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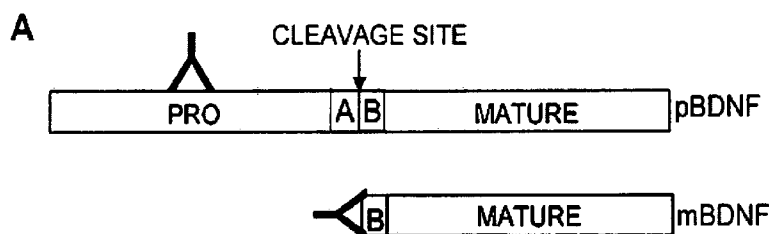
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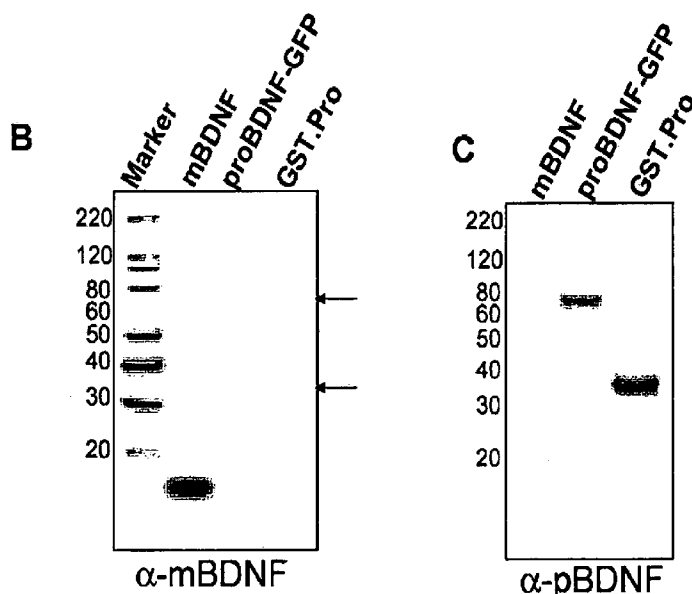
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(54) Title: METHOD FOR MEASURING MATURE BRAIN DERIVED NEUROTROPHIC FACTOR



(57) Abstract: A method of obtaining antibody specific for mature brain-derived neurotrophic factor (mBDNF), and uses thereof.





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METHOD FOR MEASURING MATURE BRAIN DERIVED NEUROTROPHIC FACTOR

5 BACKGROUND

Brain derived neurotrophic factor (BDNF) is a member of the neurotrophin family of secretory proteins. It is synthesized as a precursor protein (proBDNF), which is then proteolytically processed to form mature BDNF (mBDNF). These two forms of BDNF differ substantially in size, with unglycosylated proBDNF having an empirical molecular weight of about 27.8 kDa and mBDNF of about 13.5 kDa [1, 2, 3, 4]. Soon after synthesis, proBDNF enters the endoplasmic reticulum (ER) lumen through its signal peptide and processed subsequently by the signal peptidase and N-glycosylated [1, 5]. ProBDNF can be cleaved to form mBDNF by intracellular proteases including the serine protease furin in the trans-Golgi network and the prohormone convertases (PC1/3) in the secretory granules [1, 5]. Recent studies have demonstrated that proBDNF can also be processed extracellularly by matrix metalloproteinases (MMP3 or MMP7) and the serine protease plasmin [6, 7]. Because of the lack of specific tools to detect mBDNF, it remains unclear the relative contributions of intracellular and extracellular proteases in different tissues and different types of cells. It is also unclear how the intracellular and extracellular cleavages are regulated in various biological processes. Unlike other neurotrophins, BDNF can be sorted into one of the two secretory pathways: constitutive (passive) or regulated (induced or active) [2, 8, 9]. While ProBDNF can be measured by antibodies specific for the pro-domain of proBDNF, tools to detect mBDNF alone are lacking. Therefore, the ratio of proBDNF/mBDNF that is secreted through the constitutive or regulated pathway remains unknown.

Until a few years ago, only mBDNF was considered to be biologically active, and the pro-domain was suggested to play a passive role in folding and the maturation process [10, 11,12]. However, proBDNF can be detected in the extracellular milieu. Recent findings on proNGF and mature NGF have led to the "Yin-Yang hypothesis", which suggests that pro- and mature neurotrophins could elicit opposite biological effects through the activation of two different receptor systems: the p75 neurotrophin receptor (p75^{NTR}) and the Trk receptor tyrosine kinases, respectively [7]. The Yin-Yang hypothesis explains well the biological functions of proBDNF and mBDNF. In the peripheral nervous system (PNS), numerous studies have demonstrated the role of mBDNF in neuronal survival [13,14,15]. In contrast, proBDNF induces apoptosis in peripheral neurons [7,16,17, 18]. In the central nervous system (CNS), the major function of BDNF is to regulate synaptic plasticity [19,20,21,22].

Mature BDNF plays a critical role in early phase long-term potentiation (E-LTP) [23]. In addition, tPA, by activating plasminogen, converts proBDNF to mBDNF extracellularly in the hippocampus, and such a conversion is critical for L-LTP expression [24]. This work provided a mechanistic link between tPA and BDNF in L-LTP, and revealed a physiological role for extracellular cleavage of proBDNF. More recently, we have shown that proBDNF, if not processed, selectively enhances NMDA receptor-dependent long term depression (LTD) by activating its preferred receptor p75^{NTR} [25]. Thus, parallel to their roles in mediating cell death/survival in the PNS, proBDNF and mBDNF also elicit opposite effects on LTP/LTD in the CNS [7]. Therefore, cleavage of proBDNF by intracellular or extracellular proteases has now emerged as an important mechanism in controlling the direction of neurotrophin regulation. To further understand the differential biological functions of proBDNF and mBDNF, there is a growing demand for methods that can distinguish and, specifically detect the two isoforms of BDNF.

Numerous studies have reported changes in the levels of BDNF protein in various pathological conditions. The levels of BDNF in cerebrospinal fluid (CSF), serum or postmortem brain tissues are altered under several neurodegenerative disorders, and pathophysiological conditions. For example, BDNF levels are lowered in the serum and CSF of patients with relapsing remitting Multiple Sclerosis [26], Huntington's disease [27], and schizophrenia [28, 29], whereas the levels increase in CSF of children suffering from asphyxia [30]. In addition, reduced levels of BDNF were found in the plasma of patients with acute coronary syndromes [31, 32]. BDNF levels are also reduced in the brain of patients suffering from Alzheimer's [33][34][35]. Recently, an increase in BDNF levels in the sera of newborn infants has been shown to predict intellectual and social development abnormalities, particularly in autism [36]. None of these studies have distinguished proBDNF from mBDNF. Majority of previous methods of detecting BDNF were based on the use of antibodies raised against the mature domain of BDNF, and therefore cannot differentiate between proBDNF and mBDNF. Antibodies against proBDNF have recently been generated. This was relatively easy since the pro-domain, which is unique for proBDNF, could be used to generate the antibodies. Antibody detection of mBDNF is difficult because the sequence in mBDNF is shared by proBDNF. However, to better understand the cellular processing/secretion as well as differential biological functions of proBDNF and mBDNF, and to measure their relative levels in various neurological and psychiatric disorders, it is essential to develop new tools and methods that specifically detect mBDNF.

Based on the idea that cleavage of proBDNF will expose a new N-terminus of mBDNF, we have invented a method that allows the generation of an antibody against this previously unexposed epitope.

5 SUMMARY

Since pro- and mature BDNF elicit very different and sometimes opposite biological effects, it is important to have a method that distinguishes mBDNF from proBDNF. This method should allow measurement of the relative amounts of proBDNF and mBDNF synthesized in neurons, and detection of their secretion through either constitutive or
10 regulated pathway.

It is therefore an object of the invention to provide a method of generating antibody against mBDNF. In one embodiment, the method comprises one or more of the following steps: i) the use of the N-terminal amino acid sequence of mature BDNF to generate antibody; ii) affinity purification procedure to obtain a substantially purified antibody, and iii)
15 immuno-depletion of the substantially purified antibody using a peptide that spans the cleavage site in proBDNF. The resulting antibody is highly specific for mBDNF because the portion that reacts with proBDNF has been removed.

The expression "substantially purified" is intended to mean a compound or substance (e.g. antibody) that is isolated from its natural surroundings, for example, having been
20 removed from whole blood or serum by a process that may include affinity purification, or the like.

The N-terminal amino acid sequence (peptide) used in the initial step can be any fragment from the N-terminal of mBDNF of suitable length to effectively elicit antibody formation (e.g. an N-terminal fragment of SEQ ID NO:1). In one embodiment, a seven
25 amino acid sequence corresponding to the amino terminus of the cleaved mBDNF is used (HSDPARR, SEQ ID NO:2), preferably with a cysteine residue at the C-terminus as a linker (i.e. HSDPARRC, SEQ ID NO:4) Suitable peptides will generally be from 6 to 50 amino acid residues, more usually 6-25 or 7-15 residues in length. It will be appreciated that peptides with any integral number of amino acids between 4 and 50, inclusive, can be used
30 for this purpose. Peptides that correspond to the amino terminus of cleaved mBDNF, such as SEQ ID NO:2, are referred to herein as B-peptides. Methods for generating peptides are well known in the art.

The peptide can be administered to any animal suitable for the production of antibodies (e.g. a rabbit) using methods that are known in the art [37] the serum collected

after an interval sufficient for antibody production, and the resulting antibody isolated by affinity purification. For example, the antibody can be purified on a column conjugated with SEQ ID NO:2. Following this initial purification, the antibody is then further purified by contacting it with a peptide that spans the cleavage point in proBDNF, for example,

5 CSMRVRRHSDPARR (SEQ ID NO:3) Suitable peptides for this purpose will generally be from 12-50, more generally 12-25, and in particular 12-16 amino acid residues in length. It will be appreciated that peptides with any integral number of amino acids between 8 and 50, inclusive, can be used for this purpose. Such peptides are referred to herein as A+B peptides. The resulting antibody has a high specificity for mBDNF.

10 It is also an object of the invention to provide antibodies obtained using the methods of the invention. The antibodies of the invention can be used alone or with other agents in suitable compositions for the detection of mBDNF, for diagnostic or investigative (e.g. research) purposes. Thus, the invention includes antibodies made by the methods outlined above, and compositions comprising the antibodies.

15 It is also an object of the invention to provide a method of specific and reliable detection of mBDNF comprising the steps of contacting a sample suspected of containing mBDNF with an antibody specific for mBDNF and measuring the level of antibody-antigen complex in said sample. In one preferred embodiment, the antibody is one that has been generated by the method described herein, i.e. an antibody that has been raised against a
20 fragment of the N-terminal of mBDNF, and purified by contacting it with a peptide spanning the cleavage point of proBDNF to mBDNF. The antibody may be labeled, for example with a fluorescent label, to aid in detection of antibody antigen complexes. Many suitable means of labeling antibodies are known in the art (see, e.g. conjugation kits available from Upstate Signaling Solutions, Q-dot conjugation kits from Invitrogen etc., [38]). For example, labels
25 may be directly linked through a covalent bond or covalently through a linking molecule which typically bears reactive sites capable of forming covalent bonds with the label and the antibody respectively. Other techniques familiar in the art, for example, radiolabels, enzymatic labels, biotin-avidin labels, chemiluminescent labels, quantum dot, may also be used.

30 The terms "BDNF", "proBDNF" and "mBDNF", as used herein, mean any isoform of a mammalian BDNF, proBDNF or mBDNF, in particular a human BDNF. In addition to human BDNF, BDNF sequences for rodents (rat and mouse) and other species are known in the art. Information as to the sequences and properties of these forms can be found on the NCBI website and in other resources known to persons of skill in the art [39].

The compositions and methods of the present invention are expected to be useful for research purposes and for clinical studies. As mentioned above, most studies to date in measuring BDNF protein levels have used antibodies raised against the mature domain of BDNF, and therefore have measured both proBDNF and mBDNF. A few investigators have used proBDNF specific antibody, but to the inventors' knowledge, no one has a method to detect specifically mBDNF. The mBDNF specific antibody generated using the method described in this application will be used to revisit the expression and regulation of mBDNF protein. We therefore anticipate a large commercial usage of reagents based on the mBDNF specific antibody (Western blot, immunoprecipitations, immunocytochemistry, ELISA, etc). Moreover, we expect a wide range of application of the mBDNF-specific detection methods be used in clinical investigations. Further, an ELISA developed using the antibody of the present invention is expected to be useful for differentiating between the two forms of BDNF in these and other clinical disorders. Measurements can be made, for example, on samples of blood or cerebrospinal fluid from such patients.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Western blot detection of purified mBDNF. (A) Schematic representation of proBDNF, mBDNF, and the epitopes that the proBDNF and mBDNF antibodies are directed towards. The mBDNF antibody was raised against the "B" peptide, affinity purified using the "B" peptide conjugated column, and depleted against the "A+B" peptide. (B) Detection of purified mBDNF. Equal amounts of purified proteins (GST fused to Pro-domain of BDNF, proBDNF fused to EGFP and mBDNF) were resolved on 4 – 12% NuPAGE, transferred to PVDF membrane, and probed using the affinity-purified anti-mBDNF antibody at 1:500 dilution. Arrowhead points to the position of purified mBDNF. Note that the mBDNF antibody detects only mBDNF, and nothing else. (C) Control blot showing the existence of the GST-Pro fragment and proBDNF. The same blot was stripped and re-probed using anti-proBDNF antibody. Arrows point to the positions of the GST-Pro fragment and proBDNF-EGFP, respectively.

Figure 2. Western blot detection of mBDNF in transfected cells. (A) Schematic representation of the BDNF construct containing the N-terminal tags HA, FLAG, pro domain, mature domain, and the C-terminal tags EGFP and V5. The molecular weights of some fragments shown in C are also indicated. (B) Western analysis using the anti-mBDNF

antibody. AtT20 cells stably transfected with or without the epitope-tagged BDNF were lysed and processed for Western blot. (C) Control blot showing the existence of various fragments. The same blot was stripped and re-probed using anti-GFP antibody. While three bands of GFP containing proteins (as indicated) could be visualized using anti-GFP antibody (C), only the mBDNF was detectable in cell lysates using the mBDNF-specific antibody (B).

Figure 3. Western blot detection of endogenous mBDNF in brain tissues *in vivo*. Hippocampal and cortical tissues were dissected from the adult rat brain, and homogenized. The proteins were separated on NuPAGE gel and transferred to the PVDF membrane. The blot was probed with anti-mBDNF antibody. Note that this antibody detects only mBDNF, but not proBDNF.

Figure 4. Immunofluorescence staining of cultured hippocampal neurons expressing epitope tagged BDNF under membrane permeable conditions. Neurons transfected with HA-tagged BDNF construct (shown in Fig. 2A) were grown at low density for more than 14 days in vitro (>14DIV), fixed, permeabilized using 0.2% Triton X-100, and processed for immunocytochemistry using anti-HA (red) and anti-mBDNF (green) specific antibodies. Note the non-overlapping areas. Arrowheadss indicate mBDNF only spots, whereas arrows indicate proBDNF only spots.

Figure 5. Cell surface staining of proBDNF and mBDNF secreted under the constitutive and regulated conditions. Neurons transfected with HA-tagged BDNF construct (shown in Fig. 2A) were grown at low density for >14DIV, fixed under the non-permeable conditions, and processed for immunocytochemistry. In one group (A, top, constitutive), the neuronal activity was completely blocked by TAC (Tetrodotoxin, 2-APV and CNQX) for 12 hours, and cells were immunostained with either a monoclonal antibody against HA for proBDNF (red, 1:500) or anti-mBDNF antibody (green, 1:250). In another group (B, bottom, regulated), cells were depolarized by high KCl (50 mM) for 15 minutes, and stained in the same way as above. Superimposed images on the right show that proBDNF and mBDNF are differentially distributed on the cell surface. Arrowheadss indicate mBDNF only spots, whereas arrows indicate proBDNF only spots.

This application claims priority to U.S. provisional application no. 60/816597, filed June 27, 2006, which is hereby incorporated by reference in its entirety.

DETAILED DESCRIPTION

Materials

Antibodies:

- 5 Antibodies used in this study: (a) Mouse anti-GFP at 1/500 (Covance Research Products, Inc, 800 University Avenue, Berkeley, CA 94710, USA); (b) Rabbit anti-GFP at 1/500 (Abcam- Abcam Inc, One Kendall Square, Bldg. 200, 3rd Floor, Cambridge, MA 02139, USA) (c) Mouse anti-HA at 1/1000 (Covance); (d) mature specific antibody at 1/500 (custom made); (e) pro-BDNF specific antibody at 1:5000 (custom made); (f) Rabbit anti-BDNF at 1/1000
10 (N-20 Santacruz - 2145 Delaware Avenue, Santa Cruz, California 95060, U.S.A.); (g) Anti-rabbit Alexa Fluor 488 at 1/1500 (Molecular probes - 1600 Faraday Avenue, PO Box 6482, Carlsbad, California 92008); (h) Anti-mouse Alexa Fluor 633 at 1/1500 (Molecular Probes).

DNA constructs:

- DNA sequences corresponding to the signal peptide followed by haemagglutinin (HA) tag, FLAG tag and the pro-peptide of BDNF were synthesized with Xba I (5') and Nde I (3') ends
15 and ligated upstream between Xba I – Nde I sites in the parent BDNF-EGFP construct in pET21d(+). Subsequently, the entire open reading frame was amplified by polymerase chain reaction using primers with CACC sequence at the 5' and 3' ends to clone into a lentiviral vector using the pLenti6/V5 directional topo-cloning kit (Invitrogen). Sequence and the
20 reading frame were verified by automated DNA sequencing, and its expression was judged by Western analysis of cell lysates prepared from HEK and AtT20 cell lines transfected with the construct.

Neuronal cultures:

- Rat hippocampal/cortical neurons are prepared on day E18 of pregnancy. The animals were
25 sacrificed according to the Animal user guidelines provided by National Institutes of Health. Briefly, animals were euthanized and the embryos were removed under sterile conditions into ice cold Hanks Balanced Salt Solution (HBSS - Invitrogen) containing 10 mM HEPES pH 7.4, 1 mM sodium pyruvate, 2 mM glutamine and antibiotics. After removing the meninges from the embryonic brain, hippocampus or cortex was removed into HBSS and subjected to
30 trypsin (0.05%) and DNase I digestion (0.1 µg/ml) for 15 min at 37°C. The tissues were then washed three times with Dulbecco's Modified Eagles Medium containing 10% heat inactivated Fetal Bovine serum (DMEM-FBS - Invitrogen) followed by trituration using a blunt ended transfer pipette. Cells were then clarified using a 40 µm cell strainer and on a 4% Bovine serum albumin fraction V (Invitrogen) diluted in DMEM-FBS. When nucleofected

with DNA constructs, 5×10^6 cells were mixed with a cocktail of $5 \mu\text{g}$ endotoxin-free DNA (prepared using Qiagen columns - 27220 Turnberry Lane, Suite 200, Valencia, CA 91355) in the Amaxa reagent mix, and nucleofected using the protocols specified for rat or mouse neurons provided by the manufacturer (Amaxa, 205 Perry Parkway, Suite 7, Gaithersburg, MD 20877, USA). Cells were immediately diluted in DMEM-FBS and seeded according to the required dilutions (20000 – 30000 cells on a 18mm diameter No.1 Carolina glass cover slips of 0.17mm thickness coated with Poly-D-Lysine in 0.1M Borate Buffer followed by Laminin I (ATCC – P.O. Box 1549, Manassas, VA 20108, USA) at $10 \mu\text{g/ml}$ for low-density seeding or 0.6×10^6 cells/well in a 6 well plate for high-density seeding) and maintained at 37°C in the presence of 5% CO_2 . To measure the endogenous BDNF release, 100 million cortical neurons were seeded at the density of 0.2×10^6 cells/ cm^2 on 10 tissue culture dishes. In all cases, the media was replaced with Neurobasal supplemented with B27 and 2mM Glutamax I (Invitrogen) after 12 – 18 hours. The cells were grown for at least 14 days with continuous replacement of the Neurobasal medium with supplements after every 2 days. All the experiments were performed on mature cultures that are $\geq 4\text{DIV}$.

Surface immunofluorescence staining:

Neurons grown on cover slips were washed three times with phosphate buffered saline (PBS) and then fixed with 4% paraformaldehyde in PBS containing 100 mM sucrose for 3-5min on ice. All the procedures were performed on ice. The cells were then washed three times and incubated with 100 mM glycine for 10min. Blocking was done using 3% BSA (Pierce - Pierce Biotechnology, Inc., P.O. Box 117, Rockford, IL. 61105 U.S.A) in PBS for 60 min, followed by incubation for 2 hours with primary antibody diluted in blocking buffer. Excess antibody was then washed using PBS and incubated with secondary antibody (Alexa Fluor antibodies from Molecular probes) diluted in blocking buffer for 1 hour. Following three washes with PBS, the cover slips were rinsed with water and then mounted on medium containing Mowiol 4-88 and the antifade DABCO, and left overnight in dark for curing. The cells were scanned using a 63XApoChromat lens in the Zeiss confocal microscope LSM 510 Meta.

Generation of mBDNF antibody:

A seven amino acid synthetic peptide sequence corresponding to the amino-terminus of the cleaved mature BDNF (or B-peptide: HSDPARRC) was used for immunizing New Zealand White Rabbits after conjugating the peptide to Keyhole limpet hemocyanin (KLH) through the thiol (-SH) group of Cysteine at the C-terminus. Before immunization, 25 ml of blood was collected from the central ear artery using a 19 gauge needle for pre-immune serum.

About 1mg of the carrier conjugated peptide was mixed with Freund's complete adjuvant and subcutaneously injected in rabbits to induce the primary immune response. After 3rd and 7th week, two more doses of the carrier conjugated peptide was administered subcutaneously along with Incomplete Freund's adjuvant [40]. The animals were then bled to check the immune response towards the injected antigen using the BSA conjugated peptide in the Enzyme linked immunosorbent assay [37]. Rabbits that gave good immune response were further boosted with the carrier conjugated peptide before collecting the antiserum for affinity purification.

The antibodies were diluted 1:1 with 0.01M Tris buffer pH7.2 and 1mM EDTA and allowed to bind to the first affinity column (also called B peptide column). This column was prepared by mixing B-peptide with thiopharose 6B beads at the ratio of 6:1 for 2 hours at room temperature, and then washing the beads with Phosphate buffered saline (PBS) several times until the free peptide is completely removed from the column [38]. After three boost injections with KLH conjugated B-peptide, the rabbit serum was collected and affinity purified on the column conjugated with B-peptide. Briefly, the antibody solution was loaded onto the B-peptide column, and excess proteins from the serum were washed with PBS until no protein was detected in the flow through. The B-peptide column was then eluted using 0.1 mM Glycine buffer pH 2.5 to collect fractions with all antibodies against the B-peptide. The fractions were pooled and dialyzed against PBS. The antibodies against the B-peptide were then passed through the second affinity column (also called A+B peptide column, see Fig. 2A, as described above), which was generated the same way as the B peptide column except A+B peptide (CSMRVRRHSDPARR) (SEQ ID NO: 3) was used instead of B peptide. The flow through in this procedure contains the antibody which specifically recognizes the cleaved end of the mBDNF, but not the uncleaved proBDNF were used in this study.

Example 1

Specific detection of mBDNF

Because proBDNF and mBDNF elicit different and often opposite biological effects [7], it is essential to distinguish whether secreted BDNF is in pro- or mature isoform. Although Western blot can distinguish proBDNF and mBDNF based on their molecular weights, ELISA and immunocytochemistry are better methods because they are much more sensitive and can be used when very small number of cells/tissues are available. Therefore, we developed a method that allows specific and reliable detection of mBDNF, with high sensitivity. The method is based on the premise that cleavage of proBDNF will generate a

previously unexposed epitope, which can be used to generate a specific antibody. An antibody was raised against the synthetic peptide corresponding to the amino termini of the cleaved mBDNF (peptide B, Fig1A), affinity-purified using the B peptide column, and depleted against the peptide spanning the cleavage sequence (peptide A+B, Fig. 1A). Using this strategy, we generated a B-antibody that specifically detects only mBDNF, but not proBDNF. On a Western blot with equal amounts of purified proteins, the B-antibody specifically recognized mBDNF, but not proBDNF or the pro-fragment (pro-domain of BDNF fused to glutathione-S-transferase at the N-terminus) (Fig. 1B). Re-probe of the same blot with an anti-proBDNF antibody detected proBDNF and the pro-fragment, but not mBDNF (Fig. 1C). We also tested the specificity of the B-antibody in cell lysates from a stable cell line expressing BDNF with the epitope tags (Fig. 2A). While the B-antibody specifically recognized the mBDNF (Fig. 2B), polyclonal anti-GFP antibody detected proBDNF, mBDNF and EGFP in cell lysates (Fig. 2C).

Example 2

Detection of mBDNF in brain tissues

To ascertain the specificity of the mBDNF antibody in vivo, tissue homogenates are prepared from specific subregions of the brain, such as cortex and hippocampus. Briefly, the cortex and the hippocampus were removed from the adult rat brain and immediately homogenized in the presence of protease inhibitors in RIPA buffer containing 1% SDS. The homogenates were then sonicated repeatedly and then centrifuged. The protein concentration of the supernatant was estimated and 30µg of total protein from cortex or hippocampus were resolved on a 4-12% NuPAGE, transferred to PVDF membrane and probed using the anti-mBDNF (B-peptide) antibody. Even in brain tissue, the B-peptide antibody specifically detects only the mature BDNF, but not proBDNF or any other proteins (Fig. 3).

Example 3

Immunofluorescence staining of Hippocampal neurons for pro- and mBDNF

Having established the specificity of the anti-mBDNF antibody by Western blotting in purified proteins, cell lysates and in brain tissues, we then checked the specificity of the antibody by immunofluorescence under membrane permeable conditions to observe the intracellular distribution of proBDNF and mBDNF in neurons. Since endogenous mBDNF in the cultured hippocampal neurons was extremely low, a HA-tagged construct was transfected into these neurons to aid detection. Staining with the B-peptide antibody specifically detected

mBDNF (Fig. 4). There are subcellular compartments specifically recognized by the B-antibody (mBDNF, indicated by arrowheads), as well as those by the HA antibody (proBDNF, indicated by arrows). These results validate the specificity of the B-antibody, and its potential to be used to study subcellular distribution of mBDNF.

5

As demonstrated by the results in the examples above, the methods of the invention are effective in generating useful antibodies for measuring levels of mBDNF, and should accordingly be useful for generating antibodies that can be used both in research and clinical settings.

10

It is to be understood that this invention is not limited to particular embodiments described, as such may, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting, since the scope of the present invention will be limited only by the appended claims.

15

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Listing of sequences:

The complete sequence of mature human BDNF is

HSDPARRGELSVCDSEWVTAADKKTAVDMSGGTVTVLEKVPVSKGQLKQYFYE
5 **TKCNPMGYTKEGCRGIDKRHWNSQCRTTQSYVRALTMDSKKRIGWRFIRIDTSCVC**
TLTIKRGR (SEQ ID NO:1)

Example of B-peptide: **HSDPARR (SEQ ID NO:2)**

10 Example of A+B peptide with cysteine at N-terminus for conjugation:
CSMRVRRHSDPARR (SEQ ID NO:3)

Example of B-peptide with cysteine: **HSDPARRC (SEQ ID NO:4)**

15 Sequence corresponding to the pro-domain of BDNF:
MKEANIRGQGGLAYPGVRTHGTLESVNGPKAGSRGLTSLADTFEHVIEEL
LDEDQKVRPNEENNKDADLYTSRVMLSSQVPLEPPLLFLLEEYKNYLDAANMSMRV
RR (SEQ ID NO:5)

20

WE CLAIM:

1. A method of generating antibody against mBDNF comprising the steps of:
 - i) providing a substantially purified antibody to the N-terminal amino acid sequence of mature BDNF; and
 - ii) immunodepletion of the substantially purified antibody by contacting it with a peptide that spans the furin cleavage point of proBDNF (A+B peptide), such that antibody that reacts with said A+B peptide is removed; to obtain an antibody that is specific for mBDNF.
2. The method of claim 1, wherein the substantially purified antibody is obtained by a process that includes affinity purification.
3. The method of claim 1 wherein the mBDNF antibody is against human mBDNF.
4. The method of claim 1 wherein the N-terminal amino acid sequence of BDNF is between 6 and 50 amino acid residues.
5. The method of claim 1 wherein the N-terminal amino acid sequence of BDNF is between 6 and 25 amino acid residues.
6. The method of claim 1 wherein the N-terminal amino acid sequence of BDNF is B-peptide (e.g. SEQ ID NO:2).
7. The method of claim 1 wherein the peptide that spans the cleavage point of proBDNF is between 12 and 25 amino acid residues.
8. The method of claim 1 wherein the peptide that spans the cleavage point of proBDNF is SEQ ID NO:3.
9. The method of claim 1 wherein the antibody is generated by immunizing a nonhuman mammal with the N-terminal amino acid sequence of mature BDNF.
10. The method of claim 9 wherein the mammal is immunized with SEQ ID NO:4.

11. The method of claim 9 wherein the mammal is a rabbit.
12. A method of detection of mBDNF comprising contacting a sample suspected of containing mBDNF with an antibody obtained by the method of one of claims 1-11 under conditions suitable for antibody-antigen complexes to form, and measuring the level of antibody-antigen complex in said sample.
13. The method of claim 12 wherein the sample is a serum sample.
14. The method of claim 12 wherein the sample is a sample of cerebrospinal fluid.
15. An antibody obtained by the method of one of claims 1-11.

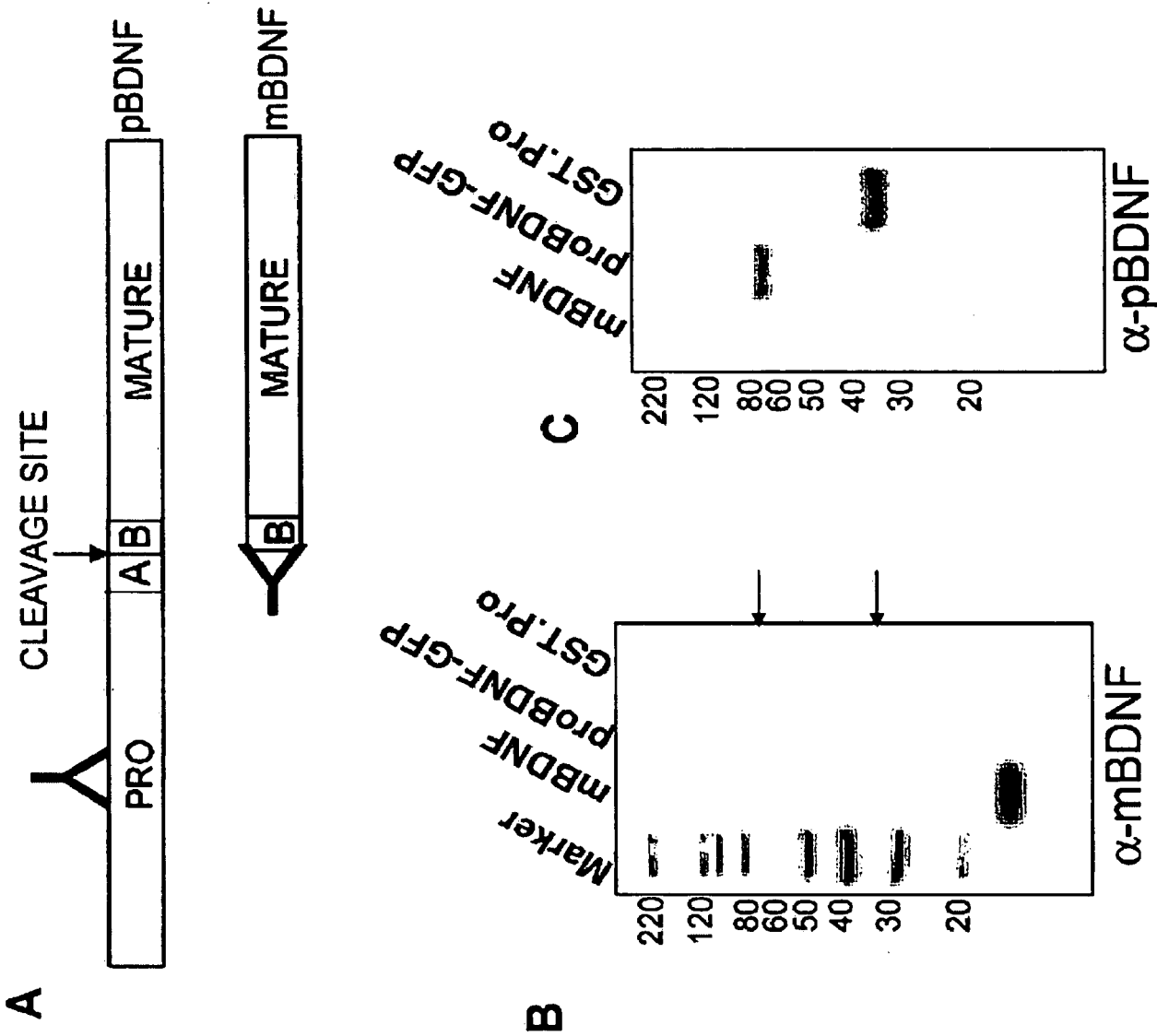


Figure 1

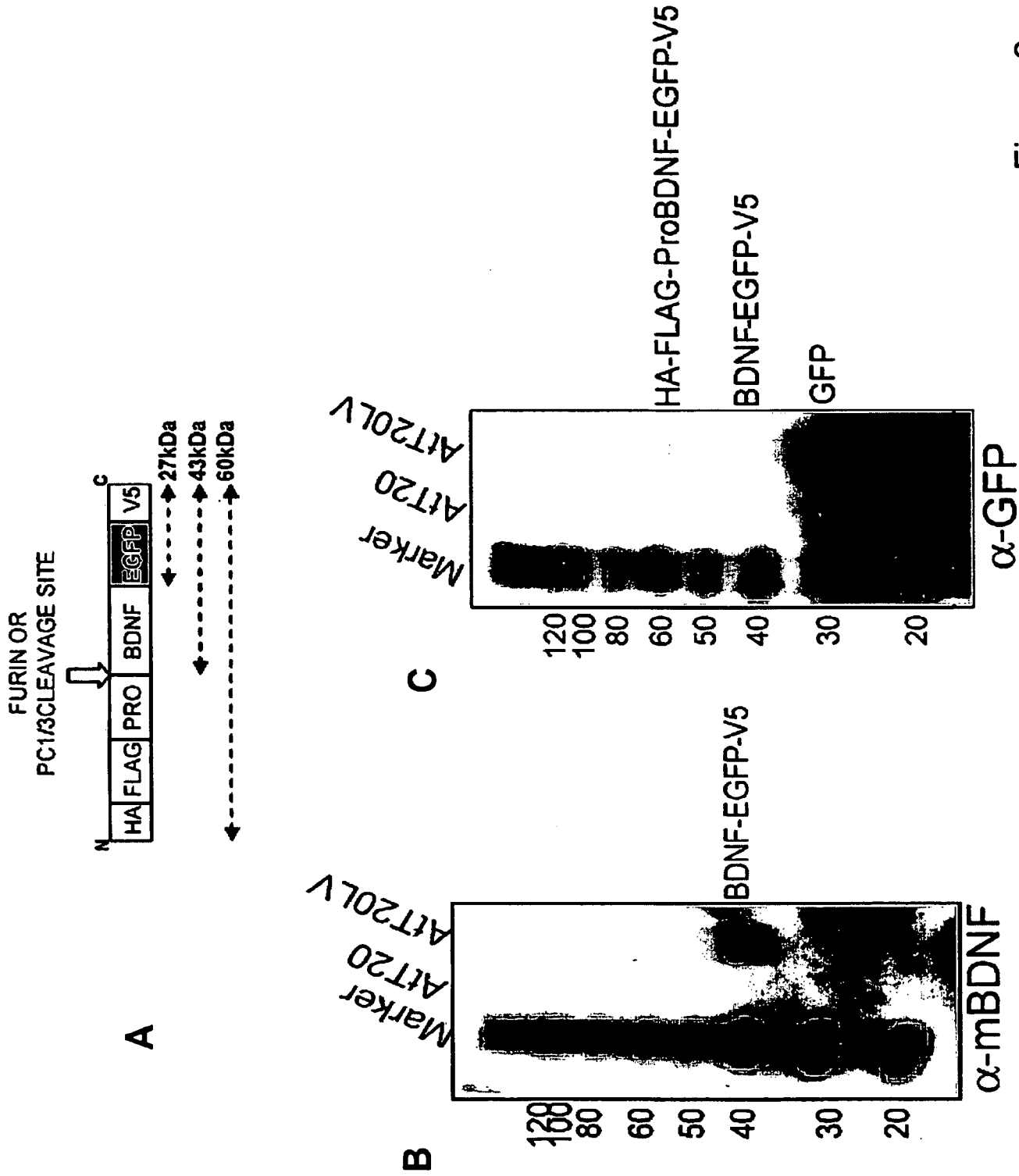


Figure 2

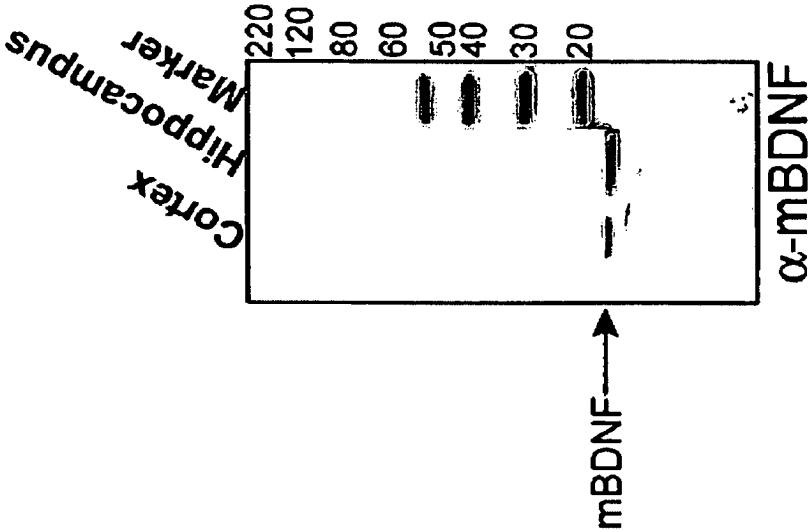


Figure 3

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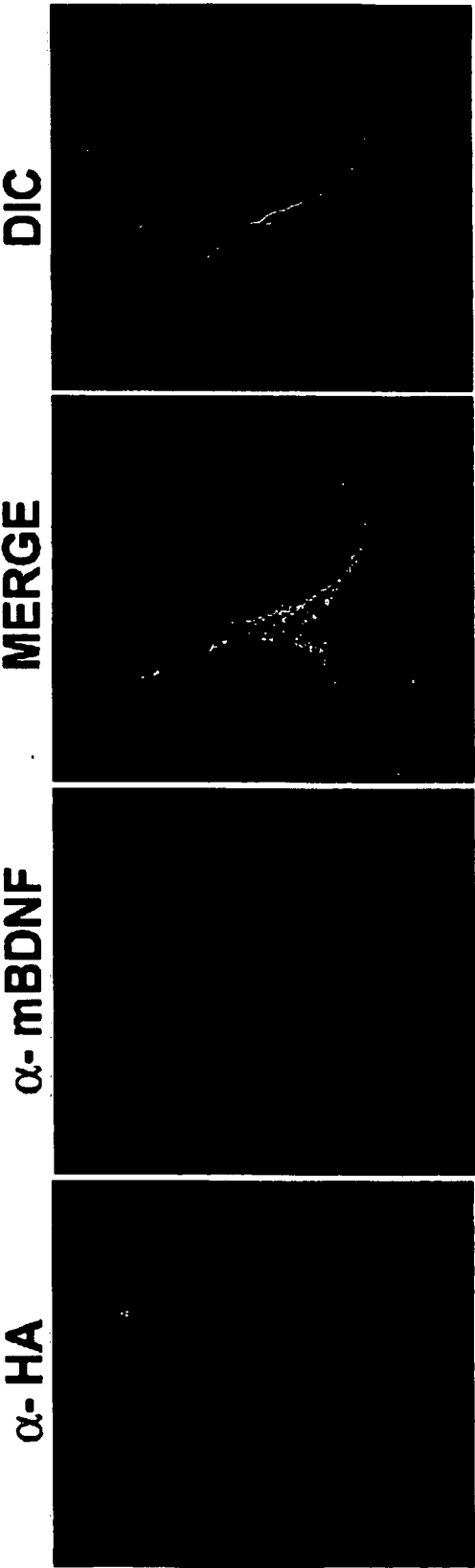


Figure 4

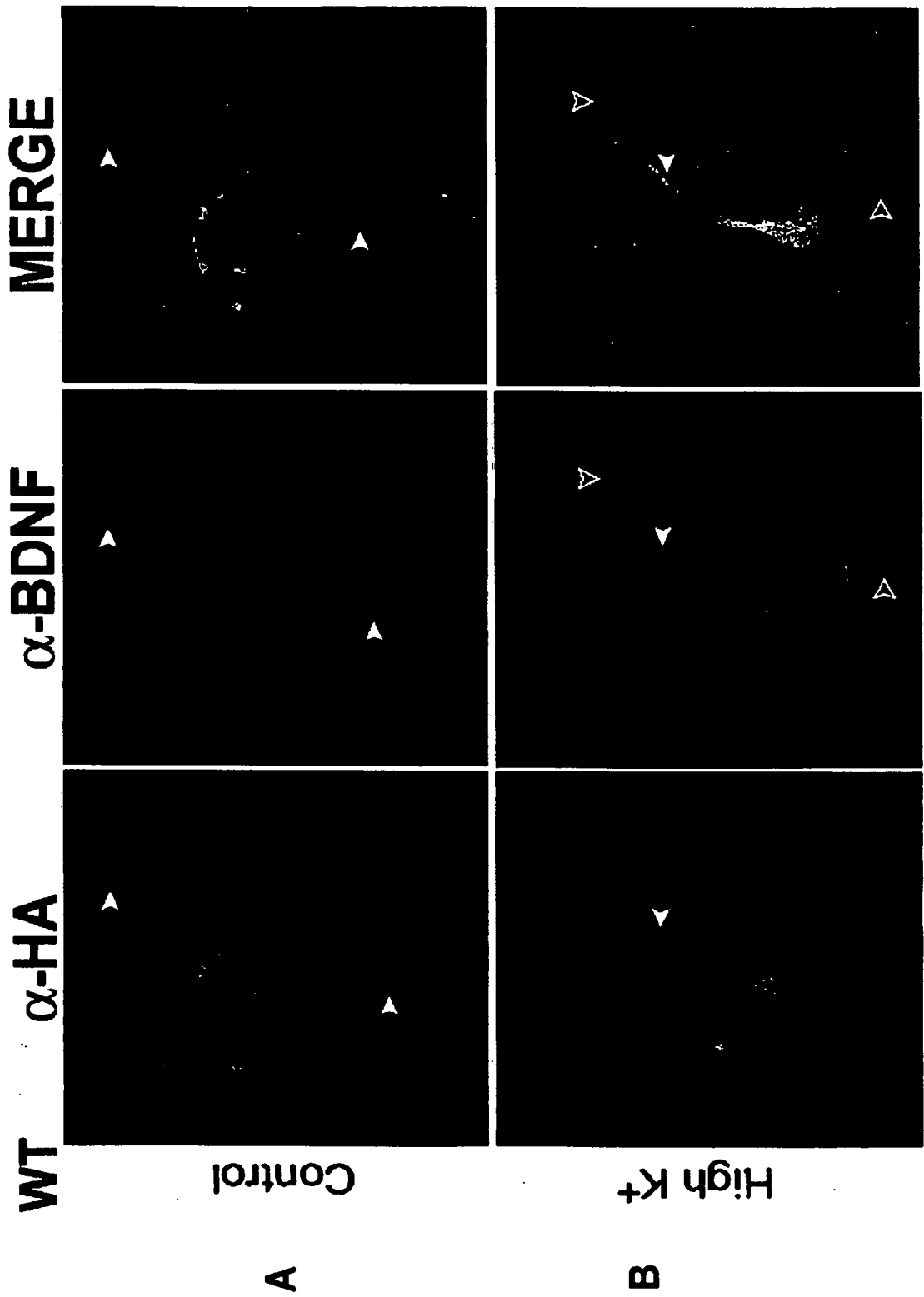


Figure 5