The role of BDNF in depression : will the neurothrophin hypothesis sparkle on, long after the glitter of the firework is gone?

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CHAPTER 2

Determinants of serum BDNF concentrations

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SIGNIFICANCE: In this study we sketch the basic determinants of serum BDNF concentrations. Herewith we offer an improved base to understand inter-individual differences in serum BDNF concentrations and knowledge that is essential in preventing erroneous inferences from data.

ABSTRACT

Brain-Derived Neurotrophic Factor (BDNF) belongs to the neurotrophin family of growth factors and affects the survival and plasticity of neurons in the adult central nervous system. The high correlation between cortical and serum BDNF concentrations has led to many human studies on BDNF concentrations in various populations, however knowledge about determinants that influence BDNF is lacking. To gain insight into the factors that influence BDNF concentrations in humans, we measured in 1,168 people aged 18 through 65, free of antidepressants and current psychiatric disease four categories of determinants (sampling, socio-demographics, lifestyle indicators and diseases) were measured as well as serum BDNF concentrations. We used univariable analyses and multivariable linear regression analyses in particular to determine which of the possible determinants significantly influenced serum BDNF concentrations. Our final multivariable regression analysis revealed that a non-fasting state of blood draw, later measurement, longer sample storage and being a binge drinker all were associated with attenuated BDNF concentrations. This was in contrast to smoking and living in an urban area, which was associated with increased BDNF concentrations. Moreover we found that older subjects also had higher BDNF concentrations, but this only applied to women. Future studies on serum BDNF concentrations in humans should correct for the time of blood withdrawal, duration of serum storage, urbanicity, age, gender, smoking status and food and alcohol intake.

INTRODUCTION

Brain-derived neurotrophic factor (BDNF) belongs to the neurotrophin family of growth factors and affects the survival and synaptic plasticity of neurons in the adult nervous system (Mossner *et al*., 2007). BDNF binds with the TrkB tyrosine kinase receptor (Chao, 2003), which results in intracellular phosphorylation and activation of signaling cascades that lead to activation of pro-survival pathways (Ullrich and Schlessinger, 1990). BDNF is expressed throughout the central nervous system (Binder and Scharfman, 2004) and significant concentrations are also found in peripheral blood (Sen *et al*., 2008). BDNF crosses the blood-brain barrier by a saturable transport system (Pan *et al*., 1998). In animal models, cortical BDNF concentrations are highly correlated with peripheral serum BDNF concentrations (Sartorius *et al*., 2009), but evidence on the contrary has been reported as well (Elfving *et al*., 2010). Inconsistencies might be explained by the fact that BDNF-expression and its TrkB receptor is not specific for neuronal cells, but can also be found in endothelial cells in peripheral tissues (Esteban *et al*., 1995; Hiltunen *et al*., 1996; Donovan *et al*., 2000). The supposed importance of neuroplasticity in both the etiology and recovery of psychiatric disorders, has led to many studies linking serum BDNF concentrations to a wide variety of psychiatric and neurodegenerative diseases (Nakazato *et al*., 2003; Azoulay *et al*., 2005; Yasutake *et al*., 2006; Ciammola *et al*., 2007; Ikeda *et al*., 2008). For proper interpretation of serum BDNF concentrations in humans, however, knowledge about determinants of serum BDNF concentrations is essential for adequate control of confounding factors.

As touched above, BDNF is not only considered to be a neurotrophin, but also an immunotrophin, epitheliotrophin and metabotrophin (Chaldakov *et al*., 2007). As of yet somatic conditions that have been correlated to BDNF mainly include cardiovascular disease or cardiovascular risk factors or disease (Ejiri *et al*., 2005; Fujinami *et al*., 2008; Hristova and Aloe, 2006; Suwa *et al*., 2006; Geroldi *et al*., 2006). For example, patients with acute coronary syndrome as well as patients with diabetes mellitus do have lower plasma concentrations of BDNF (Manni *et al*., 2005; Fujinami *et al*., 2008). However, spurious associations may be explained due to (unknown) confounding factors, since health indicators like alcohol use, smoking and physical exercise have also been linked to BDNF (Chan *et al*., 2008; Tang *et al*., 2008; Umene-Nakano *et al*., 2009). Even more basic determinants, like sampling characteristics have not specifically been examined regarding their association with serum BDNF concentrations. Although most human studies control for gender and age effects, reported effects of gender and age are contradictory. These mixed results may be explained by small sample sizes ranging from as low as 10 (Marano *et al*., 2007) through a maximum of 465 participants (Ziegenhorn *et al*., 2007), as well as failure to control for potentially confounding factors (Aydemir *et al*., 2007). Finally, possible effects of sampling characteristics on serum BDNF concentrations have hardly been examined. The few studies available, however, do suggest a decrease of BDNF concentrations after longterm storage (Trajkovska *et al*., 2007) and a diurnal variation within individuals (Piccinni *et al*., 2008). Considering the broad interest in BDNF and the many factors associated with serum BDNF concentrations, it is surprising that no studies have been conducted to determine the effects of potential determinants of serum BDNF concentrations. Therefore, we examined the determinants (sampling characteristics, socio-demographic variables, lifestyle indicators and [chronic] diseases) of serum BDNF in a large cohort of people without current psychiatric diseases.

METHODS

Data are from the baseline measurement of the Netherlands Study of Depression and Anxiety (NESDA). NESDA is a multisite naturalistic cohort study aimed to describe the 8-year course and consequences of depressive and anxiety disorders and to integrate biological and research paradigms

within an epidemiological approach. Recruitment took place in the general population, in general practices and in mental health organizations. The baseline sample consists of 2,981 participants aged 18 through 65 years of which 2,625 were selected on the basis of a current or life-time history of depression and/or anxiety disorder, belonging to a high-risk group because of a family history or sub-threshold depressive or anxiety symptoms, and 356 healthy controls. Patients with a primary psychiatric disorder not subject of NESDA, such as psychotic disorder, obsessive-compulsive disorder, bipolar disorder, or severe addiction were excluded as well as those not being fluent in Dutch. The Medical Ethics Commission of the participating institutes approved of this study and all respondents provided written informed consent.

All respondents received a structured diagnostic interview using the Composite International Diagnostic Interview (CIDI version 2.1) in order to assess current and lifetime DSM-IV diagnoses. The 4 hour baseline assessment further included written questionnaires, interviews, a medical examination, and collection of blood and saliva samples, in order to gather extensive information about key (mental) health outcomes and demographic and clinical determinants. All measurements and interviews took place were carried out by our specially trained staff according to a previously determined protocol. For a detailed description of the objectives and methods of NESDA, see Penninx *et al*. (2008). Based on our previous study (Molendijk *et al*., 2010) showing that BDNF was associated with both current psychopathology and antidepressant usage, but not with trait psychopathology or sub-threshold symptoms, we excluded only participants with a current DSM-IV diagnosis of a depressive disorder or anxiety disorder in the last 6 months (n = 1,688) and those who were using any kind of antidepressant medication (n = 736). This resulted in a study population of 1,198 participants. Of these, 30 did not have serum BDNF assessment and 3 samples were below the detection limit. These persons therefore were excluded, leaving a final sample of 1,165 for the present study.

BDNF assessment

Blood was collected (between 06:20 and 12:30 h) after an overnight fast and immediately transferred to one of the five local laboratory-sites to start processing within 1 h. Serum samples for BDNF assessment were stored at -85 C˚. After concluding the baseline assessment, serum samples were sent (ranging from 22 to 60 months after withdrawal) to the Department of Psychiatry and Neuropsychology in Maastricht (The Netherlands) for BDNF measurements. Serum BDNF protein concentrations were measured within 3 months after their receipt using the Emax Immuno Assay system from Promega according to the manufacturer’s protocol. The undiluted serum was acid treated, which in a dilution-dependent way reliably increased the detectable BDNF. Subsequently, serum samples were diluted 100 times and stored again at -85 C˚ for BDNF assay the next day. After dilution, the BDNF concentrations were well within the range of the standard curve. The assay sensitivity threshold was ascertained at 1.56 ng/ml reflecting the minimum level of BDNF in the serum that could be reliably determined. Three samples were below this threshold and deleted for all subsequent analyses (see also above). In our pilot study we had found that BDNF concentrations of acid-treated samples with subsequent dilution the day preceding the BDNF assay did not differ from the concentrations obtained in samples following acid treatment only on the preceding day, or from those derived after acid treatment conducted the day of the BDNF measurement. The serum samples used in our pilot study were stored samples of six individuals who did not participate in the present study. The samples’ coefficients of variance ranged from 2.9% to 8.1%. To gauge the intra-assay variance for the present study, we analyzed two of our current samples on two different plates on the same day. The resultant coefficients of variance of 0.1% and 3.1% were both well below the maximum

intra-assay variance of 8.8% as specified by the manufacturer. Greiner Bio-One high affinity 96-well plates were used and the resulting absorbance was read in duplicate using a Biorad Benchmark microplate reader at 450 nm.

Potential determinants of BDNF

Sampling variables

The sampling variables that were tested consisted of time of blood withdrawal (minutes after 6.00 a.m.), the number of minutes a sample was kept in a cool box before being processed in the local laboratory, the duration of sample storage at -85 C˚ (days) and finally non-adherence to the pretest fasting protocol.

Socio-demographic variables

The socio-demographic variables that were tested consisted of gender (male/female), age (years), urbanicity of living environment of participant (urban/not urban) and years of education. An urban environment was defined according to the classification of the Dutch office for statistics (CBS) and dichotomized in more or less than 1000 addresses per square kilometer. As a previous study of our group suggested the presence of an age effect only in women (Bus *et al*., unpublished observations), we were also interested in the interaction between age and gender a priori.

Health indicators

The health indicators that were tested in this study included presence of metabolic syndrome, smoking, physical activity (met-minutes) and drinking (abstainer, mild, moderate, excessive) and body mass index (BMI). Metabolic syndrome was defined according to the updated Adult Treatment Panel III (ATP III) guidelines of the US *national* cholesterol education program (Grundy *et al*., 2005) in which metabolic syndrome is considered present if a participant meets at least three of the following criteria: (1) elevated waist circumference (men > 102 cm; women > 88 cm); (2) elevated triglycerides (> 150 mg/dl); (3) reduced HDL cholesterol (men < 40 mg/dl; women < 50 mg/dl); (4) elevated blood pressure (> 130/85 mm Hg or use of medication for hypertension); or (5) elevated fasting glucose (> 5.6 mmol/l or use of medication for hyperglycemia). As a measure for physical activity we used Metabolic Equivalent of Task (MET)-minutes. A MET-minute is a ratio of the amount of energy expenditure during an activity to the expenditure at rest, which was calculated on the basis of the international physical activity questionnaire (Craig *et al*., 2003). Smoking was dichotomized into current smokers versus non-smokers. In addition to current smoking, we also collected information on smoking status in the past (*i.e.,* age of starting, age of quitting) and the average number of cigarettes smoked a day. The number of package years was calculated with one package year defined as smoking 25 cigarettes a day for the period of 1 year (= 9,125 cigarettes). The Fagerstrom questionnaire was used to measure the severity of nicotine dependence (Heatherton *et al*., 1991). Alcohol use was evaluated by creating four groups: abstainers, mild drinkers (drinking less than 7 units a week), moderate drinkers (drinking 8-13 units a week) and excessive (drinking ≥ 14 units a week). Alcohol categories were entered as dummy variables. In addition the AUDIT was used to measure alcohol dependence (Babor *et al*., 1989). BMI was calculated by dividing weight by the squared height and entered as a continuous variable.

Disease indicators

Based on previous associations with serum BDNF concentrations in humans, we evaluated the presence of Chronic Non- Specific Lung Disease (CNSLD; including asthma, chronic bronchitis and

chronic emphysema) and coronary artery disease (Fujinami *et al*., 2008). The latter was defined as self-reported vascular events due to atherosclerotic disease (*i.e.,* stroke, angina pectoris, myocardial infarction or a history of percutaneous transluminal coronary angioplasty or coronary artery bypass grafting). Stroke, myocardial infarction and angina pectoris were only considered present if supported by appropriately prescribed medication use.

Statistical analysis

Serum BDNF concentrations were normally distributed, with the exception of 12 (0.5%) positive outliers (*i.e.,* level higher than 3 standard deviations (SD) above mean). Outliers were handled by trimming all serum BDNF concentrations above 3 *SD*s of the mean to the 3 *SD*s value. Univariable analyses were carried out using linear regression. We performed multiple linear regression analyses to assess independent determinants of BDNF. To facilitate the interpretation of the age-gender interaction, rather than taking absolute ages, we calculated and included the deviation from the mean, so that the participants' ages were centered round the sample's mean age. We generated multivariable models within each domain first by entering sampling characteristics, socio-demographic characteristics, health indicators and disease variables in four separate models. Subsequently, the independent predictors from all domain-specific models with *P*-values less than .15 were fitted into a final multivariate model. No collinearity or heteroscedasticity problems emerged. All tests were two-sided. A *P*-value of 0.05 was considered statistically significant. All analyses were carried out using SPSS 16.0 (SPSS, Chicago, IL, USA).

RESULTS

Sample characteristics

The 1,165 participants had a mean age of 42.5 years (SD = 14.1) and 65.0% (757) were female. The mean BDNF level was 8.98 ng/ml (SD 3.1 mg/ml) with a range from 1.56 ng/ml through 18.50 ng/ml. **Table 1 ↓** presents all further characteristics as well as the univariable associations of these characteristics with serum BDNF concentrations. As shown in **Table 1 ↓**, a lower serum BDNF level was associated with non-adherence to pretest fasting protocol (ß = 0.07; *P* = .01), sampling at a later day-time (ß = 0.07; *P* = .02), a longer time of storage (ß = 0.09; *P* = .002), high alcohol intake (excessive drinkers versus others (ß = 0.073; *P* = .020) and finally increased physical activity (ß = 0.06; *P* = .03). A higher BDNF serum level was found in older patients (ß = 0.21; *P* < .001), a higher degree of urbanicity (ß = 0.15; *P* < .001), current smokers (ß = 0.07; *P* = .01) and in patients suffering from the metabolic syndrome (ß = 0.06; *P* = .03) or coronary artery disease (ß = 0.08; *P* = .005).

Multivariable analyses

From the four models, categorized by variable type, we identified eleven significant determinants. Participants that had eaten prior to blood withdrawal had significantly lower serum BDNF concentrations (ß =0.07; *P* = .02). Sampling later during the morning resulted was associated with lower serum BDNF level (ß = 0.07; *P* = .01) as well as longer sample storage (ß = 0.08; *P* = .005). Serum BDNF concentrations in women significantly increased with age (ß = 0.23; *P* < .001), but also in men (ß = 0.10; *P* = .03). The age change was significantly different in women compared to men, as was indicated by the significant interaction between gender and age (ß = 0.08; *P* = .02).

Subjects who were living in a more urbanized area had higher BDNF concentrations (ß = 0.09; *P* = .002). Excessive drinkers had significantly lower serum BDNF concentrations compared with subject with more restraint drinking habits, as was indicated by the significant dummy variable (ß = 0.08; *P* =

.009). Current smokers had a significantly higher BDNF level compared to non-smokers (ß = 0.09; P = .003). Subjects with more physical activity had lower serum BDNF concentrations (ß = 0.06; P = .04).

Patients with cardiovascular disease had higher serum BDNF concentrations (ß = 0.08; P = .005). All these variables were entered in the final model. The presence of metabolic syndrome did not reach statistical significance (ß = 0.05; P = .12), but was entered into our subsequent model as well on the basis of a statistical significance at a $P < .15$ level. Our final multivariate regression analysis revealed that, when accounting for all potential determinants at once, having eaten prior to blood withdrawal (ß = 0.07; P = .01), measurement later on the day (ß = 0.06; P = .02), longer sample storage (ß = 0.08; P = .004), and being an excessive drinker (ß = 0.06; P = .03) all were associated with attenuated serum BDNF concentrations. This was in contrast to smoking (ß = 0.10; P = .001) and living in an urban area (ß = 0.11; $P < .001$), which resulted in increased BDNF concentrations. Moreover we found that older subjects also had higher BDNF concentrations, but this was only true for women (ß = 0.23; $P < .001$). The age change did not reach statistical significance for men (ß = 0.06; P = .26) and was significantly different from age change in women (ß = 0.10; P = .007)(see **Table 2 ↓**).

Table 1. Sample characteristics (N = 1,165) and univariable associations with serum BDNF concentrations

	Mean (*SD*) value or % (*n*)	Linear regression	
		ß	*P*-value
Sampling variables			
% fasting on withdrawal day	95.3 (*n* = 1,110)	-0.07	0.01
Time of sampling (mean minutes past 0600 h)	168 (*SD* = 20)	-0.07	0.02
Time in coolbox (mean minutes)	60 (*SD* = 43)	0.03	0.35
Duration of sample storage (#days)	1286 (*SD* = 228)	-0.09	< 0.01
Sociodemographics			
% female	65.0 (*n* = 757)	0.02	0.42
Age (mean in years)	42.5 (*SD* = 14.1)	0.21	< 0.01
% living in urban area	85.9 (*n* = 1,001)	0.15	< 0.01
Education level (mean in years)	12.7 (*SD* = 3.1)	0.05	0.12
Lifestyle indicators			
% currently smoking	30.3 (*n* = 353)	0.07	0.01
% with metabolic syndrome	19.1 (*n* = 222)	0.06	0.03
Physical activity (#mean MET-minutes)	3708 (*SD* = 3108)	-0.06	0.03
% abstinent of alcohol	44.8 (*n* = 522)	Ref.	
% mild drinker; <1U/day	34.2 (*n* = 399)	-0.07	0.82
% moderate drinkers; 1-2U/day	5.9 (*n* = 69)	-0.02	0.57
% excessive drinkers; >2U/day	3.6 (*n* = 42)	-0.07	0.02
BMI (height/weight^2)	25.3 (*SD* = 4.5)	0.04	0.22
Disease			
% with coronary artery disease	5.3 (*n* = 62)	0.08	< 0.01
% with CNSLD	10.0 (*n* = 117)	< 0.01	0.98

Abbrevations: SD = standard deviation; U = unit; BMI = body mass index; CNSLD = chronic non-specific lung disease; MET = metabolic equivalent of task

Post-hoc analyses

Significant associations in the univariable or multivariable analysis were analyzed in more depth by post hoc analyses using other definitions for the variable or including potentially explanatory variables. For example, in order to explain gender differences, we examined the use of oral contraception.

Gender differences

In order to further explore the gender differences, we assessed the influence of menopausal status and contraceptive pill use in women on BDNF concentrations. Since age and menopausal status are interwoven variables, we selected women in a narrow age range of 48-52 years of age. Since pre- and postmenopausal women were evenly dispersed (premenopausal n = 41; postmenopausal n = 40) in this age range, we minimized the confounding influence of age. The age variable was entered in a multiple regression together with a dichotomous menopausal status variable and the interaction between these two variables. We corrected for variables previously found to be of influence. Premenopausal women showed a significant increase in serum BDNF concentrations with age (ß = 0.46; P = .02) and differed significantly (P = .03) from post-menopausal women who showed a non-significant decrease (ß = -0.19; P = .43). Pre- and postmenopausal women's BDNF serum concentrations did not significantly differ (ß = 0.12; P = .43) at the age of 50.1 years (mean age of this sample). To test whether contraceptive pill use influenced BDNF concentrations we selected all premenopausal women and entered the variable: yes versus no contraceptive pill use) in our final model. This, however, revealed a non-significant result for contraceptive pill use (ß = 0.03; P = .53).

Table 2. Multiple linear regression analyses (per block and overall analyses)

	Multivariate per block				Final multivariate model			
Variables in four blocks	*B*	*SE*	β	*P*-value	*B*	*SE*	Β	*P*-value
Sampling variables								
Fasting on withdrawal day (0 = no, 1=yes)	-10.03	0.443	-0.07	0.02	-10.01	0.43	-0.07	0.02
Time of sampling (minutes after 0600h)	-0.01	< 0.01	-0.07	0.01	-0.01	< 0.01	-0.07	0.02
Time in coolbox (minutes)	< 0.01	< 0.01	0.01	0.75	-	-	-	-
Duration of sample storage (days)	-0.001	< 0.01	-0.08	< 0.01	< -0.01	< 0.01	-0.08	< 0.01
Sociodemographics								
Gender (0 = female, 1 = male)	0.10	0.19	0.02	0.58	0.06	0.19	0.01	0.74
Age (years deviating from mean age)	0.05	< 0.01	0.23	< 0.01	0.05	< 0.01	0.23	< 0.01
Interaction age times gender	-0.03	0.01	-0.08	0.02	-0.04	0.01	-0.10	< 0.01
Living in urban area (0 = no, 1=yes)	-0.84	0.27	0.09	< 0.01	-0.98	0.26	0.11	< 0.01
Education level (years)	0.04	0.03	0.04	0.19	-	-	-	-
Lifestyle indicators								
Current smoking (0 = no, 1 = yes)	0.599	0.20	0.09	< 0.01	0.66	0.19	0.10	< 0.01
Metabolic syndrome (*n*)	0.419	0.27	0.05	0.12	< 0.01	0.24	< 0.01	0.99
Physical activity (MET-minutes)	< -0.01	< 0.01	-0.06	0.05	< -0.01	< 0.01	-0.05	0.07
Alcohol drinking dummy (0 vs > 2U/day)	-10.31	0.50	-0.08	< 0.01	-10.03	0.49	-0.06	0.04
Alcohol drinking dummy (0 vs 1-2U/day)	-0.58	0.40	-0.04	0.15	-0.477	0.40	-0.04	0.23
Alcohol drinking dummy (0 vs < 1U/day)	-0.21	0.21	-0.03	0.31	0.05	0.20	< 0.01	0.80
Body mass index (weight/height^2)	< 0.01	0.02	0.01	0.77	-	-	-	-
Disease								
Coronary artery disease (0 = no, 1 = yes)	10.13	0.40	0.082	< 0.01	0.54	0.41	0.04	0.18
CNSLD (0 = no, 1 = yes)	< 0.01	0.30	< 0.01	0.99	-	-	-	-

Abbrevations: SD = standard deviation; *SE* = standard error; U = unit; CNSLD = chronic non-specific lung disease; MET = metabolic equivalent of task

Smoking

We created dummy variables for former and current smokers and subjects who have never smoked and entered these into our final model, thus correcting for potentially confounding factors. This

revealed that current smokers had significantly higher BDNF concentrations compared to those who have quit smoking (ß = 0.15; $P < .001$). However, no significant difference was found between current smokers and those who have never smoked (ß = 0.05; $P = .11$). Furthermore, no effect on serum BDNF concentrations was found of package years, actual number of cigarettes a day among smokers, as well as the severity of nicotine dependence as assessed with the Fagerstrom questionnaire (data not shown).

Alcohol use

In order to check whether the effect of alcohol intake on serum BDNF concentrations could be explained by the degree of alcohol dependence, we replaced the alcohol variables in our final model with the sum score of the AUDIT. No significant effect was found of the total AUDIT score on serum BDNF concentrations (ß = 0.05; $P = .10$).

Cardiovascular disease

Since the metabolic syndrome and cardiovascular disease were associated with serum BDNF concentrations in the univariable analysis, we further investigated this by repeating our multivariable analysis in a stepwise manner. We found that significance for both metabolic syndrome and cardiovascular disease was lost after the introduction of age as a variable. Moreover we investigated the individual components of metabolic syndrome, by adding these to our final regression model as described above. Only hyper-triglyceridemia significantly contributed to the variation in serum BDNF level (ß = 0.07; $P = .018$). The four other individual criteria did not reach statistical significance: low HDL cholesterol (ß = 0.01; $P = .91$), abdominal obesity (ß = 0.02; $P = .44$), hypertension ß = 0.01; $P = .87$), hyperglycemia (ß = 0.04; $P = .17$).

DISCUSSION

Main findings

Within a large cohort of people free from psychiatric disorders, we identified eight independent determinants of serum BDNF concentrations: time of blood withdrawal, time of storage, food intake before sampling, urbanicity, age, gender, smoking status and drinking behavior. Below we will discuss these determinants in more depth.

Sampling variables

Three sampling variables had an independent effect on serum BDNF concentrations in our study. First, although the overall decline was small, BDNF concentrations were significantly lower after long-term storage over a mean period of about 3.5 years. This is in line with previous results showing that serum BDNF had significantly decreased after 5 years of storage, but not yet after 12 months (Trajkovska *et al*., 2007). In our study, however, this decline of serum BDNF was far less pronounced. Most likely, this can be explained by storage temperature, which was much lower in our study (*i.e.,* -85 C˚ compared to -20 C˚). It is important to know, especially for epidemiological studies taking blood samples outside the hospital that the time kept in a cool box before initial processing does not affect BDNF serum concentrations. Secondly, we found attenuated serum BDNF concentrations when blood was drawn later in the morning. Diurnal variation has been reported for plasma BDNF concentrations, but not for serum BDNF concentrations (Piccinni *et al*., 2008). Acknowledging the limited time-interval in which serum BDNF concentrations were measured in our study, a much larger diurnal variation in BDNF can be expected. This should be explored in more detail in subsequent studies. Finally, to our knowledge,

we are the first to show that food intake prior to sampling results in lower serum BDNF concentrations. Although we cannot exclude that these results were caused by selection bias, it does plead for taking this into consideration in future research.

Socio-demographic variables

In contrast with men, in whom change in BDNF concentrations by age is far less pronounced, in our sample of people aged between 18 and 65 years we found increasing serum BDNF concentrations in women with advancing age. In a previous study of our group among community-dwelling older people aged 50 through 72 years we found constant BDNF serum concentrations in men, whereas in women an age-related decrease was found. At a first glance, these results seem puzzling, but the difference between men and women, as well as the opposite age related effects in younger and older women, might be explained by gender-hormone differences between men and women. Post-hoc analyses found a significant interaction of age and menopausal state in women, with an age-related increase of serum BDNF concentrations in premenopausal women and an age-related decrease in postmenopausal women. As estrogen concentrations are significantly associated with BDNF concentrations (Monteleone *et al*., 2007), the postmenopausal drop in estrogen concentrations could possibly result in decreasing BDNF serum concentrations. These results thus suggest that in women serum BDNF concentrations peak at the climacteric age. However, since age and menopausal status are inextricably linked, it remains difficult to draw any conclusions in this cross-sectional approach, hence warranting future longitudinal research. Furthermore, participants living in an urban environment had significantly higher serum BDNF concentrations compared to their rural counterparts. The effect of urbanicity might be hypothesized to be an indirect effect mediated by different factors. On the one hand, one may expect decreased BDNF concentrations due to a higher exposure to stress factors (Godfrey and Julien, 2005) and higher frequency of psychopathology (Peen *et al.,* 2009). On the other hand, one may expect increased BDNF concentrations due to a higher environmental enrichment, which has shown to induce BDNF gene expression (Kuzumaki *et al.,* 2010). As we excluded participants with current psychopathology (and associated the highest stress concentrations), this latter effect might have outweighed the effect of stress and psychopathology.

Health indicators

Only two health indicators were associated with serum BDNF concentrations. First, current smoking was associated with higher BDNF concentrations. Interestingly, an opposite effect has been reported on plasma BDNF concentrations in two previous studies (Bhang *et al*., 2010). Assuming that previous results are not chance findings due to low patient numbers, these opposite effects might be explained by dysfunctioning platelets in smokers resulting in impaired BDNF-release from platelets, which would affect plasma but not serum concentrations of BDNF (Nowak *et al*., 1987). Despite the clear effect of current smoking, we neither detected a dose-response effect measured by package years (in smokers and ex-smokers) nor a dose-response effect measured by number of cigarettes a day (in current smokers). Different hypotheses seem valid. First these findings might indicate a direct toxic effect of smoking, as in animal research a direct relationship between nicotine and BDNF expression in the brain has been reported (Kim *et al*., 2007).

Secondly, the association might be caused by an underlying third factor related to serum BDNF concentrations as well as smoking habits (vulnerability for starting smoking or being able to quit when started), as preliminary evidence has found an effect of val^{66}met polymorphism of the BDNF gene on smoking habits (Lang *et al*., 2007), although a replication was negative (Montag *et al*., 2008).

Moreover, in our sample we could not find an association between the degree of nicotine dependence and serum BDNF concentrations and previous studies (performed in plasma BDNF) showed that subjects had BDNF concentrations similar to non-smokers after 12 weeks of smoking cessation (Bhang *et al*., 2010). This evidence points towards a direct involvement of the inhalation of smoke in influencing BDNF concentrations. High alcohol intake, but not the severity of alcohol dependence, was associated with lower BDNF serum concentrations in our sample. BDNF has been hypothesized to be implicated in ethanol-induced neuro-degeneration in the adult brain, but to date, the role of BDNF in alcohol use disorders is still a matter of debate (Davis 2008). The only study of serum BDNF concentrations in patients with alcohol dependence found only diminished BDNF concentrations in case of co-morbid depression, but not in patients with alcohol dependence without co-morbid depression (Umene-Nakano *et al*., 2009). This picture becomes even more complicated acknowledging the possible role of the val^{66}met polymorphism of the BDNF gene in relapse of alcohol use disorders (Wojnar *et al*., 2009). More research on the role of BDNF in alcohol use disorders is clearly warranted. Thus far, studies investigating BDNF outside the area of alcohol use disorder should exclude people who use more than 14 standardized units of alcohol a week. The small inverse relationship between physical activity and BDNF serum concentrations showed a tendency toward statistical significance. Previous studies reported increased serum BDNF concentrations directly after exercise (Tang *et al*., 2008), but decreased basic BDNF concentrations at rest in physically more active humans (Currie *et al*., 2009).

Chronic disease

Although there is evidence that BDNF may play a role in the pathogenesis of cardiovascular disease (Chaldakov *et al*., 2004; Cai *et al*., 2006), BDNF serum concentrations were not associated with prevalent cardiovascular diseases or the metabolic syndrome. A closer look on previous studies of BDNF in cardiovascular disease suggests that positive results can be explained by methodological differences. First, some studies have reported an elevated expression of BDNF in atherosclerotic plaques (Ejiri *et al*., 2005), but to date it is not clear to what extent BDNF-expression in atherosclerotic plaques contributes to overall BDNF serum concentrations. Secondly, others have reported lower plasma BDNF concentrations in patients in the acute phase of coronary syndromes (Manni *et al*., 2005), which may be explained by both the acute phase of the coronary syndrome as well as the differences between BDNF measurements in plasma versus serum. The lack of an association between BDNF serum concentrations and prevalent cardiovascular disease is relevant for future studies on the role of BDNF serum concentrations in late-life depression and neurodegenerative disorders in which cardiovascular diseases are highly prevalent. Lung diseases were not associated with BDNF level either. Elevated serum BDNF concentrations have been reported in patients with asthma (Lommatzsch *et al*., 2005), but even when we investigated asthma and Chronic Obstructive Pulmonary Disease (COPD) separately, no effect was found (data not shown).

Strengths and weaknesses

Although we conducted the largest study to date on serum BDNF concentrations in human and included a large number of possible determinants, one important methodological issue has to be addressed for proper interpretation. This issue is that results for serum BDNF concentrations cannot be generalized to studies of BDNF in plasma or platelets. Because of the storage of BDNF in platelets, the concentration of BDNF in serum and plasma differs by a factor of 200 (Rosenfeld *et al*., 1995). As BDNF in platelets does not originate from mega-karyocytes or other precursor cells of the mature

platelet (Fujimura *et al*., 2002), it is likely that most of the BDNF in human platelets is sequestered from blood (Nakahashi *et al*., 2000). Furthermore, BDNF is released by platelets during the clotting process (Rosenfeld *et al*., 1995). This means that differences in platelet functioning, either by their ability to release BDNF or sequester BDNF from blood, may result in differences between serum and plasma BDNF concentrations. A possible disadvantage of measuring BDNF in serum may be a decline in BDNF concentrations after long-term storage of serum, which may not occur for BDNF stored in platelets (Trajkovska *et al*., 2007). In our study, serum was stored at -85 C˚ for a period varying between 22 and 60 months. Although we have found that serum BDNF concentrations significantly declines in long-term storage, this decline was relatively small and was corrected for. Furthermore, only one sample had a BDNF level below the lower detection limit. Therefore, we can conclude that reliable conclusions can be made after a long-term storage at -85 C˚.

Final conclusions

Future studies on serum BDNF concentrations in humans should correct for the time of day of blood withdrawal, storage, age, gender, urbanicity, smoking status and alcohol use. Although effect sizes are generally small and clinical relevance needs to be tested in subsequent clinical samples, we would suggest to exclude subjects who did not adhere to the pretest fasting protocol from clinical studies and to keep storage time limited. Moreover, for diseases with a diurnal variation, like mood disorders, it would be interesting to examine whether other parameters of BDNF serum concentrations (*e.g.,* change of BDNF over the day) would be of more relevance than one measure in the morning.