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NOTE

Activation of signaling pathways following localized delivery of systemically administered neurotrophic factors across the blood–brain barrier using focused ultrasound and microbubbles

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Abstract

The brain-derived neurotrophic factor (BDNF) has been shown to have broad neuroprotective effects in addition to its therapeutic role in neurodegenerative disease. In this study, the efficacy of delivering exogenous BDNF to the left hippocampus is demonstrated in wild-type mice (n = 7) through the noninvasively disrupted blood–brain barrier (BBB) using focused ultrasound (FUS). The BDNF bioactivity was found to be preserved following delivery as assessed quantitatively by immunohistochemical detection of the pTrkB receptor and activated pAkt, pMAPK, and pCREB in the hippocampal neurons. It was therefore shown for the first time that systemically administered neurotrophic factors can cross the noninvasively disrupted BBB and trigger neuronal downstream signaling effects in a highly localized region in the brain. This is the first time that the administered molecule is tracked through the BBB and localized in the neuron triggering molecular effects. Additional preliminary findings are shown in wild-type mice with two additional neurotrophic factors such as the glia-derived neurotrophic factor (n = 12) and neurturin (n = 2). This further demonstrates the impact of FUS for the early treatment of CNS diseases at the cellular and molecular level and strengthens its premise for FUS-assisted drug delivery and efficacy.

(Some figures may appear in colour only in the online journal)
1. Introduction

The presence of the highly selective blood–brain barrier (BBB) has hindered the delivery of many therapeutic agents to the brain, prompting researchers to investigate various methods to circumvent this protective homeostatic mechanism, such as focused ultrasound (FUS), intra-arterial injection of hyperosmotic solutions (Kroll and Neuwelt 1998), convection-enhanced diffusion (Bobo et al 1994), intracranial injection (Weaver and Laske 2003), or chemical modification of drugs by lipidization or conjugation to genetically-engineered molecular Trojan horses (Pardridge 2005). Recently, the technique of FUS in conjunction with systemically administered microbubbles has been shown to induce BBB opening noninvasively, transiently, and with high spatial selectivity (Choi et al 2007b, 2010a, 2010b, Hynynen et al 2001, Liu et al 2008, McDannold et al 2005). The delivery of many large agents using FUS and microbubbles have been demonstrated in previous studies by our group and others: MRI contrast agents such as Omniscan (573 Da) (Choi et al 2007b) and Magnevist® (938 Da) (Choi et al 2007a), Evans Blue (Kinoshita et al 2006b), Trypan Blue (Raymond et al 2008), Herceptin (148 kDa) (Kinoshita et al 2006a), horseradish peroxidase (40 kDa) (Sheikov et al 2008), doxorubicin (544 Da) (Treat et al 2007), and rabbit anti-Aβ antibodies (Raymond et al 2008). However, despite the promise shown by the delivery of such a variety of compounds, to the authors’ knowledge, there is only one FUS study that has addressed the bioactivity of the molecules after they have crossed the BBB (Jordao et al 2010).

Until now FUS together with the use of microbubbles has been shown to open the BBB in vivo and allow certain molecules to permeate it. Prior reports on BBB disruption indicate opening but do not show whether the molecule stays in the extracellular space or enters the cell, an important and immense difference in brain drug delivery. Unless the neurotrophic factor enters the neuronal cell, its therapeutic effect cannot be triggered. This note reports two novel findings that are critical toward both the basic understanding of the physical mechanism and its biological/clinical translation. For the first time we show that (1) neurotrophic or growth factors can cross through the FUS-opened BBB, and (2) cellular and molecular cascades are triggered which demonstrate both the uptake of the molecules through the cell membrane and into the nucleus of the neurons in the hippocampus, the memory center of the brain and the target of early Alzheimer’s disease. Until now it was not known where or how the molecules diffuse i.e. whether they stay in the extracellular matrix or if and how they enter the cells. These are some of the questions that are addressed in this note. For the first time we demonstrate that this technique can deliver the drugs to the intended target into the cell nucleus triggering the required molecular cascades. In addition to what was stated above, Alzheimer’s disease can now be detected at increasingly earlier stages using PET imaging i.e. before any onset of symptoms. Therefore, targeted and noninvasive techniques such as FUS are warranted for treatment starting with the hippocampus and the brain-derived neurotrophic factor (BDNF), one of the most promising compounds shown to reverse the disease in cell cultures and direct injection (Hagg 1998, Nagahara et al 2009).

In this study, our first goal was to show preserved bioactivity of a delivered compound following FUS-mediated BBB opening. Our second goal was to demonstrate such feasibility using a compound that has substantial research utility and potential therapeutic value, but possesses size and stability attributes that make it difficult to cross the BBB when systemically administered. Such a compound exerting influence on proliferation and differentiation of neurons, and promotes neuronal growth and survival in the nervous system (Waterhouse and Xu 2009), including hippocampal neurons (Yan et al 1997).
BDNF, along with its TrkB receptor, is widely expressed and densely distributed throughout the CNS (Altar et al. 1994) in both the developing and the mature brain (Hofert et al. 1990). It has been proposed that an insufficient supply of endogenous neurotrophic factors for selective neuronal populations may lead to the development of neurodegenerative diseases (Connor et al. 1997). Decreased levels of BDNF protein have been demonstrated in the substantia nigra in Parkinson’s disease (Howells et al. 2000), and in the hippocampus and the entorhinal cortex of an Alzheimer’s disease brain (Lee et al. 2005). BDNF signaling and downregulation also appear to play a crucial role in Huntington’s disease (Ciammola et al. 2007, Simmons et al. 2009) and neuropsychiatric diseases and depression (Martinowich et al. 2007, Shirayama et al. 2002).

Despite its importance in health and disease, the role of BDNF as a therapeutic agent has been diminished due to its BBB impermeability and short half-life in the systemic circulation. BDNF’s molecular weight (27 kDa) exceeds the ~180 Da limit (Kroll and Neuwelt 1998) of BBB permeability. Moreover, BDNF has a half-life of less than 10 min in the blood and is rapidly cleared from the circulation mostly by the liver (Sakane and Pardridge 1997). Considering the neuroprotective properties of BDNF and the growing interest in this compound as well as the aforementioned problems associated with its transport across the BBB, intravenously administered BDNF is an ideal candidate to study the FUS-mediated delivery to the brain.

The aim of our study was to demonstrate the efficacy of the FUS method by accomplishing two objectives after BBB opening: (1) diffusion of BDNF across the BBB and containment within the targeted region, i.e. the hippocampus, and (2) associated activation of the BDNF receptor and its downstream signaling molecules in neurons, showing bioactivity of the functional BDNF upon delivery.

In order to discuss BDNF bioactivity, a brief review of the BDNF signaling pathway is deemed essential. On the surface of cell soma, dendrites, and axons of hippocampal neurons, BDNF binds to the tyrosine kinase (Trk) receptor with high affinity, initiating TrkB dimerization and autophosphorylation at the intracellular catalytic domain (Y705/6) (Huang and Reichardt 2001). This event in turn induces the autophosphorylation of tyrosines 515 and 816 on the TrkB receptor. Phosphotyrosine 515 activates the Ras/MAPK and phosphoinositide 3 kinase pathways and ultimately enhances neuronal survival and differentiation. Phosphotyrosine 816, on the other hand, activates phospholipase Cγ, leading to upregulation of intracellular Ca^{2+} levels and activation of the calcium/calmodulin kinase pathway. Both Ras/MAPK and calcium/calmodulin pathways are considered responsible for the activation of the transcription factor cAMP-response-element binding protein (CREB), which induces gene expression and is involved in the development and synaptic plasticity (Lonze and Ginty 2002).

2. Methods

To visualize the passage of BDNF across the BBB, the fluorescent dye Alexa Fluor 594 was conjugated to BDNF prior to the experiments. The fluorescent tag was not expected to modify the transport properties of BDNF given its small molecular weight (~0.3 kDa) compared to the BDNF’s (27 kDa). Following successive application of FUS sonication (figures 1(a) and (b)), microbubble injection, and intravenous injection of BDNF, the mice (n = 3) were sacrificed 20–30 min post-sonication for histological analysis (1) to allow sufficient time for the BDNF to accumulate in the BBB-opened region and facilitate detection of the compound (higher fluorescent intensity) at the sonicated areas; (2) to allow most of the compound to circulate through the microvasculature before being cleared by the venous system, since
circulating BDNF has been shown to have a half-life of less than 10 min (Sakane and Pardridge 1997); and (3) to allow sufficient time for the downstream signaling cascade to be activated. The activation of the BDNF receptor, TrkB, occurs within seconds of BDNF delivery (Boulanger and Poo 1999).

2.1. Animals

A total of seven C57Bl6 male mice were used for this study (17.6–23.0 g, Harlan Laboratories). The animals were anesthetized with a mixture of oxygen (0.8 L min\(^{-1}\) at 1.0 Bar, 21 °C) and 1.5–2.0% vaporized isoflurane (Aerrane, Baxter Healthcare) using an anesthesia vaporizer (SurgiVet, Smiths Group). The mouse’s vital signs were monitored continuously and isoflurane was adjusted throughout the experiment as needed. The Columbia University Institutional Animal Care and Use Committee gave approval for the mouse studies.

The total number of mice used in the BDNF study was seven \( (n=7) \) as follows: three mice were injected with BDNF, sonicated, and sacrificed after 20–30 min for both IHC and fluorescence analysis, one mouse was injected with BDNF, sonicated, and sacrificed but died after 3 min (IHC and fluorescence analyses were also performed but discounted in the statistical analysis), and three controls were sonicated and sacrificed after 20–30 min (no BDNF) for IHC analysis. We used three types of ‘controls’ to show the effect of sonication on the BDNF permeation through the BBB and subsequent activation of the downstream signaling cascade as follows:

(a) to demonstrate the difference between the left (sonicated) hippocampus and the right (non-sonicated) hippocampus in four mice (three mice sacrificed after 20–30 min, one died after 3 min) following BDNF injection and sonication;
(b) to demonstrate no difference between the left (sonicated) hippocampus and the right (non-sonicated) hippocampus in three control mice following only sonication (no BDNF) so as to rule out the effect of sonication alone on signaling cascade activation;
(c) to obtain negative controls for the IHC analysis in all studied mice, where no primary antibody was added to the sections.
Table 1. FUS parameters used in the case of GDNF and NTN. PL is the pulse length and P-N denotes peak-negative.

<table>
<thead>
<tr>
<th>Compound</th>
<th>GDNF</th>
<th>NTN</th>
</tr>
</thead>
<tbody>
<tr>
<td>PL (cycles)</td>
<td>15 000</td>
<td>30 000</td>
</tr>
<tr>
<td>P-N pressure</td>
<td>0.30 0.45 0.60</td>
<td>0.30 0.45 0.60</td>
</tr>
<tr>
<td>Circulation time</td>
<td>45 180 180 60 60</td>
<td>6.5 45 30 60 60</td>
</tr>
</tbody>
</table>

The statistical analysis was based on the three BDNF-injected mice that were sacrificed after 20–30 min, so it did not include the 3 min mouse case. The results for the case of 3 min BDNF-injected mouse are also presented merely for comparison in terms of the downstream signaling activation and its time dependence in order to support previous reports on the temporal sequence of signaling cascades downstream of BDNF. It is important to note that the BDNF was injected into the femoral vein 10 min after sonication while the mice were still anesthetized. There were at least 3 min (one mouse) and 20–30 min (three mice) of circulation before PBS transcardial perfusion was started.

2.2. Neurotrophic factors

2.2.1. BDNF. The BDNF conjugated to Alexa Fluor® 594 dye was used in our experiments (Invitrogen Corp., Carlsbad, CA, USA). BDNF Human Recombinant was produced in Escherichia coli and is a homodimer, non-glycosylated, polypeptide chain containing $2 \times 119$ amino acids with a total molecular mass of 27 kDa. According to the supplier, it was purified by proprietary chromatographic techniques and the sequence of the first five N-terminal amino acids was determined and found to be Met-His-Ser-Asp-Pro. Biological activity was determined by evaluating ED50 ($50 \text{ ng ml}^{-1}$), calculated by the dose-dependent induction of ACHE (acetylcholine esterase) in rat basal forebrain primary septal culture. The compound (6.0 mg) was custom conjugated to Alexa Fluor® 594 dye (~1:1 molar ratio) and provided in a fine lyophilized powder. The vials were stored under $-18^\circ \text{C}$ until use.

2.2.2. Glia-derived neurotrophic factor and neurturin. A total of 12 mice received FUS followed by glia-derived neurotrophic factor (GDNF) (40–90 mg kg$^{-1}$ in 0.15 ml PBS, $n = 10$), as well as neurturin (NTN) (20 mg kg$^{-1}$ in 0.2 ml PBS, $n = 2$) injections. Only 2 mice were used for the NTN study due to the limited amount provided for the purpose of this study. Both GDNF and NTN were conjugated with Alexa Fluor® 488 fluorescent dye. Four sites within a 1 mm square in the caudate were sonicated at a frequency of 1.5 MHz, with a pulse length of 15 000 cycles ($n = 3$) and 30 000 cycles ($n = 8$), at varying pressures. Detailed acoustic parameters are shown in table 1.

In the case of 6.5 min circulation time, blood was drawn after 45 s to confirm the circulation and fluorescence of the protein. Brain, liver, kidney, and testes were extracted and fixed for frozen section. Organs were then frozen into blocks and sectioning at 100 $\mu$m and used to locate the GDNF.

2.3. Ultrasound

A single-element spherical segment FUS transducer (center frequency: 1.525 MHz; focal depth: 90 mm) was driven by a function generator (Agilent Technologies) through a
50 dB power amplifier (ENI) to generate the FUS beam (Choi et al 2007b). A pulse-echo transducer (center frequency: 7.5 MHz; focal length: 60 mm) was positioned through a center hole of the FUS transducer so that the foci of the two transducers were aligned. It was driven by a pulser–receiver system (Panametrics) connected to a digitizer (Gage Applied Technologies) and was used for imaging. A cone filled with degassed and distilled water and capped with an acoustically transparent polyurethane membrane (Trojan; Church & Dwight) was mounted on the transducer system (figure 1(a)). The transducers were attached to a computer-controlled 3D positioning system (Velmex). The FUS transducer’s pressure amplitude reported in this study was previously measured with a needle hydrophone (needle diameter: 0.2 mm; Precision Acoustics) in degassed water while accounting for 18.1% attenuation by the mouse skull. The dimensions of the beam were measured to have a lateral and axial full-width at half-maximum intensity of approximately 1.32 and 13.0 mm, respectively.

2.4. Targeting procedure

The head of each anesthetized mouse was immobilized using a stereotaxic apparatus. The fur on top of the head was removed with an electric razor and a depilatory cream. After applying ultrasound gel, a water bath with its bottom made of an acoustically and optically transparent membrane was placed on top of the head and gel. A grid positioning method to target the mouse hippocampus was then used as previously described (Choi et al 2007b). In brief, a metallic grid was placed in alignment with the mouse skull sutures, which were visible through the intact scalp of the mouse after hair removal. The left hippocampus was localized by identifying the sagittal suture and then moving 2.5 mm to the left of that suture and subsequently 3 mm in depth from the top of the skull. The grid was removed immediately after targeting was completed and prior to FUS application as it would otherwise interfere with the sonication. Four target sonication locations were identified relative to the sutures. The first target overlapped the medial portion of the hippocampus, the lateral portion of the thalamus, and the posterior cerebral artery. The transducer was then moved 1 mm anterior and 1 mm lateral for the second target and then 1 mm posterior for the third. The fourth and final target was 1 mm medial and 1 mm anterior. In the end, four different locations were targeted at the corners of a 1 mm \( \times \) 1 mm square.

2.5. Sonication protocol

2.5.1. BDNF. Definity® microbubbles (diameter: 1.1–3.3 \( \mu \)m, vial concentration: 1.2 \( \times \) 1010 bubbles ml\(^{-1}\); Lanthus Medical Imaging) composed of octafluoropropane gas encapsulated in a lipid shell were diluted (1:20) in phosphate-buffered saline (PBS) and then administered into the tail vein (final administered concentration: 50 \( \mu \)l kg\(^{-1}\) of body mass). This dosage was selected to be consistent with previous studies by our group. However, it is important to note that different bubble concentrations have not shown any significant differences (Choi et al 2010a). Therefore, in future studies when we plan on using the clinical dose, which is five times lower, we do not expect significant differences. 1 min after injection, pulsed-wave FUS (peak-rarefractional pressure: 0.46 MPa; pulse repetition frequency: 10 Hz; pulse length: 20 ms) was applied. Each of the four target locations was sonicated twice, resulting in a total of eight sets of 30 s sonication with a 30 s delay between each set.

2.5.2. GDNF and NTN. The sonication parameters are provided in table 1. All other parameters used were the same as in the case of BDNF.
2.6. Administration, perfusion, and sectioning

A bolus injection of BDNF via the femoral vein was administered 10 min after sonication (40–90 mg kg\(^{-1}\) of mouse body mass in 0.2 ml PBS). Except for one animal that died 3 min after injection and was perfused immediately, the rest of the animals were sacrificed 20–30 min after the injection to allow for adequate circulation. The animals were transcardially perfused with PBS (4–5 min) and 4% paraformaldehyde (7–8 min) at a flow rate of 6.8 ml min\(^{-1}\). Next, the skulls were removed and immersion-fixed for 24 h before extracting the brains. Extracted brains were fixed again in 4% paraformaldehyde for 24 h, and transferred to 10% (30 min), 20% (60 min), and 30% (24 h) sucrose solutions for cryoprotection. Brain samples were then embedded in an optimal cutting temperature medium and were frozen using dry ice and isopentane. Frozen blocks were sectioned horizontally at 10–150 \(\mu\)m thickness for fluorescent imaging and at 5–10 \(\mu\)m thickness for immunohistochemistry. Slices covering the entire hippocampus were selected, placed on a slide, and stored in –18 °C freezer for later analysis. In every case, the right hippocampus is not sonicated and therefore serves as the control to the left hippocampus, which is always the sonicated one.

2.7. Immunohistochemistry

Immunohistochemistry was performed only in the case of BDNF according to standard procedures. We used five primary antibodies: two against phosphorylated TrkB receptor (p-Y816 rabbit polyclonal (ab75173) and p-Y515, rabbit polyclonal (ab51187)) purchased from Abcam Inc. (Cambridge, MA), and three against phosphorylated Akt (p-S473 rabbit monoclonal (#4060)), phosphorylated MAPK (p-T202/T204 rabbit monoclonal (#4370)), and phosphorylated CREB (p-S133 rabbit monoclonal (#9198)) (Cell Signaling Technology, Danvers, MA). Slides containing thin frozen sections (5–10 \(\mu\)m) were dried and placed in a citrate buffer (pH 6.0) for antigen retrieval using a microwave. Slides were allowed to cool for 20 min prior to a PBS rinse (3 × 5 min) and then incubated in 0.3% hydrogen peroxide in PBS (5 min) to block endogenous peroxidase activity. Slides were washed again in PBS (3 × 5 min) and blocked in 10% normal goat serum with 0.1% BSA for 20 min. After the blocking solution was removed, the primary antibodies were diluted in a DAKO antibody diluent solution (1:50–1:300) and incubated for 60 min at room temperature. Slides were washed in PBS for 5 min and incubated with biotinylated secondary antibody (goat anti-rabbit 1:200; Vector Laboratories, Burlingame, CA) for 30 min at room temperature. Slides were washed again in PBS (3 × 5 min) and VECTASTAIN® ABC reagent was added to the sections for 30 min (A: 1:60, B: 1:60 in PBS mixed 30 min prior to use). Slides were washed in PBS (3 × 5 min) and a peroxidase substrate solution diaminobenzidine (DAB) (DAKO, Carpinteria, CA) was added to sections (one drop of DAB in 1 ml buffer). Slides were immersed in \(\text{dH}_2\text{O}\) as soon as color developed. Sections were counterstained with hematoxylin, cleared and dehydrated with alcohols and xylene, and covered with Permount™ mounting medium (Thermo Fisher Scientific, Inc. Waltham, MA, USA), and a glass coverslip.

2.8. Bright-field and fluorescent microscopy

Bright-field and fluorescent images were acquired using a light and fluorescence microscope (BX61; Olympus, Melville, NY, USA) with a filter set at excitation and emission wavelengths of 595 and 615 nm, respectively.
2.9. Quantification

Bright-field images were white corrected with the same correction for both right (control) and left (sonicated) images. The DAB stain density was then extracted from the bright-field images using a color deconvolution method and implemented in Matlab (Mathworks, Natick, MA) by the Open Microscopy Environment project (OME, www.openmicroscopy.org). H&E DAB built-in vectors were used for the deconvolution step. Where DAB is developed, the cellular region in question turns brown. For each image, the mean stain intensity was computed using the logarithm of the stain intensity. Standard image artifacts (folded tissue, holes, or stain droplets) were manually segmented in each image and removed from the analysis. For each mouse and each antibody, the percentage change (PC) of the stain intensity between the left and the right sides was then computed as follows: PCI = 100 [(ILeft – IRight)/IRight].

2.10. Statistical analysis

Statistical analysis was performed using a two-tailed Student’s $t$-test to determine whether the BDNF concentration is significantly increased in the sonicated (left hippocampus) region compared to the BDNF concentration in the unsonicated (right hippocampus) region. A $p < 0.05$ was considered significant in all comparisons.

3. Results

Figure 2(a) shows the diffusion of BDNF at the sonicated region in the left hippocampus as detected by the fluorescent intensity of Alexa Fluor 594 (mouse sacrificed 20 min after BDNF injection). A considerable difference was evident in fluorescent intensities between the sonicated hippocampus (left) and the control unsonicated hippocampus (right). Regions of greater intensity included parts of the thalamus, the transverse hippocampal artery and its branches inside the hippocampus, the neurons in the pyramidal (CA1–CA3) layers of the hippocampus proper, and the neurons in the hilus and granular layers of the dentate gyrus. Figures 2(b) and (c) depict the extent of immunoreactivity to phosphorylated MAPK (activated molecule downstream of BDNF signaling; discussed below) in a DAB-stained section that was sectioned ∼300 µm dorsally from the frozen section imaged in figure 2(a). The DAB-stained regions closely matched the areas of BDNF diffusion, providing a multi-modality confirmation of BDNF delivery across the BBB. No difference in DAB intensity was observed in the case of the mouse sacrificed 3 min after sonication (figure 2(d)) or the negative control for the case shown in figure 2(a), i.e. no primary antibody added (figure 2(e)).

To demonstrate post-delivery bioactivity of the BDNF compound, we utilized immunohistochemical techniques to detect activated downstream signaling molecules using two primary antibodies against the activated TrkB receptor (pTrkB Y816 and pTrkB Y515), and three primary antibodies against the phosphorylated MAPK (T202–T204), phosphorylated CREB (S133), and phosphorylated Akt (S473). We quantified the relative activation of these signaling molecules in sonicated versus non-sonicated hippocampal BDNF-administered ($n = 3$) and control mice ($n = 3$) by measuring the DAB stain intensity as described in the methods.

Across all BDNF-administered mice (sacrificed 20–30 min post-sonication), immunoreactivity to each activated signaling molecule displayed a distinct characteristic stain that was unique and easily identifiable among various brain slices. Figure 3 demonstrates the greater presence of immunoreactivity to pTrkB Y816 (figure 3(a)–(c)) and pTrkB Y515 (figure 3(d)) in the sonicated brain regions (left column) compared to the non-sonicated
Figure 2. (a) Fluorescent image of a 100 µm frozen brain section from a mouse sacrificed 20 min after sonication. The sonicated hippocampus (left) shows much higher fluorescent intensity than the unsonicated hippocampus (right), depicting BBB opening and the extravasation of fluorescent-tagged (Alexa Fluor 594) BDNF in the sonicated region; (b) a 5 µm frozen section from the same mouse was immunohistochemically stained using a primary antibody against phosphorylated MAPK (pMAPK). Consistent with the fluorescent image in (a), the intensity of DAB staining is much greater in the left sonicated hippocampus compared to the right control; the black box indicates the enlarged area in (c), where immunoreactivity to pMAPK is shown in mossy fiber terminals (arrowhead), suprapyramidal CA3 dendrites (black star), and the axons of the Schaffer collateral system (hollow star); (d) immunohistochemical staining of a 5 µm frozen section from a mouse that was sacrificed 3 min after sonication; the same primary antibody against pMAPK was used. No difference in the DAB intensity is observed between the sonicated and the control hippocampus, i.e. in this case no significantly greater immunoreactivity to MAPK was shown in the sonicated region unlike in all three 20–30 min mice ((b), (c)); (e) negative control performed at the same time and for the same mouse as in (a); no primary antibody (against pMAPK) was added to this 5 µm frozen section during the staining procedure. All magnifications are 40 × and scale bars are 500 µm except for (c), which is 100 × and 200 µm, respectively.
Figure 3. Immunohistochemical staining of 5 µm frozen sections using a primary antibody against (a)–(c) phosphorylated TrkB 816 and a primary antibody against (d) phosphorylated TrkB 515. Mice were sacrificed 20–30 min (a), (b), (d) or 3 min (c) after sonication. The difference in DAB intensity between the sonicated hippocampus (left column) and the contralateral control hippocampus (right column) is detectable in all the sections (a)–(d). In (a) and (b), the 20–30 min mice show increased DAB intensity in the presence of TrkB 816, although, in contrast to MAPK and CREB, a lower proportion of the sections showed notable differences between the left and right hippocampus. (b) clearly shows a difference in the DAB intensity in the choroid plexus of left versus right hippocampus given that the former is stained in brown (examples of DAB staining are indicated by the arrows) while the latter is only stained in blue. In (c), the 3 min circulation case shows increased DAB intensity in the presence of TrkB 816. The immunoreactivity to pTrkB is shown at the plasma membrane of neuronal cells in CA1 region (the arrows in (a)), ependymal cells of choroid plexus (the arrowheads in (b)), neuronal cells in hilus and granular layers of dentate gyrus (the arrowheads in (c)), and at the plasma membrane of pyramidal neurons (the arrow in (d)) and axons (the stars in (d)). Magnifications and scale bars are 400 × and 50 µm (b), 200 × and 100 µm (a), (c), and 100 × and 200 µm (d), respectively.
contralateral regions (right column). On the sonicated side, DAB staining is clearly visible on neuronal cell membranes in the hilus and granular layers of the dentate gyrus (figure 3(c)), CA1 (figure 3(a)), and CA3 (figure 3(d)) regions of the hippocampus, and on the ependymal cell membranes of the choroid plexus in the adjacent lateral ventricle (figure 3(b)). It should be noted that such difference in DAB intensity was not apparent in all the sections, but only in some, probably due to loss of TrkB phosphorylation state after the 20–30 min delay between sacrificing the mice post-sonication and the BDNF injection. On the other hand, there was strong immunoreactivity to pMAPK and pCREB in the sonicated left hippocampus of BDNF-administered mice in most of the sections analyzed 20–30 min post-injection. The temporal sequence of phosphorylation in downstream signaling molecules can be especially appreciated when the results are compared to those of the mouse expiring 3 min post-sonication. In those 3 min cases, the difference in DAB intensity between the left sonicated hippocampus and the control was observed only in the case of the pTrkB antibody (figure 3(c)), but not in pMAPK (figure 2(d)), pAkt (figure 4(b)), and pCREB (figure 4(e)) cases, suggesting that the BDNF-mediated phosphorylation of the TrkB receptor is rapid compared to phosphorylation of downstream molecules. As mentioned previously, immunoreactivity to downstream molecules was not only more pronounced, but also unique and distinct in the sonicated left hippocampus of >20 min samples. Phosphorylated MAPK was clearly detected in axons and dendrites of the pyramidal and granular neurons, but not in the neuronal cell bodies. pMAPK immunoreactivity was especially present in the mossy fibers of the CA3 hippocampal neurons (figures 2(b) and (c)). As expected, the phosphorylated CREB immunoreactivity was only observed in the nuclei and cytoplasm of the neuronal soma in all CA regions and layers of the sonicated hippocampus. With respect to the right (unsonicated) hippocampus (in both the control and BDNF-administered cases), much lower levels of pMAPK and pCREB immunoreactivity were detectable, suggesting basal levels consistent with other literature (figures 2(b) and (c) and figures 4(c) and (d), right columns). In the case of pCREB, however, the CA1 region and parts of the granular layers of the dentate gyrus in the right unsonicated hippocampus showed complete absence of immunoreactivity (figures 4(c) and (d), right columns).

In the case of antibodies against pTrkB (Y515 and Y816 results combined), pMAPK, and pCREB, mean per cent difference in DAB stain intensity between the left and right hippocampal regions were significantly greater in the BDNF-administered mice (25.22%, 60.58%, and 56.91%) compared to that in the control mice (−1.36%, −9.20%, and −8.67%), respectively ($p < 0.05$; figure 4(f)). In the case of the pAkt antibody, no significant difference between the mean per cent differences in DAB stain intensity of the BDNF-administered mice and the control mice was observed, although parts of the sonicated thalamus in the BDNF-administered mice showed greater DAB stain intensity than the unsonicated side in certain sections (figure 4(a)).

4. Discussion

Despite the indisputable potential of the exogenous neurotrophic factors, their systemic use is hindered by their BBB impermeability, short half-life in the systemic circulation and the risks involved with repeated injections. Therefore, no recommended clinical dosage levels for systemic administration are available. Several groups have attempted to use alternative delivering methods, including carboxy-directed pegylation and chimeric peptide technology (Sakane and Partridge 1997) of the compound for greater transport across the BBB. Despite the success of such methods, the mode of delivery is non-specific and unsuitable for target-specific interventions within the brain. The combination of FUS and microbubbles allows for non-invasive and localized opening of the BBB that is transient
Figure 4. Immunohistochemical staining of 5 μm frozen sections using primary antibodies against phosphorylated Akt (a), (b) and phosphorylated CREB (c)–(e). Mice were sacrificed 20 min (a), (c), (d) or 3 min (b), (e) after sonication. The box in (c) shows the enlarged area in (d). Difference in DAB intensity between sonicated regions (left images) and the contralateral control (right images) is only observable in the 20 min samples (a), (c), (d); black stars). The greater intensity of the DAB stain in the sonicated region is especially noticeable in the thalamus in the case of pAkt (the stars in (a)), and in the CA1 region of hippocampus (the left two stars in (c)), and in neuronal cells of the hilus and granular layers of dentate gyrus in the case of pCREB (the arrows in (d), left image). These findings are contrasted with the minimal or lack of DAB staining in neuronal cells of the hilus and granular layers of dentate gyrus in the control unsonicated hippocampus (the arrows and arrowheads in (d), right image, respectively). Magnifications and scale bars are 40 × and 500 μm (c), 100 × and 200 μm (a), (b), (e), and 200 × and 100 μm (d), respectively. In (f), immunohistology stain intensity analysis shows PC between the left (FUS) and the right (no FUS) sides of the mice brains. A significant difference ($p < 0.05$, $n = 3$; depicted by the asterisks) was found between the BDNF-administered animal group and the control (no BDNF) animal group for the TrkB, MAPK, and CREB antibodies. The bars represent mean ± standard deviation.
and potentially safe (based on short-term histological assessment (Baseri et al 2010)). In addition, the FUS method provides greater control over the delivery and time of BBB recovery based on the acoustic pressure and bubble size (Sakane and Pardridge 1997). Finally, as this study demonstrated, BDNF bioactivity in the targeted brain regions is preserved following FUS-mediated delivery, as indicated by the increased immunoreactivity to the activated BDNF TrkB receptor, and to the activated downstream signaling molecules MAPK and CREB in the hippocampal neurons in the sonicated regions.

Regarding the reversibility of the BBB opening, a recently published report by our group (Samiotaki et al 2011) has shown the reversibility of the BBB opening using the same parameters as in this study. As far as the large animal or clinical feasibility of transcranial FUS is concerned, we have recently demonstrated BBB opening through the human and monkey skulls, the former in vitro (Deffieux and Konofagou 2010) and the latter both in vitro and in vivo (Tung et al 2011, Marquet et al 2010, 2011). The dependence of the BDNF bioactivity on the acoustic pressure will constitute a topic of future studies.

One limitation of this study is that only three BDNF-injected mice with a circulation time of 20–30 min were used to demonstrate feasibility compared to the three controls in the statistical analysis. The higher costs involved in acquiring BDNF, required a limited number of mice that could be used as a trade-off for allowing sufficient amounts of the compound to reach the brain to observe significant difference in the outcome. Hence, feasibility was deemed worthwhile given the translational potential of therapeutic applications with this neurotrophic factor. Another limitation was the different BDNF concentrations that were administered in different mice. As this was a feasibility study, an increasingly lower dosage was used as the amount of BDNF available was limited and a maximum number of mice with the available concentration needed to be secured. However, the circulation time of BDNF was kept the same (20–30 min) across all three mice used compared to the controls. According to the statistical results, more significant cascade effects were shown to be triggered in the sonicated regions compared to the contralateral (control) side in all cases, indicative of the fact that a critical BDNF concentration was reached sufficient for triggering these effects. Also, two additional neurotrophic factors that have shown promise in treating Parkinson’s disease (Benisty et al 1998, Eisch et al 2003), were also tested, namely GDNF (figure 5(i)) and NTN (figure 5(ii)) using the same sonication parameters as in the BDNF study but targeting the caudate putamen instead of the hippocampus in two mice as the former is more relevant to Parkinson’s disease. It is clearly shown that, although all these three proteins are of similar molecular weight and overall constituency, not all neurotrophic factors will successfully cross the BBB after opening with FUS. NTN does behave similarly to BDNF permeating through the opened barrier and into the parenchyma (figure 5(ii)), however, GDNF does not (figure 5(i)). The latter finding was verified in 12 mice while the former in two. The noted discrepancy may lie in the fact that there are fewer receptors in the brain for GDNF than for BDNF, i.e. the two proteins on two different transport mechanisms. Also, we found that the GDNF gets broken down in circulation within the first 45 s, and upon imaging post-mortem, no fluorescence was found in the brain (figure 5(ii-b)), kidneys, liver, bladder, or testes. Therefore, not only was GDNF not detected in the brain parenchyma but was also absent from the other organs, pointing to significant differences in the GDNF systemic circulation compared to those of BDNF and NTN.

Using MRI-based permeability assessment techniques, it was recently shown that the permeability of the sonicated region increases by at least 100-fold compared to the contralateral (control) side (Vlachos et al 2010). Nevertheless, future studies are needed to determine the exact amount of BDNF that diffuses through the BBB via FUS (percentage of administered dose), and ultimately, determine the therapeutic dose of exogenous BDNF in animal models.
Figure 5. (a) T1-weighted MR image of the entire mouse head verifying BBB opening using gadolinium enhancement and (b) fluorescence image magnified in the region of interest where the highest gadolinium enhancement was detected (white rectangle) in two separate murine brains; one with fluorescently tagged (Alexa Fluor) (i) GDNF (Invitrogen, Inc.) and the other with (ii) NTN were systemically administered using the same methods as for the BDNF study except for the target being the caudate putamen instead of the hippocampus. No statistical or immunohistochemistry studies were conducted in these two cases to determine the downstream effects.

of disease. In addition, despite the fact that increased BDNF expression or supply may lead to effective neuronal regeneration, exogenous BDNF could also have adverse effects such as a pro-epileptic effect (Koyama and Ikegaya 2005) depending on the underlying propensity. Hence, both the beneficial and harmful effects of exogenous BDNF need to be adequately addressed prior to therapeutic clinical applications.

5. Conclusion

Neurotrophic delivery to the brain has been proven essential in reversing the neuronal degeneration process but so far has been hindered by the BBB. In this study, not only was it shown that the BDNF can cross the ultrasound-induced BBB opening but that it can also trigger signaling pathways in the pyramidal neurons of the hippocampus in mice in vivo from the membrane to the nucleus. As shown with two additional neurotrophic factors, namely GDNF and NTN, these preliminary findings do not automatically apply to all such proteins and will depend on the pharmacokinetics and other properties of the molecular uptake of the molecule in the brain. However, the fact that a therapeutic molecule after permeation through
the opened BBB triggered a molecular cascade and entered the neuronal nucleus, indicates for the first time that FUS in conjunction with microbubbles can generate downstream effects at the cellular and molecular level and thus increase the drug’s efficacy and potency in controlling or reversing disease.

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