Neuropsychopharmacology (2004) 29, 805–812

www.neuropsychopharmacology.org

Drug-Induced Decrease of Protein Kinase A Activity Reveals Alteration in BDNF Expression of Bipolar Affective Disorder

Félicien Karege*[,1], Michèle Schwald[1] and Rachid El Kouaissi[1]

[1]*Geneva University Hospitals (Belle-Idéé), Division of Neuropsychiatry, Chêne-Bourg (Geneva), Switzerland*

Bipolar affective disorder (BAD) is a severe disease whose molecular and cellular bases are not well known. The aim of the present study was to probe the cAMP signaling downstream targets by pharmacologically manipulating the protein kinase A (PKA) enzyme, along with the assessment of brain-derived neurotrophic factor (BDNF) expression in lymphoblasts. The time course of lymphoblast PKA activity (up to 72 h) revealed optimal activity at 24 h. Then, the enzyme activity and protein levels of PKA Cα subunit and phopsho-cAMP responsive element binding (CREB) were assayed in lymphoblasts derived from 12 BAD and 12 control (CT) subjects and cultured for 24 h in the presence of cAMP analog drugs. The results indicated that basal PKA activity and PKA Cα subunit immunolabeling are increased in cells from BAD compared with controls. Enzyme activity was increased by Sp-isomer in BAD and in CT's cells, without change in protein levels. In contrast, the Rp-isomer decreased enzyme activity and protein levels. In drug-naive conditions, there was no change in BDNF expression of BAD cells compared with CT cells. Treatment with Sp-isomer induced increased BDNF in both groups, while treatment with Rp-isomer induced a significant decrease in BDNF expression of BAD compared with CT. The p-CREB changes followed changes in BDNF levels, with increased and decreased Sp-isomer and Rp-isomer treatment, respectively. Our results suggest that mood disorder is associated with PKA upregulation and this could mask alteration in BDNF expression, because slowing down of PKA signaling results in a decrease of BDNF expression. These findings, combined with previous reports, provide a new insight to explain pharmacological features in different diagnostic groups.
Neuropsychopharmacology (2004) **29,** 805–812, advance online publication, 21 January 2004; doi:10.1038/sj.npp.1300384

Keywords: bipolar affective disorder; cAMP-dependent PKA; BDNF; lymphoblast; Sp-8-Br-cAMPS and Rp-8-Br-cAMPS

INTRODUCTION

Bipolar affective disorder (BAD) is an incapacitating chronic disease that affects approximately 1% of the world's population and requires an appropriate treatment (Goodwin and Jamison, 1990). Unfortunately, its molecular and cellular bases are far from being elucidated, although important breakthroughs have recently been achieved: a number of outstanding reports consider affective disorder as being due to transduction mechanisms abnormally regulated, associated with impairment of structural plasticity and cellular resilience (Manji *et al*, 2000; Rajkowska, 2000; Duman, 2002).

Among the multiple cell signal transduction pathways, the cAMP signaling has drawn particular interest (Tardito *et al*, 2000; Stewart *et al*, 2001; Bezchlibnyk and Young, 2002), and hyperfunction of cAMP and downstream targets has been postulated for the etiopathology of BAD (Young *et al*, 1993; Perez *et al*, 1999). The first cAMP signal target is protein kinase A (PKA), an important phosphorylating enzyme that activates the transcription factors, that is, the cAMP responsive element binding (CREB) protein, and controls the expression of critical genes such as brain-derived neurotrophic factor (BDNF) (Shieh and Ghosh, 1999). Elevated PKA activity was reported both in discrete post-mortem brain regions (Fields *et al*, 1999; Chang *et al*, 2003) and in peripheral cells from BAD patients (Perez *et al*, 1995, 1999; Tardito *et al*, 2003; Karege *et al*, 2003).

The BDNF, a member of the neurotrophin family involved in neuronal development and cell survival (Ghosh *et al*, 1994), has been shown to be one of the main downstream targets of the cAMP/PKA signaling in cell culture (Condorelli *et al*, 1994; Nakahashi *et al*, 2000) and in rat brain (Morinobu *et al*, 1999). The CREB factor has also been implicated in the upregulation of BDNF expression, and DNA sequence analysis identified a putative CREB in the promotor region of the BDNF gene (Finkbeiner *et al*, 1997). CREB function is regulated by its state of phosphorylation at serine-133, which results in activation of CRE-mediated gene transcription (Mayr and Montminy, 2001).

*Correspondence: Félicien Karege, Geneva University Hospitals (Belle-Idéé), Division of Neuropsychiatry, ch. Petit Bel.Air, 2; CH-1225 Chêne-Bourg (Geneva), Switzerland, Tel: +4122-305 53 14, Fax: +4122-305 53 09, E-mail: felicien.karege@biolo.unige.ch
Received 19 June 2003; revised 09 October 2003; accepted 16 December 2003
Online publication: 18 December 2003 at http://www.acnp.org/citations/Npp12180303268/default.pdf

Human studies have also provided data that support a role for BDNF in affective disorders (Chen *et al*, 2001). Therefore, evidence has emerged that the alteration of the cAMP/BDNF pathway could be involved in the biological basis of depression, with a pivotal role in regulating stress and therapeutic responses (Duman, 2002). This role of BDNF in mood disorder has recently been strengthened by family-based association studies, which showed that the BDNF gene is a risk locus for depression (Neves-Pereira *et al*, 2002; Sklar *et al*, 2002). Moreover, studies with animal models of depression strongly support a role for BDNF in the biology of mood disorder (Shirayama *et al*, 2002; Roceri *et al*, 2002).

However, working with the peripheral cell model of lymphoblasts, in a recent study, our group did not observe any change in BDNF expression in a BAD population compared with a control (CT) group, thus conflicting with the above hypothesized depression-associated BDNF alteration (Karege *et al*, 2003). This lack of alteration in BDNF expression was ascribed to the upregulation of the cAMP signal that characterized the group that we have studied. The upregulation could probably rectify change in BDNF gene capacity. In order to test this hypothesis, we have undertaken to explore the pathway of PKA activity/BDNF expression in BAD patients by manipulating PKA activity with specific drugs with the aim of slowing down the cAMP signal in the BAD group.

Due to inaccessibility to the human brain, a peripheral cell model, the EBV-transformed lymphoblast, which presents several advantages, has been used in BAD studies (Wright *et al*, 1984; Kay *et al*, 1994; Shamir *et al*, 1998; Emamghoreishi *et al*, 2000): this cell, easy to culture, expresses BDNF protein and its cognate receptor, TrkB (Schenone *et al*, 1996). In addition, it has repeatedly demonstrated trait-dependent defects in signal transduction of BAD either in cAMP or in Ca^{2+} signaling (Kay *et al*, 1994; Wright *et al*, 1984; Shamir *et al*, 1998; Emamghoreishi *et al*, 2000). It offers an advantage over the blood platelet as neurohormonal and medication influences were removed by several passages in cell culture.

As drugs, two powerful cAMP analogs, Rp-8-Br-cAMPS and Sp-8-Br-cAMPS, were used to manipulate PKA activity. These cAMP analogs are more membrane-permeant than other modulators such as 8-bromo-cAMP, and have much higher specificity for PKA than does forskolin (Schaap *et al*, 1993; Gjerstsen *et al*, 1995). The Rp-8-Br-cAMPS is a potent competitive inhibitor of PKA, particularly in cells expressing mostly type I kinase such as leukemia cells (Gjerstsen *et al*, 1995), whereas the Sp-8-Br-cAMPS is a potent activator (Schaap *et al*, 1993) and both drugs are more resistant to phosphodiesterases than other cAMP analogs. Here we demonstrate that artificial slowing down of cAMP signaling with PKA inhibitor results in disclosing a defect in BDNF expression of BAD, but not in control subjects, suggesting the existence of a cAMP-linked masking process in bipolar disorder. The activation of CREB might be the connection between PKA and BDNF.

This study provides a framework for understanding the signal transduction hypothesis of BAD at the level of gene expression and the neurotrophic hypothesis and supports recent data obtained with post-mortem brain tissue.

MATERIALS AND METHODS

Subjects and Lymphocyte Transformation

The lymphocytes were obtained from 12 BAD (6M + 6F), euthymic patients, and 12 healthy control (CT) subjects (6M + 6F). The study was approved by this hospital's ethics committee, and all persons gave informed consent prior to their inclusion in the study. Both patient and control populations were balanced with respect to age (37 ± 6 *vs* 40 ± 7 years for BAD and CT, respectively). The criteria of inclusion for BAD were at least one manic episode; no comorbidity was revealed in BAD, and the CT subjects had no known psychiatric or neurological history. The cells were isolated with the Ficoll-Hypaque kit (Sigma, Buchs, CH) and transformed with the Epstein–Barr virus supernatant (Walls *et al*, 1995). Immortalized cells were cultured at 37°C in a humidified atmosphere at 5% CO_2 on RPMI-1640 medium supplemented with 2 mM L-glutamine, 10% fetal calf serum, and antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin). The cell lines were subcultured every 3–5 days depending on the confluence, and harvesting was carried out between 8 and 20 passages.

Cell Stimulation and cAMP-Dependent PKA Assays

Six million cells (6×10^6) were transferred in low serum content (2%) medium and the following drugs were added 24 h before cell harvesting: 10 μM Sp-8-Br-cAMPS or 10 μM Rp-8-Br-cAMPS. These drugs were purchased from Biolog, Life Science Institute (Bremen, Germany). At 24 h after drug addition, culture was stopped, cells were counted and then rinsed in phosphate buffer (PBS, pH 6.5), and finally homogenized in 0.5 ml ice-cold 25 mM Tris-EDTA buffer, pH 7.4 (containing 1 μM IBMX and 100 μM leupeptin, phosphodiesterase, and protease inhibitor, respectively). For all assays, the cytosol fraction was used as per the methods previously published (Karege *et al*, 2001, 2003).

The cAMP-PKA Activity Assay

Briefly, the cytosol extract was isolated by centrifugation at 14 000 *g*, 4°C, 30 min. Enzyme assay was performed within 30 min with the PepTag© kit from Promega Corp (Madison, WI), which uses a fluorescent peptide (f-kemptide) as PKA substrate. Phosphorylated peptide was resolved on agarose gel (0.8%) as adapted in our laboratory (Karege *et al*, 2001). Two assay conditions were performed, namely the nonspecific PKA phosphorylation (with addition of the 5 μM PKI, a specific PKA inhibitor) and the total cAMP-activated phosphorylation (without addition of the 5 μM PKI, in the parallel tubes). Specific enzyme activity was estimated as the difference between the two measures. Enzyme activity was assayed as the concentration of the fluorescent substrate read as relative fluorimetric units (RFU) in a Versa-Fluor fluorimeter (Bio Rad Laboratories, Glattburgg, CH) set at 540 nm excitation and 590 nm emission light in 250 μl microcuvettes. A set of tubes containing commercial catalytic PKA C subunit (10 ng), instead of the samples, was used as positive control and for standard curve in the presence of increasing levels of f-peptides. Protein assay was performed with the BCA kit (Pearse Chemical, Rockford, USA). The results were

expressed in nanomoles of phospho-kemptides formed in unit time (min) per mg of protein.

Western Immunoblotting of Cα Subunit of PKA

In order to assess the changes in catalytic PKA subunit and p-CREB levels, aliquots of cytosol homogenates (PKA Cα) and whole-cell lysate (p-CREB) from lymphoblasts of BAD and their respective controls were subjected in triplicate to electrophoresis (SDS-PAGE; 100 V at 4°C; 2 h) with equal amounts of protein (40 and 60 μg protein for PKA and p-CREB, respectively), and then transferred onto PVDF membranes (Amersham Pharmacia Biotech, Zurich, CH). Blots were blocked in PBS containing 0.5% Tween 20, 3% BSA for 1 h and incubated overnight at 4°C with PKA Cα antiserum (dilution: 1 : 1000; sc-903, Santa Cruz Biotechnology, USA) or with p-CREB antiserum (dilution 1 : 500; Chemicon Int., Temecula, USA). Membranes were then incubated with secondary antibody IgG conjugated to horseradish peroxidase (diluted to 1 : 5000) in blocking buffer for 2 h at room temperature. The Western blots were detected with the ECL (Amersham Pharmacia Biotech) and exposed to film for 10 s to 5 min. The specificity of the PKA antibody was checked with a commercial sample of PKA and a human cortical extract. As control, β-actin antiserum was used by reprobing the same blots after a careful stripping with Re-Blot plus kit (Chemicon Int., Temecula, CA). The reprobing also allows to reduce the interblot variability.

Quantitation of the immunoreactivities was performed by densitometric scanning using an image analysis system (Molecular Analyst, BioRad Laboratories). The optical density (OD) units obtained from each immunoreactive band were normalized against the OD of reference bands (from nonstimulated cells of control subjects). Before starting the experiment with samples, the procedure was standardized with increasing levels of protein (10–100 μg per well) for checking the linearity, and standard curves were repeatedly performed.

Total RNA Extraction and RT-PCR Assay

Total RNA was extracted from a fixed number (6×10^6) of lymphoblasts using RNeasy kit (Qiagen, Basel, CH) and the concentration was read in a spectrophotometer at 260 nm. First-strand cDNA was synthesized from 2 μg of tRNA with the RT Omniscript enzyme in 20 μl mixture, according to Qiagen's instructions. The reaction mixture was incubated at 37°C for 1 h. For PCR assay, a fragment of 594 bp was amplified from 2 μl of the above cDNA mixture (sequence corresponding to exon 5; Genebank Accession XM-006027) in 50 μl mixture containing 0.4 μM of each BDNF primer (F-primer: 5′-agagtgatgaccatccttttcc-3′ and R-primer: 5-gcagccttcttttgtgtaacc-3′), PCR buffer, and 2.5 U of Taq DNA polymerase made up to 50 μl with nuclease-free water. The PCR program started with a 2 min denaturation phase (94°C) followed by an amplification phase of 32 cycles (94°C for 1 min; 55°C for 30 s and 72°C for 1 min and ended by 5 min at 72°C). As control, an 848 bp sequence of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was amplified in 28 cycles (F-primer: 5′-*atcaatg-gaaatcccatcacc*-3′ and R-primer: *tctcttcctcttgtgctcttgc*-3′) using the above PCR parameters. To ascertain the unique band of double-strand cDNA, an aliquot of PCR product was separated on 1.5% agarose gel and stained with ethidium bromide.

Double-Strand cDNA Quantitation of PCR Products

The PCR products were quantitated with the fluorescent PicoGreen® dye (Molecular Probes, Eugene, OR), a fluorochrome that selectively binds dsDNA and has been previously used with accuracy to quantitate PCR products (Romppanen *et al*, 2000). This labeling enables a higher dynamic range than the coloration with ethidium bromide. Fluorescence was measured with the Versafluorometer© in 250 μl microcuvettes with the following dilutions: 10 μl of Microcon 30-filtered PCR products were added to 90 μl of 50 mM Tris-EDTA buffer, pH 7.5, mixed with 100 μl of 1 : 200 diluted PicoGreen® dye. After 2–5 min in a dark room, fluorescence was measured (480 and 520 nm of excitation and emission light, respectively). A standard curve with serial dilution of commercial dsDNA was previously established. The results, expressed in ng/μl of BDNF cDNA synthesized in 32 cycles, were normalized to GAPDH PCR products of the same samples, and finally expressed as the ratio of BDNF/GAPDH values. The CT's value in drug-naive culture was taken as reference (100%).

Statistical and Data Analysis

Preliminary tests for the linear range of different immunoblots with respect to protein concentration were performed, and sample loading was in triplicate. β-actin protein and GAPDH mRNA were used as references, respectively, for Western immunoblot and RT-PCR assays. The results were expressed as ratio to these references. For p-CREB, data were expressed as % change to control (untreated CT cells). Due to the small sample sizes ($N = 12$), nonparametric analyses were used (Friedman test for analysis of variance and Mann–Whitney *U*-test to compare groups). The statistical significance was set at $p < 0.05$.

RESULTS

Figure 1 displays a time course of PKA activity from 3 to 72 h culture of control cells. Friedman test of analysis of variance (nonparametric) yielded a significant variance among parameters ($\chi^2 = 6.3$, $p < 0.04$). Minor changes were observed before 24 h (no change in Rp-treated cells), and above 24 h there was significant cell death and a decrease in overall activity. Subsequent measures were taken at 24 h of drug manipulations.

Table 1 displays the mean values of PKA activity, and Friedman test of all groups indicated significant differences among groups ($\chi^2 = 35.9$; df = 5, $p < 0.001$). *Post hoc* analyses with Mann–Whitney *U*-test showed that lymphoblast cells from BAD subjects present significantly higher PKA activity, either in naive-drug ($Z = -2.31$; $p < 0.02$) or in Sp-treated PKA activity, than controls ($Z = -2.19$ $p < 0.01$). The Sp-isomer increased the PKA activity both in BAD ($Z = -3.41$; $p < 0.01$) and in CT-derived cells ($Z = -2.68$; $p < 0.02$). The Rp-isomer decreased PKA activity in both groups of populations compared to their respective naive

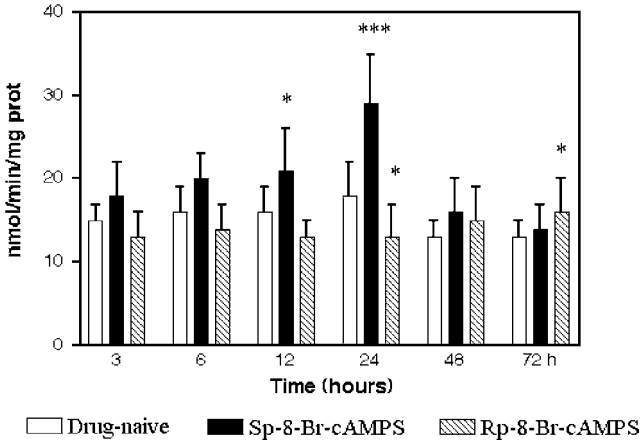

Figure 1 Time course of cytosolic PKA activity of EBV-transformed lymphoblast cells cultured either in RPMI-1640 (drug-naive), in RPMI-1640 supplemented with 10 μM Sp-8-Br-cAMPS (activator), or 10 μM Rp-8-Br-cAMPS (inhibitor), from 3 to 72 h. During the activation period, the serum content (FCS) was decreased from 10 to 2%. PKA assay was performed by the nonradioactive PepTag© kit from Promega Corp. The non-PKA phosphorylation was estimated by adding in parallel tubes a PKA inhibitor (5 μM PKI). The Friedman test revealed significant changes between groups ($p<0.04$), and the optimal was observed at 24 h. Mann–Whitney test, drug-treated *vs* untreated cells: *$p<0.05$; ***$p<0.001$.

Table 1 PKA Activity in Bipolar and Control Cells

Subjects (number)	Drug-naive	Sp-8-Br-cAMPS	Rp-8-Br-cAMPS
Controls (12)	18.3±4	29.5±9*	13.6±4*
Bipolars (12)	23.6±6¶	41.4±11¶¶**	15.5±6**

PKA activity mean values (±SD) from 12 bipolar- and 12 control-derived cells cultured either in drug-naive, in 10 μM Sp-8-bromo-cAMPS or in 10 μM Rp-8-bromo-cAMPS for 24 h. Results are expressed in nmol/min/mg prot of phosphokemptide levels. Friedman test's analysis of variance yielded: $\chi^2=35.9$; df = 5, $p<0.001$. Comparison between bipolar and control cells with nonparametric Mann–Whitney *U*-test: ¶¶$p<0.01$ and ¶$p<0.02$. Comparison between drug-treated and naive cells **$p<0.01$ and *$p<0.02$.

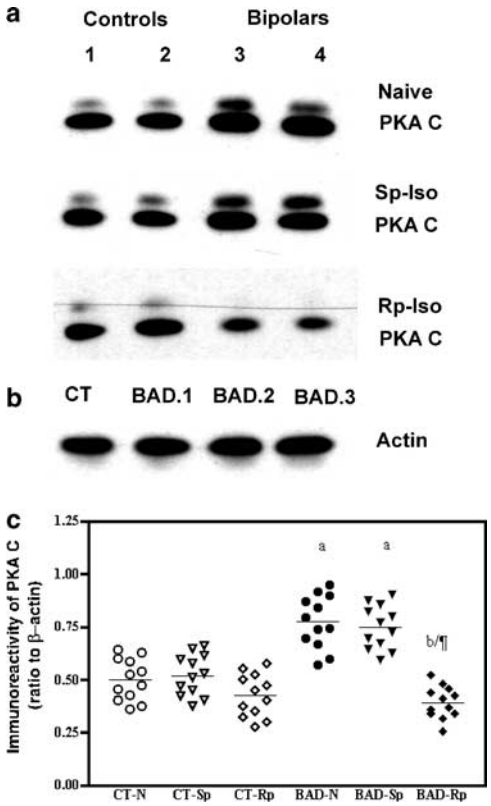

Figure 2 Representative immunoblots. (a) Lymphoblast PKA Cα subunits from two control (1-2) and two bipolar (3-4) derived cells cultured either in naive conditions (top), in Sp-isomer (middle) or in Rp-treated cells for 24 h. (b) Lymphoblast β-actin from a control subject cultured in naive conditions (CT), and from bipolar-derived cells cultured either in drug-naive (BAD.1), in Sp-isomer-treated (BAD.2), or Rp-treated (BAD.3) cells. (c) Relative immunoreactivity of PKA Cα subunits in lymphoblasts from 12 control (open) and 12 bipolar (closed) subjects, cultured either in drug-naive (circle), in PKA activator (Sp-isomer: triangle), or in inhibitor (Rp-isomer: diamond-shaped) conditions. Results were expressed as ratio of PKA to β-actin immunolabeling. Friedman test indicated significantly different groups ($p<0.001$). Mann–Whitney test: BAD *vs* reference: (a) $p<0.001$; (b) $p<0.01$ and BAD-Rp *vs* BAD-N: ¶$p<0.001$.

conditions: BAD's cells ($Z=-3.00$; $p<0.01$) and control group's ($Z=-2.16$ $p<0.02$). Figure 2a, b displays representative immunoblots of PKA Cα subunit and β-actin proteins, located at 40 and 46 kDa of molecular weight, respectively. The antibody used is known to be crossreactive with Cβ and Cγ, and the upper band may represent another PKA C subunit. No significant change was observed either in β-actin (Figure 2b) or in Sp-treated CT, while the PKA Cα immunoreactivity increased in bipolar subjects, both in drug-naive and in Sp-isomer-treated cells. In Rp-isomer-treated cells, the PKA C immunolabeling decreased particularly in BAD cells. The ODs of standardized immunolabeling are displayed in Figure 3c (ratio to β-actin). The Friedman test indicated significant variations between groups ($\chi^2=47.2$; $p<0.001$). The Mann–Whitney *post hoc* analysis compared BAD to CT cells in naive-drug culture: the BAD cells displayed more PKA Cα protein immunoreactivity than CT cells ($Z=-3.78$; $p<0.001$). The addition of 10 μM Sp-8-Br-cAMPS in BAD cells increases PKA C levels over reference ($Z=-3.17$; $p<0.001$), but not

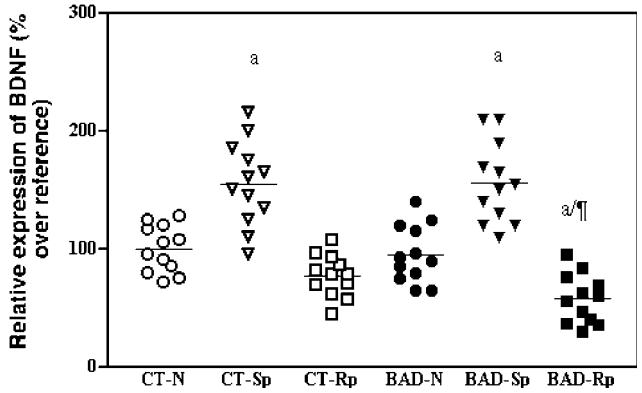

Figure 3 BDNF gene expression in lymphoblasts from 12 control (open) and 12 bipolar (closed) subjects, cultured either in drug-naive (N: circle), in PKA stimulator (Sp-isomer: triangle), or inhibitor (Rp-isomer: diamond-shaped) conditions. Results were expressed in ratio of BDNF/GAPDH PCR products. Friedman test yielded significant changes ($p<0.001$) and Mann–Whitney test: drug-treatment *vs* control (CT-N): (a) $p<0.001$; comparison between BAD-Rp and CT-Rp: ¶$p<0.02$.

over BAD's cells in drug-naive culture. The use of Rp-8-Br-cAMPS significantly decreased the immunoreactivity in BAD samples with respect to reference ($Z=-2.5$; $p<0.01$) and to BAD in naive cells ($Z=-2.98$; $p<0.01$), but not significantly in CT-treated cells (NS). Following the decrease with Rp-treatment, there was no further difference between BAD and CT populations. The relative levels of BDNF mRNA as expressed in ratio to GAPDH mRNA levels are shown in Figure 3. The Friedman test for analysis of variance indicated significant changes among groups ($\chi^2=39.3$; $p<0.001$), while no change was observed in GAPDH mRNA (not shown). The Mann–Whitney *post hoc* analyses of BDNF levels yielded no difference between BAD *vs* respective CT cells, either in drug-naive (NS) or in Sp-treated cells (NS). The Sp-isomer drug induced an increase in BDNF levels in BAD ($Z=-3.81$; $p<0.001$) and in CT cells ($Z=-3.31$; $p<0.001$). The Rp-isomer decreased the BDNF expression in BAD cells ($Z=-3.84$; $p<0.001$), but not in CT subjects ($Z=-1.1$; NS). In Rp-isomer culture, BAD express significantly less BDNF than CT subjects ($Z=-2.8$; $p<0.02$).

To obtain more accurate information the active form of CREB, the p-CREB, was assayed in the cell lysate of the same samples. The phospho-CREB blot was found, as expected, in the range of molecular weight of 43. Representative immunoblots are displayed in Figure 4a (the antibody also detected the phospho-ATF-1); the mean values of % change over control (unstimulated CT cells were taken as reference) of their ODs are displayed in Figure 4b. The Friedman test between the six p-CREB categories indicated significant changes ($\chi^2=47.2$; $p<0.001$). The Mann–Whitney test between treated samples and controls indicated that the p-CREB levels were increased in Sp-isomer-treated cells, both in CT and in BAD cells with respect to reference (*U*-test: $Z=-3.12$; $p<0.01$ and $Z=-2.98$; $p<0.01$, for BAD and CT, respectively). The Rp-isomer treatment results in a significant decrease of immunoreactivity only in BAD cells ($Z=-2.28$; $p<0.02$). There was a nonsignificant trend to increase ($p<0.08$) in untreated BAD and decrease in Rp-isomer-treated cells ($p<0.09$) compared with reference.

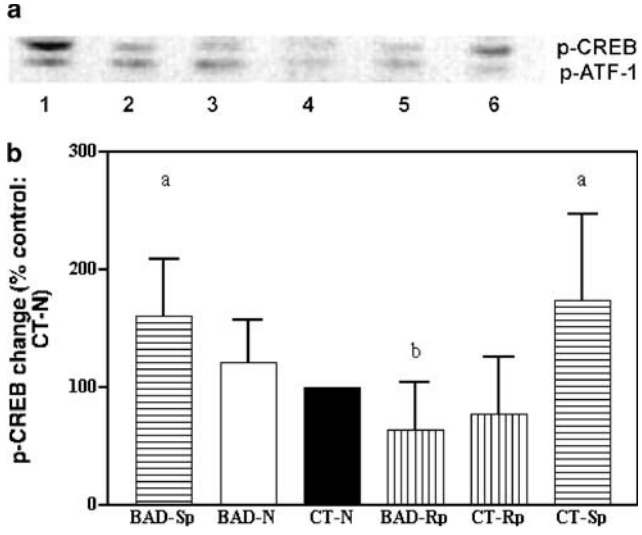

Figure 4 p-CREB levels in lymphoblasts. (a) Representative immunoblots of: lymphoblast p-CREB levels from three control- (blot 3-5-6) and three bipolar- (blot 1-2-4) derived cells cultured either in drug-naive (2-3), in Sp-isomer (1-6), or in Rp-isomer (4-5) for 24 h. (b) Relative change in immunolabeling of p-CREB protein levels in lymphoblasts from 12 bipolar and 12 control subjects, cultured either in drug-naive, in PKA activator, or inhibitor conditions. Results were expressed as percent of control (p-CREB immunolabeling of CT-N, taken as reference: black bar). Friedman test indicated significantly different groups ($p<0.0001$) and Mann–Whitney test between treated *vs* control cells: (a) $p<0.01$; (b) $p<0.02$.

DISCUSSION

This study has shown that basal and drug-manipulated lymphoblast PKA activity undergoes alteration in a time course assay, with optimal changes at 24 h of cell culture period. At that time, the enzyme activity and the protein levels of PKA Cα and the p-CREB are elevated in lymphoblasts from BAD compared to cells from CT subjects. The treatment with a PKA activator, Sp-8-Br-cAMPS, increased the activity both in BAD and CT, but did not affect the Cα subunit levels. Conversely, the use of a PKA inhibitor, Rp-8-Br-cAMPS, resulted in a decrease of activity and the protein levels, as well. In basal conditions, there was no difference in BDNF expression between BAD and CT subjects. The activation of PKA increased the expression of BDNF while its inhibition decreased it, specifically in BAD population. Thus, in lowered PKA activity and protein levels, BAD patients express less BDNF compared with control subjects. The p-CREB assay, the active form of CREB, aimed to prove the connection between PKA signaling and BDNF expression in this cell model, also changed with an increase in Sp-isomer-treated BAD cells (but not naive cells), and a significant decrease in Rp-isomer-treated subjects.

To our knowledge, this is the first study that shows that BDNF expression could be reduced by slowing down the PKA activity and protein levels in BAD. In a preliminary study, our group has shown that PKA activity is upregulated in BAD without change in BDNF levels compared with controls (Karege *et al*, 2003). Moreover, in a recent report on human post-mortem brain, no difference was observed in hippocampal BDNF levels between BAD and CT subjects, while BDNF levels were decreased in major depression with respect to controls (Chen *et al*, 2001). In the latter study, BDNF expression was found to be increased in antidepressant-treated major depression subjects, without change in ATD-treated BAD. In two reports, alteration in BDNF expression and CREB levels was shown in post-mortem brains of suicide subjects, most of them with an ante-mortem diagnosis of major depression (Dwivedi *et al*, 2003a, b). Taken together, these results indicate that there is a difference between depression diagnostic groups, in their respective cAMP/BDNF pathways. While recent genetic studies reported BDNF gene as a risk locus in BAD (Sklar *et al*, 2002; Neves-Pereira *et al*, 2002), no change has been observed, until now, in its expression in this illness. Our explanation is that due to the link between cAMP/PKA signaling and BDNF, the defect in BDNF gene could be rectified by the upregulation in cAMP signaling. Disclosure of dysfunction in BDNF expression can be achieved by downregulating the cAMP signal. To test this explanation and to establish a link between our results and genetic studies, we are planning to genotype all the subjects used in

this study to confirm this postulated change in the BDNF gene.

The effects of the cAMP analogs on PKA activity were, as expected, opposed (downregulation by Rp-isomer and upregulation by Sp-isomer), but as far as PKA C protein levels are concerned, some dissonance was observed. The Sp-isomer did not change the PKA Cα protein, while the Rp-isomer reduced PKA Cα levels. Actually, the two isomers interact differently with PKA holoenzyme. The Sp-isomer binds to the PKA tetramer and promotes its dissociation as does the cAMP (Francis and Corbin, 1999). We have recently reported that the addition of Sp-cAMPS in cell culture increased PKA activity and decreased the PKA R regulatory subunit levels (Karege *et al*, 2003). In this respect, the increase in C subunit abundance accords well with the previous findings of elevated cAMP/PKA in post-mortem BAD brain and platelet cells (Fields *et al*, 1999; Perez *et al*, 1999; Chang *et al*, 2003). Such an observation suggests that the high PKA C found in BAD lymphoblasts reflects an adaptation pattern to high levels of BAD-associated cAMP signaling. Alternatively, the R subunits could be inactivated after their release from the holoenzyme, resulting in freeing more PKA levels and activity without *de novo* synthesis. Consistent with this, it was recently shown that changes in PKA are due to a post-translational mechanism rather than gene transcription (Chang *et al*, 2003).

The effect of Rp-8-Br-cAMPS on PKA Cα subunit levels is different. The Rp-isomer binds competitively to the holoenzyme but does not dissociate it. How this binding also results in the decrease of PKA C levels is not understood. Possibly, an additional effect of Rp-isomer is to decrease the synthesis of new molecules of PKA C by an indirect process that is not yet known. It has been demonstrated that changes in intracellular levels of cAMP can cause cell type-specific changes in the expression and abundance of PKA subunits (see review Spaulding, 1993). Alternatively, a prolonged inactive state of the holoenzyme due to the nonhydrolyzable Rp-isomer finally renders the PKA C protein inaccessible to the antibody. Whatever the cause of PKA blunting, the cAMP downstream signaling is downregulated and becomes unable to compensate the altered expression of BDNF.

We have also assessed the state of CREB activation by evaluating the p-CREB levels, and found a close relationship between changes in PKA and changes in BDNF expression. The most important finding is the decrease of CREB levels in Rp-isomer-treated patients than in control subjects, and its subsequent reflection in BDNF expression. A previous study on post-mortem cortices has shown that cortical CREB levels are lowered in anticonvulsant-treated subjects than in drug-free BAD subjects (Dowlatshahi *et al*, 1999). Our study, by examining the alteration in downstream cAMP signal components, confirms the connection between PKA and BDNF and supports its importance in mood disorder.

The present data, compared to studies on major unipolar depression, suggest a different dysfunction of the cAMP signal and its target, BDNF. It is worth mentioning that PKA activity was mostly reported to be decreased in major depression (Shelton *et al*, 1996; Perez *et al*, 2001), whereas BDNF levels were found to be decreased either in post-mortem brain tissue (Chen *et al*, 2001) or in serum of major depression patients (Karege *et al*, 2002; Shimizu *et al*, 2003). Moreover, the antidepressant drugs induce an increase in the cAMP signal and BDNF levels (Duman *et al*, 1997) while lithium, the most used mood stabilizer, induces a decrease in the cAMP signal (reviewed by Li *et al*, 2000). Therefore, our findings, combined with those reports, provide a new insight to explain some pharmacological features of different depression diagnostic groups.

However, caution must be exercised. We are aware that our assay using a peripheral cell is an indirect means. Moreover, the importance of lymphoblast BDNF is not known. As was previously stated, the cellular distribution and regulation of PKA subunits are tissue-specific. Lastly, it is unknown whether these changes are primary or secondary to a disturbance in crosstalk regulation originating from dysfunction of another signaling system. The possibility of a subtle regulation, not detectable by our method, cannot be excluded.

In summary, our study has observed an upregulation in the basal PKA activity of BAD-derived lymphoblasts. This sustained elevation in cellular PKA activity can affect subsequent downstream targets, such as the activation of CREB and the expression of BDNF, a critical protein in neuronal plasticity, by masking its possible alteration. It is proposed that basal PKA activity in bipolar disorder exerts a tonic upregulation on BDNF expression, which can be removed by slowing down the PKA signaling. Therefore, it is probable that the cAMP/BDNF pathway is not that used by mood stabilizers in the treatment of BAD, in contrast to the ATD drugs known to boost this pathway in major depression. Other molecular factors such as GSK-3β, for instance, are currently being explored (Manji *et al*, 2000). All these studies on signal transduction and gene expression in psychopathology have implications for development of novel treatments.

ACKNOWLEDGEMENTS

We gratefully acknowledge the help of Mrs Pascale Marin and Monique Vessaaz in establishing lypmphoblast cell lines, and Mrs Jean Gunn in editing the English manuscript.

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