GSK3β, CREB, and BDNF in peripheral blood of patients with Alzheimer's disease and depression

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Background: Glycogen synthase kinase-3β (GSK3β), cAMP-response element-binding protein (CREB) and brain-derived neurotrophic factor (BDNF) play critical roles in neuronal survival, synaptic plasticity and memory and participate in the pathophysiology of both depressive disorder and Alzheimer’s disease (AD).

Methods: This study was designed to determine the association of GSK3β activity, CREB activity and BDNF concentration in peripheral blood of patients with AD with or without depressive symptoms and in depressive patients without AD. GSK3β activity in platelets, CREB activity in lymphocytes and BDNF concentration in plasma, platelet-rich plasma or platelets were measured in 85 AD patients (36 of whom displayed co-morbid depressive symptoms), 65 non-AD patients with depressive disorder and 96 healthy controls. AD patients were clinically assessed for stage of dementia, cognitive impairment and severity of depressive symptoms. Depressive patients were clinically assessed for severity of depression.

Results: We observed increased CREB activity and GSK3β activity in AD with depressive symptoms or in AD at mild stage of dementia. Decreased BDNF concentration was found in platelet-rich plasma of AD patients at moderate to severe stages of dementia or in AD without depressive symptoms. An association was revealed of the severity of cognitive impairment with the increase of GSK3β in the platelets of AD patients with mild dementia. In depressive patients, a lower concentration of phosphorylated GSK3β was associated with a higher severity of depression. Association was confirmed between severity of depression, CREB activation, and BDNF concentration in drug-naïve depressive patients.

Conclusion: Our data demonstrated that AD is accompanied by increased CREB activity in lymphocytes and a decreased concentration of BDNF in platelet-rich plasma. The decreased BDNF concentration appears to correlate with moderate to severe stages of dementia in AD. Observation of decreased phosphorylation of GSK3β in platelets of both AD patients with depressive symptoms and depressive patients after treatment confirms the role of increased GSK3β activity in the pathophysiology of both AD and depressive disorder. Associations were confirmed between AD and platelet GSK3β activity, lymphocyte CREB activity and plasma BDNF. CREB activity and platelet BDNF concentration seems to be related to depressive disorder.

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1. Introduction

Alzheimer’s disease (AD) and depressive disorder are major neuropsychiatric diseases. Depression is a co-morbidity with a similar prevalence in both AD and control subjects (Heun et al., 2013). Depressive symptoms in the elderly are primarily symptoms of genuine depression rather than prodromes of AD (Mossaheb et al., 2012). Nevertheless, depressive symptoms are frequent psychiatric complications of AD, affecting as many as 50% of patients (Lyketsos and Olin, 2002; Lyketsos et al., 2011; Migliorelli et al., 1995; Starkstein et al., 2005). Moreover, meta-analysis of the correlation of depression with AD revealed that persons with a history of depression were more likely to be diagnosed with AD later in life (Ownby et al., 2006). In addition, the depressive state escalates the pre-existing suffering of AD patients. In a previous study, depression with dementia appeared to
lower performance on cognitive tests, and treatment of depression led to improvement in test performance (Greenwald et al., 1989). A link between AD and depression could be provided by hypothalamic–pituitary–adrenal (HPA) axis overdrive, decreased serotonin levels (Sierksma et al., 2010) and disturbances in several other signaling pathways involved in pathophysiological processes in AD, depressive disorder and AD with co-morbid depression (Maes et al., 2011).

Dementia in AD is associated with progressive neurodegeneration, which is characterized by synaptic injury and neuronal loss. The principal neuropathological features of AD include formation of senile plaques, which are composed of amyloid-β (Aβ) oligomers, and intracellular neurofibrillary tangles, which are composed of hyperphosphorylated tau protein. Nonetheless, a growing body of evidence shows that other factors could be included in AD etiology. Neurodegeneration in AD consists of synaptic damage, neuronal loss (Chen, 2009) and neurogenesis defects, which in turn contribute to cognitive dysfunction. Mechanisms leading to neurodegeneration that are studied in this context include the following: formation of pore-like structures with channel activity (Lin et al., 2001), alterations in glutamate receptors and excitotoxicity (Li et al., 2011), aberrant excitatory network activity (Palop and Mucke, 2010), mitochondrial dysfunction (Bosetti et al., 2002; Cardoso et al., 2004; Chatturvedi and Flint, 2013; Gibson et al., 1998), lysosomal dysfunction (Lee et al., 2011) and alterations in neuroplasticity and neurogenesis (Lilja et al., 2013).

Signal molecules involved in the neurodegenerative progression of AD include glycogen synthase kinase-3β (GSK3β), CAMP-response element-binding (CREB) protein (Scott, 2012), and cyclin-dependent kinase-6 and Fyn kinase (Crews and Masliah, 2010). Common pathophysiological mechanisms of depression and AD could involve other pathways, such as hyperactivation of the HPA axis, chronic inflammation and induction of oxidative and nitrosative stress, as well as disturbed neuroplasticity and neurogenesis, which involve factors such as glucocorticoids, proinflammatory cytokines, brain-derived neurotrophic factor (BDNF) and transforming-growth-factor-β (TGF-β).

1.1. GSK3β

GSK3β is a constitutively active serine/threonine protein kinase involved in a variety of cellular processes, such as glycolysis synthesis, gene transcription regulation, protein synthesis, synaptic plasticity, cell cycle regulation, and programmed cell death (Hooper et al., 2008). Its activity is negatively regulated by the insulin and Wnt signaling pathways (Gould et al., 2007; Logan and Nusse, 2004). For example, insulin inhibits GSK3β activity via sequential activation of phosphatidylinositol 3-kinase and protein kinase B (Akt), the latter of which phosphorylates GSK3β at the critical serine residue 9 (Cross et al., 1995).

There is evidence suggesting the involvement of GSK3β in the pathophysiology of mood disorders, AD and schizophrenia. Recent studies demonstrated that GSK3β plays a pivotal role in regulating the production of pro- and anti-inflammatory cytokines through both the innate and the adaptive immune systems (Jope et al., 2007; Wang et al., 2011). GSK3β inhibitors may reduce neuroinflammation (Beurel, 2011). Modulation of GSK3β activity is implicated in either a direct or a downstream mechanism of action of many mood stabilizers and antidepressants (Maes et al., 2012).

AD is accompanied by memory deficits that could be related to alterations in the hippocampus (degeneration of dentate gyrus), as well as neurogenesis. It was found that neurogenesis is increased in the AD hippocampus, where it may give rise to cells that replace neurons lost in the disease (Jin et al., 2004). Adult neurogenesis is a process controlled by several kinases and phosphatases, among which GSK3β exerts important functions. Increased activity of GSK3β is believed to play a key role in both familial and sporadic AD due to neurotoxic alterations of substrates such as tau, β catenin, amyloid precursor protein (APP), Bax protein, phosphoprotein phosphatase 1 inhibitor and c-Jun N-terminal kinase (Hernández et al., 2009). In vivo overexpression of GSK3β causes alterations in adult neurogenesis, leading to depletion of neurogenic niches (Fuster-Matanzo et al., 2013) and reversible alterations of postsynaptic densities and dendritic morphology (Llorens-Martín et al., 2013). Because GSK3β plays important roles in the regulation of key factors of AD neuropathology, including Aβ neurotoxicity, synaptic plasticity, memory, inflammation and neuronal survival, GSK3β inhibitors are being studied as therapeutics for AD (Medina and Avila, 2010).

1.2. CREB

CREB is a ubiquitous and constitutively expressed transcription factor that has been widely implicated in long-lasting synaptic plasticity underlying learning and memory (Kandel, 2012), as well as neuronal survival (Walton and Dragunow, 2000). The CREB protein has multiple roles in different brain areas (Carlezon et al., 2005), and its signaling has been studied in both affective (Schmidt and Duman, 2010) and neurodegenerative (Rouaux et al., 2003; Saura and Valero, 2011) disorders.

To activate expression of a target gene, CREB requires phosphorylation at the critical serine residue 133, representing a point at which several major signaling pathways converge (Pláteník et al., 2000). Multi-site phosphorylation of CREB by a variety of protein kinases has been described. It is believed that CREB phosphorylation is responsible for transcriptional activation, leading to the production of numerous gene products, such as BDNF, that play key roles in synaptic plasticity and cognitive function (Scott, 2012). However, Transducer of Regulated CREB activity proteins (TORCs) can independently activate CREB upon its phosphorylation (Conkright et al., 2003).

It is hypothesized that the long-term effects of treatment with antidepressants include induction of transcription factors, including CREB, leading to an increase in the expression of neurotrophic factors, including BDNF, and their receptors (Nibuya et al., 1996). CREB appears to be involved in the mechanism of action of antidepressants, as well as in the depressive disorder itself (Blendy, 2006).

A deleterious role of CREB signaling pathway disturbances in synaptic alteration in AD has been described (Smith et al., 2009). The Aβ peptide alters hippocampus-dependent synaptic plasticity and memory and mediates synapse loss via the CREB signaling pathway (Saura and Valero, 2011). Chen et al. (2012) stated that “overexpression of β-site APP-cleaving enzyme 1 (BACE1) reduces CREB phosphorylation, protein kinase A activity, and cAMP levels, whereas downregulation of BACE1 has the opposite effect”. Recently, it was found that in the AD brain, CREB is cleaved by calpain I, producing a truncated form of CREB that has a reduced ability to promote neuronal glucose transporter 3 (GLUT3) expression, resulting in impaired glucose uptake and metabolism (Jin et al., 2013).

1.3. BDNF

BDNF and its receptor, TrkB, are broadly expressed in the brain and participate in a range of intracellular signaling processes, neuronal protection and survival, axonal and dendritic morphology and synaptic plasticity (Numakawa et al., 2010). BDNF expression is also regulated in part by phosphorylation of CREB. In addition to TrkB, a low-affinity p75 neurotrophin receptor is also involved in neuronal survival and plasticity (Hashimoto, 2010; Pittenger and Duman, 2008).

BDNF is essential for brain development, neuroplasticity and neuronal survival. Decreased levels of BDNF and decreased levels of CREB phosphorylation are among the most frequently validated biomarkers of depressive disorder. BDNF has been implicated in a number of
neuropsychiatric disorders, including affective disorders, schizophrenia, addiction, eating disorders and neurodevelopmental disorders (Autry and Monteggia, 2012; Carrard et al., 2011; Hashimoto, 2010; Koenigsberg et al., 2012). Electrophysiological and molecular biological studies performed in aged animals, as well as animal models of AD, have demonstrated that cognitive decline is associated with significant modifications in synaptic plasticity (Baietti et al., 2012), i.e., in BDNF-regulated processes.

There is evidence linking changes in BDNF to core psychopathological features of AD (Diniz and Teixeira, 2011). Recent data suggest that plasma BDNF is a biomarker of impaired memory and general cognitive function in aging women (Komulainen et al., 2008).

BDNF can be stored in platelets, which may be a confounding factor when assessing the relationship between serum and brain BDNF. There is currently no data regarding the quantity of BDNF stored in platelets in AD patients.

A significant decrease of BDNF serum concentration has been found in AD patients compared to healthy controls (Forlenza et al., 2010). However, BDNF serum concentrations do not correlate with cerebrospinal fluid levels, age or Mini-Mental State Examination (MMSE) scores in AD patients (Laske et al., 2007). Higher BDNF serum levels appeared to be associated with a slower rate of cognitive decline in AD patients (Laske et al., 2011). However, opposing data on serum and brain levels of BDNF have been reported in AD patients as well, likely reflecting differences in patient recruitment and stage of the disease. For example, significantly increased BDNF serum concentrations were found in patients at early stages of probable AD compared to patients at more severe stages of AD and age-matched healthy controls (Laske et al., 2006), which may be explained by a compensatory increase in BDNF in early stages of AD. In another study, it was found that serum BDNF levels were significantly increased in mild cognitive impairment and AD patients compared to healthy controls, and this increase in AD patients was not dependent on illness severity (Angelucci et al., 2010).

Major depression is characterized by low BDNF in serum (Karege et al., 2002) and plasma (Lee et al., 2007). BDNF levels increase significantly after antidepressant treatment, and there is a significant correlation between the change in BDNF levels and the change in depression scale scores (Brunoni et al., 2008; Sen et al., 2008). It is hypothesized that peripheral BDNF administration produces antidepressant-like effects, and measurements of serum BDNF can provide new insights into structures and functions of the brain that are important in the pathophysiology and treatment of mood disorders (Schmidt and Duman, 2010; Schmidt et al., 2011). Peripheral BDNF can be used as a biomarker of mood states and disease progression for mood disorders, including major depressive disorder and bipolar disorder (Fernandes et al., 2011; Hashimoto, 2010).

It has been suggested that the alteration of serum or plasma BDNF observed in depression is not caused by a difference in total blood BDNF, but instead is likely related to a decreased ability of platelets to release BDNF that is unrelated to platelet reactivity (Karege et al., 2005). Recently, depressed patients were shown to have decreased platelet BDNF levels compared to healthy controls (Lee and Kim, 2009; Serra-Millás et al., 2011). A decrease in BDNF gene expression was reported in the peripheral cells of depressed patients (Pandey et al., 2010a). This result indicates that the decreased serum levels are not simply due to the failure of platelets to release accumulated BDNF.

1.4. Biological model

Blood-based biomarkers have been investigated extensively for both depressive disorder (Fišar and Raboch, 2008) and AD (Doecke et al., 2012). Plasma, platelets and peripheral blood mononuclear cells are the key components of peripheral blood in studies of pathophysiology of neuropsychiatric diseases, such as mood disorders and AD (Maes et al., 2009). Platelets share some metabolic pathways with neurons (e.g., synthesis and transport of neurotransmitters) and contain high levels of BDNF (Burnouf et al., 2012). It is hypothesized that the difference between the serum and plasma levels of BDNF reflects the amount of BDNF stored in platelets (Lee and Kim, 2009). GSK3β is present in human platelets (Barry et al., 2003), where it acts as a negative regulator of platelet function (Li et al., 2008). Moreover, platelets are significant sources of Aβ peptide in human blood (Chen et al., 1995). A great deal of evidence indicates that vascular impairment is a fundamental contributor to AD pathology, and platelets are generally considered a key element in vascular impairment because they represent the link between Aβ deposition, peripheral inflammation and endothelial senescence (Laske et al., 2012). Platelets may be considered as a valuable cellular model to evaluate peripheral inflammatory biomarkers in AD (Casoli et al., 2010, 2013).

1.5. Aim of the study

Important reasons persist for the examination of peripheral CREB, BDNF and GSK3β in AD, depression and AD with co-morbid depression. Our working hypothesis consists of three sections: (1) GSK3β, CREB and BDNF levels in peripheral blood reflect changes of these parameters in the brain; (2) The mean activity of GSK3β increases, CREB activity decreases and BDNF concentration decreases in both AD and depressive disorder, and there is a synergistic effect of depressive symptoms co-morbid with AD on changes in these levels; and (3) GSK3β activity, CREB activity and BDNF concentration in the course of these diseases are associated with depression severity, characterized by depression scale scores, as well as with AD progression, characterized by dementia stage and cognitive impairment. We aimed to examine the association of CREB in lymphocytes, GSK3β in platelets and BDNF in plasma, platelet-rich plasma (PRP) and platelets from AD patients with or without current depressive symptoms, as well as depressive patients without AD. This study is designed to analyze the association between blood concentrations of these biochemical parameters and scores on diagnostic questionnaires measuring cognitive impairment and dementia stage and depression severity.

2. Materials and methods

2.1. Subjects

Two patient groups were established for this study. The first group included AD patients, and the second group included patients with major depressive disorder in a current depressive episode. All patients were recruited from the Department of Psychiatry of the First Faculty of Medicine and General University Hospital in Prague. Demographic data were collected for each person, and participants were asked to complete a questionnaire regarding their medical history, personal habits and use of medication. The clinical diagnoses were established by trained specialists.

The inclusion criteria for AD patients were as follows: age > 50 years; diagnosis of probable AD according to the NINCDS-ADRDA Alzheimer’s Criteria; brain imaging (magnetic resonance imaging) measuring cortico-subcortical atrophy (atrophy in the hippocampus and temporal corners of the side chambers) and excluding any other organic brain lesions (vascular changes, tumors, intracranial hemorrhage, etc.); MMSE score > 26; and no serious unstable somatic disease. Other causes of dementia were excluded, including pseudo-dementia. The following test assessments were used: the Addenbrook’s Cognitive Examination (inclusive of MMSE), the AD Assessment Scale—Cognitive (ADAS cog), and the Barthel activities of daily living (ADL) scale. Depressive symptoms, which are included in behavioral and psychological symptoms of AD dementia, were assessed by the Geriatric Depression Scale (GDS). AD dementia stage (mild, moderate or severe) was determined according to patterns of cognitive impairment.

AD patients (N = 85) were sub-grouped according to the severity of co-morbid clinically relevant depressive symptoms: those with
depressive symptoms and GDS ≥ 7 (N = 36) and those without marked depressive symptoms and GDS < 7 (N = 49). Another division of AD patients was in two groups according to their MMSE score and stage of dementia: AD patients with mild dementia and MMSE score ≥ 20 (N = 40) and AD patients with moderate to severe dementia and MMSE score < 21 (N = 45). Milder, moderate and severe dementia stages were recognized in 40, 33 and 12 AD patients, respectively.

AD patients were treated with reversible acetylcholinesterase inhibitors and/or NMDA receptor antagonists, as well as other drugs according to their somatic illnesses. Those with co-morbid depression were typically treated with antidepressants as well.

In patients with depressive disorder, severity of depression was assessed using the 21-item Hamilton Rating Scale for Depression (HRSD-21) and Clinical Global Impression—Severity scale (CGI-S). Patients with depressive disorder were included if they were above 18 years old and suffering from either single or recurrent current depressive episode without psychotic symptoms. Diagnosis of a depressive episode or a recurrent depressive episode was confirmed using a structured clinical interview according to the 10th revision of the International Statistical Classification of Diseases and Related Health Problems (ICD-10). Serious somatic disease or chronic somatic pharmacotherapy was not present, and patients were without organic brain disease, without cognitive impairment and without abuse of psychoactive substances. Depressed patients had to have a HRSD-21 score greater than 10 and a CGI-S score of 2 or higher at baseline (before treatment). A negative screen for bipolar disorder was performed for all tested subjects using the Mood Disorder Questionnaire (MDQ).

Patients with depressive disorder were assayed at two different time points: first upon admission to the inpatient or outpatient treatment and beginning of treatment (baseline; N = 65), and again at the end of the trial, i.e., after treatment for 28 ± 15 days (N = 50; 15 subjects did not finish the study). At the baseline, 16 depressive patients were drug-naïve; i.e., they were not treated with antidepressants for more than 6 weeks or they had never been treated with antidepressants.

Pharmacotherapy was administered according to the guidelines of the Czech Psychiatric Association. Out of the 65 depressive patients, 44 were treated primarily with selective serotonin reuptake inhibitors (escitalopram, sertraline, paroxetine, fluoxetine). However, antidepressants of other classes were also used (mirtazapine, venlafaxine, trazodone, agomelatine). Balanced pharmacotherapy often included benzodiazepines; antidepressants were co-administered exceptionally (in 8 patients). Most patients demonstrated a marked decrease in the severity of their depressive symptoms after treatment. Upon completion of the trial, 75% (49 of 65) of the patients were responders, defined as ≥ 50% improvement in HRSD score compared to baseline (Hirschfeld et al., 2002).

The control group consisted of 96 age-matched healthy volunteers who underwent a psychiatric examination equivalent to that of AD patients, and it was confirmed that they were non-demented, non-depressed and without any organic brain disorder.

This study was carried out in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki), and the study protocol was approved by the Ethical Review Board of the First Faculty of Medicine and General University Hospital in Prague. Written informed consent was obtained from all subjects.

2.2. Blood sample collection and treatment

Following an overnight fast, blood samples of patients and healthy controls were drawn from the antecubital vein between 7:00 and 8:00 a.m.; no subjects used cigarettes or coffee, and morning medications had not yet been administered to patients. Seven milliliters of blood was drawn into BD Vacutainer® blood collection tubes (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) with K3 EDTA as the anticoagulant. PRP was separated by centrifugation at 150 × g for 20 min at 25 °C. Platelets in PRP were counted by microscopy using a hemocytometer.

Samples for the BDNF assay were obtained by centrifugation (14,000 × g, 10 min, 25 °C) of 500 μl PRP; 450 μl plasma was separated for plasma BDNF measurement, and the remaining plasma containing the platelet pellet (50 μl) was used for BDNF determination in PRP.

The sample for the GSK3 assay was obtained by centrifugation (14,000 × g, 10 min, 25 °C) of 500 μl PRP; 450 μl plasma was separated, and the remaining plasma containing the platelet pellet (50 μl) was diluted in 450 μl chilled deionized water, followed by denaturation with 125 μl 50 g/l sodium dodecyl sulfate (SDS) and 0.5 M dithiothreitol at 100 °C for 10 min.

Lymphocytes for the CREB assay were isolated using the density gradient medium Histopaque®-1077 (Sigma-Aldrich). Following rinsing in phosphate-buffered saline (PBS), lymphocytes were counted by microscopy using a hemocytometer, diluted and denatured in the same manner as described for platelets in the GSK3 assay.

All samples were stored at −85 °C until analysis.

2.3. Antibodies and reagents

The following commercial antibodies were obtained from Cell Signaling Technology, Inc. (Danvers, MA): phospho-CREB (Ser133) (87G3) rabbit monoclonal antibody (#9198), CREB (48H2) rabbit monoclonal antibody (#9197), phospho-GSK-3α/β (Ser21/9) antibody (#9331), GSK-3β (27C10) rabbit monoclonal antibody (#9315) and anti-rabbit IgG, HRP-linked antibody (#7074). The PVDF Western blotting membrane Immobilon-P was from Millipore (Billerica, MA). The Restore™ Western Blot Stripping Buffer, SuperSignal® West Pico chemiluminescence substrate, the X-ray film and the M-PER® Mammalian Protein Extraction Reagent were manufactured by Pierce Biotechnology, Inc. (Rockford, IL). For the measurement of BDNF, the following kits and reagents produced by R&D Systems, Inc. (Minneapolis, MN) were employed: human BDNF DuoSet ELISA development kit (cat. no. DY248), Reagent Diluent (1% BSA in PBS, cat. no. DY995), and Human BDNF Quantikine® Immunoassay (cat. no. DBD00). All other chemicals used were from Sigma-Aldrich (St. Louis, MO) and were of analytical purity or better.

2.4. Assay for CREB in lymphocytes by Western blot

Samples of SDS-denatured lymphocytes (starting amount 53,000 cells; approximately 3 μg total protein per well) were applied on 4.5%/ 10% SDS-polyacrylamide gels. A suitable constant amount of a standard prepared from THP-1 cells (human monocytic cell line) was applied to the marginal wells of each gel. The proteins from the samples and standards were resolved by electrophoresis (15 mA/gel, approximately 2 h at room temperature). A semi-dry electro-blotting procedure was then employed to transfer the proteins onto PVDF membrane. The resulting bands were blocked for 1 h with 5% (w/v) skim milk dissolved in Tris-buffered saline with 0.05% (w/v) Tween 20 (TBST) and then incubated in the phospho-CREB (pCREB) primary antibody diluted 1:1000 in 5% (w/v) skim milk in TBST overnight at 4 °C on a shaking platform. The following day, the blots were washed with TBST and incubated in the secondary HRP-conjugated anti-rabbit antibody diluted 1:2000 in 1% (w/v) skim milk in TBST for 1 h at room temperature. The blots were washed with TBST, the SuperSignal® West Pico chemiluminescence substrate was applied as directed by the manufacturer, and the blots were wrapped in plastic foil and exposed to X-ray film. The antibodies were then stripped from the blots by incubation in the Restore™ Western Blot Stripping Buffer for 5 min at room temperature, followed by extensive washing with TBST. An analogous immunodetection procedure was performed again, starting from the blocking step, this time employing the CREB primary antibody (1:1000).
The developed films were scanned with ImageScanner (Amersham Pharmacia Biotech, now GE Healthcare Life Sciences, Little Chalfont, UK), and the resulting digitized images were analyzed with Scion Image software version Beta 4.0.3 (Scion Corporation, downloaded from http://www.scioncorp.com). The linear range of the chemiluminescence signal on the X-ray film was determined by preliminary experiments, and only those films whose exposure was in the linear range were evaluated. When the pCREB and/or CREB signal of the sample differed too much from the signal of the standard, the analysis was repeated with a different sample amount. Because the need for re-analysis was relatively frequent, for the most recent samples this tedious procedure was replaced by the filmless densitometry method described for the GSK assay below.

In both methods of analysis, the integrated densities (equivalent to volumes of detected bands) from 2 to 3 films or the band volumes from 2 to 3 images were averaged. Both pCREB and CREB immunoreactivities were normalized to the THP-1 standards present on each gel and reported as the pCREB/CREB ratio.

It should be noted that the pCREB antibody employed in this study was virtually free from confounding cross-reactivity with pyruvate dehydrogenase, which had been found previously with other pCREB antibodies (Platenik et al., 2005).

2.5. Assay for GSK3β in platelets by Western blot

The PRP samples for Western blot analysis consisted of platelets at 80% of their in vivo concentration together with 8% (v/v) blood plasma, corresponding to approximately 5.6 μg/ml of ‘carrier’ protein. The Western blot procedure used for the detection of GSK was analogous to the one described above for CREB, but in this case, no stripping was employed, and instead, the phospho-GSK3β (pGSK3β) and GSK3β signals were obtained separately from different blots processed in parallel. Nine microliters of the denatured sample (approximately 50 μg of the ‘carrier’ protein) was applied on a 4.5%/10% SDS-polyacrylamide gel for analysis of pGSK3β and GSK3β signals were obtained separately from different blots processed in parallel. Nine microliters of the denatured sample (approximately 50 μg of the ‘carrier’ protein) was applied on a 4.5%/10% SDS-polyacrylamide gel for analysis of pGSK3β and GSK3β. The western blot signal was detected using a Fusion FX7 imaging system (version 5.04 for Windows (GraphPad Software, Inc., San Diego, CA) was employed for data analysis). The results from duplicate wells were averaged, corrected for the blank reading, and the concentrations of BDNF in the samples were interpolated from a third order polynomial calibration curve. Dilution of samples was taken into account, and the results were ultimately expressed as ng BDNF per ml PRP.

The samples of EDTA-containing plasma for measurement of BDNF were also stored at −85 °C until use. After thawing, they were centrifuged at 10,000 × g for 10 min to remove any eventual chylosity. Human BDNF Quantikine® Immunoassay kits were used precisely as directed by the manufacturer. Both the samples and the standards (0–4000 pg/ml recombinant BDNF) were assayed in duplicate. The absorbance readings at 540 nm were subtracted from those at 450 nm. Using GraphPad PRISM software, the duplicates were averaged and corrected for the blank reading, and the second or third order polynomial calibration curve was calculated to determine the concentration of BDNF in each sample. Approximately one-third of the samples yielded values above the highest standard (4000 pg/ml) and were subsequently re-assayed following a suitable dilution.

The values of BDNF in PRP (ng/ml) and plasma (ng/ml) were used for calculation of the level of BDNF in platelets (ng/10⁸ platelets).

2.7. Statistical analysis

Statistical analyses were performed using the STATISTICA data analysis software system (version 10.0, StatSoft, Inc., Tulsa, OK, USA). The data normality was tested by Shapiro–Wilk’s W test. All data are presented as the mean ± standard deviation except for pGSK3β/GSK3β, pCREB/CREB, BDNF_plasma, BDNF_PRP, and BDNF_platelet, which did not fulfill the criteria of a normal distribution. The hypothesis that the logarithmic distribution of these parameters is a normal distribution was not rejected. Therefore, parametric statistics were used for analysis of log-transformed data, and the mean values and SD-derived error intervals were determined by inverse transformation of log(mean ± SD).

The general linear model was used to analyze all measurements, including those that can be conducted via analysis of variance (ANOVA) or analysis of covariance (ANCOVA) and Scheffé or Dunnett post-hoc comparison between means.

A factor analysis was performed for multiple comparison of clinical and biochemical data to determine groups of parameters related to common factors. Data for AD and depressive patients were analyzed separately. Method of principal components was used for factor extraction. According to Kaiser criterion we retained factors...
with an eigenvalue greater than 1, then varimax rotation method was used to yield a factor structure that is simplest to interpret. Factor loadings were calculated and interpreted in terms of association of clinical parameters (GDS, MMSE, HRSD-21, and CGI-S) and potential biomarkers (pCREB/CREB, pGSK3β/GSK3β, BDNF_plasma, BDNF_PRP, and BDNF_platelet).

Stepwise multiple regression analysis for the continuous dependent variable on the continuous predictors was used with backward elimination of predictors. Biochemical parameters (GSK3β, pGSK3β, pGSK3β/GSK3β, pCREB/CREB, BDNF_plasma, BDNF_PRP, and BDNF_platelet) were used as dependent variables; age, BMI, education, platelet concentration in PRP and clinical variables (HRSD-21 and CGI-S for depressive patients, GDS and MMSE for AD patients) were used as predictors. The partial correlation coefficients (p) were calculated to characterize relationships between biochemical and clinical variables, controlling for age, education, BMI, and platelet concentration.

3. Results

Personal and clinical parameters were acquired from patients and controls, including age, education, body mass index (BMI), MMSE, GDS, HRSD-21 and CGI-S (Table 1). Next, biochemical parameters were determined in blood samples of AD patients, depressive patients and controls: GSK3β, pGSK3β and pGSK3β/GSK3β ratio in platelets, pCREB/CREB ratio in lymphocytes (all in arbitrary units), BDNF in plasma (BDNF_plasma; ng/ml), BDNF in PRP (BDNF_PRP; ng/ml), and BDNF in platelets (BDNF_platelet; ng/10^8 platelets).

BDNF_platelet was calculated from BDNF_PRP by subtraction of BDNF in plasma (BDNF_plasma; ng/ml), BDNF in platelets (BDNF_platelet; ng/10^8 platelets). BDNF_platelet was calculated from BDNF_PRP by subtraction of BDNF in plasma (BDNF_plasma; ng/ml), BDNF in platelets (BDNF_platelet; ng/10^8 platelets). BDNF_platelet was calculated from BDNF_PRP by subtraction of BDNF in plasma (BDNF_plasma; ng/ml), BDNF in platelets (BDNF_platelet; ng/10^8 platelets).

While a slightly lower mean BDNF_PRP was found in men compared to women in the control group, statistical analysis (ANCOVA and post-hoc Dunnett test) were used to determine indicated p-values compared to controls; *p < 0.05, **p < 0.01, ***p < 0.001.

BDNF PRP was observed in AD patients compared to controls (p = 0.0089) and depressive patients (p = 0.0072). The decrease in BDNF_PRP in AD patients with moderate to severe stages of dementia (p = 0.0027) was responsible for this effect (Table 2). The significant decrease of BDNF PRP was observed in AD patients and depressive patients (expressed as ng per ml) and not in platelets (expressed as ng per 10^8 platelets) in AD patients can be explained by a slightly lower (approximately 15%) mean platelet concentration in AD patients compared to controls.

Compared to controls, no significant difference was found in patients with depressive disorder for BDNF_plasma, BDNF_PRP and BDNF_platelet. Thus, the mean values of these parameters are not displayed in Table 2.

3.2. Association between variables

Factor loadings were calculated for clinical and biochemical data from AD and depressive patients respectively (Table 3).

There were three factors found in depressive patients before and after treatment with cumulative percent of total variance 65%. Factor 2 evidenced correlation of GDS with pCREB/CREB, pGSK3β/GSK3β, and BDNF_plasma; Factor 3 indicated association of MMSE with GDS and pGSK3β.

Three main factors were found in depressive patients before and after treatment with cumulative percent of total variance 66% and 64% respectively. Factor loadings indicate low association of both HRSD-21 and CGI-S with biochemical parameters. There is some association between HRSD-21 and PCREB/CREB or pGSK3β/GSK3β before treatment. Subgroup of drug-naive depressive patients showed three factors with cumulative percent of total variance 84% and 85% respectively; factor loadings indicate association of HRSD-21 with PCREB/CREB and BDNF_PRP.

The associations between age and the measured biochemical parameters were determined. Using data from controls, we calculated partial correlation coefficients (controlled for BMI, education, and platelet concentration in PRP) between age and the following parameters: GSK3β (ρ = −0.327; p = 0.013), pGSK3β (ρ = −0.296; p = 0.025), pGSK3β/GSK3β (ρ = −0.224; p = 0.093), pCREB/CREB (ρ = 0.140; p = 0.24), BDNF_plasma (ρ = −0.085; p = 0.48), BDNF_PRP (ρ = −0.238; p = 0.046) and BDNF_platelet (ρ = −0.302; p = 0.010). Thus, there was a significant association between age and GSK3β, pGSK3β, BDNF_PRP and BDNF_platelet. The age dependency of pGSK3β/GSK3β, pCREB/CREB, and BDNF concentrations in plasma, PRP and platelets are shown in Fig. 1.

Table 1

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Alzheimer’s disease</th>
<th>Depressive disorder</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>All</td>
<td>With depression</td>
<td>Without depression</td>
</tr>
<tr>
<td>Age (years)</td>
<td>75.6 ± 7.7</td>
<td>76.1 ± 6.7</td>
<td>75.2 ± 8.4</td>
</tr>
<tr>
<td>Education (years)</td>
<td>13.9 ± 2.8</td>
<td>14.0 ± 2.9</td>
<td>13.8 ± 2.7</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>28.1 ± 3.4</td>
<td>25.6 ± 4.0</td>
<td>22.9 ± 2.3</td>
</tr>
<tr>
<td>MMSE</td>
<td>20 ± 6.9</td>
<td>19.7 ± 6.4</td>
<td>18.2 ± 7.2</td>
</tr>
<tr>
<td>GDS</td>
<td>60 ± 3.6</td>
<td>60 ± 2.1</td>
<td>60 ± 1.4</td>
</tr>
<tr>
<td>HRSD-21</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>CGI-S</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>N (women/men)</td>
<td>85 (51/34)</td>
<td>36 (22/14)</td>
<td>49 (29/20)</td>
</tr>
</tbody>
</table>

Data are displayed as the mean ± SD. ANOVA and post-hoc Dunnet test were used to determine indicated p-values compared to controls; *p < 0.05, **p < 0.01, ***p < 0.001. BMI—body mass index; MMSE—Mini-Mental State Examination; GDS—Geriatric Depression Scale; HRSD-21—21-item Hamilton Rating Scale for Depression; CGI-S—Clinical Global Impression—Severity scale.
Table 2
GSK3β activity, CREB activity and BDNF concentrations in blood components of patients with Alzheimer’s disease and controls.

<table>
<thead>
<tr>
<th>Characteristic (blood component)</th>
<th>Alzheimer’s disease</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>All</td>
<td>With depression</td>
</tr>
<tr>
<td>pGSK3β/(GSK3β)</td>
<td>2.00</td>
<td>1.62</td>
</tr>
<tr>
<td>(platelet)</td>
<td>(0.91; 4.36)</td>
<td>(0.69; 3.82)</td>
</tr>
<tr>
<td>pCREB/CREB</td>
<td><strong>272</strong></td>
<td><strong>309</strong></td>
</tr>
<tr>
<td>(lymphocyte)</td>
<td>(169; 440)</td>
<td>(179; 531)</td>
</tr>
<tr>
<td>BDNF_plasma (ng/ml)</td>
<td>1.72</td>
<td>1.23</td>
</tr>
<tr>
<td>(plasma)</td>
<td>(0.55; 5.38)</td>
<td>(0.42; 3.63)</td>
</tr>
<tr>
<td>BDNF_PRP (ng/ml)</td>
<td>2.66</td>
<td>2.13</td>
</tr>
<tr>
<td>(platelet-rich plasma)</td>
<td>(16.2; 43.8)</td>
<td>(17.0; 43.6)</td>
</tr>
<tr>
<td>BDNF_platelet (ng/10^9 platelets)</td>
<td>6.7</td>
<td>6.9</td>
</tr>
<tr>
<td>(platelet)</td>
<td>(4.5; 9.8)</td>
<td>(4.8; 10.0)</td>
</tr>
</tbody>
</table>

Data are displayed as the mean and an error interval determined as (mean ± SD; mean + SD); mean values and SD-derived error intervals of logarithmically transformed data (log(GSK3β)/pGSK3β), log(pCREB/CREB), log(BDNF_plasma), log(BDNF_PRP), and log(BDNF_platelet)) were determined by inverse transformation of log(mean + SD). ANOVA and post-hoc Dunnett test were used to determine indicated p-values compared to controls, controlling for age; *p < 0.05, **p < 0.01, ***p < 0.001.

GSK3β—glycogen synthase kinase 3β; pGSK3β—phosphorylated GSK3β; CREB—cAMP response element-binding protein; pCREB—phosphorylated CREB; BDNF—brain-derived neurotrophic factor.

Partial correlation coefficients, characterizing relationships between biochemical variables and clinical scores, were rated using backward stepwise multiple regression analysis. The dependence of the measured biochemical parameters on education or BMI was not significant; however, adjustment for age, BMI, education and platelet concentration in PRP was used when relationships were analyzed between biochemical and clinical variables. Adjustment for age was necessary because the mean age of AD patients was significantly higher compared to controls or depressive patients. An adjustment for platelet concentration in PRP was required because a significant difference was found between BDNF_platelet and platelet concentration in PRP in both AD patients with depression (p = 0.600; p < 0.001) and AD patients without depression (p = 0.641; p < 0.001).

In AD patients, the only detected significant association was between pGSK3β and GDS (p = 0.252; p = 0.049) or between GSK3β and MMSE (p = 0.019) in AD patients with mild dementia (Table 4).

In depressive patients before treatment (baseline measurement), a significant association was found between pGSK3β and HRSD-21 (p = 0.0013) (Table 5). In depressive patients after treatment, a significant association was found between GSK3β and HRSD-21 (p = 0.0034).

When only the data from responders (N = 49) were evaluated, a significant association was found between BDNF_plasma and HRSD-21 (p = 0.0025) in depressive patients after treatment. In non-responders (N = 16), pGSK3β (p = 0.0061) and pGSK3β/GSK3β (p = 0.0049) were significantly associated with HRSD-21 before treatment. The subgroup of drug-naïve depressive patients (N = 16) displayed significant associations between HRSD-21 and BDNF in both PRP (p = 0.040) and platelets (p = 0.0080) before treatment.

4. Discussion

Apparent negative correlations that were observed between age and BDNF concentration in PRP or in platelets (Fig. 1) may indicate decreased natural neurotrophic action of BDNF in the elderly and possibly link to age-related neurodegenerative disorders.

4.1. AD patients

Hye et al. (2005) reported an increase in GSK3β protein without a compensatory decrease in activity in white blood cells obtained from...
patients with AD or mild cognitive impairment compared to healthy controls. This report is in agreement with our finding of a decreased pGSK3β/GSK3β ratio in AD with co-morbid depression (Table 2). Factor analysis (Table 3) indicates that presence of depressive symptoms in AD patients may be responsible for association of GSK3β activity with AD. A significant negative correlation between MMSE and total platelet nuclear GSK3β activity in AD patients with mild dementia (Table 4) supports also the finding of a decreased pGSK3β/GSK3β ratio in AD patients with mild dementia (Table 2). Determination of the activity of both GSK3β and CREB in lymphocytes of AD patients with depressive symptoms or with mild stage of dementia. It appears that a higher CREB activity in lymphocytes of AD patients could be associated both with the AD and the presence of co-morbid depressive symptoms (Tables 2 and 3). Determination of the underlying mechanism is likely to be difficult because approximately 300 different stimuli that can provoke CREB phosphorylation have been described in the literature (Johannessen et al., 2004). We hypothesize that chronic inflammatory processes accompanying depressive

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Table 4
Partial correlation coefficients characterizing relationships between biochemical variables and Mini-Mental State Examination (MMSE) score in patients with Alzheimer’s disease (AD).

<table>
<thead>
<tr>
<th></th>
<th>GSK3β</th>
<th>pGSK3β</th>
<th>pGSK3β/GSK3β</th>
<th>pCREB/CREB</th>
<th>BDNF_plasma</th>
<th>BDNF_PRP</th>
<th>BDNF_platelet</th>
</tr>
</thead>
<tbody>
<tr>
<td>All</td>
<td>−0.136</td>
<td>0.024</td>
<td>−0.080</td>
<td>−0.001</td>
<td>−0.045</td>
<td>0.013</td>
<td>0.007</td>
</tr>
<tr>
<td>With depressive symptoms</td>
<td>0.024</td>
<td>−0.285</td>
<td>−0.321</td>
<td>−0.089</td>
<td>0.061</td>
<td>0.251</td>
<td>0.180</td>
</tr>
<tr>
<td>Without depressive symptoms</td>
<td>−0.186</td>
<td>0.097</td>
<td>0.031</td>
<td>0.039</td>
<td>−0.087</td>
<td>0.000</td>
<td>−0.042</td>
</tr>
<tr>
<td>With mild dementia</td>
<td>−0.516</td>
<td>−0.071</td>
<td>−0.224</td>
<td>−0.070</td>
<td>−0.070</td>
<td>0.079</td>
<td>0.039</td>
</tr>
<tr>
<td>With moderate to severe dementia</td>
<td>−0.140</td>
<td>−0.030</td>
<td>−0.020</td>
<td>−0.076</td>
<td>−0.035</td>
<td>0.026</td>
<td>0.015</td>
</tr>
</tbody>
</table>

Partial correlation coefficients, characterizing relationships between biochemical variables and MMSE score, were rated using backward stepwise multiple regression analysis, controlling for age, education, BMI, concentration of platelets in platelet-rich plasma and GDS score. Significance level: *p < 0.05.

GSK3β—glycogen synthase kinase 3β; pGSK3β—phosphorylated GSK3β; CREB—cAMP response element-binding protein; pCREB—phosphorylated CREB; BDNF—brain-derived neurotrophic factor; PRP—platelet-rich plasma; BMI—body mass index; GDS—Geriatric Depression Scale.
disorder (Maes et al., 2011) and AD (Balldin et al., 2012; Rubio-Perez and Morillas-Ruiz, 2012) lead to lymphocyte activation (Ichiki, 2006) and the observed increase of CREB activity in lymphocytes.

A significantly lower BDNF concentration in PRP was detected in AD patients with moderate to severe stages of dementia, but not in AD patients with a mild stage of dementia (Table 2). This result supported earlier findings that the BDNF serum concentration is decreased in AD patients (Forlenza et al., 2010; Laske et al., 2007) and that changes in BDNF serum concentrations depend on the severity of AD (Laske et al., 2006). Our results confirm that there is no significant correlation between CREB activation in lymphocytes and BDNF concentration in PRP (Table 3). Therefore, there is no direct connection between CREB activity and BDNF concentration in peripheral blood, and these biochemical parameters should be assessed separately.

Contrary to the decrease of BDNF in PRP of AD patients, there was no significant difference in BDNF concentration in either plasma or platelets of AD patients compared to controls (Table 2). This result can be explained by a slightly lower platelet concentration (approximately 15%) in blood of AD patients compared to controls. While subtle, an age-dependent decrease of BDNF concentration in PRP (Fig. 1) may also participate in this effect because controls were age-matched to depressive patients, but not to AD patients (Table 1). We hypothesize that this possible confounder was resolved by adjustment for age in our statistical analysis. Recently, it was demonstrated that mean platelet volume, a marker of platelet activation, is decreased in AD patients (Wang et al., 2013), which indicates that BDNF may be more concentrated in platelets of AD patients than controls.

4.2. Depressive disorder patients

Platelet GSK3β activity and lymphocyte CREB activity were increased in depressive patients after treatment. Observation of decreased phosphorylation of GSK3β in platelets of depressive patients after treatment confirms the role of increased GSK3β activity in the pathophysiology of the depressive disorder. We suppose that the increase of CREB activity in depressive subjects after treatment reflects effect of pharmacotherapy.

BDNF plasma concentrations were not significantly changed in depressed patients either before or after treatment compared to controls. This result may be explained by an earlier treatment, since most of the patients studied were already being treated with antidepressants prior to their admission into this study. In our study, only 16 depressive patients had not been treated by any antidepressants for at least 6 weeks. However, these drug-naïve patients did not show any significant differences in the mean values of the measured biochemical parameters compared to controls.

We found that lower pGSK3β concentration may be associated with a higher severity of depression (higher HRSD-21) in depressed patients before treatment, and this effect was more pronounced in non-responders (Tables 3 and 5). Phosphorylation of GSK3β on the regulatory residue serine-9 generates an intra-molecular pseudo-substrate, which then blocks the enzyme active site (Hooper et al., 2008). Most likely, the level of phosphorylated GSK3β reflects the activity of the upstream kinase Akt. If a decreased level of pGSK3β in platelets indicates increased GSK3β activity, our observation indicates an association of higher GSK3β activity with a greater severity of depression, which is in accordance with the hypothesis that inhibition of GSK3β by lithium (Diniz et al., 2013) and modulation of GSK3β by other psychotropic drugs contribute to the effects of these compounds (Li and Jope, 2010; Maes et al., 2012).

The significant negative correlation between HRSD-21 and BDNF in the plasma of responders after treatment (Table 5) indicates that higher plasma BDNF is associated with lower severity of residual depression after several weeks of therapy in depressive patients. This result supports the previous finding that a partial normalization of serum BDNF levels occurs in remitted patients (Gervasoni et al., 2005).

Interestingly, even in the small group of 16 drug-naïve patients, we observed a significant negative correlation between the severity of depression and BDNF levels in both PRP and platelets (Table 5). Some association between HRSD-21 score and BDNF concentrations in PRP was confirmed by factor analysis in drug-naive depressive patients before treatment (Table 3). This result is entirely consistent with the previous finding of a negative correlation between serum BDNF levels and the severity of depression (Karege et al., 2002).

Some limitations of this study involve the unresolved effect of pharmacotherapy on platelet GSK3β activity, lymphocyte CREB activity and BDNF concentration in patients with depressive disorder. The majority of patients were treated primarily with selective serotonin reuptake inhibitors. However, balanced pharmacotherapy was used, which involved a combination of several antidepressants and benzodiazepines for treatment. Mood stabilizers or antipsychotics were used in all patients which involved a combination of several antidepressants and benzodiazepines for treatment. Mood stabilizers or antipsychotics were used in some cases. Therefore, our data did not allow for analysis of the effects of different classes of drugs on the measured biochemical parameters. However, it was demonstrated previously that there is no significant effect of treatment with antidepressants on GSK3β protein expression in platelets from patients with major depressive disorder (Pandey et al., 2010b). Consistent with these findings, we did not detect significant differences in the mean values of the measured biochemical parameters between drug-naïve and antidepressant treated patients.

5. Summary and conclusions

Factor analysis revealed that depressive symptoms co-morbid with AD contribute to the association of GSK3β activity with the AD. Decreased phosphorylation of GSK3β in platelets of both AD patients with depressive symptoms and depressive patients after treatment confirms the role of increased GSK3β activity in the pathophysiology of both AD and depressive disorder. A significant correlation between cognitive impairment and total GSK3β in AD patients with mild dementia indicates that platelet GSK3β may reflect the pathophysiological changes of AD during the early stages of the disease. A significant partial correlation between severity of depressive disorder and GSK3β activity in
nonresponders indicates potential role of platelet GSK3β activity as a marker of response to pharmacotherapy.

We hypothesize that the increase of CREB activity in lymphocytes of AD patients with depressive symptoms or with a mild stage of dementia is linked to activation of cellular defense mechanisms related to neuroinflammation.

Supposing that the lower amount of BDNF in PRP reflects a decrease in brain BDNF, our data confirms the role of BDNF in pathophysiology of AD. Association of severity of depressive disorder with CREB activity and BDNF concentration was confirmed only for drug-naïve patients.

In summary, we examined the association of CREB activity, GSK3β activity and BDNF concentration in peripheral blood with clinical parameters of AD and depressed patients. Our findings indicate that mean value of some of the measured blood-based biochemical parameters are altered in AD, but not in depressive disorder. However, associations of these biochemical parameters with disease severity were observed in both AD and depression. Platelet GSK3β activity and lymphocyte CREB activity and plasma BDNF seem to be related to both AD; CREB activity and platelet BDNF concentration seem to be related to depressive disorder.

Disclosure of interest

The authors declare that they have no conflicts of interest concerning this article.

Acknowledgments

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References


Neurotrophins and inflammation, dis-