

Blood BDNF concentrations reflect brain-tissue BDNF levels across species

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Abstract

Brain-derived neurotrophic factor (BDNF) is involved in synaptic plasticity, neuronal differentiation and survival of neurons. Observations of decreased serum BDNF levels in patients with neuropsychiatric disorders have highlighted the potential of BDNF as a biomarker, but so far there have been no studies directly comparing blood BDNF levels to brain BDNF levels in different species. We examined blood, serum, plasma and brain-tissue BDNF levels in three different mammalian species: rat, pig, and mouse, using an ELISA method. As a control, we included an analysis of blood and brain tissue from conditional BDNF knockout mice and their wild-type littermates. Whereas BDNF could readily be measured in rat blood, plasma and brain tissue, it was undetectable in mouse blood. In pigs, whole-blood levels of BDNF could not be measured with a commercially available ELISA kit, but pig plasma BDNF levels (mean 994 ± 186 pg/ml) were comparable to previously reported values in humans. We demonstrated positive correlations between whole-blood BDNF levels and hippocampal BDNF levels in rats ($r^2 = 0.44$, $p = 0.025$) and between plasma BDNF and hippocampal BDNF in pigs ($r^2 = 0.41$, $p = 0.025$). Moreover, we found a significant positive correlation between frontal cortex and hippocampal BDNF levels in mice ($r^2 = 0.81$, $p = 0.0139$). Our data support the view that measures of blood and plasma BDNF levels reflect brain-tissue BDNF levels.

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Introduction

Brain-derived neurotrophic factor (BDNF) is the most abundant neurotrophin in the brain and essential for neuronal survival during development and for integration of neurons in the adult brain (Waterhouse & Xu, 2009). BDNF is also expressed in liver (Cassiman *et al.* 2001), skeletal muscle (Matthews *et al.* 2009) and is pivotal for normal development of the cardiovascular system (Donovan *et al.* 2000). In blood, BDNF is mainly stored in thrombocytes, with only a minor free fraction in plasma (Fujimura *et al.* 2002). Whole-blood, serum and plasma BDNF protein levels can be determined with commercially available ELISA kits;

these kits can also reliably be used to measure CSF and brain-tissue levels (Aznar *et al.* 2010; Laske *et al.* 2007; Trajkovska *et al.* 2007).

A large number of studies have examined and reported blood BDNF levels in patients with neurodegenerative and neuropsychiatric disorders, including major depression (reviewed in Allea & Francia, 2009; Zuccato & Cattaneo, 2009; Martinowich *et al.* 2007, respectively). However, it is uncertain whether BDNF levels measured in blood reflect BDNF brain levels across species since BDNF is also produced in the periphery, although there is some evidence that BDNF crosses the blood–brain barrier in mice (Pan *et al.* 1998). Until now, only three studies have directly compared serum BDNF levels with brain-tissue levels of BDNF in rat brain tissue. In the first study, the association was only evident in young rats (Karege *et al.* 2002) while in a more recent study, it was reported that serum BDNF also correlated with brain-tissue

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BDNF in adult animals (Sartorius *et al.* 2009). In contrast, Elfving and co-workers looked for an association in Flinders rats, but found a negative correlation between BDNF in blood and hippocampus (Elfving *et al.* 2009). To our knowledge, there are no studies available that have investigated this relationship in species other than rats. Intriguingly, even though BDNF shows a high-degree of homology between species, it was observed that BDNF was undetectable in mouse blood, despite comparable brain-tissue levels of BDNF in rat and mouse (Radka *et al.* 1996). Thus, some discrepancies between species in BDNF detection and/or transport between the different compartments may exist.

There is a growing interest for including the pig (*Sus scrofa*) in neurobiological research due to the vast anatomical and genetic similarities between human and porcine neurobiology; furthermore, pigs are increasingly used to model human brain disorders (Lind *et al.* 2007). Contrary to the lissencephalic brain of rodents, the pig brain has sulci and gyri providing a structural organization which is more comparable to what is found in the primate brain. To our knowledge, no studies have measured BDNF in pig blood and only a few studies have investigated BDNF protein expression in pigs (e.g. Peiris *et al.* 2004). Moreover, in the present study, we included data from the pig to detect possible differences between rodents and other mammals.

Here, we investigated BDNF levels in the blood and brain in three different mammalian species to decide how accurately BDNF in the blood reflects BDNF expression in brain tissue.

Material and methods

Animals

Mice (NMRI, males, aged 8 wk) and rats (Sprague-Dawley, aged 8–12 wk) were housed 5–6 and 2 per cage, respectively, and maintained under a 12-h light/dark cycle (lights on 07:00 hours) with food and water available *ad libitum*. Following acclimatization for 1 wk before the start of the experiments, animals were euthanized by cervical dislocation (mice) or decapitation (rats), trunk blood was collected and the frontal cortex and hippocampus were dissected on a plate cooled with dry ice and immediately frozen at -80°C .

Pigs (Danish Landrace, females, aged 8–12 wk) were housed in pairs under standard conditions and were allowed to acclimatize for 1 wk before the start of experiments. Blood samples were taken after anaesthesia with 0.1 ml/kg i.m. injections of Zoletil veterinary mixture, and plasma was obtained and

stored at -20°C . Following an injection of sodium pentobarbital (2000 mg), the animals were quickly decapitated, and the brain was removed for immediate dissection. Representative pieces from frontal cortex and hippocampus were obtained and immediately frozen at -80°C .

BDNF^{2L/2L}Cre mutants were generated as previously described (Rios *et al.* 2001). Animals were of a mixed C57Bl6 and 129 background and wild-type littermates were used as controls to avoid problems interpreting the data due to differences in background. Animals were aged 10–14 wk and individually housed in the Tufts University Behavioral Core Facility, habituated to a reversed 12-h light/dark cycle for a minimum of 1 wk with free access to water and standard chow.

All experimental procedures were approved by the Danish Animal Experiments Inspectorate and the experiments involving conditional BDNF knockout (cBDNF KO) mice were approved by the Institutional Animal Care and Use Committee at Tufts University and were in compliance with the NIH Guide for the Care and Use of Laboratory Animals.

Tissue and blood

Tissue extracts were homogenized in RIPA buffer [50 mM Hepes (pH 7.5), 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 10% glycerol, 1% NP-40, 1% DOC, 0.1% SDS, protease inhibitor cocktail, 2 mM sodium orthovanadate, all reagents from Sigma, Denmark], sonicated for 5×6 s on ice followed by centrifugation in a cooled centrifuge (4°C , 10000 *g* for 10 min). The supernatant was stored at -80°C until further processing. The protein concentration was measured with the modified Lowry method (DC Protein Assay, Bio-Rad Laboratories, Denmark). Blood samples were collected in K₂EDTA-coated containers and either immediately frozen (whole blood) or centrifuged for 15 min at 3000 *g* to obtain plasma. For serum preparation, the blood was collected in additive-free containers and left to coagulate for 1 h. The lysis of whole-blood samples was performed as previously described (Trajkovska *et al.* 2007).

BDNF ELISA

Brain, blood and plasma samples were assayed for BDNF levels with two different immunoassay kits. For mouse samples we used a commercially available sandwich ELISA kit from Promega (Sweden) according to the manufacturer's instructions. In brief, 96-well Nunc-ImmunoTM MaxiSorpTM plates (Denmark) were coated with anti-BDNF monoclonal antibody and

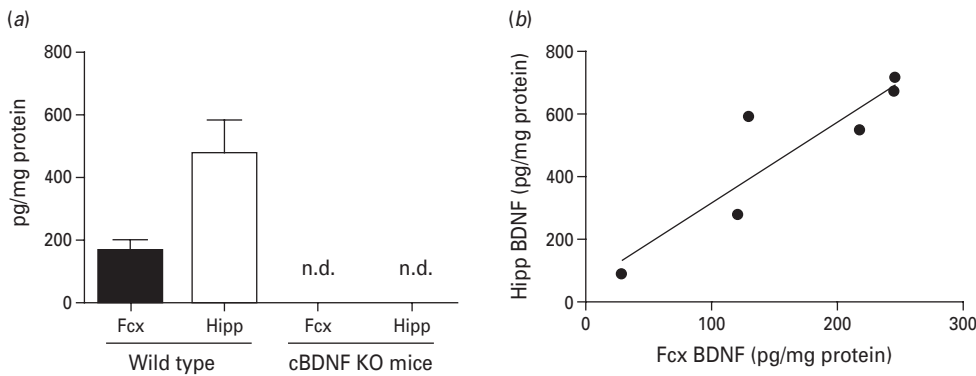


Fig. 1. (a) Brain-derived neurotrophic factor (BDNF) levels in the frontal cortex (Fcx) and hippocampus (Hipp) from wild-type and conditional BDNF knockout (cBDNF KO) mice, and (b) a correlation analysis of the levels in frontal cortex and hippocampus. BDNF levels were within the detection range in wild-type mice being ~3-fold higher in hippocampus than frontal cortex. In cBDNF KO mice, which are centrally depleted of BDNF, BDNF levels were not detectable in either the frontal cortex or hippocampus. There was a significant correlation between BDNF levels in the frontal cortex and hippocampus ($p = 0.0139$, $r^2 = 0.81$, Pearson's correlation test, $n = 6$). n.d., Non-detectable. Error bars indicate s.e.m.

incubated overnight at 4 °C. The plate was washed with TBS-T (Tris-buffered saline containing 0.1% Tween 20) (Sigma) and incubated with block and sample buffer at room temperature (RT) for 1 h before being washed again. All dilutions of samples and standard curve were made using block and sample buffer. Diluted samples and standards were added in duplicate. The plate was incubated with standards and samples for 2 h at RT, then washed and incubated with anti-human BDNF polyclonal antibody. After 2 h, the plate was washed and incubated with anti-IgY antibody conjugated to horseradish peroxidase (HRP) for 1 h at room temperature. Finally, TMB (3,3',5,5'-tetramethylbenzidine) and hydrogen peroxide solution was added to the plate and after 10 min, the reaction was stopped with 1 M HCl and the absorbance was immediately measured on an ELISA reader using a 450-nm filter (MicroPlate Reader 550, Bio-Rad Laboratories, Denmark).

For rat and pig samples we used a commercially available sandwich ELISA kit thoroughly described and validated in one of our previous studies (Trajkovska *et al.* 2007). Briefly, 100 µl of BDNF standards (7.82–500 pg/ml recombinant human BDNF) were applied in duplicate to the rabbit anti-human BDNF pre-coated 96-well plates. The samples were diluted (1:220) and 100 µl was added to the plate in duplicate. The plate was covered and incubated overnight at 4 °C. Subsequently, the plate was washed for 4 × 2 min in wash-buffer, incubated with another biotinylated primary antiserum for 3 h, washed, incubated with a streptavidin-HRP complex for 1 h and developed in a solution of TMB as described above.

Samples and standards were run in duplicate and BDNF concentrations were calculated using the standard curve and the content was expressed as equivalent of human recombinant BDNF protein in the standards. The BDNF standards contain BDNF concentrations within the range 7.82–500 pg/ml. However, to obtain valid measurements all samples were diluted at least 1:2 in sample buffer resulting in a detection limit of ~16 pg/ml.

Statistical analyses

The correlation analyses were performed using Pearson's correlation. The level of statistical significance was set to $p < 0.05$. GraphPad Prism 5 (GraphPad software, USA) was used for statistical and graphical presentations.

Results

Validation of ELISA BDNF measurements

To ensure specificity of the ELISA method used in the present study, we obtained tissue samples from cBDNF KO mice to validate that the ELISA method can be used for detection of BDNF without cross-reactivity with related proteins. This is particularly important when measuring BDNF in brain tissue where the other neurotrophins are present as well. Unmistakably, we showed that while BDNF is detectable in the frontal cortex and hippocampus in wild-type mice, brain BDNF levels in KO mice were undetectable (Fig. 1a). Additionally, we found a significant correlation between BDNF levels in the frontal

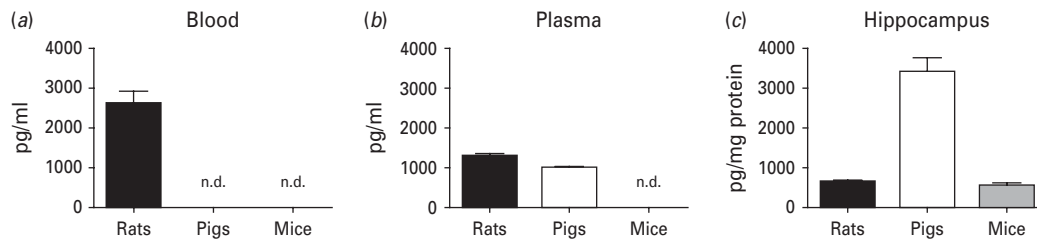


Fig. 2. Brain-derived neurotrophic factor (BDNF) levels in (a) blood, (b) plasma and (c) hippocampus in rats, pigs and mice. (a) BDNF levels in whole blood could be measured in rats, but were undetectable in mice and pigs. (b) In plasma, we were able to measure BDNF in rats and pigs, but not in mice. (c) In hippocampus, pigs showed 4- to 5-fold higher BDNF concentrations than rats and mice. n.d., Non-detectable. Error bars indicate S.E.M.

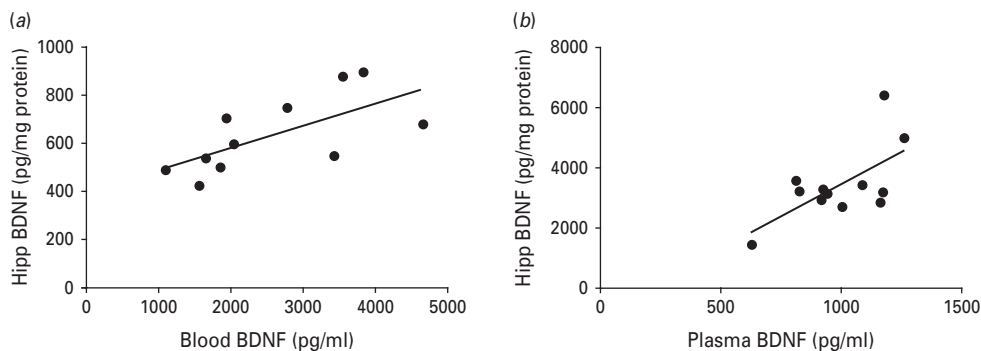


Fig. 3. Brain-derived neurotrophic factor (BDNF) levels in hippocampus (Hipp) vs. blood and plasma in rats and pigs. (a) There was a significant positive correlation between blood BDNF and BDNF in the hippocampus in rats ($p=0.025$, $r^2=0.44$), and (b) a significant positive correlation between BDNF levels in plasma and hippocampus in pigs ($p=0.025$, $r^2=0.41$, $n=12$); Pearson's correlation test.

cortex and hippocampus in wild-type mice ($p=0.0139$, $r^2=0.81$, $n=6$) (Fig. 1b). These findings support the use of sandwich ELISA to measure BDNF protein levels. Furthermore, it shows that cross-reactivity with other neurotrophins is negligible.

BDNF measurements in rats, pigs and mice

Using the ELISA kit from Millipore (USA) (rats and pigs) and Promega (rats, pigs, mice), BDNF levels could be measured in whole-blood samples from rats, but not in whole blood from mice and pigs (Fig. 2a). In plasma, we were able to measure BDNF in rats and pigs, but not in mice (Fig. 2b). In the hippocampus, a high-expression region for BDNF, BDNF was measurable in all three species when using the Millipore ELISA kit for rat and pig samples and the Promega ELISA kit for mouse samples. The hippocampal BDNF levels in rats and mice were comparable, while hippocampal BDNF levels in pigs were 5-fold higher (636.1 ± 158 , 559.9 ± 171.2 and 3415 ± 1228 pg BDNF/mg total protein for rats, mice and pigs, respectively) (Fig. 2c).

Correlation of BDNF in blood and brain in rats and pigs

A correlation analysis of BDNF levels in blood and hippocampal tissue showed a significant positive correlation between BDNF in rat blood and BDNF in rat hippocampal brain tissue ($p=0.025$, $r^2=0.44$, $n=11$) (Fig. 3a). In pigs, we found a significant correlation between plasma BDNF levels and hippocampal BDNF levels ($p=0.025$, $r^2=0.41$, $n=12$) (Fig. 3b). Because BDNF is undetectable in mouse blood, plasma or serum, a correlation analysis was not obtainable.

Discussion

Our main findings in this study are (1) BDNF levels in blood and plasma correlate with BDNF in hippocampus, underlining the potential for peripheral measures of BDNF as a biomarker, (2) despite high homology in the protein sequence across species, the detection properties of blood BDNF in rats, mice and pigs are different.

The ELISA kits used in this study had previously been evaluated (Elfving *et al.* 2010; Trajkovska *et al.* 2007). In these studies, however, there was no evaluation of the specificity of the ELISA method for brain-tissue BDNF measurements. As stated in Gass & Hellweg (2010) it will be of great importance to obtain an estimation of the cross-reactivity with other neurotrophins, e.g. NGF, NT-3 and NT-4/NT-5. We found in our study that an analysis of tissue obtained from cBDNF KO mice and their wild-type littermates yielded the expected outcomes. In cBDNF KO mice, BDNF is gradually depleted post-natally and absent in adult mice (Rios *et al.* 2001). The signal we detected in tissue samples from the cBDNF KO mice was similar to background measurements, i.e. the absorbance without any added protein. This illustrates that the cross-reactivity with other members in the neurotrophin family in the applied ELISA kits is negligible.

We found a positive correlation between BDNF in hippocampus and BDNF in plasma and blood in pigs and rats, respectively. A correlation between BDNF in brain and BDNF in blood was first shown by Karege *et al.* (2002). In young rats, they found a correlation between BDNF in the cortex and serum, while serum BDNF and tissue levels in adults did not correlate. Recently, in a more elaborate study, a significant correlation between serum BDNF and brain tissue in adult animals was presented, but results from pre-frontal cortex and hippocampus were not presented individually (Sartorius *et al.* 2009). The origin of BDNF in blood is not entirely clear, although supposedly the brain is the main contributor to blood BDNF. This was elegantly demonstrated in an exercise study, illustrated by a change in BDNF arterial-to-internal jugular venous difference after a single bout of exercise (Rasmussen *et al.* 2009).

We did not detect significant correlations in either rats or pigs when correlating BDNF in the frontal cortex with BDNF in blood and plasma, respectively. This could either be because the major BDNF output from the brain hypothetically originates from the hippocampus (Yan *et al.* 1997) and is thereby the key determinant for blood BDNF levels. However, it can also be due to intercortical differences in BDNF output leaving dissections from frontal cortex misleading for the whole cortex. Another more plausible explanation may be that BDNF is more difficult to extract from the frontal cortex and therefore the total yield is subject not only to a bias but also to higher variation. Elfving *et al.* (2010) showed that the extraction efficiency of BDNF after spiking the homogenates was ~30% for frontal cortex, but ~70% for hippocampus. On the other hand, the data from the present study in mice

tissue showed a highly significant correlation between BDNF levels of hippocampus and frontal cortex, suggesting fairly low variation in extraction yield from these two compartments, at least in mice.

Intriguingly, we were unable to detect BDNF in blood from pigs albeit the method utilized has previously been validated for human samples (Trajkovska *et al.* 2007). Indeed, both ELISA kits used in this study were capable of detecting pig BDNF in plasma and brain tissue, with plasma values comparable to the values seen in most human studies (Brunoni *et al.* 2008). To our knowledge, no studies have quantified pig BDNF levels. Apparently, as exemplified in the present study, the transport of BDNF in blood is different across species. It may be speculated that BDNF binds strongly to a transporter protein in pig blood and this binding persists despite the relatively harsh lysis protocol applied to these samples. Another explanation could be non-specific binding of other proteins hindering binding of BDNF to the antibodies in the ELISA. Since this binding is suggested to be much weaker than specific binding of BDNF, it would be washed off during the subsequent comprehensive washing steps (see Methods section) leaving end-point measurements similar to background measurements (see above).

In mice, BDNF levels in blood, serum and plasma were undetectable using different commercially available BDNF ELISA kits. This is puzzling, however, since BDNF levels in brain tissue were easily detectable using the Promega ELISA kit. In a previous study, a similar observation was made using a home-made enzyme-linked immunoassay (EIA) for BDNF measurements (Radka *et al.* 1996). Correspondingly, these authors were unable to detect BDNF in either mouse serum or plasma, but did so readily in rats and humans and in mouse brain. Here, we elaborated on these findings using a well-validated ELISA kit. We analysed whole-blood lysates to get a complete release of BDNF from thrombocytes and we performed measurements on undiluted serum. Nevertheless, we were unable to detect any signal above background measurements in plasma, serum or blood. Apparently, this is not due to lack of BDNF transport capacity as it was shown that mice readily transport iodine-labelled BDNF and recombinant BDNF in blood (Pan *et al.* 1998). It is also possible that BDNF is rapidly cleared from plasma, but this needs to be validated. The important message here is that blood BDNF cannot be measured in mice with the most commonly used commercially available ELISA kit.

In conclusion, we show here that blood BDNF concentrations correlate positively with BDNF levels in

the hippocampus of rats and pigs. These findings further demonstrate the close linkage between blood and brain BDNF levels and heighten the potential of BDNF blood measurements as a predictor of BDNF levels in high-expression brain regions.

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Statement of Interest

None.

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