Binding of the Receptor Tyrosine Kinase TrkB to the Neural Cell Adhesion Molecule (NCAM) Regulates Phosphorylation of NCAM and NCAM-dependent Neurite Outgrowth*

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Recognition molecules and neurotrophins play important roles during development and maintenance of nervous system functions. In this study, we provide evidence that the neural cell adhesion molecule (NCAM) and the neurotrophin receptor TrkB directly interact via sequences in their intracellular domains. Stimulation of TrkB by brain-derived neurotrophic factor leads to tyrosine phosphorylation of NCAM at position 734. Mutation of this tyrosine to phenylalanine completely abolishes tyrosine phosphorylation of NCAM by TrkB. Moreover, the knockdown of TrkB in hippocampal neurons leads to a reduction of NCAM-induced neurite outgrowth. Transfection of NCAM-deficient hippocampal neurons with mutated NCAM carrying an exchange of tyrosine by phenylalanine at position 734 leads to promotion of NCAM-induced neurite outgrowth. Furthermore, TrkB mediates the tyrosine phosphorylation of NCAM at position 734. Thus, the interaction of the two proteins regulates NCAM-mediated neurite outgrowth. Our observations indicate a functional relationship between TrkB and NCAM.

Development and maintenance of nervous system functions depend on molecular cues that interact with each other in morphogenetic processes, such as cell proliferation, migration, differentiation and survival, neuritogenesis, and synaptogenesis, as well as in regulation of synaptic activity in the adult. Recognition molecules have been shown to play an important role in these interactions. Equally important are neurotrophic factors that regulate neuronal survival and neuritogenesis not only during development, but also in the adult, when the nervous system is under constraint to regenerate after injury and during synaptic plasticity, underlying learning and memory (1).

Among the recognition molecules, the neural cell adhesion molecule (NCAM) has been recognized to be a major player in neuronal cell interactions, including formation and modulation of synaptic contacts (2–6). Overlapping in some functions with NCAM, the neurotrophic receptor TrkB (receptor tyrosine kinase B), a receptor tyrosine kinase that is triggered by its ligand, the brain-derived neurotrophic factor (BDNF), regulates neuronal cell survival, neurite outgrowth, synaptogenesis, and synaptic activity (7–13). It has been suggested that NCAM via its a2,8-linked polysialic acid (PSA) presents BDNF to TrkB, thus concentrating the neurotrophin close to the site of its action (14, 15). On the other hand, a recent study showed that PSA removal increases the binding of BDNF to septal neurons and thereby causes an increase in choline acetyltransferase activity (16). Here we show that NCAM and TrkB directly interact with each other via their intracellular domains and that the interaction of the two proteins regulates NCAM-mediated neurite outgrowth. Furthermore, TrkB mediates the tyrosine phosphorylation of NCAM, thus playing a regulatory role in NCAM-mediated neurite outgrowth.

EXPERIMENTAL PROCEDURES

Mice—C57BL/6 mice or NCAM-deficient (NCAM<sup>−/−</sup>) mice (17) that had been back-crossed onto the C57BL/6 background and their wild-type (NCAM<sup>+/+</sup>) littermates were bred and maintained at the animal facility of the Universitätsklinikum Hamburg-Eppendorf.

Antibodies—The monoclonal NCAM antibodies P61 and 5B8 reacting with the intracellular domain of the 140- and 180-kDa isoforms of NCAM (NCAM140-ID and NCAM180-ID), the mouse monoclonal NCAM180-ID specific antibody D3, the mouse monoclonal NCAM antibody H28 and the polyclonal NCAM antibody 1B2 both against the extracellular domain of NCAM, and the mouse monoclonal antibody 735 against PSA have been described (18–21). Mouse anti-penta-His antibody was from Qiagen (Hilden, Germany). The pan-Trk rabbit polyclonal antibody C-14 raised against a peptide in the C terminus (8, 22), the TrkB-specific rabbit polyclonal antibody H-181 raised against amino acids 160–340 in the extracellular domain of human TrkB (7, 8, 22), the TrkB-specific rabbit polyclonal antibody 794 against the cytoplasmic domain of TrkB, the mouse phosphotyrosine antibody PY20, and TrkA- and TrkC-specific antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). All horseradish peroxidase (HRP)-coupled secondary antibodies were purchased from Dianova (Hamburg, Germany) and Jackson Immunoresearch (West Grove, PA).

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§ The abbreviations used are: NCAM, neural cell adhesion molecule; BDNF, brain-derived neurotrophic factor; ID, intracellular domain; PSA, polysialic acid.

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Germany), and streptavidin-HRP was from Sigma-Aldrich (Deisenhofen, Germany).

DNA Constructs—Rat pcDNA3-NCAM140 and rat pcDNA3-NCAM180 were a kind gift of Patricia Maness (University of North Carolina, Chapel Hill, NC). Cloning of the intracellular domain of mouse CHL1 (close homolog of L1) into pcDNA3 was described (23). Rat full-length TrkB cDNA was cloned into the pcDNA3 plasmid (Invitrogen). To generate the NCAM140-Y734F and NCAM140-Y734E mutants, the sense primer 5'-G GTG ATC ATG GAC ATC ACC TGC TTC TCT CGT AAC AAG TGT GGC C-3' or the sense primer 5'-G GTG ATC ATG GAC ATC ACC TGG AGC TAC CAC GCC C-3' and the antisense primer 5'-G GCC ACA CTT GTT CAG GAA GGA GCA GGT GAT GTC CAT GAC C-3' or the antisense primer 5'-G GCC ACA CTT GTT CAG GAA GGA GCA GGT GAT GTC CAT GAC C-3' were used, respectively (sequence differences from wild-type NCAM140 are given in boldface type).

Production and Purification of Recombinant Intracellular Domains—Sequences coding for NCAM140-ID (bp 2135–2550) and NCAM180-ID (bp 2135–2850) or CHL1-ID (bp 3315–3627) were amplified and cloned into pQE30. Protein expression in Escherichia coli strain M15/pREP4 and purification of soluble proteins under native conditions using a French pressure 20K chamber (Spectronic Instruments/SLM Aminco) at 10,000 p.s.i. for 5 min on ice and nickel-chelate resin (Ni2+-NTA beads, Qiagen) was conducted according to the manufacturer's instructions. Protein solutions were dialyzed against phosphate-buffered saline, pH 7.3 (PBS) and concentrated using Centricon filter devices (Millipore Corp., Bedford, MA). Native TrkB-ID prepared in a baculovirus expression system (24) was a kind gift of Shinichi Koizumi and Motohiko Kometani (Novartis Pharma K.K., Tsukuba Research Institute, Ibaraki, Japan).

Phage Display—A phage library (New England Biolabs, Frankfurt, Germany) displaying 1010–1011 random 12-mer peptides at the pili of M13-like phage particles in fusion with the N terminus of the pVIII major coat protein was used (25). All in vitro selection steps were performed according the Ph.D.-12™ phage display peptide library kit instruction manual version 2.0 (New England Biolabs). NCAM180-ID immobilized on Ni2+-NTA beads (Qiagen) was used for biopanning. After three rounds of biopanning, bound phages were eluted using 0.2 m glycine-HCl, pH 2.2, and single phage clones were selected, amplified in Escherichia coli strain ER2738, and subjected to DNA sequencing.

Biochemical Cross-linking—0.2 mg of Sulfo-SBED (Perbio Science, Bonn, Germany) dissolved in 5 μl of DMSO was incubated with 0.2 mg of NCAM180-ID or CHL1-ID in 0.5 ml of PBS for 1 h at room temperature in the dark. Unbound cross-linker was removed by overnight microdialysis in PBS. Brains from 2–3-month-old C57BL/6J mice were homogenized at 4 °C in PBS containing 1 mM MgCl2, 1 mM MnCl2, 1 mM EGTA, 1 mM NaF, 0.5 mM Na2VO4, 1 μM N-[3,5-difluorophenyl]acetyl-l-αlanyl-2-phenylglycine-1,1-dimethylethyl ester (Calbiochem), 20 μM GM6001 (Calbiochem), 1× Complete™ EDTA-free protease inhibitor mixture (Roche Applied Biosciences). After centrifugation for 45 min at 25,000 × g and 4 °C, the resulting membrane pellet was resuspended in RPMI medium (PAA Laboratories, Pasching, Austria) and preincubated at 37 °C for 2 h. After the addition of protein-cross-linker complexes, the samples were incubated for 30 min at room temperature and exposed to UV light (365 nm) for 15 min on ice. Triton X-100 was added at a final concentration of 1%, and after a 45-min incubation on ice, the samples were centrifuged for 5 min at 200 × g and 4 °C. The supernatants were incubated with 350 μl of Ni2+-NTA beads for 1 h at 4 °C. After washing the beads with PBS, bound protein was eluted by 0.25 m imidazolium, pH 8.0, 300 mM NaCl, and 50 mM NaH2PO4 and incubated with 50 μl of magnetic streptavidin Dynabeads (Dynal Diagnostics, Hamburg, Germany) for 1 h at 4 °C. The beads were washed with PBS, and bound proteins were eluted by boiling the beads in SDS-PAGE sample buffer (60 mM Tris/HCl, pH 6.8, 2% SDS, 1% β-mercaptoethanol, 10% glycerol, 0.02% phenolphlor blue).

Immunoprecipitation—To isolate brain membranes, 2–3-month-old NCAM-deficient or wild-type littermate mice were homogenized in HOMO buffer (5 mM Tris-HCl, 0.32 m sucrose, 1 mM MgCl2, 1 mM CaCl2, 1 mM NaHCO3, 1× Complete™ EDTA-free protease inhibitor mixture, pH 7.5) and centrifuged at 17,000 × g for 20 min at 4 °C. The pellet was resuspended in 9 volumes of ice-cold H2O plus 1× Complete™ EDTA-free protease inhibitor mixture and adjusted to 5 mM Tris-HCl, pH 7.5. After centrifugation at 25,000 × g for 20 min at 4 °C, the pellet was resuspended in immune precipitation buffer (25 mM Tris-HCl, 150 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS, pH 7.6). The detergent extracts were centrifuged for 1 h at 100,000 × g and 4 °C and subjected to preclearing by incubation with Protein A/G-agarose Plus (Santa Cruz Biotechnology). Triton X-100 (final concentration 0.5%) and antibody were added to the precleared supernatant and incubated for 3 h at 4 °C. Protein A/G-agarose beads were added and incubated overnight at 4 °C under constant agitation. NCAM antibody H28 was immobilized to Protein A/G-Sepharose beads by incubating 200 μg of antibody with 400 μl of beads overnight at 4 °C under constant agitation followed by incubation with 200 mM sodium tetraborate, pH 9.0, for 3 h and with 0.2 m ethanolamine, pH 8.0, for 2 h at room temperature. Transiently transfected CHO cells or neurons were lysed in immune precipitation buffer and were subjected to the same treatment as above, or anti-phosphotyrosine agarose beads (Millipore, Schwalbach, Germany) were added and incubated overnight at 4 °C under constant agitation. Beads were washed once with washing buffer containing 750 mM NaCl in 10 mM Tris-HCl, pH 7.5, and 0.5% Triton X-100 and washed three times with PBS and finally treated with 2× SDS-PAGE sample buffer for 5 min at 95 °C.

Peptide Coupling and Biotinylation of Peptide/BSA Conjugate—BSA (10 mg; Sigma-Aldrich) dissolved in 2 ml of conjugation buffer (83 mM NaH2PO4, 0.9 mM NaCl, pH 7.2) was incubated with 140 μl of a mixture of 13 mg/ml m-maleimido-benzoyl-N-hydroxysuccinimidyl ester (Sigma-Aldrich) in DMSO and 2.5 mg/ml biotinamidocaproate-N-hydroxysuccinimide ester (Sigma-Aldrich) in DMSO for 1 h at room temperature and gentle agitation. After gel filtration through a PD-10 column (GE Healthcare), the flow-through was incubated with 2 mg of synthetic peptide bearing a cysteine residue at the C
terminus in conjugation buffer for 3 h at room temperature. Cysteine (10 mg/ml) was added, and the sample was dialyzed overnight at 4 °C against PBS and concentrated by using Vivaspin centrifugation tubes (Vivaspin 20, Vivascience, Göttingen, Germany) with a 30 kDa membrane cut-off.

Western Blot Analysis and ELISA—Western blot analysis and ELISA were performed as described (26). For ELISA, proteins and peptides were coated on microtiter plates (Maxisorb F8, Nunc, Wiesbaden, Germany) at a concentration of 10–20 μg/ml and 10–100 μg/ml overnight at 4 °C under constant agitation, respectively. In case of competition ELISA, the competitor was preincubated for 10 min at different concentrations (5–400 μg/ml) with a constant amount (25–50 μg/ml) of the indicated binding protein. Specific binding was quantified by measuring the absorbance at 405 nm using an ELISA reader. As a control for nonspecific binding, BSA instead of the indicated protein/peptide was used. Absorbance values from nonspecific binding were always subtracted from the absorbance values of the respective sample measurements.

Microexplant Cultures—Preparation of cerebellar microexplants and measurements of neurite outgrowth on NCAM immunoaffinity-purified from adult mouse brain was as described (27).

Transfection of CHO Cells—CHO cells were grown in Glasgow modified Eagle’s medium supplemented with 10% fetal calf serum (Invitrogen) in 6-well plates at 37 °C in a humidified incubator gassed with 5% CO2 and transiently transfected at 90% confluence using the Lipofectamine Plus kit (Invitrogen) according to the protocol of the manufacturer.

Transfection of Hippocampal Neurons and Neurite Outgrowth Measurements—Hippocampal neurons were prepared, cultured, and transfected as described (28). For analysis of neurite outgrowth, neurons were co-transfected with EGFP expression vectors (1 μg/10^6 cells). Transfected neurons were seeded on 96-well plates coated with 100 μg/ml poly-L-lysine (Sigma-Aldrich) or 6 μg/ml laminin (Sigma-Aldrich) or 6 μg/ml NCAM-Fc (28). BDNF was added to the cultures 4 h after cell plating. After 24–36 h, cells were washed with PBS, fixed with 4% formaldehyde in PBS, and stained with toluidine blue. For image acquisition of neurons stained with toluidine or co-transfected with enhanced GFP and for operator-controlled tracing of neurites, the inverted microscope Axiosvert 135 and Kontron imaging system (Kontron Elektronik) or the laser-scanning confocal microscope LSM510-based imaging system (Zeiss, Jena, Germany) were used, respectively. The mean lengths of neurites were measured.

Immunocytochemistry and Confocal Laser-scanning Microscopy—Dissociated hippocampal neurons were seeded on coverslips coated with poly-L-lysine and maintained in serum-free medium for 4–5 days. Cultures were placed on ice and incubated in fresh medium with NCAM antibody H28 or PSA antibody 735 for 50 min, washed three times with PBS, incubated with secondary anti-mouse antibody coupled to the fluorescent dye Cy2 for 30 min in the dark, washed again three times with PBS, and fixed using 4% formaldehyde in PBS. After fixation, the neurons were incubated with polyclonal TrkB antibody 794, washed three times with PBS, incubated with secondary antirabbit antibody coupled to Cy3, and washed again with PBS.

Coverslips were embedded in Aqua PolyMount medium (Polysciences Inc., Eppelheim, Germany) and analyzed with a Zeiss LSM510 confocal laser-scanning microscope.

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RESULTS

Identification of an Interaction between the Intracellular Domains of TrkB and NCAM—In search of binding partners for the intracellular domain of NCAM, we screened a random 12-mer peptide library by phage display using the recombinant intracellular domain of the 180-kDa isoform of NCAM (NCAM180-ID) as substrate-coated bait. Phages that bound to immobilized NCAM180-ID were eluted, and single clones were selected and further investigated by ELISA. Phage clones that showed a strong concentration-dependent binding to substrate-coated NCAM180-ID were subjected to sequence analysis. Of 67 clones, one phage coded for the peptide QHFVH-KHPLGLSA. This sequence has a pronounced similarity to the sequence QHFVHHR, which is part of a sequence stretch within the intracellular domain of TrkB (Fig. 1A). This short sequence stretch comprising amino acids 673–684 is present in the membrane-proximal catalytic domain of TrkB and contains the functionally pivotal aspartic acid, which is required for the enzymatic activity of TrkB.

Scanning the Swiss-Prot protein data base (available on the World Wide Web) using the sequence motif QHFVH(R/K) revealed that this motif occurs only in TrkB. This motif is present in human, monkey, bovine, pig, mouse, rat, and chicken TrkB and in the putative TrkB homologue of fruit fly and snail (data not shown). The shorter sequence motif QHFVH is also found in the inositol 1,4,5-triphosphate receptor types 1, 2, and 3 from different species but not in other proteins. Sequence motifs similar to QHFVH(K/R), such as QHXH, QXXXH, HXXH, or HXXHHK were observed in 3, 11, 28, and 3 peptides, respectively, binding to NCAM180-ID (data not shown). The specificity of the binding of the synthetic phage-derived peptide QHFVH-KHPLGLSA was analyzed by ELISA using substrate-coated NCAM180-ID and a biotinylated peptide/BSA conjugate. The intracellular domain of the close homolog of L1 (CHL1-ID), an immunoglobulin superfamily member like NCAM, served as a control for specificity. A concentration-dependent, saturable binding of the peptide/BSA conjugate to NCAM-ID but not to the CHL1-ID was observed (Fig. 1B).

To ascertain whether the intracellular domain of NCAM also binds to a synthetic TrkB peptide comprising amino acids 673–684 and/or to the entire TrkB-ID, an ELISA was performed. Both recombinant NCAM140-ID and NCAM180-ID but not CHL1-ID bound to the substrate-coated synthetic TrkB peptide (Fig. 1C) and, in addition, to the substrate-coated recombinantly expressed TrkB-ID (Fig. 1D). Because NCAM140-ID and NCAM180-ID both interact with TrkB, further experiments were performed with either of the two molecules.
To investigate the interaction between NCAM and TrkB in neurons and to obtain indications that this interaction occurs in vivo, we examined localization of NCAM and TrkB in primary cultured neurons after antibody-induced capping of NCAM using confocal immunofluorescence microscopy. For co-capping, live cultured hippocampal neurons were incubated first with a monoclonal antibody either against NCAM or PSA, which is predominantly present on NCAM, and then with secondary mouse antibodies. After fixation, neurons were incubated with a rabbit antibody against the intracellular domain of TrkB and a secondary rabbit antibody. A pronounced punctated co-immunostaining of TrkB with NCAM or PSA-NCAM was seen along neurites (Fig. 2), indicating that NCAM and TrkB have a chance to interact with each other in hippocampal neurons.

Next, co-immunoprecipitation experiments were performed to verify that TrkB and NCAM are associated with each other. Using pan-Trk antibodies for immunoprecipitation, NCAM180 was found to co-immunoprecipitate from brain detergent extracts of adult wild-type mice but not from detergent extracts of NCAM-deficient mice, as shown by the Western blot analysis with the NCAM180-ID-specific antibody (Fig. 3A). Western blot with a TrkB-specific antibody showed that the pan-Trk antibody, which recognizes the intracellular domains of TrkA, TrkB, and TrkC, immunoprecipitated only the full-length 155-kDa TrkB isoform and not the truncated 95-kDa TrkB isoform, which is devoid of most of the intracellular domain. Because several TrkA-, TrkB-, and TrkC-specific antibodies were not effective in immunoprecipitation, TrkB specificity could not be proven. However, when the monoclonal NCAM antibody H28 was immobilized to Protein A/G beads and used for immunoprecipitation, a specific co-immunoprecipitation of NCAM and the full-length 155-kDa TrkB isoform from brain detergent extracts of adult wild-type but not NCAM-deficient mice was observed (Fig. 3B). No co-immunoprecipitation of the 95-kDa TrkB isoform, TrkA or TrkC was detectable (data not shown), whereas all three major isoforms of NCAM were immunoprecipitated from detergent extracts of wild-type but not NCAM-deficient mouse brains (Fig. 3B). These results confirm that TrkB and NCAM interact via their intracellular domains.

To further characterize the specificity of the interaction between TrkB and NCAM, a chemical cross-linking approach was performed. Recombinant NCAM180-ID and, as a control, recombinant CHL1-ID were coupled to the trifunctional cross-linker sulfo-SBED containing a biotin moiety, and these conjugates were incubated as baits with crude mouse brain homogenates. After UV cross-linking, target proteins from the brain homogenate that bound to the bait protein were isolated using streptavidin-coupled magnetic beads and separated by SDS-PAGE under reducing conditions, leading to the transfer of the biotin label from the bait proteins to the bound target proteins. Western blot analysis using streptavidin conjugated to horse-radish peroxidase allowed to detect biotinylated binding partners. Using CHL1-ID as bait, no biotinylated band was detectable, whereas a biotinylated protein with an apparent molecular mass of ~155 kDa was detected as a binding partner of the NCAM-ID bait (Fig. 3C). This molecular mass resembled that...
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of full-length TrkB, and by Western blot analysis using a pan-Trk and a TrkB-specific antibody, only a 155 kDa band was detected (Fig. 3C), implying that the cross-linked 155-kDa protein was indeed full-length TrkB.

To identify the binding site for TrkB within NCAM140-ID and NCAM180-ID, peptides comprising consecutive sequences derived from NCAM140-ID and NCAM180-ID were probed in a competition ELISA for inhibition of binding of NCAM140-ID or NCAM180-ID to substrate-coated TrkB peptide. The peptide comprising the membrane-proximal sequences (amino acids 730–764) of NCAM140-ID and NCAM180-ID, but not the two other peptides deduced from the more distal intracellular sequences, reduced binding of NCAM180-ID to the TrkB peptide (Fig. 4). These observations indicate that the intracellular domains of TrkB and NCAM directly interact with each other in functionally important domains: the catalytic domain of TrkB and the membrane-proximal part of NCAM140-ID and NCAM180-ID that carries the functionally important tyrosine residue that, when phosphorylated, has been implicated in neurite outgrowth (29).

**Determination of Tyrosine Phosphorylation of NCAM by TrkB**—Considering the function of TrkB as a receptor tyrosine kinase, we investigated whether TrkB is required for the phosphorylation of the intracellular domain of NCAM at a single transmembrane-proximal tyrosine that is important for neurite outgrowth and whether this phosphorylation is dependent on BDNF. We generated a mutation in full-length NCAM140 (NCAM140-Y734F) by exchanging this tyrosine with phenylalanine and analyzed tyrosine phosphorylation in the presence or absence of BDNF after transfection of NCAM140 or NCAM140-Y734F and/or TrkB into fibroblast-like CHO cells that do not express detectable levels of NCAM. Upon co-transfection of TrkB and NCAM140 or NCAM140-Y734F, immunoprecipitation of tyrosine-phosphorylated proteins by immobilized phosphotyrosine antibodies and Western blot analysis using NCAM antibodies showed co-immunoprecipitation of NCAM140 with the phosphotyrosine antibody in the presence of BDNF but not in its absence (Fig. 5A). However, even in the presence of BDNF and upon co-transfection of TrkB, NCAM140-Y734F was not co-immunoprecipitated by the phosphotyrosine antibody (Fig. 5A). Furthermore, neither NCAM140-Y734F nor NCAM140 was co-immunoprecipitated by the phosphotyrosine antibody when transfected alone without TrkB (Fig. 5A), indicating that TrkB was necessary for tyrosine phosphorylation of NCAM. However, the binding of TrkB to NCAM was not influenced by phosphorylation because they co-immunoprecipitated with or without BDNF triggering of TrkB to the same degree (data not shown).

To exclude the possibility that NCAM is co-immunoprecipitated with other tyrosine-phosphorylated proteins, we also performed co-immunoprecipitations using an NCAM antibody and probed the immunoprecipitates with the phosphotyrosine antibodies. Upon co-transfection of TrkB and NCAM140 or NCAM140-Y734F, immunoprecipitation using NCAM antibodies and Western blot analysis using phosphotyrosine antibodies revealed co-immunoprecipitation of tyrosine phosphorylated NCAM140 but not of NCAM140-Y734F, even in the absence of BDNF (Fig. 5B). In the presence of BDNF, the amount of tyrosine-phosphorylated NCAM140 was significantly increased (Fig. 5B). When cells were mock-transfected or transfected with NCAM140 alone, no co-immunoprecipitation...
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Finally, to investigate whether BDNF-triggered tyrosine phosphorylation of NCAM takes place in neurons, primary cultures of non-transfected hippocampal neurons were incubated in the absence or presence of BDNF and subjected to immunoprecipitation of tyrosine-phosphorylated proteins by immobilized phosphotyrosine antibodies. Western blot analysis using NCAM antibodies showed an increase in co-immunoprecipitation of NCAM with the phosphotyrosine antibody in the presence of BDNF relative to that observed in its absence (Fig. 5C). In summary, these results show a TrkB-dependent tyrosine phosphorylation of NCAM.

NCAM-mediated Neurite Outgrowth Is Modulated by Phosphorylation/Dephosphorylation of Tyr734—Because it has been shown that neurite outgrowth of neuroblastoma cells transfected with NCAM180-Y734F was increased by 47 ± 8% compared with those transfected with wild-type NCAM180 (29), we analyzed whether transfection of NCAM-deficient hippocampal neurons with NCAM140-Y734F had the same effect on neurite outgrowth. In addition, we analyzed neurite outgrowth of neurons transfected with NCAM140-Y734F mutant carrying an exchange of tyrosine with glutamate, which mimics the phosphorylation of tyrosine. Transfection of NCAM140-Y734F led to an increase of neurite outgrowth on the NCAM substrate by 38 ± 12% relative to that observed after transfection with wild-type NCAM140, whereas after transfection of NCAM140-Y734E, neurite outgrowth on the NCAM substrate decreased by 41 ± 6% and was similar to that observed with mock-transfected neurons on NCAM substrate (Fig. 6A). On poly-L-lysine substrate, neurite outgrowth was not affected by either transfection (Fig. 6A). These results suggest that phosphorylation of Tyr734, which is mimicked by the Y734E mutation, reduces NCAM-mediated neurite outgrowth, whereas dephosphorylation of Tyr734, which is mimicked by the Y734F mutation, enhances NCAM-mediated neurite outgrowth.

To test whether BDNF-induced phosphorylation of NCAM by TrkB had the same inhibitory effect on NCAM-mediated neurite outgrowth as the Y734E mutation, neurite outgrowth of wild-type cultured hippocampal neurons was determined in the absence or presence of BDNF. No significant effect of BDNF was observed on neurite outgrowth on the NCAM substrate with dissociated primary cultures of hippocampal neurons (data not shown). However, with explants from cerebellum, we observed that the enhanced neurite outgrowth on the NCAM substrate relative to that on the poly-L-lysine substrate was abolished in the presence of BDNF, whereas neurite outgrowth on poly-L-lysine was enhanced in the presence of BDNF, and neurite outgrowth on laminin was not altered by BDNF (Fig. 6B). The results indicate that NCAM-mediated neurite outgrowth is modulated by tyrosine phosphorylation of NCAM by TrkB, which is triggered by BDNF under certain conditions.

Effect of Knockdown of TrkB Expression on NCAM-mediated Neurite Outgrowth—Because NCAM-mediated neurite outgrowth depends on tyrosine phosphorylation of NCAM on the one hand, and, on the other hand, TrkB phosphorylates NCAM...
at Tyr\(^{734}\), we investigated the functional relationship between NCAM-dependent neurite outgrowth and TrkB expression. Therefore, neurite outgrowth on substrate-coated NCAM was determined upon ablation of TrkB expression in cultures of dissociated early postnatal hippocampal neurons by using siRNA interference. The transfection of hippocampal neurons with TrkB siRNA led to a knockdown of TrkB protein expression by about 50% after 24 h (Fig. 7A). In parallel, control siRNA was applied, and poly-L-lysine or laminin were used as negative or positive control substrate coats, respectively. Cultures were maintained in the absence or presence of BDNF to investigate whether activation of TrkB affected neurite outgrowth. In the absence of BDNF, neurons transfected with control siRNA showed enhanced neurite outgrowth on NCAM or laminin substrate coats relative to that observed on the poly-L-lysine substrate (Fig. 7B). In the presence of BDNF, neurite lengths were slightly increased on poly-L-lysine and on NCAM but not on laminin relative to that observed in the absence of BDNF (Fig. 7B). The enhanced neurite outgrowth on NCAM in the presence and absence of BDNF was completely abolished upon transfection of neurons with TrkB siRNA. The presence of TrkB siRNA did not change the extent of neurite outgrowth on poly-L-lysine or on laminin when compared with the negative control siRNA values (Fig. 7B). These observations indicate a functional interdependence of NCAM and TrkB in enhancement of NCAM-mediated neurite outgrowth. Integrin-dependent neurite outgrowth as triggered by laminin was not affected by reduced expression of TrkB.

**DISCUSSION**

Our present study has revealed a direct association of the neural cell adhesion molecule NCAM and the tyrosine receptor kinase TrkB. The functional relationship between TrkB and NCAM is evidenced by the ability of TrkB, particularly in the presence of its ligand BDNF, to enhance tyrosine phosphorylation of NCAM. Homophilic interaction of NCAM in a transposition promotes neurite outgrowth from hippocampal neurons (30). This NCAM-induced neurite outgrowth depends on TrkB because reduction of TrkB expression does not allow neurite outgrowth triggered by the homophilic NCAM interaction. It is interesting in this context that an increased rather than decreased NCAM-dependent neurite outgrowth of B35 neuroblastoma cells (29) or cultured hippocampal neurons (this study) was observed, when tyrosine phosphorylation of NCAM
was prevented by mutation of the single tyrosine in the intracellular domain of NCAM at position 734. On the other hand, we observed a decreased NCAM-dependent neurite outgrowth from hippocampal neurons under conditions that mimic phosphorylation of Tyr \(^{734}\) by using the NCAM140-Y734E mutant or a decreased neurite outgrowth from cerebellar explants after BDNF-triggered TrkB-mediated phosphorylation. In neurons, the interaction between TrkB and NCAM and the phosphorylation of NCAM by TrkB may alter the NCAM-associated signaling associated with neurite outgrowth. The co-localization of NCAM and TrkB in cultured neurons after antibody-induced clustering of NCAM or PSA-NCAM supports the notion that both proteins interact even in the absence of BDNF and, thus, without activation of TrkB by BDNF. The \(v\) in vitro binding assays confirm that the direct interaction of TrkB and NCAM does not require TrkB activation and does not depend on NCAM phosphorylation. The interplay between BDNF, TrkB, and NCAM depends not only on NCAM tyrosine phosphorylation but also on the neuronal cell type because (i) BDNF stimulated outgrowth of hippocampal neurons on the NCAM substrate but inhibited outgrowth of axons from cerebellar explants on the NCAM substrate and (ii) expression of phosphotyrosine-deficient NCAM stimulated outgrowth of hippocampal neurons on the NCAM substrate, whereas silencing of TrkB inhibited this outgrowth. Furthermore, we have to postulate additional factors affecting the interplay between the three molecules, such as the FGF receptor 1 and Erk1, the activities of which are up-regulated by phosphotyrosine-deficient NCAM (29) and the neurotrophin receptor p75, the expression levels of which are cell type-specific and controlled by NCAM (31).

The role of the functional interplay between TrkB and NCAM in development is exemplified by similar alteration of the lamination of the hippocampus. Normally, most of the mossy fibers grow superficial to the CA3 pyramidal neurons in the suprapyramidal bundle, which forms the major constituent of stratum lucidum (32, 33). In NCAM-deficient mice, the ordered laminar organization is severely altered, despite the fact that all axons initially grow in the right direction and appear to make synapses with CA3 pyramidal cells (34). Similarly, the thickness of the stratum lucidum as well as the density of mossy fiber terminals and their synaptic contacts are reduced in the absence of TrkB (35, 36). This appears to be due to a significant reduction of the surface area and the perimeter of mossy terminals. Thus, the lack of TrkB signaling results in smaller and less complex mossy axon terminals and in reduced numbers of synaptic vesicles. As these deficits increase with postnatal ages (37), it is plausible to assume that NCAM and TrkB signaling is necessary for the presynaptic maturation of mossy fiber terminals. These findings are interesting in view of the fact that mice constituted deficient in NCAM expression show abnormal responses in fear conditioning and addictive responses (38–40). Furthermore, both NCAM and the TrkB ligand BDNF are involved in major depressive disorder by being significantly down-regulated in the hippocampus and the prefrontal cortex of afflicted patients (41). These observations suggest a role of TrkB in regulating important functions of NCAM not only during nervous system development and in synaptic plasticity but also in regeneration after injury (42, 43).

Our findings that TrkB binds directly to NCAM through its intracellular domain and triggers, through activation by its ligand BDNF, tyrosine phosphorylation of NCAM and thereby modulates NCAM-mediated neurite outgrowth represent novel evidence that a neurotrophin receptor can modulate the functions of a neural cell adhesion molecule by direct interaction. The ways by which NCAM cooperates with TrkB in synaptic plasticity, learning, and memory will be interesting to investigate in future experiments using neuron-specific conditionally ablated TrkB- and NCAM-deficient mice.

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