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Phosphoproteomic Analysis of Neurotrophin Receptor TrkB Signaling Pathways in Mouse Brain

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SUMMARY

1. The signaling pathways activated by trkB neurotrophin receptor have been studied in detail in cultured neurons, but little is known about the pathways activated by trkB in intact brain. TrkB is a tyrosine kinase and protein phosphorylation is a key regulatory process in the neuronal signal transduction pathways.

2. We have investigated trkB signaling in the transgenic mice overexpressing trkB in postnatal neurons (trkB.TK) using phosphoproteomics.

3. We found that several proteins are overphosphorylated on tyrosine residues in the brain of trkB.TK mice and identified some of these proteins.

4. We demonstrate that the well characterized signaling molecules mitogen-activated protein kinase (MAPK) and cyclic AMP responsive element binding protein (CREB) were phosphorylated at a higher level in the brain of trkB.TK mice when compared to the wild type littermates. Furthermore, we found that β-actin was tyrosine phosphorylated in the brain of the transgenic mice.

5. Our results demonstrate that phosphoproteomics is a sensitive approach to investigate signaling pathways activated in mouse brain.

KEY WORDS: BDNF; phosphorylation; MAPK; CREB; actin; proteomics.

INTRODUCTION

Neurotrophins are critical regulators of neuronal survival, connectivity and plasticity in the peripheral and central nervous systems (Huang and Reichardt, 2001; Lu, 2003). Among neurotrophins, the brain-derived neurotrophic factor BDNF is relatively abundantly expressed in brain and it has been recently connected to a variety of brain functions, such as learning and memory, anxiety, depression and feeding control (Castrén, 2004; Huang and Reichardt, 2001; Lu, 2003).

BDNF binds to and activates the receptor tyrosine kinase trkB (Barbacid, 1994; Huang and Reichardt, 2003; Kaplan and Miller, 2000; Klein *et al.*, 1991). Upon ligand binding, two trkB receptors dimerize and become autophosphorylated

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on specific tyrosine residues. Phosphorylated tyrosine serve as docking sites for adaptor and signaling molecules which activate intracellular signaling cascades and thereby mediate the effects of BDNF to cellular functions and gene transcription (Huang and Reichardt, 2003; Kaplan and Miller, 2000).

Experiments in cultured neurons and cell lines show that trkB autophosphorylation activates at least three well-characterized signaling pathways: the interaction of the shc adaptor molecule with the phosphorylated tyrosine 515 leads to the activation of the small GTP binding protein ras and further to the activation of the mitogen-activated protein kinase (MAPK) pathway, which is considered a critical pathway for the effects of BDNF on cellular morphology and plasticity (Huang and Reichardt, 2001, 2003; Kaplan and Miller, 2000). Second, shc binding also activates the phosphoinositol-3-kinase (PI3K) and thereby activates the AKT pathway, which has been suggested to mediate the effects of BDNF on neuronal survival (Huang and Reichardt, 2001, 2003; Kaplan and Miller, 2000). Third, phosphorylation of tyrosine 816 at the C terminus of trkB serves as the docking site for the phospholipase C-γ (PLCγ), which regulates intracellular calcium levels trough the release of inositol phosphates from phospholipids in the plasma membrane (Huang and Reichardt, 2001; Kaplan and Miller, 2000).

Although the importance of these pathways is well established in cultured cells, it is less clear which pathways are activated by BDNF and trkB in intact brain. For example, even though the shc binding site is considered to be of critical importance for the MAPK and AKT pathways, an *in vivo* mutation of the tyrosine 515 of trkB to phenylalanine (thereby preventing the phosphorylation of this site) in mice yielded viable and healthy mice with no apparent impairments in any of the BDNF mediated functions in brain, although effects mediated by the neurotrophin-4, another ligand of trkB, were compromised (Minichiello *et al.*, 1998). In contrast, mutation of the PLCγ binding tyrosine to phenylalanine yielded live mice with compromised long-term potentiation (LTP) (Minichiello *et al.*, 2002), which suggests that the PLCγ pathway mediates the impairments in learning and memory which are produced by the reduced BDNF or trkB activity (Linnarsson *et al.*, 1997; Lu, 2003; Minichiello *et al.*, 1999; Saarelainen *et al.*, 2000). Nevertheless, it remains unclear which molecular pathways are critical for the various effects of BDNF in brain.

We have recently characterized a mouse model of increased trkB signaling in adult brain. These mice overexpress functional trkB in neurons postnatally under the thy1 promoter and show increased trkB expression and autophosphorylation as well as the activation of the PLCγ pathway in brain (Koponen *et al.*, 2004a,b). Furthermore, the mice show better learning and memory and reduced anxiety when compared to their wild-type littermates in several different behavioral tests, which indicates that increased trkB expression also produces behavioral effects which reflects enhanced trkB signaling (Koponen *et al.*, 2004b). We have reasoned that examining the signaling molecules activated in the brain of these transgenic mice might reveal those signaling pathways which are utilized by trkB signaling in normal brain. In this study, we have used phosphoproteomic methods to compare phosphorylated proteins in the brain extracts from trkB overexpressing mice and their wild type littermates.

MATERIALS AND METHODS

Animals

The production of transgenic mice overexpressing the N-terminally FLAG-tagged full length rat TrkB receptor (trkB.TK) cDNA under the Thy1 promoter has been described in detail previously (Koponen *et al.*, 2004b). Mice were maintained in the National Laboratory Animal Center, University of Kuopio and the National Public Health Institute, Kuopio, Finland. Mice were housed in metal cages under standardized animal room conditions (12 h light/dark cycle, temperature $22 \pm 1^{\circ}$C, humidity 50–60%) and free access to food and water. For the experiments described below, heterozygous transgenic male mice were mated with CD2F1 (BALB/c $\times$ DBA/2) wild-type females to produce the heterozygous transgenic trkB.TK (tg) and the wild-type control mice (wt) from the same litters. All animal procedures were conducted in accordance with the guidelines issued by the Society for Neuroscience and approved by the Experimental Animal Ethics Committee of the National Laboratory Animal Center, University of Kuopio, Finland.

Protein Samples

Mice were killed by cervical dislocation, and parietal cortex and hippocampus were quickly dissected and collected into a lysis buffer containing 9.5 M Urea, 2% Triton X-100, 1% CHAPS, 2% ampholyte 3-10 pH, 100 mM DTT, 2 $\times$ Complete Mini^{TM} (Roche Diagnostics GmbH, Mannheim, Germany), 1 mM sodium vanadate, 1 nM Microcystin-LR^{TM} (ALEXIS Biochemicals, Switzerland) and homogenized with Pellet Pestle motor (Kontes, Vineland, NJ). Samples were incubated with rotation at $+22^{\circ}$C for 30 min, centrifuged at 12 000 g for 10 min and the supernatants were subjected to protein determination by 2-D Quant Kit^{TM} using the bovine serum albumin (BSA) as standard (both from Amersham Biosciences, SF, USA). The protein concentration was approximately 8–9 mg/ml, and the samples were stored at -70°C before using.

Isolation of the Acidic Protein Fraction from Parietal Cortex Lysates

Proteins isolated from the parietal cortex of trkB.TK mice were separated by isoelectric focusing in glass capillary tubes. Lysates were loaded (up to 60 μg per capillary) on an acrylamide gel (4% acrylamide total monomer, 9.2 M urea, 2% Triton X-100, 1% CHAPS, 2% ampholyte 3–10 pH) preformed in glass capillaries (int. diameter 1 mm, length 100 mm). Isofocusing was performed on Mini-Protean II 2-D Cell (Bio-Rad Laboratories, Hercules, CA) in accordance with instruction manual recommendations. Isofocused gels were extracted from tubes, the acidic zones (pH 3–pH 5) of gels were cut each into 3 sections (as shown on Fig. 4A). Proteins were electroeluted from the combined gel pieces of each section separately on Model 422 Electro-Eluter (Bio-Rad Laboratories, Hercules, CA). The electroeluted proteins were precipitated in cold methanol. Cold methanol (-20°C) was added to protein solution (1–4 volumes), samples were vortexed and incubated at -20°C for

1 h. The precipitated proteins were centrifuged at 14 000 *g* for 10 min, supernatants were discarded, pellets were washed once by 50% (v/v) methanol and dissolved either in TBS pH 7.4 for chromatography analysis or in 1 × Laemmli sample buffer (62.5 mM Tris-HCl, pH 6.8, 1% SDS (w/v), 5% 2-mercaptoethanol, 10% glycerol (v/v), and 0.001% bromophenol blue (w/v)) for immunoblotting. Proteins from sections 1 and 2 were combined and separated by reverse-phase chromatography on a 0.21 cm × 10 cm C1 column (TSK-TMS250; TosoHaas, Tokyo, Japan). The chromatography was performed using a linear gradient of 3–100% (v/v) acetonitrile in 0.1% (v/v) trifluoroacetic acid over 60 min at a flow rate of 200 μl/ min.

SDS-PAGE, Immunoblotting and Quantification

Tissue lysates in the required amounts were mixed with either 1 × Laemmli sample buffer for resolving by standard SDS-PAG electrophoresis or IPG reswelling buffer for resolving by two-dimensional electrophoresis. Samples were resolved by 10% SDS-PAGE or 2-D electrophoresis and transferred onto Immobilon-P^{TM} polyvinylidene fluoride (PVDF) transfer membranes (Amersham Biosciences). Membranes were blocked with 4% BSA in TBS-Tween 20 (0.1%). The blots were first probed with antibodies against the phosphorylated forms of the protein and then stripped and probed with antibodies against total proteins of the same type. Antibodies used were as follows: mouse anti-phosphotyrosine (4G10 Upstate Biotechnology, Lake Placid, NY, 1:3000), mouse anti-phospho-p44/42MAPK (E10, Cell Signaling Inc., 1:2000), anti-phosphoSer133-CREB (#9191, Cell Signaling Inc., 1:1000), rabbit anti-MAPK (#9102, Cell Signaling Inc., 1:5000), mouse anti-CREB-1 (X12, Santa Cruz Biotechnology, 1:2000), mouse anti b-actin (AC-15, Sigma 1:10.000).

Blots were developed using the enhanced chemiluminescence detection method ECL Plus^{TM} (Amersham Biosciences, CL, UK). Multiple exposures were taken at different times to ensure that nonsaturated ECL film (Amersham) was used for quantitation. Films were digitized by densitometry with a flatbed transparency scanner and quantified by image analysis software MCID/M4, version 3 (Imaging Research Inc.). All blots had 3–6 samples of both genotypes and each experiment was repeated at least three times for each antibody with similar results. Individual blots were analyzed by comparing WT–TG samples within the same blot and combined results of all blots presented as percentage of wild type (mean ± SD).

Two-Dimensional Polyacrylamide Gel Electrophoresis and Mass Spectrometry

The first dimension of the two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) was performed on an IPGphorTM (Amersham Pharmacia Biotech AB, Sweden) isoelectric focusing apparatus. Non-linear, 13 cm long, pH 3–10 Immobiline gels (IPG-strips; Amersham Pharmacia Biotech AB) were rehydrated in a strip holder for 6 h in 250 μl rehydration buffer, containing 9 M urea (Promega), 0.5% CHAPS (Fluka), 0.2% DTT (Sigma), 0.5% Pharmalytes 3–10 (Pharmacia) and 150 μg protein extract. Isoelectric focusing was carried out at 20°C with the following settings: 150 V, 1 h; 300 V, 1 h; 600 V, 1 h; linear gradient 600–5000 V,

3 h; and 5000 V, 2 h or until 20 000 Vh was reached. Prior to the second dimension, the IPG-strips were equilibrated for 10 min in 30 ml of 50 mM Tris-HCl, pH 8.8, 6 M urea (Promega), 30% glycerol, 2% SDS and 1% (w/v) DTT (Sigma) and then a further 10 min in the same buffer in which DTT was replaced with 4.5% (w/v) iodoacetamide (Sigma). The second dimension, 10% SDS-PAGE (Laemmli, 1970), was carried out in a Hoefer DALT electrophoresis tank (Pharmacia). After the electrophoresis, the gels were fixed for 1.5 h in 30% ethanol and 0.5% acetic acid and silver-stained using the method of O'Connell and Stults (O'Connell and Stults, 1997).

Protein Identification

Protein spots were excised from the gel and the proteins were destained, reduced, alkylated and digested in-gel with trypsin, as described previously (Pandey *et al.*, 2000). The recovered peptides were, after desalting, subjected to matrix-assisted laser desorption/ionization- time of flight (MALDI-TOF) mass spectrometric analysis.

MALDI-TOF mass spectra for mass fingerprinting and MALDI-TOF/TOF mass spectra for identification by fragment ion analysis were acquired using an Ultraflex TOF/TOF instrument (Bruker-Daltonik GmbH, Bremen, Germany). Protein identifications with the generated data were performed using Mascot® Peptide Mass Fingerprint and MS/MS Ion Search programs (http://www.matrixscience.com).

RESULTS

To evaluate the activation of the different TrkB signaling pathways and to search for new phosphorylation targets, we have investigated the proteome of parietal cortex and hippocampus of TrkB TK+ overexpressing (tg) and wild type mice (wt) using two-dimensional electrophoresis and phosphotyrosine (pTyr) immunodetection by Western blotting. We have observed distinct differences in pTyr protein profile between the wt and tg mice. Several proteins were phosphorylated at a higher level in the tg brain when compared to the brain extracts from the wt mice. (Fig. 1).

One of the extensively phosphorylated proteins (spot 1 on Fig. 1B) was identified and confirmed by immunoblotting as the mitogen-activated protein kinase (MAPK 42/44) (Fig. 2A). The expression and phosphorylation of MAPK42/44 in cortical extracts of tg and wt mice was further studied using 1D-gels and Western blotting. These experiments revealed that phosphorylation level of MAPK was, indeed, significantly increased in brains of tg mice when compared to the wt mice. Furthermore, the level of the total MAPK 42/44 protein was reduced in the tg brains, although the difference was not significant (Fig. 2B).

The transcription factor CREB (Cyclic AMP response element-binding) is able to mediate signals from various signaling pathways, one of them is MAPK pathway (Shaywitz and Greenberg, 1999). CREB has been shown to be activated

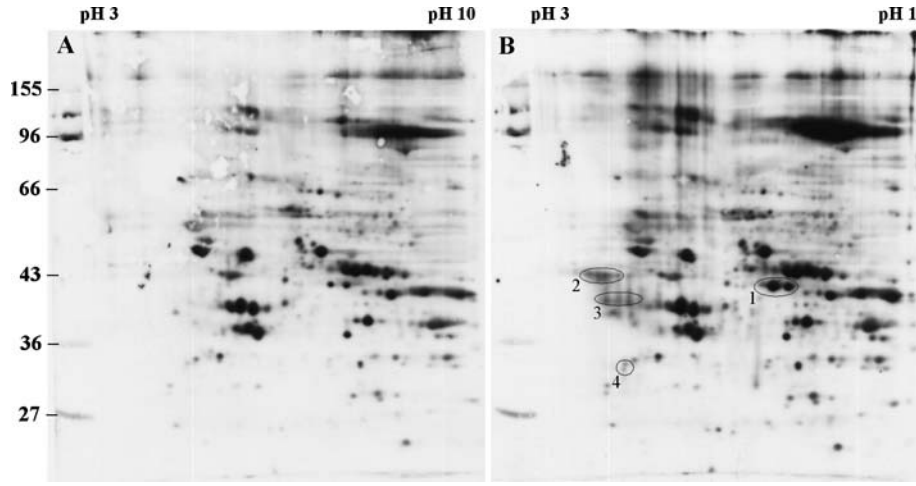

Fig. 1. The patterns of mouse parietal cortex proteins separated by 2-D gel electrophoresis using a wide-range non-linear pH gradient (pH 3–10). (A) wild type mouse, (B) TrkB.TK mouse. The proteins were immunoblotted with anti-phosphotyrosine antibody 4G10. The differentially phosphorylated proteins are marked on the gel.

by phosphorylation at Ser133 in response to BDNF stimulation in cultured neurons *in vitro* (Finkbeiner *et al.*, 1997). To elucidate the possible changes in activation of CREB, we have examined its phosphorylation and expression level in parietal cortex of tg and wt mice using 2-D electrophoresis and immunoblotting. This study has revealed significant increasing of CREB activation in tg mice in comparison to wt (Fig. 3).

Protein from the spots 2 (Fig. 1B) was identified by mass spectrometric analysis as a β-actin. The spots at the location 2 are located at acidic side (pH 3.0–4.0), distinctly separated from actin location on 2D gel. We have detected a very weak signal from these spots in direct detection by immunoblotting with β-actin antibody (data not shown). To investigate the β-actin from acidic part of the gel, we have purified acidic proteins from cortical lysates of tg mice as described (see Methods) (Fig. 4A). β-actin was clearly detected in the most acidic part (pH 3.0–4.0) and its position on blot coincided with a pTyr positive protein (Fig. 4B). Proteins from acidic fraction (sections 1 and 2 on Fig. 4) were combined and separated by reverse phase chromatography. Analysis of chromatography fractions by immunoblotting has revealed that β-actin and pTyr positive protein elution profiles are similar (Fig. 4C). Furthermore, immunoprecipitation using antibodies against β-actin followed by western blotting detection with the anti-pTyr antibody produced a very weak band in both tg and wt brains (data not shown). These results indicated that β-actin is (or can be) phosphorylated on tyrosine in tg mice. However, the minute fraction of the phosphorylated actin from the total actin precluded any further characterization of the phosphorylation site.

Proteins from spots 3 and 4 (Fig. 1B) could not be reliably identified by mass spectrometry and were therefore not further investigated.

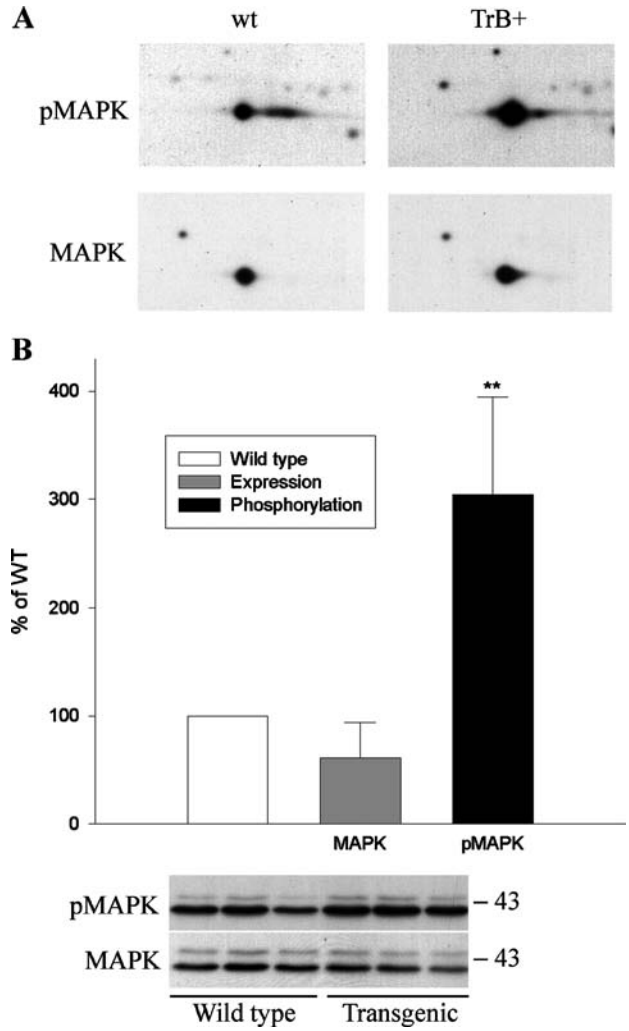

Fig. 2. Mitogen activated protein kinase (MAPK) pathway is activated in mice overexpressing trkB.TK. The patterns of mouse parietal cortex proteins separated by 2-D gel electrophoresis using a wide-range non-linear pH gradient (pH 3–10) (A) and 1-D electrophoresis (B) are shown. Proteins were immunoblotted with antibody recognizing the phosphorylated forms of p42 and p44 MAPK and reprobed with panMAPK antibody. Blots are presented as mean ± SD, $^{**}P < 0.01$.

DISCUSSION

The signaling pathways activated by neurotrophins have been extensively investigated in cell lines and cultured neurons *in vitro* (Huang and Reichardt, 2001; Kaplan and Miller, 2000) and many of these pathways have also been verified in

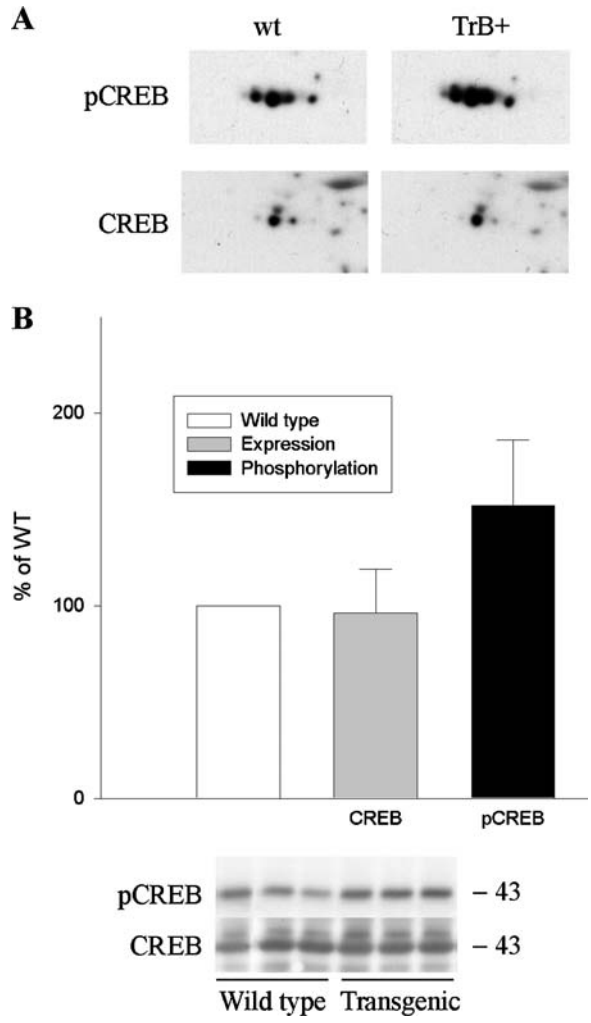

Fig. 3. Mouse parietal cortex proteins separated by 2-D gel electrophoresis using a narrow-range linear pH gradient (pH 3–7) (A) and 1-D electrophoresis (B). Proteins were immunoblotted with an antibody recognizing the phosphorylated CREB and reprobed with the CREB antibody. Quantitation revealed ~1.5-fold increase in phosphorylation of CREB after normalization against total protein expression. 1-D and 2-D Blots are presented as mean ± SD, $^{*}P < 0.05$.

in vivo settings. However, in many cases, transfected trk receptors and saturating neurotrophin concentrations have been used. While such conditions reveal the pathways which can be activated through trk signaling, they tell little about which neurotrophin-activated pathways are actually used in the CNS of living animals. We have produced a transgenic mouse which overexpress trkB under a promoter

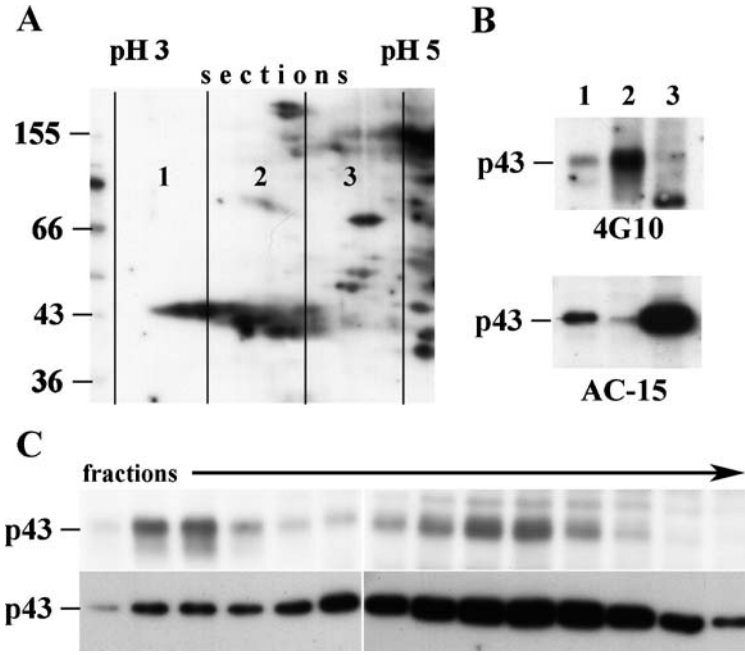

Fig. 4. Tyrosine phosphorylation of β-actin. Proteins of parietal cortex of trkB.TK transgenic mice were separated by isoelectric focusing in glass capillary tubes. The acidic zones of the gels were cut each on 3 sections (as shown on A). (B) Proteins were electroeluted from the combined sections, precipitated by methanol, separated by electrophoresis and immunoblotted with phosphotyrosine (4G10) and β-actin (AC-15) antibodies. C: Proteins from the 1 and 2 sections were combined and separated by reverse phase chromatography, the fractions were immunoblotted with 4G10 (upper panel) and AC-15 (lower panel).

which directs expression to postnatal neurons and observed increased trkB activity in the brain of these mice (Koponen *et al.*, 2004b). In agreement with the neuron-specific characteristics of the thy1 promoter used to drive the transgene expression (Aigner *et al.*, 1995; Ingraham and Evans, 1986), we have previously shown using the Flag epitope inserted to the N-terminus of the transgene that the transgene is expressed only in neurons and not in glial cells (Koponen *et al.*, 2004a,b), and the expression of the transgene is turned on during the third postnatal week (Koponen *et al.*, 2004b). The late activation of the promoter has prevented us from using cultured neurons derived from the brains of E15 embryos of these mice in *in vitro* experiments, since the promoter is not active in these cultures under our experimental conditions (E.C., unpublished observation). We have shown that these mice express a behavioral phenotype which is largely opposite to that described in mice with reduced BDNF levels in brain (Gorski *et al.*, 2003; Linnarsson *et al.*, 1997) which is consistent with the increased BDNF signaling in brains of these mice (Koponen *et al.*, 2004b). Specifically, mice learn better in several hippocampus-dependent learning paradigms and show reduced anxiety

(Koponen *et al.*, 2004b). We therefore reasoned that these mice might be of use in an attempt to investigate which signaling pathways are activated by trkB activation in brain *in vivo*. By using phosphoproteomic approach, we have here revealed several proteins which are overphosphorylated in the cortical samples of trkB.TK mice and identified some of them by mass spectrometry and immunoblotting.

Proteomics is a rapidly expanding field of systems biology and phosphoproteomics is a newly emerged subfield of proteomics (Mann *et al.*, 2002; Pandey and Mann, 2000). Phosphoproteomics utilizes either phospho-specific antibodies or the possibility to label phosphorylated proteins by using radioactively labeled orthophosphate as substrate for activated kinases. Both of these methods are exceedingly sensitive and can detect minute amounts of phosphorylated proteins. As typically only a small fraction of the total protein gets phosphorylated, and phosphorylation often alters the migration of the protein in 2D gels in one or both directions, the site where phosphorylated proteins are detected represents usually only a small fraction of the protein present in neurons (Mann *et al.*, 2002). Even though methods for protein detection by mass spectrometry have become very sensitive in recent years, detection of minute spots recognized by very sensitive antibodies is often still very difficult.

One of the proteins found to be overphosphorylated in the brain of transgenic mice was MAPK42/44. MAPK is a critical component in the pathway which mediates extracellular signals to transcriptional changes in nucleus. MAPK has been previously shown to be altered by trkB activation both in cell lines and in cultured neurons *in vitro* (Huang and Reichardt, 2001; Kaplan and Miller, 2000). However, the role of MAPK pathway in the signal transduction of trkB *in vivo* is less clear. Overexpression of BDNF in hippocampus fails to increase phosphorylation of MAPK (Tolwani *et al.*, 2002) and genetic mutation of the tyrosine 515, which is considered the mediator of MAPK pathway activation, does not interfere with trkB signaling in transgenic mice *in vivo* (Minichiello *et al.*, 1998). We have previously observed only a slight and non-significant increase in the phosphorylated form of MAPK in the hippocampus of the trkB.TK mice (Koponen *et al.*, 2004b). In the current study, a careful analysis using both 2D and 1D gels revealed that the phosphorylation of MAPK is, indeed, increased in the parietal cortex of trkB.TK mice. In our hands, 2D gels appear more sensitive in detecting changes in MAPK phosphorylation than western blots, nevertheless, we also detected a significant increase in MAPK phosphorylation using westerns, although we previously reported only a non-significant increase (Koponen *et al.*, 2004b). Thus, improved methodology appears as the most plausible explanation for the apparent discrepancy between our current observations and our previous report. In our previous studies, we observed that total protein levels of components of two other signaling pathways, PLCγ and AKT, were reduced while the levels of phosphorylated proteins were increased or not changed, respectively (Koponen *et al.*, 2004b), which might represent a downregulation of the total protein to compensate for the increased fraction of the phosphorylated, active form. In agreement with those observations, the increased levels of phosphorylated MAPK were paralleled with a small reduction in the total MAPK protein.

CREB is phosphorylated and activated in response to several different intracellular signaling pathways (Shaywitz and Greenberg, 1999). Among these,

neurotrophins NGF and BDNF have been shown to increase phosphorylated CREB in cultured neurons (Finkbeiner *et al.*, 1997). On the other hand, CREB is the major regulator of BDNF gene expression in neurons (Shaywitz and Greenberg, 1999). We show here that CREB is overphosphorylated at the Ser131 in the brains of transgenic mice overexpressing trkB in neurons in vivo. We have previously shown that increased CREB phosphorylation in response to antidepressant drug treatment is attenuated, when trkB signaling is inhibited in transgenic mice thorough the overexpression of a dominant-negative trkB isoform (Saarelainen *et al.*, 2003). These data suggest that the level of CREB phosphorylation is delicately and bidirectionally regulated by the activity of trkB receptor in brain.

Another protein which is phosphorylated on tyrosine at a higher level in trkB overexpressing mouse cortex than in the wild type cortex was identified as β-actin. Actin is one of the most abundant proteins in the cell and a key component of cellular cytoskeleton. The level of tyrosine phosphorylation of β-actin turned out to be very low in our samples and was found in acidic fractions, well separated in 2D gels from the main body of β-actin expression in cells. Unfortunately, this very low fraction of phosphorylated form of β-actin precluded the further experiments required for the identification of the phosphorylated tyrosine within β-actin. There are only few reports on tyrosine phosphorylation of β-actin and its physiological consequences in mammalian cells (Baba *et al.*, 2003; Lim *et al.*, 2004), whereas in slime molds and plants, tyrosine phosphorylation of actin has been demonstrated to be an important regulator of actin filament rearrangement and cell shape (Howard *et al.*, 1993; Kameyama *et al.*, 2000). In agreement with our findings, actin phosphorylation in Dictyostelium and plants was found to occur at a minor acidic isoform of actin (Howard *et al.*, 1993; Kameyama *et al.*, 2000). Since the structure of β-actin is highly conserved in evolution, our data suggest that tyrosine phosphorylation could regulate the reorganization of actin filaments also in mammalian brain. In agreement with this notion, it has recently been shown that phosphorylation and dephosphorylation of β-actin is crucial for the reorganization of actin filaments in mammalian cells (Baba *et al.*, 2003), and that stimulation of a human fibroblasts cell line with epidermal growth factor, which activates a receptor tyrosine kinase related to trkB, induces tyrosine phosphorylation of actin (Lim *et al.*, 2004). These data suggest that growth factors, including BDNF, might regulate cellular architecture by inducing tyrosine phosphorylation of β-actin. However, due to the low levels of phosphorylated actin in our samples, the verification of this will have to wait for further experiments.

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