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Brain-derived Neurotrophic Factor in Human Saliva: ELISA Optimization and Biological Correlates

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Abstract

In order to assess the physiological significance of human salivary BDNF, we have optimized a sensitive and specific enzyme-linked immunosorbent assay (ELISA). We determined the range of salivary BDNF concentrations, the impact of saliva collection method, and the association of salivary BDNF with several biological characteristics. The ELISA had a detection limit of 62.5 pg/ml, and intra-assay and inter-assay precisions of 4.2% and 8.2%, respectively. Salivary BDNF concentrations were highly variable between individuals (median= 618 pg/ml) and were affected by collection method. Women had significantly higher levels of salivary BDNF than men. There was no relationship, however, between salivary BDNF levels and the other biological characteristics examined.

Keywords

BDNF; ELISA; saliva; human; optimization; Val66Met

Introduction

Saliva contains a wide variety of proteins which contribute to the health of the oral cavity and gastrointestinal tract. One essential role of these proteins is the maintenance, cytoprotection and repair of oral and gastric soft tissues, a function supported by the plethora of growth factors found in saliva. Numerous growth factors have been identified in saliva, including epidermal growth factor (EGF), transforming growth factor (TGF), nerve growth factor (NGF), and insulin-like growth factor (IGF) (See Zelles et al., 1995, for review).

Brain-derived neurotrophic factor (BDNF) is a member of the neurotrophin family, a subset of the larger growth factor family. BDNF was initially isolated from porcine brain (Barde et al., 1982) and found to have a highly conserved sequence across mammalian species (Maisonpierre et al., 1991). BDNF exists in both an N-glycosylated pro-form, which induces cellular apoptosis (Lee et al., 2001), and a mature form, which promotes cell development and differentiation, survival and plasticity (Egan et al., 2003).

BDNF and its receptors are widely distributed throughout the brain (Ernfors et al., 1990), heart, spleen, submandibular gland, skin, and lung tissue (Yamamoto et al., 1996; Tirassa et al., 2000). BDNF is also found in measurable levels in blood platelets (Yamamoto and Gurney, 1990) and circulating plasma (Rosenfeld et al., 1995). Recently, we demonstrated

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through immunoblotting and enzyme digestion that pro- and mature BDNF are present in human saliva (Mandel et al., 2009).

Alterations in blood BDNF concentrations due to environmental and genetic factors have been increasingly implicated in a variety of psychiatric disorders. There is evidence that decreased serum BDNF occurs in depression (Karege et al., 2002), bipolar disorder (Cunha et al., 2006), eating disorders (Nakazato et al., 2003), and schizophrenia (Ikeda et al., 2008), and that treatment of these disorders with medication upregulates BDNF levels (Karege et al., 2002; Gonul et al., 2005; Gama et al., 2007). Low serum BDNF concentrations may also be associated with depression-related personality traits in healthy subjects, suggesting a possible role as a risk marker (Lang et al., 2004). Finally, the presence of the common Val66Met single nucleotide polymorphism (SNP), which produces an amino acid substitution in the pro-BDNF sequence, has been associated with decreased episodic memory function (Egan et al., 2003) and mood disorders (Neves-Pereira et al., 2002).

In assessing the relationship between BDNF levels and psychological state, every study has required drawing blood from patients/participants, a process that can be stressful and time consuming. Therefore, it would be beneficial to both patients and researchers to develop a less invasive means of determining BDNF concentrations. Collection and analysis of *salivary* BDNF may therefore present a noninvasive and cost-effective alternative to blood BDNF analysis.

The aims of the current study were to optimize an ELISA technique for accurate quantitation of salivary BDNF, as well as to analyze the effects of saliva collection method on detectable BDNF concentrations. Furthermore, we sought to determine if a relationship exists between salivary BDNF concentrations and serum BDNF concentrations, sex, age, BMI, and the presence of the Val66Met SNP.

Materials and Methods

Participants

Thirty-six healthy volunteers (20 female and 16 male) from Cornell University in Ithaca, NY, participated in this study. This study was approved by the Institutional Review Board for Human Participants at Cornell University and all participants gave written informed consent for participation. Participants had not consumed any food or drink, nor brushed their teeth, for two hours before sample collection. They were instructed not to consume alcoholic beverages for the 24 hours prior to sample collection. All participants were asked about their prescription and OTC medication use, as well as smoking habit. Information about general and oral health, age, weight and height was also collected.

Sample Collection

Blood and two saliva samples were collected from each participant. All samples were collected within a 10 minute period between 12 and 1 p.m. to minimize any possible effect of diurnal variation. For serum samples, 5 ml of blood were collected and allowed to clot. The tubes were centrifuged at 3000 rpm for 10 minutes and the serum was aliquotted. For genotyping, 5 ml of venous blood were collected from each participant into a tube coated with EDTA to prevent coagulation. The tubes were centrifuged in the same manner as above. The buffy coat was suctioned off with bulb pipette and placed into another tube for genetic analysis.

Saliva samples were collected by both passive expectoration and a Salivette collection device (Sarstedt, Newton, NC). For the passive expectoration samples, approximately 3 ml of unstimulated whole resting saliva were collected from each participant into a 15-ml

conical tube on ice. The Salivette samples were collected according to the manufacturer instructions. Briefly, participants were instructed to chew on the cotton roll for 2 minutes or until the cotton was fully saturated with saliva, and then spit the cotton into the Salivette tube. Participants were asked not to handle the cotton roll in order to prevent possible contamination. All saliva samples were stored on ice until handling (approximately 1 hour), at which point the tubes were centrifuged at 4000 rpm for 15 minutes at 4°C and the samples aliquotted. Protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO) was added to all samples (serum and saliva) before storage at -80°C. Upon thawing, the samples were centrifuged once more to ensure complete debris removal.

Optimization of Salivary BDNF ELISA

We initially attempted to use commercial kits (Chemicon and Promega), that were optimized for BDNF detection in serum and plasma, to quantify salivary BDNF. The results, however, were rarely above the minimum detection level of the kits, most likely due to matrix complexity. Therefore, a sandwich ELISA optimized for salivary BDNF was developed. All antibodies were tested in a checkerboard fashion until the optimized combination, which gave the highest sensitivity and lowest background, was achieved. A 96-well microtiter plate (Nunc Maxisorp; VWR, West Chester, PA) was incubated overnight at 4°C with 100 µl of monoclonal mouse anti-human BDNF (clone 35928.11; Calbiochem, San Diego, CA), diluted to 1 µg/ml in filter-sterilized PBS, pH 7.4. The plate was manually washed three times with TBS + 5% tween (TBST), allowing the plate to soak for 1 minute each time, and blocked with 300 µl of 3% BSA in PBST for 2.5 hours at room temperature. Samples were acidified with 1M HCl to pH 3 for 20 minutes, re-neutralized with 1M NaOH, and then diluted 1:4 in filter-sterilized buffer (1% BSA in PBST). Standards ranging from 15.63 to 500 pg/ml were prepared fresh for each assay using a full-length, homodimeric recombinant BDNF (rBDNF; Peprotech, Rocky Hill, NJ) diluted in sample buffer. Following blocking, the plate was washed 5 times and 100 µl of sample/standard were added to the wells in duplicate measure. The plate was incubated for 2 hours at RT with agitation and then washed 5 times. Subsequently, 100 µl of polyclonal chicken anti-human BDNF (2.5 µg/ml; Promega) were added to the plate for 2.5 hours. The plate was washed 5 times and 100 µl of anti-chicken IgY-HRP (1 µg/ml; Promega) were added to each well for a 1 hour incubation. Following this incubation, the plate was washed 5 times and 100 µl of room temperature TMB (tetramethylbenzidine; Promega) were added to each well. After 15 minutes, 1M HCl was added to stop the reaction. The assay was read at 450 nm. The background level of the 0 pg/ml standard was subtracted from all other standards and samples. The amount of BDNF in each sample was calculated using the regression equation from the standard curve. Samples with a coefficient of variation (CV) above 10% were reanalyzed. If a CV above 10% occurred in one of the standards, the entire plate was rerun.

ELISA Assay Validation

The validity of the salivary BDNF ELISA was tested in several ways. Cross reactivity for the other members of the neurotrophin family, NT-3, NT-4 and NGF, was analyzed. Each neurotrophin was assayed at a concentration of 100 ng/ml. The limit of detection (LOD) for the ELISA was determined from 10 different assays by calculating the mean value + 3 SD of the 0 pg/ml standard absorbance and comparing that to the mean value - 3 SDs of the other standards.

Saliva pooled from six subjects was used for all validation experiments. In order to determine the recovery efficiency of BDNF in the ELISA, pooled saliva was spiked with rBDNF in amounts ranging from 5-25 pg. A control (un-spiked) sample was always run on the same plate and the amount of endogenous BDNF in the sample was subtracted from the spiked sample before calculating recovery efficiency.

In order to evaluate matrix effects, linearity of sample dilution was determined by serially diluting pooled saliva to 1:4, 1:8 and 1:16 in sample buffer. The intra-assay precision was determined as the mean coefficient of variation (CV) of 5 analyses in one assay, and test reproducibility was determined as the mean CV of analyses from 6 different assays.

ELISA for Serum BDNF

The amount of BDNF in each serum sample was quantified using the Emax Immunoassay kit (Promega) according to the manufacturer's instructions. Serum samples were diluted 1:100 in buffer, following acidification and neutralization. This kit utilizes the same antibody detection reagents as the salivary BDNF ELISA. According to the manufacturer, this kit detects a minimum of 15.6 pg/ml of BDNF and demonstrates less than 3% cross-reactivity with the other neurotrophins.

Total Protein Quantification

BDNF concentration in each saliva sample was calculated not only by the amount of protein per milliliter saliva, but also by the amount per milligram of total salivary protein. Total protein in each sample was measured using a modified version of Lowry's procedure, which includes a Na-deoxycholate (DOC) and trichloric acetic acid (TCA) protein precipitation. This modification enables accurate protein determination in samples that are dilute and/or contain interfering substances (Bensadoun and Weinstein, 1976).

Genotyping for the Val66Met Polymorphism

Participants were genotyped for the Val66Met SNP (rs6265) according to a previously published method (Neves-Pereira et al., 2002). This assay indicates if an individual is homozygous for the val allele (val/val), or if they possess at least one met allele (val/met, met/met). Genomic DNA was extracted from buffy coats using a DNeasy tissue kit (Qiagen Inc., Valencia, CA). The target sequence was amplified using a HotStar Hi Fidelity Polymerase kit (Qiagen). The 50 µl reaction solution contained 150 ng DNA, 2.5 U DNA polymerase, 1 µM of each primer (Forward: 5'-GAGGCTTGACATCATTGGCT-3'; Reverse: 5'-CGTGTACAAGTCTGCGTCCT-3') and reaction buffer, containing 200 µM dNTPs and 7.5 mM MgSO₄. The DNA templates were denatured for 5 min at 95°C and then 30 PCR cycles were performed, each consisting of 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s. After the last cycle, samples were incubated at 4°C. Samples were then digested overnight with 2 U of Eco721 (Fermentas Life Sciences, Glen Burnie, MD). The fragments were separated on a 2.5% agarose gel at 100 V and visualized with ethidium bromide. A low molecular weight DNA ladder (New England Biolabs Inc., Ipswich, MA) was used to monitor movement down the gel. Allele A (methionine substitution) was the uncut product at 113 bp, and allele G (valine) comprised the cut bands of 78 and 35 bp.

Statistics

Statistical analyses were performed using Statistica 8.0 software (Statsoft, Inc, Tulsa, OK). As the Shapiro-Wilk test for normality of salivary BDNF levels produced significant results ($P < 0.001$), the salivary BDNF values were log-transformed. The transformed data were normally distributed ($P > 0.08$), and were used for analysis. Correlations between data sets were analyzed using the nonparametric Spearman's rank method. Comparisons between BDNF concentrations in the different sample types were analyzed using the nonparametric Wilcoxon signed-rank test for paired samples, while the Mann-Whitney U test was used to compare unpaired samples. A P value (two-tailed) of < 0.05 was considered significant.

Results

Participant Characteristics

Subjects were excluded from analysis if they were taking medication known to affect BDNF levels, including antidepressants and hormone replacement therapy. Subjects who smoked or reported the presence of oral mucosal inflammation and/or bleeding were also excluded. Furthermore, if a saliva sample appeared to be contaminated by blood, the subject was excluded from analysis. Based on these criteria, five participants were removed from the initial experimental group. The remaining participants (N=31; 16 female) had a mean age of 26 years $\pm$ 4.2 years and a mean BMI of 25.1 $\pm$ 5 kg/m². Ten participants (32%; 9 females and 1 male) were either hetero- or homozygous for the met allele at position 66, while the remaining 21 subjects were val/val. These results are consistent with the reported allelic frequency of the Val66Met SNP in mixed-ethnicity studies (i.e. Li et al, 2005).

Salivary BDNF Concentrations

All samples with a concentration of salivary BDNF below the lower LOD (see “Assay Validation” below) were excluded from analysis. When the amount of BDNF per milliliter saliva was analyzed, 94% (N=29) of the passive samples and 65% (N=20) of the Salivette samples were above the lower LOD. When analyzed per milligram of total salivary protein, 88% (N=28) and 71% (N=22) of the passive and Salivette samples, respectively, were above the detection limit. See Table 1 for the salivary BDNF concentrations of the samples above the lower LOD, as measured by ELISA.

There was a significant relationship between the two types of saliva samples in the amount of BDNF per milliliter ($\rho=0.64$; $P<0.01$) and milligram total protein ($\rho=0.53$; $P<0.05$). Saliva collected by passive expectoration contained more BDNF than that collected by Salivette in terms of amount per volume ($P<0.001$; Figure 1A) and amount per milligram of protein ($P<0.05$; Figure 1B).

Association of Salivary BDNF with Biological Variables

The association of BDNF concentrations in passively collected saliva samples with sex, serum BDNF, age, BMI, and presence of Val66Met SNP was examined (Table 2). Women had significantly higher levels of salivary BDNF than men (1017 and 513 pg/ml, respectively; $P<0.05$). There was no significant relationship between BDNF concentrations in saliva and serum (median BDNF= 12.3 ng/ml; range of 0.57 to 33.5 ng/ml) samples. We also did not find a relationship between salivary BDNF levels and age or BMI. There was a trend for subjects with the Val66Met SNP to have higher levels of BDNF ($P=0.06$). However, 90% of subjects with the SNP were female and the relationship was no longer present when sex was taken into account ($P=0.2$).

Assay Validation

There was no detectable cross-reactivity with NT-3, NT-4 or NGF, even at concentrations as high as 100 ng/ml. The LOD of the assay was 62.5 pg/ml, since the mean value + 3 SDs of the blank buffer samples was less than the mean value – 3 SDs of this standard concentration. The average recovery efficiency of pooled saliva spiked with rBDNF was 94%. Recovery of a 5 pg spike was 92.8%, while recovery of 25 pg was 94.5%.

rBDNF was used to produce a standard curve with concentrations ranging from 15.63 to 500 pg/ml. Generally, a typical standard curve included seven points and had correlation coefficients of $r^2>0.99$ (Figure 2). Serial dilutions of pooled saliva in sample buffer produced a linear correlation coefficient of $r^2=0.996$ (Figure 3). The repeatability of the BDNF ELISA as measured by intra-assay precision was 4.2% and the reproducibility as

measured by inter-assay precision was 8.2%. Furthermore, out of 400 samples run in duplicate, only ten (2.5%) had CVs above 10%.

Discussion

In the present study, a sensitive and specific ELISA for the quantitation of BDNF in human saliva was developed and validated. This assay enabled us to analyze salivary BDNF concentrations in a healthy population using a small amount of saliva. Furthermore, we were able to determine the effects of saliva collection method on BDNF levels and assess the relationship between salivary BDNF levels and several biological factors, including age, sex, BMI, presence of the Val66Met SNP, and serum BDNF levels.

Salivary BDNF concentrations were highly variable between subjects in this study. This result is common in salivary protein research and also consistent with previous studies of blood BDNF in healthy individuals (Lommatzsch et al., 2005). There are numerous factors known to affect salivary protein levels, including circadian rhythms, flow-rate, stress, and infection (Rudney, 1995). In the current study, it is not likely that diurnal variation contributed to the observed variance, since all samples were collected between approximately 12 and 1 pm. We also analyzed the salivary BDNF proportion of total salivary protein, in addition to the amount per milliliter of saliva, which should diminish the effect of variation in saliva flow. Since stress levels (Mitoma et al., 2008) and infection (Lommatzsch et al., 2007) are known to also alter blood BDNF levels, the effect of these factors on salivary BDNF levels will need examination. Furthermore, the stability of salivary BDNF over time within an individual needs to be explored, as studies of other salivary proteins indicate a fair amount of longitudinal stability (Rudney, 1995).

It is notable that we found decreased BDNF concentrations in samples collected with the Salivette collection device. Altered salivary protein levels have been observed previously with cotton-based sample collection. The collection devices appear to differentially effect concentrations of different proteins. For example, Shirtcliffe et al. (2001) found artificially high levels of testosterone, progesterone, and estradiol, while levels of sIgA were artificially low. While the reason for these differences is not totally understood, it is thought that the cotton is cross-linking or non-specifically binding to proteins (Shirtcliffe et al., 2001). In the case of salivary BDNF, the number of samples in which we were able to measure the protein decreased from 94 to 65% based on collection method. Accordingly, such cotton devices may not be suitable for saliva collection in future studies of salivary BDNF.

Altered blood BDNF concentrations have been widely studied in individuals with mood disorders, as well as in healthy populations at risk for such disorders. For example, depressed patients have been found to have lower serum BDNF levels, which correlate with the severity of the disorder (Karege et al., 2002). Accordingly, one objective of the current study was to determine the feasibility of using salivary BDNF as an alternative to blood in future studies of the protein. Our findings, however, indicate that salivary BDNF levels do not reflect systemic BDNF levels and are, therefore, unlikely to be useful as a diagnostic tool. We chose to examine this relationship following reports by Tsukinoki et al. (2007) that BDNF levels in plasma are affected by levels in the submandibular salivary gland in the rat. It is possible that we did not observe a relationship in the current experiment due to our use of different assays, with different primary antibodies, for quantifying the two sources of BDNF, or that we should have measured BDNF in plasma instead of serum. However, our results are consistent with previous research on salivary and serum growth factor concentrations. For example, Ino et al. (1993) did not find a correlation between salivary and serum levels of EGF.

The BDNF receptors, trkB and p75, are widely distributed throughout the oral cavity and GI tract. It is therefore likely that salivary BDNF is playing some role in the maintenance and regulation of cells in these tissues. The current ELISA technique will be useful in elucidating the role of salivary BDNF, as well as the source of the protein in saliva. As is the case with all BDNF ELISAs, however, the assay cannot differentiate between pro- and mature BDNF levels. Since the pro- and mature forms of BDNF appear to elicit distinct, and often opposite, effects it will be necessary to develop an assay that can quantify each form.

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Figure 1. Effect of Collection Method on Salivary BDNF Concentrations

Figure 2. BDNF Standards for ELISA

Figure 3. Linearity of Sample Dilution

Table 1
Salivary BDNF Concentrations from ELISA

	Passive, per ml saliva *	Passive, per mg protein	Salivette, per ml saliva	Salivette, per mg protein
N	29	28	20	22
Median	618	476	207	307
Min	77	132	71	73
Max	2737	1709	2020	2285

* All saliva concentrations are in pg

Table 2

Biological Correlates of Salivary BDNF

	Serum BDNF	Age	BMI	Sex	Val66Met SNP
BDNF/ml	$\rho = -0.1$	$\rho = -0.23$	$\rho = 0.12$	$P < 0.05$	$P = 0.09$
saliva	$P = 0.57$	$P = 0.24$	$P = 0.54$		
BDNF/mg	$\rho = -0.1$	$\rho = -0.04$	$\rho = 0.18$	$P = 0.24$	$P = 0.49$
protein	$P = 0.82$	$P = 0.85$	$P = 0.37$		