of benzodiazepines were allowed: diazepam equivalent <15 mg) was at least of 2 wk and subsequently patients were treated with escitalopram over a period of 3 months. The Montgomery–Asberg Depression Rating Scale (MADRS) was administered at baseline (T0) to assess the illness severity, and during escitalopram treatment, at T8 (after 8 wk treatment) and T12 (after 12 wk treatment). Blood sample collection was performed at the same time as clinical evaluation.

A control group (16 females, 7 males; mean ± S.D. age 45.68 ± 10.21 yr; BMI 24.64 ± 2.44), consisting of 23 subjects with a negative anamnesis for any Axis I disorder (confirmed by the MINI interview), a negative family history for psychoses and mood disorders and without any regular treatment for a medical condition was enrolled for the study. Moreover, no control participant was on the oral contraceptive pill.

The study was approved by the local ethics committee and all subjects enrolled gave their informed consent to participation. Venous blood samples for both patients and controls were collected in the morning after an overnight fast in anticoagulant-free tubes for serum sample preparation and in PaxGene tubes for RNA isolation.

BDNF serum levels determination

Both patient and control blood samples were taken in the morning after an overnight fast (between 08:00 and 09:00 hours) in anticoagulant-free tubes, they were kept at room temperature for 2 h followed by 1 h at 4 °C before the serum was separated by centrifugation (1268 *g* for 15 min). The serum samples were then stored at −80 °C until the time of assay. BDNF levels were diluted 1:100 and measured in duplicate using the ELISA method with the human BDNF Quantikine kit (cat. DBD00, R&D Systems, datasheet available at: www.rndsystems.com) according to the manufacturer's instructions. The ELISA plate templates were planned so that patient samples at baseline, T8 and T12 and the corresponding control samples were run in the same assay. BDNF content is expressed as the equivalent of the human recombinant protein. The detection limit of the assay was 20 pg/ml and the data are expressed as ng protein/ml of blood serum.

Blood sample preparation, RNA isolation, and cDNA synthesis

After blood samples were withdrawn, PAXgene tubes were stored at −80 °C until they were processed. RNA isolation was performed using the PAXgene blood RNA kit (Qiagen, USA) according to the manufacturer's protocols. RNA samples were treated with DNase solution in order to eliminate any possible DNA contamination. RNA quantity and quality were assessed by evaluation of the $A_{260/280}$ and $A_{260/230}$ ratios using a Nanodrop spectrophotometer (NanoDrop Technologies, USA) and an Agilent Bioanalyzer (Agilent Technologies, USA). Two micrograms of total RNA were used for cDNA synthesis using random hexamer primers (Invitrogen, USA) and the Superscript II Reverse Transcriptase (Invitrogen) according to the manufacturer's protocols.

Analyses of BDNF mRNA levels in peripheral leukocytes

The expression levels of the total BDNF mRNA and of the housekeeping genes *GAPDH*, *β-actin*, *β_2 microglobulin* (*B2M*), *cytochrome c-1* (*Cyc1*) and *ATP synthase*, *H^+ transporting mitochondrial F1 complex*, *β subunit* (*Atp5b*) have been analysed by real-time PCR using a StepOne Real-Time System (Applied Biosystems, USA). PCR reactions were carried out with TaqMan Gene Expression Master Mix (Applera Corp., USA) and TaqMan Gene Expression assays. After an initial incubation step, 40 cycles (95 °C for 15 s and 1 min at 60 °C) of PCR were performed. Each sample was assayed in duplicate using two independent retrotranscription products. The real-time experimental templates were planned so that patient samples at baseline, T8 and T12 and the corresponding control samples were run in the same assay. Data analyses have been performed according to the comparative C_t method by the use of Applied Biosystems Real Time software, that automatically determines, using the auto C_t determination feature, the optimal baseline and threshold settings.

Statistical analysis

Demographic and clinical characteristics in the experimental samples are described either in terms of their mean ± S.D. or in terms of proportions. Qualitative variables were tested by means of χ^2 and Fisher's tests. After checking for normality, Student's *t* tests were used when appropriate to evaluate the differences in quantitative variables. Pearson's coefficient was used to evaluate bivariate correlations.

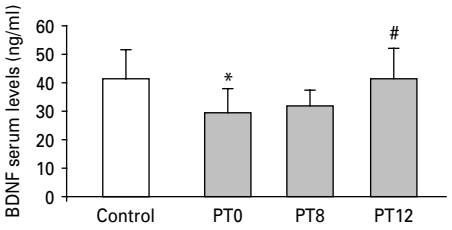

Fig. 1. Brain-derived neurotrophic factor (BDNF) serum levels (ng protein/ml blood serum) in controls and in depressed patients (P) at the three different sampling times including T0 (baseline, PT0), T8 (after 8 wk treatment, PT8), and T12 (after 12 wk treatment, PT12). Values, expressed as mean ± standard deviation for control, PT0, PT8, and PT12 samples are 40.92 ± 10.05, 30.58 ± 9.13, 31.92 ± 8.58, and 41.38 ± 10.49, respectively (* $p < 0.05$ in PT0 *vs*. control group; # $p < 0.05$ in PT12 *vs*. PT0).

Univariate analysis of variance was used for comparing the mean values of the serum and mRNA BDNF levels in drug-free patients *vs*. controls. Clinical and biological changes that occur during drug treatment were analysed by means of the General Linear Model (GLM) according to a repeated-measures design with time (T0, T8, T12) as a within-subjects factor. The Greenhouse–Geisser (GG) correction was applied to degrees of freedom when the sphericity assumption was violated. All of the statistical evaluations were performed using the SPSS version 13.0 software package (website: http://www.spss.com).

Results

Drug treatment improved the depressive symptomatology as measured by MADRS scores with T0, T8 and T12 values of 21.42 ± 3.17, 11.23 ± 7.07 and 7.23 ± 5.15, respectively (mean ± S.D.). The GLM indicated a significant decrease in MADRS scores during escitalopram treatment (GG correction $p < 0.001$) with a percentage improvement at T12 of 67.71% (% MADRS score reduction). In particular, the planned 'repeated' contrasts indicated a significant decrease in MADRS scores between T8 and T0 ($p < 0.001$) and between T12 and T0 ($p < 0.001$).

BDNF serum measurement

Univariate analyses of variance for BDNF serum levels indicated a significant decrease in the drug-free MD patients (PT0) compared to controls (values for controls = 40.92 ± 10.05 ng/ml; PT0 = 30.58 ± 9.13 ng/ml; $p = 0.001$, Fig. 1). No influence of gender, BMI or age was evidenced on BDNF serum content ($p = 0.560$, $p = 0.272$, $p = 0.414$, respectively). In the patient group,

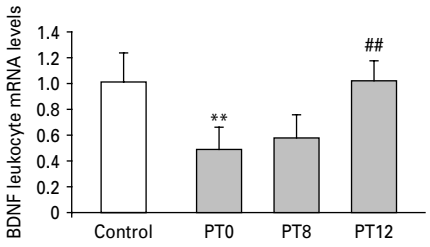

Fig. 2. Brain-derived neurotrophic factor (BDNF) mRNA levels in leukocytes of controls and in depressed patients (P) at the three different sampling times including T0 (baseline, PT0), T8 (after 8 wk treatment, PT8), and T12 (after 12 wk treatment, PT12). Values, expressed as mean ± standard deviations for control, PT0, PT8, and PT12 samples are 1.01 ± 0.22, 0.48 ± 0.18, 0.57 ± 0.25 and 1.02 ± 0.15, respectively (** $p < 0.001$ in PT0 *vs*. control group; ## $p < 0.001$ in PT12 *vs*. PT0).

no correlation was observed between the baseline BDNF serum levels and severity of illness measured as MADRS score at T0 ($p = 0.579$). Moreover, no correlation between platelet count and BDNF serum levels at baseline was observed ($p = 0.634$).

In the GLM a significant increase in BDNF serum levels was found during escitalopram treatment (GG correction $p = 0.001$). In particular, while changes at T8 did not reach statistical significance (T8 = 31.92 ± 8.58 ng/ml, $p = 0.350$), the BDNF serum levels at T12 increased (T12 = 41.38 ± 10.49 ng/ml, $p = 0.002$) to values similar to those observed in the controls (Fig. 1). No correlation was observed between the increase in BDNF serum levels and the reduction in the MADRS score ($p = 0.777$).

Leukocyte BDNF gene expression

BDNF mRNA levels have been normalized on five housekeeping genes (*B2M*, *GAPDH*, *β-actin*, *Cyc1*, *Atp5b*). The univariate analysis of variance on BDNF mRNA levels indicated a significant decrease in mRNA levels in drug-free MD patients compared to controls (PT0 = 0.48 ± 0.18, controls = 1.01 ± 0.22, $p < 0.001$, Fig. 2) and, no effect of gender, BMI or age on BDNF mRNA levels was found ($p = 0.525$, $p = 0.188$, $p = 0.468$, respectively).

The GLM analysis showed a significant increase in BDNF mRNA levels during escitalopram treatment (GG correction $p < 0.0001$). As for the serum values, the T8 BDNF mRNA levels were also not significantly different from the T0 values (PT8 = 0.57 ± 0.25, $p = 0.098$), while the T12 mRNA levels increased significantly (PT12 = 1.02 ± 0.15, $p < 0.001$ *vs*. PT0; Fig. 2).

No relationship was observed between the T0 BDNF mRNA levels and the baseline severity of illness ($p = 0.325$). In addition, at the baseline, BDNF mRNA levels correlated with BDNF serum levels ($r = 0.409$, $p = 0.006$) and changes in BDNF mRNA levels (ΔBDNF mRNA levels) during escitalopram treatment correlated with changes in BDNF serum levels (ΔBDNF serum levels; $r = 0.474$, $p = 0.030$) as well as with symptoms improvement, measured as reduction in the MADRS score (ΔMADRS) ($r = 0.452$, $p = 0.040$).

Discussion

There is overwhelming evidence demonstrating that BDNF serum levels are reduced in depressed patients and that these levels normalize following antidepressant treatment (Sen *et al*. 2008), suggesting that BDNF serum fluctuations may reflect neurotrophic disturbances in limbic regions and restoration processes induced by antidepressant treatment. This hypothesis has been upheld by data showing a positive correlation between BDNF serum and cortical levels in rats during neurodevelopment (Karege *et al*. 2002*b*) and by a positive association between BDNF serum content and cortical levels of *N*-acetyl aspartate (NAA), a neuronal integrity marker (Lang *et al*. 2007). To date, however, little is known about the central or peripheral origin of blood neurotrophin and data regarding a possible bi-directional crossing of BDNF through the BBB are controversial. In fact, Pan *et al*. (1998) demonstrated that blood BDNF crosses the BBB through an active transport system, whereas other studies suggested that the BBB crossover is only possible after conjugation with specific vectors (Wu & Pardridge, 1999). In support of the first hypothesis, it has been recently demonstrated that other peripheral growth factors such as the insulin-like growth factor 1 (IGF-1) which is primarily synthesized by the liver, is able to reach the brain and to regulate different neuronal functions and behaviour (Duman *et al*. 2009).

In order to clarify the origin of BDNF serum alterations in MD patients, we have examined both BDNF serum and leukocyte mRNA levels. The results obtained for serum were in line with the current literature: BDNF serum levels were significantly decreased in MD patients at baseline but not correlated with illness severity, while BDNF deficit was gradually normalized by escitalopram treatment confirming the meta-regression data reported by Brunoni *et al*. (2008).

The new finding is that BDNF mRNA levels in leukocytes were also significantly reduced in drug-free MD patients and were progressively induced to levels similar to those observed in controls during

escitalopram treatment. These data are in line with recent evidence by Otsuki and colleagues (2008) showing no alteration in leukocyte BDNF mRNA levels in MD patients treated with antidepressant drugs. We also found that the increase in BDNF mRNA levels during treatment was associated with clinical improvement, supporting the notion that amelioration of symptoms is associated with a peripheral BDNF increase. Numerous evidences have shown that in leukocytes antidepressants modulate the expression of several genes involved in brain functionality, in synaptic plasticity and in drug efficacy (Iga *et al*. 2008). Moreover, in leukocytes, it has been demonstrated that antidepressant treatments are also able to up-regulate the intracellular cAMP concentration and pCREB protein levels, well known mediators of BDNF induction (Koch *et al*. 2009).

Furthermore, a correlation was found between BDNF mRNA levels in leukocyte and BDNF serum levels both at baseline and during antidepressant treatment, suggesting that alterations in BDNF protein levels observed in MD patients may be, at least partially, related to the synthesis and secretion of BDNF from white blood cells rather than from platelet release (Karege *et al*. 2005; Lee & Kim, 2009). In fact, BDNF in the blood is mainly stored at high levels in platelets, that play a central role in the controlling of BDNF blood since they bind, store and release the neurotrophin constitutively (in plasma), upon activation after a traumatic injury and *in vitro* (in serum) during clotting processes (Fujimura *et al*. 2002). However, these cells are not able to synthesize the neurotrophin but acquire it from other compartments and a reduced capacity of platelets to release BDNF in plasma and serum has been reported in MD patients (Karege *et al*. 2005; Lee & Kim, 2009).

However, BDNF blood alterations are not specific for MD, since they are also found in several other psychiatric disorders, e.g. bipolar disorder, schizophrenia or eating disorders, indicating that peripheral BDNF can not be considered a marker of illness. More likely, the reduction in peripheral BDNF may underlie a common pathophysiological mechanism, linked to the deregulation of synaptic plasticity, shared by several mental disease processes. Moreover, it may also provide some insight into the high rates of comorbidity that exist between many of the disorders (Littrell, 2008; McEwen, 2008).

However, some limitations of this study should be mentioned. First, all the patients were treated with benzodiazepines during the wash-out period, therefore it is not possible exclude an effect of these drugs on BDNF, since recent findings reported a modulation operated by these drugs on neuroplasticity during antidepressant treatment (Wu & Castrén, 2009). Another limit is that we cannot differentiate whether the BDNF increase observed during escitalopram treatment is an effect due to the medication or might derive from an improvement of depressive symptoms, since our patient sample was mainly composed of patients that are responsive to antidepressant treatment. Replication studies in wider and more heterogeneous samples will be needed to confirm these results.

In conclusion, this study demonstrates for the first time that BDNF serum fluctuations in patients with MD are associated with an altered synthesis of BDNF in white blood cells and that leukocyte BDNF mRNA increases observed during pharmacological treatment correlates with symptoms improvement. These observations uncover a potential clinical implication suggesting that peripheral BDNF may have an effect on brain function and behaviour. In this regard, recent evidences have reported that peripheral infusion of BDNF increases neurogenesis in the brain and produces anxiolytic and antidepressant effects in rodents (RS Duman & HD Schmidt, unpublished data, cited in Sen *et al*. 2008). These effects may be due to the direct actions of BDNF which is transported into the brain or from indirect events resulting in the peripheral induction of additional factors that are more easily transported through the BBB such as IGF-1 (Duman *et al*. 2009). Finally, our results suggest that peripheral tissues might also play an active and central role in MD aetiopathogenesis and in the action of mechanisms of antidepressant drugs.

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Statement of Interest

None.

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