Development/Plasticity/Repair

Fibroblast Growth Factor-Regulated Palmitoylation of the Neural Cell Adhesion Molecule Determines Neuronal Morphogenesis

Evgeni Ponimaskin,[1]* Galina Dityateva,[2,3]* Mika O. Ruonala,[4,5] Masaki Fukata,[6,7] Yuko Fukata,[6,7] Fritz Kobe,[1] Fred S. Wouters,[4] Markus Delling,[8] David S. Bredt,[9] Melitta Schachner,[2,10,11] and Alexander Dityatev[2,3]

[1]Abteilung Neuro- und Sinnesphysiologie, Physiologisches Institut, Universität Göttingen, 37073 Göttingen, Germany, [2]Zentrum für Molekulare Neurobiologie, Universitätsklinikum Hamburg-Eppendorf, 20246 Hamburg, Germany, [3]Department of Neuroscience and Brain Technologies, The Italian Institute of Technology, 16163 Genova, Italy, [4]European Neuroscience Institute, 37085 Göttingen, Germany, [5]Center for Membrane Proteomics, University of Frankfurt am Main, 60438 Frankfurt am Main, Germany, [6]Division of Membrane Physiology, Department of Cell Physiology, National Institute for Physiological Sciences, Okazaki 444-8787, Japan, [7]PRESTO, Japan Science and Technology Agency, Chiyoda, Tokyo 102-0075, Japan, [8]Howard Hughes Medical Institute, Harvard Medical School, Boston, Massachusetts 02115, [9]Department of Integrative Biology, Eli Lilly and Company, Indianapolis, Indiana 46285, [10]W.M. Keck Center for Collaborative Neuroscience, and [11]Department of Cell Biology and Neuroscience, Rutgers University, Piscataway, New Jersey 08854

During development of the nervous system, short- and long-range signals cooperate to promote axonal growth, guidance, and target innervation. Particularly, a short-range signal transducer, the neural cell adhesion molecule (NCAM), stimulates neurite outgrowth via mechanisms that require posttranslational modification of NCAM and signaling via receptors to a long-range messenger, the fibroblast growth factor (FGF). In the present study we further characterized a mechanism which regulates the functional interplay between NCAM and FGF receptor(s). We show that activation of FGF receptor(s) by FGF2 leads to palmitoylation of the two major transmembrane NCAM isoforms, NCAM140 and NCAM180, translocation of NCAM to GM1 ganglioside-containing lipid rafts, and stimulation of neurite outgrowth of hippocampal neurons. Ablation of NCAM, mutation of NCAM140 or NCAM180 palmitoylation sites, or pharmacological suppression of NCAM signaling inhibited FGF2-stimulated neurite outgrowth. Of the 23 members of the aspartate-histidine-histidine-cysteine (DHHC) domain containing proteins, DHHC-7 most strongly stimulated palmitoylation of NCAM, and enzyme activity was enhanced by FGF2. Thus, our study uncovers a molecular mechanism by which a growth factor regulates neuronal morphogenesis via activation of palmitoylation, which in turn modifies subcellular location and thus signaling via an adhesion molecule.

Key words: **acylation; palmitoylation; cell adhesion; growth factor; lipid raft; neurite outgrowth; FLIM; FRET; hippocampus**

Introduction

Protein S-palmitoylation via a thioester linkage of 16-carbon fatty acid to a cysteine residue modulates diverse aspects of neuronal development and synaptic transmission. In particular, palmitoylation regulates the function of proteins that control neuronal differentiation, neurite outgrowth, axonal pathfinding, filopodia formation, and synaptic vesicle release (Huang and El-Husseini, 2005; Linder and Deschenes, 2007). Palmitoylation of scaffold molecules and signaling receptors targets proteins to special cholesterol- and sphingolipid-rich membrane microdomains, generally termed as lipid rafts, regulates ion channel clustering and modifies synaptic strength (Huang and El-Husseini, 2005; Linder and Deschenes, 2007). Regulation of palmitoylation occurs through the actions of protein acyltransferases and protein acylthioesterases (Smotrys and Linder, 2004).

Among transmembrane neural cell adhesion molecules, neural cell adhesion molecule (NCAM) is particular in that its intracellular domain lacks the characteristic cluster of basic residues proximal to the inner membrane but contains four closely spaced, juxta-membrane cysteines. Mutagenesis studies have indicated that the cysteine residue closest to the transmembrane domain is not acylated with either palmitate or oleate, whereas the other three cysteines are acylated to differing extents and form a segment tethered to the plasma membrane (Little et al., 1998). Mutation of the cysteine residues does not affect homophilic interaction between NCAM molecules (Little et al., 1998). How-

Received May 13, 2008; revised July 23, 2008; accepted July 29, 2008.

This work was supported by the Deutsche Forschungsgemeinschaft through the Center for Molecular Physiology of the Brain and Grant PO 732/1-2 (E.P.). M.S. is New Jersey Professor of Spinal Cord Research. We thank Harold Cremer for NCAM-deficient mice, Achim Dahlmann and Eva Kronberg for genotyping and animal care, and Patricia Maness for NCAM cDNAs. The FGF receptor inhibitor PD173074 was a gift from Parke-Davis (now Pfizer).

*E.P. and G.D. contributed equally to this work.

Correspondence should be addressed to either of the following: Alexander Dityatev, Department of Neuroscience and Brain Technologies, The Italian Institute of Technology, Via Morego 30, 16163 Genova, Italy, E-mail: alexander.dityatev@iit.it; or Melitta Schachner, Zentrum für Molekulare Neurobiologie, Universitätsklinikum Hamburg-Eppendorf, Martinistrasse 85, D-20251 Hamburg, Germany, E-mail: melitta.schachner@zmnh.uni-hamburg.de.

E. Ponimaskin's present address: Department of Neurophysiology, Medizinische Hochschule Hannover, Hannover 30625, Germany.

DOI:10.1523/JNEUROSCI.2171-08.2008

Copyright © 2008 Society for Neuroscience 0270-6474/08/288897-11$15.00/0

ever, blocking NCAM palmitoylation abolishes targeting of NCAM to lipid rafts, inhibits NCAM signaling in lipid rafts via the focal adhesion kinase (FAK), and reduces NCAM-mediated neurite outgrowth (Niethammer et al., 2002). Activation of FAK by NCAM in lipid rafts is mediated by the fyn kinase that associates with the intracellular domain of the receptor-like protein tyrosine phosphatase RPTPα, forming a complex with the cytoskeletal protein spectrin and NCAM (Bodrikov et al., 2005). Outside of lipid rafts, a direct interaction between the extracellular domain of NCAM and the fibroblast growth factor (FGF) receptor is required for NCAM-stimulated neurite outgrowth and activation of protein kinase C (PKC) (Williams et al., 1994; Saffell et al., 1997; Kolkova et al., 2000; Cavallaro et al., 2001; Anderson et al., 2005). Activation of the FGF receptor contributes to neuronal migration, neurite outgrowth, and axonal pathfinding (Ford-Perriss et al., 2001; Gill and Tsai, 2006) by a signaling cascade that involves activation of phospholipase Cγ (Williams et al., 1994).

In view of the functional interplay between NCAM and the FGF receptor and the importance of palmitoylation of NCAM for neurite outgrowth, we investigated whether FGF-mediated signaling influences palmitoylation of NCAM and whether palmitoylation of NCAM is required for FGF-stimulated outgrowth of hippocampal neurons. Furthermore, we identified an enzyme that palmitoylates NCAM in an FGF-dependent manner and stimulates neurite outgrowth.

Materials and Methods

Materials. [9,10-^{3}H(N)]Palmitic acid (30–60 Ci/mmol), [^{35}S]GTPγS (1300 Ci/mmol), and [^{35}S]methionine/cysteine label (>1000 Ci/mmol) were purchased from Hartmann Analytic (Braunschweig). Ace-glow Western Blotting Analysis System was from Peqlab, and peroxidase-conjugated secondary antibodies were purchased from GE Healthcare. Protein A-Sepharose CL-4B beads were from Sigma-Aldrich. Lipofectamine 2000 reagent was purchased from Invitrogen. Cell culture dishes were ordered from Nunc.

Rat NCAM140 and NCAM180 in the pcDNA3 vector were a kind gift from Patricia Maness (University of North Carolina, Raleigh, NC). The plasmid coding for enhanced green fluorescent protein (EGFP) was from Clontech. Plasmids for expression of aspartate-histidine-histidine-cysteine (DHHC) proteins are described previously (Fukata et al., 2004). The NCAM140Δ and NCAM180Δ constructs, in which the four cysteine residues located in the intracellular domain adjacent to transmembrane domain were replaced by serines, were described previously (Niethammer et al., 2002). The production, purification, and validation of neurite outgrowth promoting activity of NCAM-Fc are described in the same study.

The following reagents were used: FGF2 (human basic FGF; Sigma-Aldrich), FGF receptor inhibitor PD173074 (50 nM; Parke-Davis), PKC inhibitor peptide 20–28 (50 μM; Merck), and Src kinase family inhibitor PP2 (50 nM; Merck).

Metabolic labeling, immunoprecipitation, and immunoblotting. Neuroblastoma N2A cells were grown in DMEM containing 10% fetal calf serum (FCS) and 1% penicillin/streptomycin at 37°C under 5% CO_2. For transient transfection, cells were seeded at low density (8×10^5) in 35 mm dishes and transfected with appropriate vectors using Lipofectamine 2000 reagent according to the manufacturer's instruction. Six hours after transfection, cells were serum starved for 16 h. Cells were then labeled with [^{35}S]methionine/cysteine (50 μCi/ml, >1000) or [^{3}H]palmitate (300 μCi/ml, 30–60 Ci/mmol) as indicated. In some experiments, FGF2 (dissolved in H_2O), vehicle (H_2O), or PD173074 was added as indicated. After labeling, cells were washed once with ice-cold PBS (140 mM NaCl, 3 mM KCl, 2 mM KH_2PO_4, 6 mM Na_2HPO_4, pH 7.4) and lysed in 600 μl of ice-cold RIPA buffer (0.15 mM NaCl, 20 mM Tris-HCl, 10 mM EDTA, pH 7.4) containing 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride, and 10 mM iodoacetamide. For pulse-chase experiments, N2A cells transfected with NCAM140 and NCAM180 were labeled with [^{3}H]palmitate for 1 h and then chased with medium containing unlabeled palmitate for the indicated time periods. Insoluble material was pelleted (5 min, 20,000 $\times$ *g*), and anti-NCAM antibodies (Niethammer et al., 2002) were added to the resulting supernatant at a dilution of 1:100. After overnight agitation at 4°C, 30 μl of Protein A-Sepharose CL-4B was added, and samples were incubated under gentle agitation for 2 h. After brief centrifugation, the pellet was washed twice with ice-cold RIPA buffer, and the immune complexes were released from the beads by incubation for 3 min at 100°C in nonreducing electrophoresis sample buffer (62.5 mM Tris-HCl, pH 6.8, containing 20% glycerol, 6% SDS, and 0.002% bromophenol blue). Radiolabeled polypeptides were analyzed by SDS-PAGE on 10% acrylamide gels under nonreducing conditions and visualized by fluorography using Kodak X-Omat AR films. Densitometric analysis of fluorograms was performed by Gel-Pro Analyser Version 3.1 Software (Media Cybernetics), and the unpaired two-tailed *t* test was used for statistical evaluation of data.

For immunoblotting, intact or transiently transfected N2A cells expressing NCAM140, NCAM180, or DHHC-7 were harvested in ice-cold extraction buffer (10 mM Tris/HCl, pH 7.4, 1 mM EDTA, 150 mM NaCl, 1 mM DTT, 20 μM leupeptin, 2 μg/ml aprotinin) and separated by 10% SDS-PAGE under nonreducing conditions. Proteins were transferred to Hybond nitrocellulose membrane (GE Healthcare) and probed with antibodies raised against NCAM (1:600 diluted in PBS/Tween 20). Proteins were detected using the Ace-glow chemiluminescence detection reagents (Peqlab) and Chemi-Smart5000 detection system (Vilber Lourmat) and analyzed by Bio1D software (Vilber Lourmat).

Hydroxylamine and β-mercaptoethanol treatment. Polyacrylamide gels containing NCAM140 labeled with [^{3}H]palmitate were fixed (10% acetic acid, 10% methanol) and treated overnight under gentle agitation with 1 M hydroxylamine (pH 7.5) or with 1 M Tris (pH 7.5). Gels were washed in water and agitated for 30 min in dimethylsulfoxide (DMSO) before they were processed for fluorography. For the β-mercaptoethanol treatment, N2A cells transfected with NCAM140 were labeled with [^{3}H]palmitate, and NCAM140 was immunoprecipitated as described above. Immunoprecipitates were treated with the indicated concentrations of β-mercaptoethanol for 30 min at 37°C and subjected to SDS-PAGE and fluorography.

Fatty acid analysis. [^{3}H]Palmitate-labeled NCAM140 or NCAM180 was immunoprecipitated from transfected N2A cells with anti-NCAM antibody and subjected to SDS-PAGE. The bands corresponding to NCAM140 and NCAM180 were excised, and fatty acids were cleaved by treatment of the dried gel slices with 6N HCl for 16 h at 110°C. Fatty acids were extracted with hexane and separated into individual fatty acid species by thin-layer chromatography on RP-18 TLC plates (Merck) using acetonitrile/acetic acid (1:1, v/v) as solvent. Radiolabeled fatty acids were visualized by fluorography. For identification of individual fatty acid species, radiolabeled markers ([^{3}H]myristate, [^{3}H]palmitate, and [^{3}H]stearate) were run on the same plate.

Analysis of DHHC activities in transfected human embryonic kidney 293 and N2A cells. Transfected HEK293 (human embryonic kidney 293) cells were preincubated for 30 min in serum-free DMEM with fatty acid-free bovine serum albumin (5 mg/ml; Sigma-Aldrich). Cells were then labeled with 0.25 mCi/ml [^{3}H]palmitate (PerkinElmer) for 4 h in the preincubation medium. Cells were washed with PBS, removed by scraping in SDS-PAGE sample buffer (62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, and 0.001% bromophenol blue) and 10 mM DTT and boiled for 2 min. For fluorography, protein samples were separated by SDS-PAGE under reducing conditions. Gels were treated with Amplify (Amersham) for 30 min, dried under vacuum, and exposed on film (Kodak Biomax MS) at −80°C. Expression of NCAM140 was confirmed by immunoblotting with NCAM antibodies (Niethammer et al., 2002). After initial screening of 23 DHHC proteins, three candidate DHHCs (DHHC-3, DHHC-7, and DHHC-8), showing the highest levels of NCAM140 palmitoylation, were further tested for their ability to stimulate palmitoylation of NCAM in HEK293 and N2A cells. Cells were transfected with one of these DHHCs, labeled with [^{3}H]palmitate (300 μCi/ml, 30–60 Ci/mmol) for 2 h, and then subjected to palmitoylation

analysis as described above. Densitometric analysis of final fluorograms was performed by Gel-Pro Analyzer software version 3.1 (Media Cybernetics), and unpaired two-tailed *t* test was used for statistical evaluation of data.

Preparation and transfection of hippocampal neurons. Cultures of hippocampal neurons were prepared from 1- to 2-d-old C57BL/6J mice (Dityatev et al., 2000). Cells were dissociated by trypsin treatment and plated in Neurobasal-A medium supplemented with 5 μg/ml gentamycin and 0.5 mM L-glutamine (all from Invitrogen) in 96-well plates (Falcon 353072; BD Labware) coated with 100 μg/ml poly-L-lysine (Sigma-Aldrich). In some experiments, cells were transiently transfected by electroporation before plating using a nucleofection kit for mouse neurons for Nucleofector I (Amaxa) (Dityateva et al., 2003). All cells were cotransfected with the genes of interest (0.5 or 1 μg of DNA per 10^6 cells in 60 μl of transfection solution) and EGFP expression vector (0.75 μg of DNA) (Dityateva et al., 2003). Four hours after plating, one-half of the medium was replaced with fresh medium. Cultures were maintained at 37°C in a humidified incubator gassed with 5% CO_2.

Determination of neurite length. Chemicals were added to hippocampal cultures 4 h after cell plating. Twenty four hours after plating, cells were briefly washed with PBS and fixed with 4% formaldehyde in PBS. Nontransfected cultures were stained with Toluidine blue and the inverted microscope Axiovert 135 and Kontron imaging system were used for image acquisition of stained cells and operator-controlled tracing of neurites. For analysis of transfected neurons, EGFP-positive cells were visualized and traced using the laser scanning confocal microscope LSM510 based imaging system (Zeiss) (Dityateva et al., 2003; Kvachnina et al., 2005). The longest neurite per neuron was measured. Statistical evaluation was performed using the unpaired two-tailed *t* test.

Fluorescence lifetime imaging-based analysis of Förster resonance energy transfer for determination of NCAM levels in lipid rafts. Hippocampal neurons were grown on poly-L-lysine coated glass coverslips for 23 h and then stimulated with 50 ng/ml FGF2 for 1 h or left untreated. Cells were fixed with 4% formaldehyde in PBS for 30 min at room temperature, extensively washed with PBS, and quenched with 50 mM glycine in PBS. The GM1 ganglioside, used here as a lipid raft marker, was visualized by a 1 h incubation at room temperature with nonsaturating concentration (1 μg/ml) of Alexa594-conjugated cholera toxin subunit β (Molecular Probes, Invitrogen). All NCAM isoforms were labeled with polyclonal NCAM antibodies (Niethammer et al., 2002) and visualized using Alexa488-conjugated anti-rabbit secondary antibody (Molecular Probes, Invitrogen). Antibodies were incubated for 1 h at room temperature in PBS supplemented with 1% BSA and 0.1% Cold Water Fish Gelatin (Sigma-Aldrich) before mounting on glass slides with Mowiol (Polysciences Inc.).

Fluorescence lifetime (τ) images of the donor fluorochrome (Alexa488) were obtained using a previously described frequency-domain wide-field fluorescence lifetime imaging microscope (Esposito et al., 2005). Histograms of the τ distribution in each image were normalized to the total number of analyzed pixels in the image to obtain cell size-independent probability density functions (PDF). Lifetime distributions were converted to Förster resonance energy transfer (FRET) efficiencies using standard formula $E = 1 - \tau/\tau_0$, where τ_0 is the mean lifetime of the donor ($\tau_{0,}$ 2.039 $\pm$ 0.035 ns) obtained from a Gaussian fit of the PDF of the cells stained for NCAM but not for GM1 (donor-only cells) using Origin software (Origin Lab Corp.). FRET images were represented in false color ranging from blue (0%) to red (20% FRET). The same fit also provides the threshold value for the occurrence of FRET, defined as three times the SE (3σ). The PDFs of untreated and FGF2-stimulated cells (stained for both NCAM and GM1) were used to determine the probability of exceeding the 3σ threshold (pFRET, i.e., the integral of the PDF above 3σ) (Esposito et al., 2007). These values were represented in the bar graph and statistically compared using the Student's *t* test. FRET probabilities were measured in somatic and neuritic compartments using separate binary masks that were created from the fluorescence intensity image of the Alexa488 channel. These masks were applied to the average τ images using ImageJ software (http://rsb.info.nih.gov/ij/) before data analysis.

Results

Palmitoylation of NCAM isoforms

Acylation of NCAM140 and NCAM180 was studied in neuroblastoma N2A cells, which express both NCAM isoforms. Cells were metabolically labeled with [^{3}H]palmitate followed by immunoprecipitation with polyclonal antibodies against mouse NCAM. The resulting fluorogram (Fig. 1*A*, right) demonstrates that the labeled proteins comigrate with NCAM140 and NCAM180 as detected by immunoblotting (Fig. 1*A*, left) and that both NCAM isoforms incorporate [^{3}H]palmitate.

Next, we analyzed the fatty acid bond to the NCAM isoforms to distinguish between amide- and ester-type linkages. In contrast to stable amide bond linkages, thioester and hydroxyester linkages are sensitive to β-mercaptoethanol. Moreover, thioester bonds can be distinguished from the hydroxyester bonds by their sensitivity to hydroxylamine (Ponimaskin et al., 2001). Because the expression levels of NCAM140 and particularly NCAM180 in N2A cells are low, detection and analysis of acylated proteins needed very long exposure times (4–6 weeks). We therefore increased the expression levels of NCAM140 and NCAM180 by transient transfection. In N2A cells transfected with NCAM140, incorporation of [^{3}H]palmitate was sensitive to increasing concentrations of β-mercaptoethanol (Fig. 1*B*). Also, treatment of [^{3}H]palmitate-labeled NCAM140 with neutral hydroxylamine removed the label (Fig. 1*C*). Sensitivity to neutral hydroxylamine and to the reducing agent indicates that the isotope labeled fatty acid is linked to NCAM140 via a thioester bond. Similar results were obtained for N2A cells transfected with NCAM180 (data not shown). Fatty acid analysis of [^{3}H]palmitate labeled NCAM140 and NCAM180 showed that NCAM140 contains predominantly palmitic acid, with traces (<3%) of myristic and stearic acids (Fig. 1*D*). Similar results were obtained for the NCAM180 (data not shown).

Stimulation of the FGF receptor increases palmitoylation of NCAM140 and NCAM180

We studied the time course of incorporation of [^{3}H]palmitate in N2A cells transiently transfected with either NCAM140 or NCAM180 in the absence or presence of FGF2. Incorporation of radiolabel increased steadily during the labeling period in the absence of FGF2 (Fig. 2*A*). In the presence of FGF2, we observed a significant increase in palmitate labeling of NCAM140 (Fig. 2*A*). The change in radioactive NCAM140 labeling upon FGF2 stimulation was not due to general metabolic effects, because the [^{35}S]methionine/cysteine ratio in the total protein amount was not increased by FGF2 treatment (Fig. 2*B*). Note that the amount of NCAM140 was even slightly reduced by FGF2 treatment (Fig. 2*B*). The absolute increase in the rate of palmitate incorporation was then calculated by densitometry of [^{3}H]palmitate fluorograms in relation to the expression level of the NCAM140 as assessed by [^{35}S]methionine/cysteine labeling. Application of FGF2 to N2A cells transfected with NCAM140 significantly increased palmitoylation of NCAM140 (Fig. 2*C*). Neither epidermal growth factor (EGF) nor NGF nor NCAM-Fc increased NCAM140 palmitoylation (data not shown). A similar FGF2 effect was obtained when N2A cells were transiently transfected with NCAM180 (Fig. 2*D*). We also found that FGF2 elevates the levels of palmitoylation of endogenous NCAM140 and NCAM180 in N2A cells (Fig. 3). These effects of FGF2 were inhibited by an antagonist of FGF receptors, PD173074 (Figs. 2, 3). The effects of FGF2 on palmitoylation of NCAM140 and NCAM180 were also abolished by the protein synthesis inhibitor

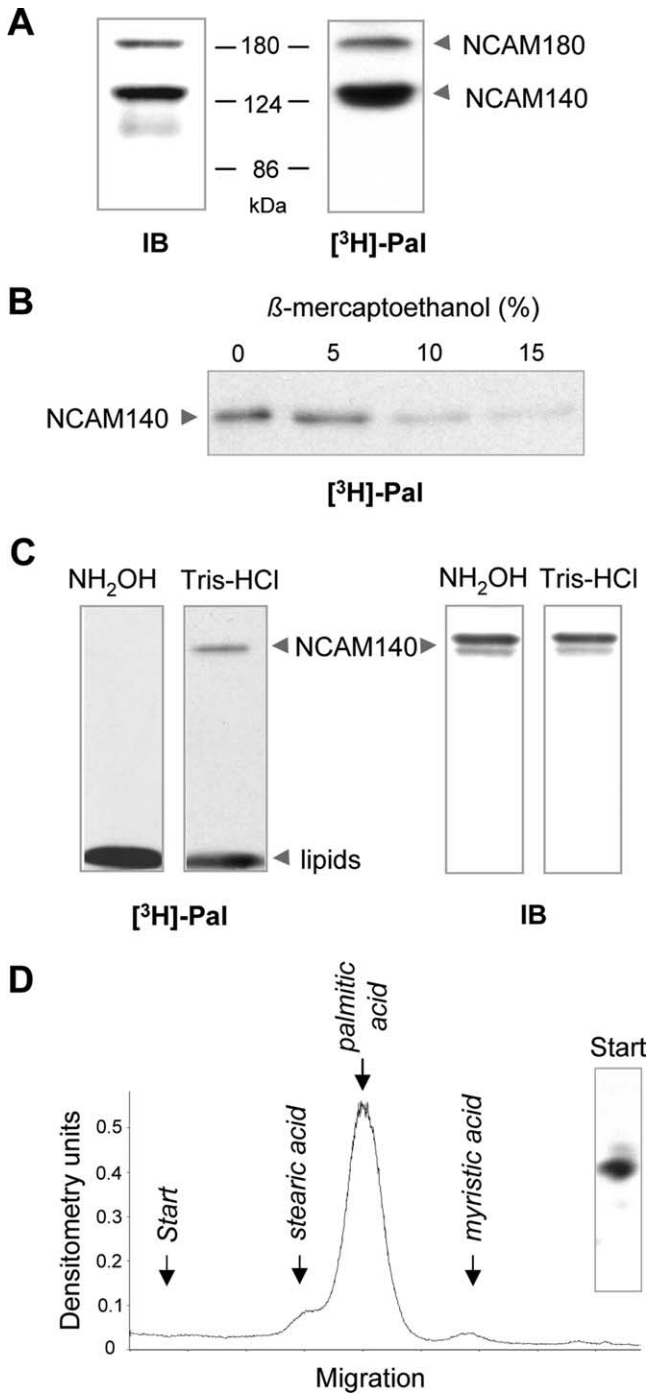

Figure 1. Acylation analysis of NCAM. ***A***, Neuroblastoma N2A cells were labeled with [^{3}H]palmitate and subjected to immunoprecipitation with anti-NCAM antibody, followed by SDS-PAGE and fluorography (right). Representative fluorograms (5 weeks exposure) from three experiments are shown. Expression of the NCAM140 and NCAM180 was documented by immunoblotting (IB, left). ***B***, N2A cells were transfected with NCAM140, labeled with [^{3}H]palmitate, immunoprecipitated with anti-NCAM antibody, and treated with nonreducing buffer or with 5%, 10%, or 15% β-mercaptoethanol before SDS-PAGE and fluorography. The fluorogram (5 weeks exposure) is representative of three experiments. ***C***, N2A cells were transfected with NCAM140, labeled with [^{3}H]palmitate, immunoprecipitated with anti-NCAM antibody, and subjected to SDS-PAGE. The gel was treated with 1 M hydroxylamine (NH_2OH) (left) or 1 M Tris-HCl (right). The fluorogram is representative of three experiments. The amount of protein was controlled by immunoblotting (IB) of the gels loaded with immunoprecipitated NCAM. ***D***, N2A cells were labeled with [^{3}H]palmitate and subjected to immunoprecipitation with anti-NCAM antibody, SDS-PAGE, and fluorography. NCAM-bound fatty acids were hydrolyzed, extracted, and separated by thin-layer chromatography. The fluorogram (6 d exposure) obtained from the TLC-plate (inset) was analyzed and is representative of three experiments.

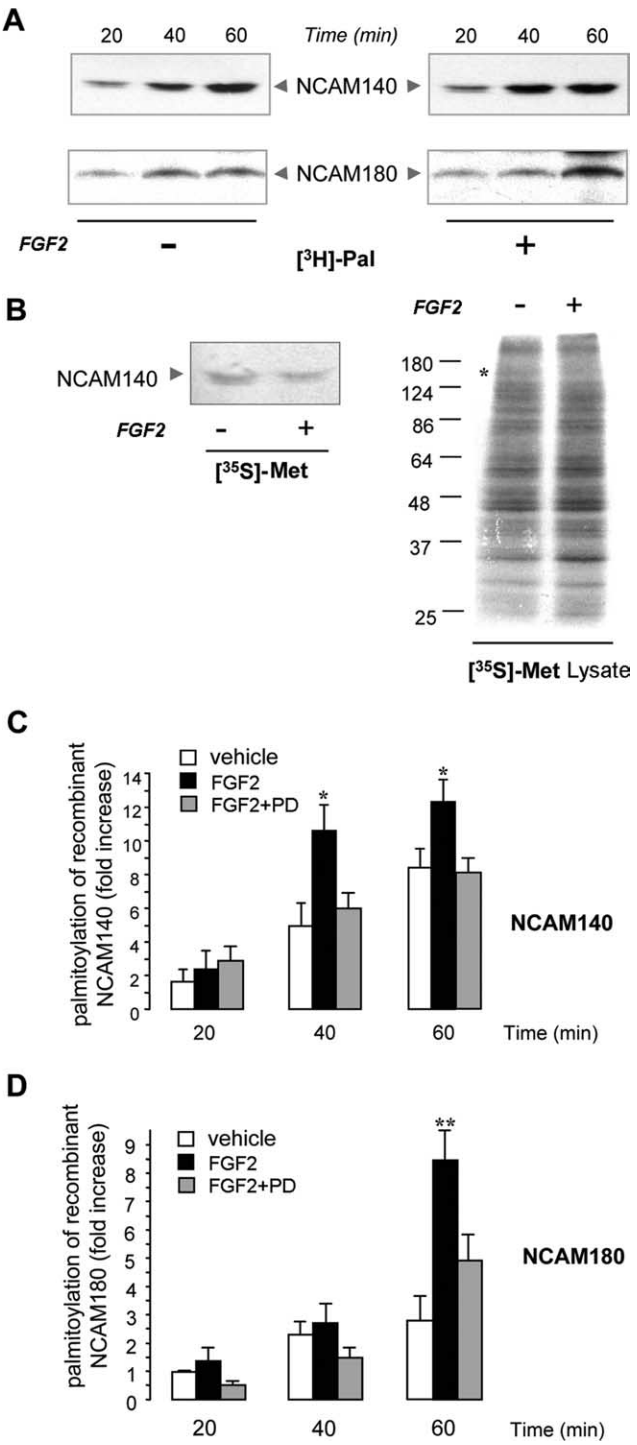

Figure 2. FGF2 treatment increases palmitoylation of transfected NCAM140 and NCAM180. ***A***, N2A cells were transfected with NCAM140 or NCAM180 and labeled with [^{3}H]palmitate in the presence of either vehicle or FGF2 (50 ng/ml) as indicated. NCAM140 (top) and NCAM180 (bottom) were immunoprecipitated, resolved by SDS-PAGE, and detected by fluorography. Representative fluorograms (4 weeks exposure) of three experiments are shown. ***B***, N2A cells were transfected with NCAM140, labeled with [^{35}S]methionine/cysteine in the presence of either vehicle or FGF2 (50 ng/ml) as indicated, and subjected to immunoprecipitation (left), SDS-PAGE, and fluorography. Incorporation of [^{35}S]methionine/cysteine into total cell proteins is shown (right). The position of NCAM140 in the blot is indicated by an asterisk. ***C***, ***D***, The intensity of [^{3}H]palmitoylated NCAM140 labeling from NCAM140 transfected (***C***) or NCAM180-transfected (***D***) cells was assessed by densitometry of fluorograms relative to [^{35}S]methionine/cysteine labeling. The value for vehicle treated cells at 20 min was set to 1. Bars represent means + SEM ($n = 3$). A statistically significant difference between FGF2-stimulated and nonstimulated palmitoylation is indicated (*$p < 0.05$, **$p < 0.01$, *t* test).

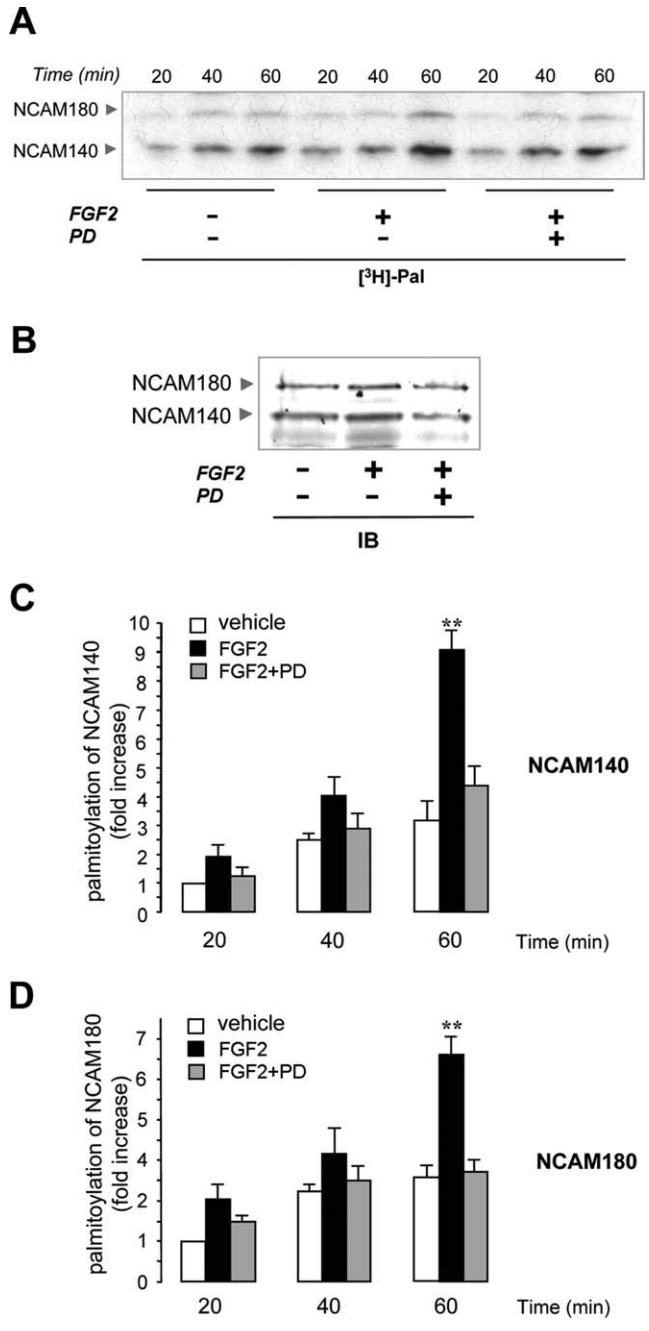

Figure 3. ***A***, ***B***, FGF2 treatment results in increased palmitoylation of endogenous NCAM140 and NCAM180. N2A cells were labeled with [^{3}H]palmitate in the presence of either vehicle, FGF2 (50 ng/ml), or FGF2 together with PD173074 (PD, 5 μM) as indicated and subjected to immunoprecipitation with anti-NCAM antibody, SDS-PAGE, and fluorography (***A***) or to immunoblotting (IB) with anti-NCAM antibody (***B***). Representative fluorogram (9 weeks exposure) of three experiments is shown. ***C***, ***D***, The intensity of the NCAM140 (***C***) and NCAM180 (***D***) [^{3}H]palmitate labeling was assessed by densitometry of fluorograms relative to protein levels estimated by immunoblotting. The value for vehicle treated cells at 20 min was set to 1. Bars represent means + SEM ($n = 3$). The statistically significant difference between FGF2-treated and vehicle groups is indicated (**$p < 0.01$, *t* test).

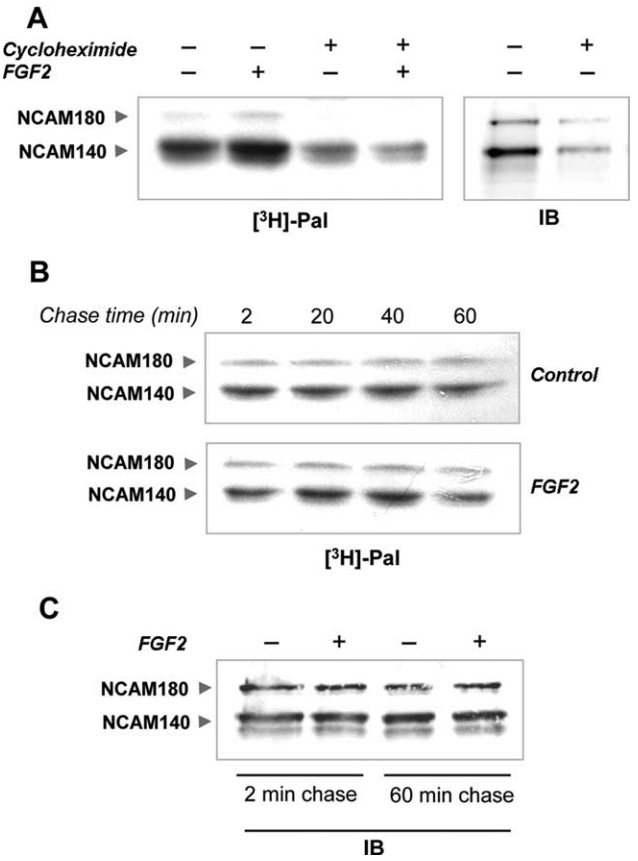

Figure 4. Palmitoylation dynamics of NCAM140 and NCAM180. ***A***, N2A cells transfected with NCAM140 and NCAM180 were incubated for 60 min with [^{3}H]palmitate in the absence or presence of cycloheximide (50 μg/ml). In parallel, FGF2 (50 ng/ml) or vehicle was added. Cell lysates were then subjected to the immunoprecipitation, SDS-PAGE, and fluorography. The fluorogram is representative of three independent experiments. Part of cell lysates was subjected to immunoblotting (IB, right) to analyze the expression of the NCAM140 and NCAM180. ***B***, N2A cells transfected with NCAM140 and NCAM180 were labeled with [^{3}H]palmitate for 1 h and then chased with medium containing unlabeled palmitate for the time periods indicated in the figure. During the chase, cells were treated with FGF2 (50 ng/ml) or vehicle. NCAM proteins were detected by fluorography after immunoprecipitation and SDS-PAGE. The fluorogram is representative of three experiments. ***C***, Expression of NCAM140 and NCAM180 proteins during pulse-chase experiments is documented by immunoblotting (IB) of these molecules.

cycloheximide (Fig. 4*A*), suggesting that protein synthesis is required for regulated palmitoylation of NCAM by FGF2.

Because palmitoylation is a reversible process, the detected increase in palmitoylation of NCAM140 and NCAM180 can be either due to an increase in palmitoylation or reduction in depalmitoylation of these molecules. To distinguish between these two possibilities, we performed a series of pulse-chase experiments. These experiments revealed a very low rate of NCAM depalmitoylation in 60 min after a pulse of [^{3}H]palmitate given in absence of FGF2 (Fig. 4*B*). Also in the presence of FGF2, we did not observe any significant depalmitoylation (Fig. 4*C*). Thus, we conclude that the observed FGF2-induced increase in NCAM palmitoylation is due to regulation of palmitoylation rather than depalmitoylation. The low depalmitoylation rate of NCAM is reminiscent of that found by Kang and coworkers for synaptotagmin I and SNAP-25 (Kang et al., 2004).

FGF2 stimulation increases the association of NCAM with lipid rafts

To investigate whether FGF2-induced palmitoylation affects the lipid raft association of NCAM, we applied fluorescence lifetime imaging (FLIM)-based FRET microscopy (Esposito et al., 2005). The FLIM-FRET technique provides a sensitive quantitative measure for protein–protein associations. Our analysis showed that at basal state, i.e., without FGF2, only a small fraction (4%) of fluorescently labeled NCAM molecules associated with GM1-

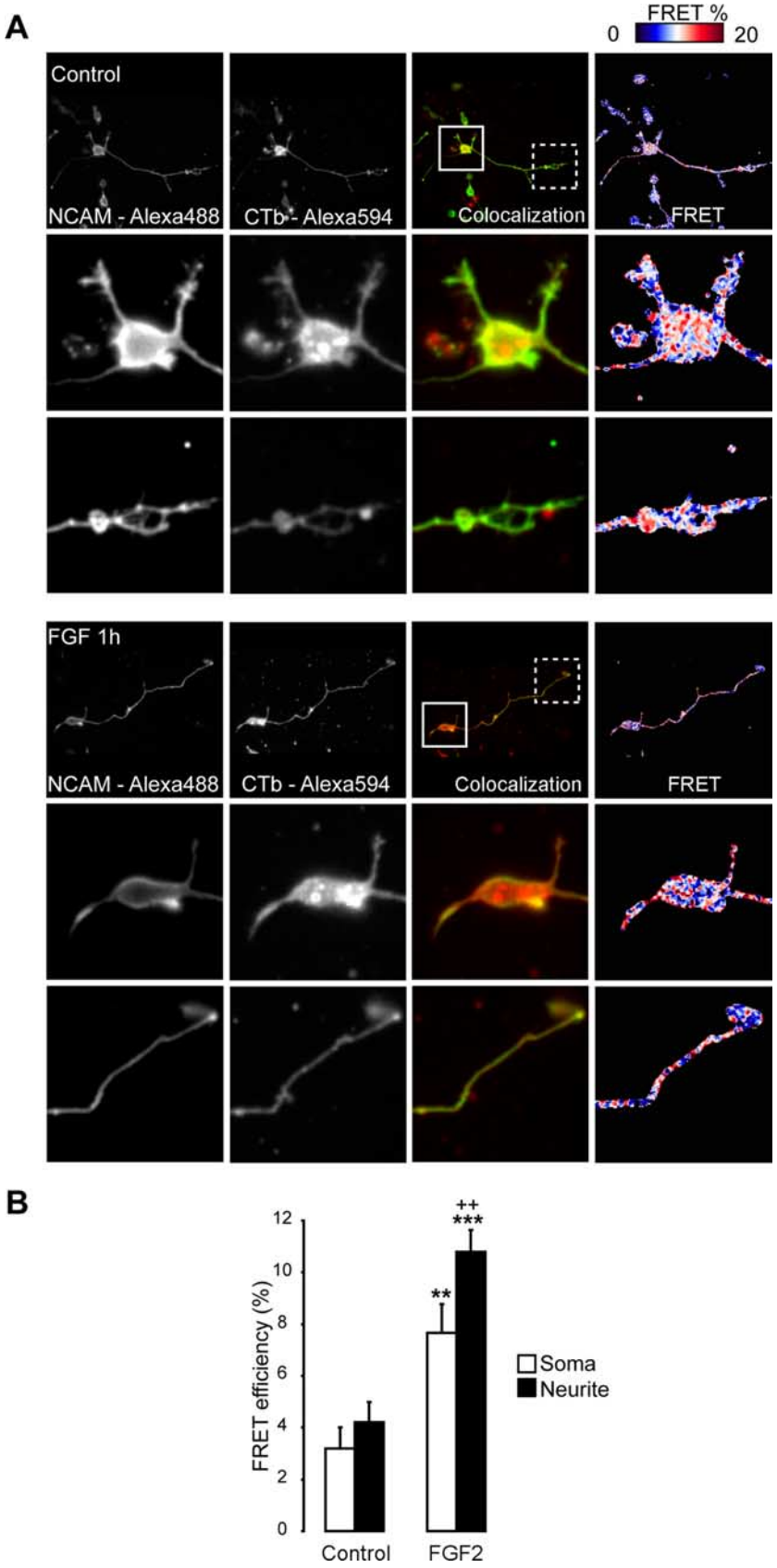

Figure 5. FGF2 stimulates the translocation of NCAM into GM1-enriched lipid rafts. ***A***, Untreated (control) and FGF2-treated cultures of hippocampal neurons were labeled using polyclonal anti-NCAM antibodies (NCAM-Alexa488; FRET donor) and for GM1-containing lipid rafts using cholera toxin subunit β (CTβ-Alexa594; FRET acceptor). The FRET efficiencies, indicative of copartitioning in the same lipid microdomain, are shown in false color ranging from blue (0%) to red (20% FRET) in the far right images in each row. Images on the left from the FRET images show a colocalization of NCAM and GM1. The second and third rows in both sets of images show the soma and the growth cone at a high resolution, the positions of which are outlined in the first row by solid and dashed lines, respectively. Notice the increase in deep red FRET signal in the FGF2-treated neuron. ***B***, Statistical evaluation of the copartitioning of fluorescently labeled NCAM with CTβ positive lipid rafts in cell bodies and neurites. Bars show means + SEM (10 cells in each group) of the occurrence of FRET (probability of obtaining FRET values that exceed the background, pFRET, see Materials and Methods). Application of FGF2 for 1 h induces a threefold increase in lipid raft-associated NCAM in neurites (***$p < 0.001$, *t* test) and a twofold increase (**$p < 0.01$, *t* test) in the cell somata. The increase in neurites was significantly higher than in somata ($^{++}p < 0.01$, *t* test).

positive lipid rafts (Fig. 5*A*). This fraction in neuronal somata and neurites (Fig. 5*B*) is close to the levels of NCAM detected in biochemically prepared lipid raft fractions (Niethammer et al., 2002). After a 1 h FGF2 stimulation, the amount of lipid raft-associated NCAM increased by twofold in the somata and by threefold in neurites (Fig. 5*B*). Prolonged FGF2-stimulation (20 h) did not increase the lipid raft association of NCAM further (data not shown).

FGF2-stimulated neurite outgrowth is NCAM dependent

We next asked whether FGF2-stimulated NCAM palmitoylation and translocation to lipid rafts may regulate neurite outgrowth. First, we investigated whether FGF2-stimulated neurite outgrowth requires NCAM by comparing longest neurite lengths of wild-type and NCAM-deficient hippocampal neurons. FGF2 stimulated neurite outgrowth of wild-type neurons maintained on a poly-L-lysine substrate for 20 h in the presence of FGF2, as compared with untreated wild-type neurons (Fig. 6*A*). The FGF2-induced effects on outgrowth were blocked by the FGF receptor antagonist PD173074. In contrast, FGF2 was ineffective when applied to NCAM-deficient neurons. Thus, not only does NCAM-stimulated outgrowth require signaling via the FGF receptor (Saffell et al., 1997; Niethammer et al., 2002), but also FGF receptor signaling activity depends on expression of NCAM. There was no difference in outgrowth of NCAM deficient and wild-type neurons in the presence of NGF or EGF (data not shown).

For NCAM-dependent neurite outgrowth, i.e., on an NCAM substrate, the NCAM140 isoform, but not NCAM180, is necessary and sufficient (Niethammer et al., 2002). Here, we investigated the importance of NCAM140 and NCAM180 for

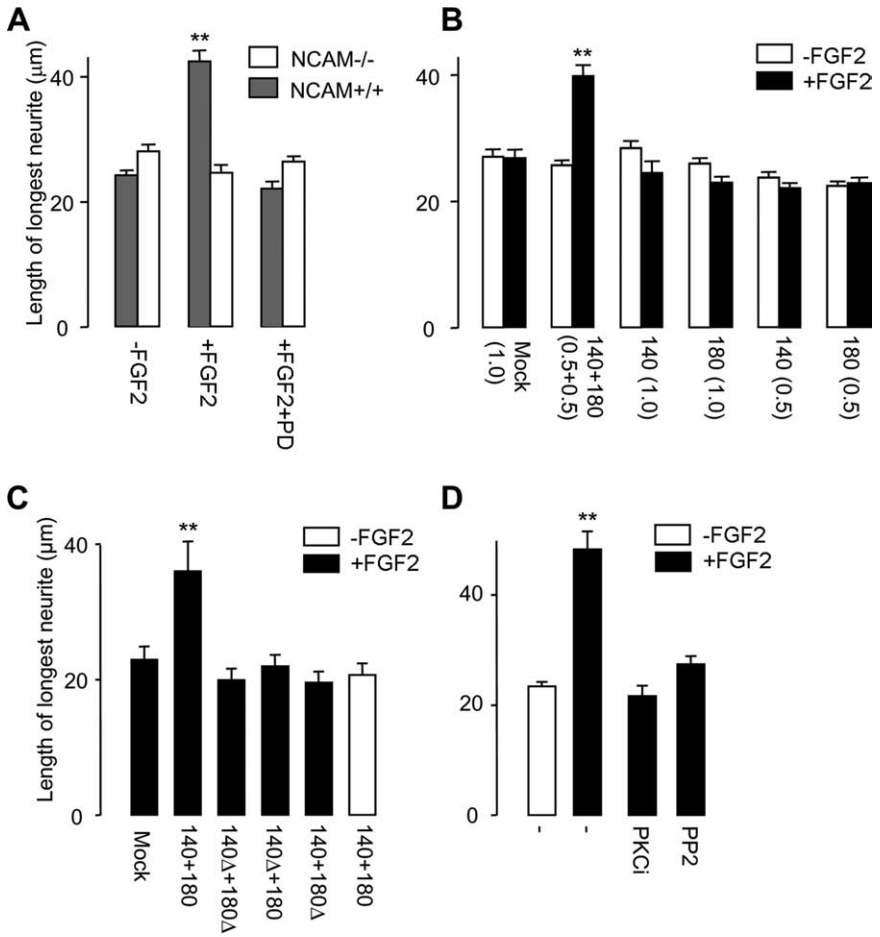

Figure 6. FGF2-stimulated neurite outgrowth requires palmitoylation of NCAM140 and NCAM180 and signaling via fyn kinase and protein kinase C. ***A***, The lengths of longest neurites were measured for hippocampal neurons from wild-type (NCAM $^{+/+}$) or NCAM deficient (NCAM $^{-/-}$) mice, maintained in the absence or presence of 12.5 ng/ml FGF2, or in the presence of FGF2 plus the FGF receptor inhibitor PD173074 (PD, 50 nM). ***B***, The lengths of longest neurites were measured in neurons from NCAM $^{-/-}$ mice, mock-transfected or transfected with NCAM140, NCAM180, or NCAM140 plus NCAM180, and maintained in the absence or presence of 12.5 ng/ml FGF2. Numbers in parentheses indicate the amount of transfected DNA (μg). ***C***, The lengths of longest neurites were measured for neurons from NCAM $^{-/-}$ mice, mock-transfected or transfected with a combination of NCAM140 and NCAM180 wild-type/acylation-deficient forms (NCAM140Δ and NCAM180Δ, respectively), and maintained in the absence or presence of 12.5 ng/ml FGF2. ***D***, The lengths of longest neurites were measured in neurons from NCAM $^{+/+}$ mice and maintained in the absence or presence of 12.5 ng/ml FGF2. Some cultures were treated with a PKC inhibitor (PKCi, 50 μM) or the inhibitor of fyn kinase PP2 (50 nM). Bars represent the means + SEM ($n = 3$). Statistically significant differences between genotypes are indicated (**$p < 0.01$, *t* test).

FGF2-stimulated outgrowth. Expression of either NCAM isoform alone was insufficient to support FGF2-stimulated outgrowth, i.e., there was no difference between FGF2-stimulated and untreated neurons and between NCAM-transfected and mock-transfected control neurons (Fig. 6*B*). Coexpression of NCAM140 and NCAM180 resulted in longer neurites in the presence of FGF2 than in the absence of FGF2 or in mock-transfected neurons. The total amount of NCAM180 plus NCAM140 plasmids used for transfections matched that used in transfections with single plasmids. These data suggest a synergistic interaction of the two major transmembrane NCAM isoforms in response to FGF2 stimulation.

Our aforementioned experiments demonstrated that both NCAM140 and NCAM180 may be palmitoylated in response to FGF2 stimulation. Both isoforms may translocate and signal in lipid rafts (Niethammer et al., 2002). Therefore, we studied whether palmitoylation of one or both isoforms is necessary to support FGF2 activity. Expression of mutated NCAM140Δ and NCAM180Δ, lacking four cysteine residues, did not lead to an increase of neurite length by FGF2 (Fig. 6*C*). Also coexpression of a mutated plus a wild-type form, i.e., NCAM140Δ plus NCAM180 or NCAM140 plus NCAM180Δ, was not sufficient to restore activity of FGF2. Thus, both NCAM isoforms must be palmitoylated to mediate the action of FGF2.

Previous studies revealed that NCAM promotes neurite outgrowth via the nonreceptor fyn tyrosine kinase and PKC, both signaling via lipid rafts (Beggs et al., 1997; Kolkova et al., 2000; Niethammer et al., 2002). Neurite length in the presence of FGF2 was strongly reduced by inhibitors of fyn and PKC (Fig. 6*D*), supporting the view that signaling via these NCAM associated molecules is necessary for FGF2-stimulated neurite outgrowth.

Identification of enzymes involved in palmitoylation of NCAM

Twenty-three mouse genes encoding proteins containing a "DHHC" (aspartate-histidine-histidine-cysteine) motif function as palmitoyl transferases (Fukata et al., 2004). Here, we individually cotransfected these DHHC proteins with NCAM140 into HEK293 cells and assessed palmitoylation of NCAM140 by metabolic labeling with [^{3}H]palmitate. In 23 measurements of NCAM palmitoylation in cells transfected with 23 different DHHCs, we detected only three DHHCs (DHHC-3, DHHC-7, and DHHC-8) with significantly increased palmitoylation (Fig. 7*A*). The highest activity was seen for DHHC-7, which enhanced NCAM140 palmitoylation nearly seven times over baseline values (Fig. 7*A*). An approximately fourfold enhanced NCAM140 palmitoylation was also seen with DHHC-3, the closest homolog of DHHC-7 and DHHC-8. To further verify which of these molecules are involved in palmitoylation of NCAM, we performed three experiments with each of DHHC-3, DHHC-7, and DHHC-8, and DHHC-1 as a negative control, in both HEK293 (Fig. 7*B*) and neuroblastoma N2A cells (Fig. 7*C*). This analysis revealed that DHHC-3 and DHHC-7, but not DHHC-8, stimulated palmitoylation of NCAM140 in both HEK293 and N2A cells. Additionally, we examined the effect of DHHC-7 on the NCAM140Δ (Fig. 7*B*) and thus verified that the detected palmitoylation signal indeed represents palmitoylation of NCAM140. Interestingly, there were two bands after [^{3}H]palmitate labeling of NCAM140 in HEK293 and N2A cells (Fig. 7*B*,*C*). The reason for the minor, more quickly migrating band is unknown. In fact, the appearance of this band was usually increased when there was strong palmitoylation of NCAM. Thus, the presence of this band may reflect changes in conformation of NCAM due to palmitoylation or other posttranslational modifications.

In additional series of experiments, we compared palmitoylation of endogenous NCAM isoforms in response to FGF2 stim-

ulation in N2A cells transfected with DHHC-7 versus a mock control. Palmitoylation of NCAM140 and NCAM180 in DHHC-7 transfected N2A cells was elevated in the presence of FGF2 by 2.6 ± 0.6-fold and 2.3 ± 0.8-fold, respectively (Fig. 8*A*,*B*). These data indicate that the activity of DHHC-7 is regulated by FGF receptor signaling. We also tested whether palmitoylation of NCAM by overexpression of DHHC-7 could mimic the effects of FGF2. Indeed, overexpression of DHHC-7 increased the length of neurites in wild-type but not in NCAM-deficient hippocampal neurons, and this increase occluded the effects of FGF2 (Fig. 8*C*), supporting the view that palmitoylation of NCAM is linked to neurite outgrowth and thus to the FGF receptor function. Transfection of neurons with DHHC-8, which did not stimulate palmitoylation of NCAM (Fig. 7*B*,*C*), had no influence on neurite outgrowth (Fig. 8*D*).

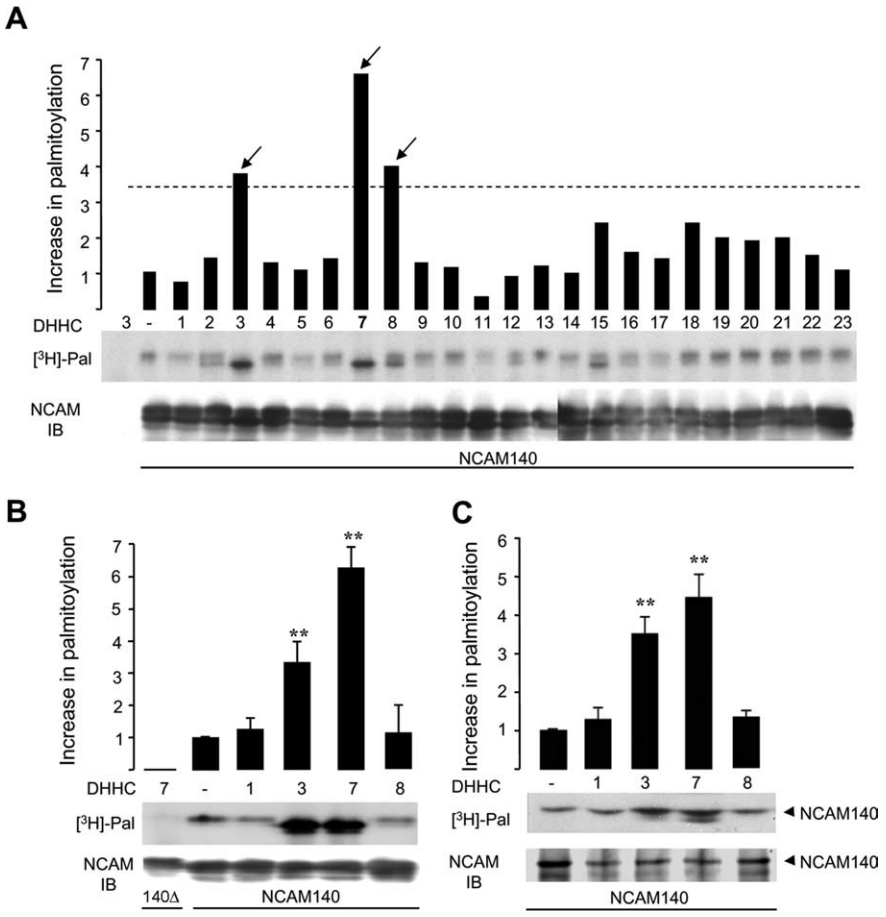

Figure 7. Screening for NCAM140 palmitoylating enzymes. ***A***, HEK293 cells transfected to express different DHHC enzymes and NCAM140 were incubated with [^{3}H]palmitate for 4 h. After metabolic labeling, proteins were separated by SDS-PAGE, followed by fluorography (top) and immunoblotting (IB) with anti-NCAM antibody (bottom). Note that several different DHHC proteins increased levels of [^{3}H]palmitate incorporation into NCAM140. Data shown in lanes 1–15 and 16–25 are derived from two blots made in parallel. The bar diagram shows the fold increase in palmitoylation obtained for the individual DHHC in relation to the expression level estimated by immunoblotting. Data are from one transfection experiment. For three DHHC proteins (indicated by arrows) the palmitoylation levels of NCAM140 are above mean + two SDs (indicated by a dashed line). Notice that the [^{3}H]palmitate signal is absent in lane 1, the only lane in which NCAM140 was not cotransfected with a DHHC protein, thus indicating that [^{3}H]palmitate signals in the other lanes represent levels of NCAM140 palmitoylation. ***B***, For these three molecules and one negative control (DHHC-1), three additional experiments were performed, which confirmed that DHHC-3 and DHHC-7, but not DHHC-8, stimulate palmitoylation of NCAM140 in HEK293 cells. Notice that no [^{3}H]palmitate signal is present when DHHC-7 was cotransfected with mutated form of NCAM140 lacking four cysteine residues (140Δ, lane 1), thus confirming that [^{3}H]palmitate signals represent levels of wild-type NCAM140 palmitoylation. ***C***, N2A cells were transfected with DHHCs as indicated and labeled with [^{3}H]palmitate for 2 h. Proteins were subjected to immunoprecipitation with anti-NCAM antibody, SDS-PAGE, and fluorography (top) or to immunoblotting (IB) with anti-NCAM antibody (bottom). Fluorogram (4 weeks exposure) is representative of three independent experiments. [^{3}H]Palmitate labeling was assessed by densitometry of fluorograms relative to protein levels estimated by immunoblotting. ***B***, ***C***, The value for cells not cotransfected with any DHHC was set to 1. Bars represent means + SEM ($n = 3$). The statistically significant difference between nontransfected and transfected groups is indicated (**$p < 0.01$, *t* test).

Discussion

Palmitoylation and neurite outgrowth

Protein palmitoylation represents a common lipid modification of proteins involved in neurite outgrowth and axon guidance. These include the growth-associated protein GAP-43, paralemmin, G-proteins, signaling proteins such as fyn and the small GTPase Ras, and cell adhesion molecules, such as NCAM, axon guidance receptor deleted in colorectal cancer (DCC), and neurofascin (for a recent review, see Huang and El-Husseini, 2005). Disruption of DCC palmitoylation prevents DCC association with lipid rafts and abolishes netrin-1-mediated axon guidance (Hérincs et al., 2005). For the integrin-tetraspanin cell adhesion molecule complex comprising CD9, CD81, and CD63, palmitoylation is required for the formation of tetraspanin-enriched microdomains, which provide a platform for regulation of integrin-dependent adhesion (Yang et al., 2004). Palmitoylation of the L1 family cell adhesion molecule neurofascin targets it to a specialized fraction of lipid rafts (Ren and Bennett, 1998). In the present study we demonstrate that NCAM140 and NCAM180 are covalently modified by thioester-linked palmitic acid. We also show that FGF2-dependent palmitoylation of NCAM promotes its translocation into lipid rafts and is required for FGF2-stimulated neurite outgrowth.

Synergistic actions of NCAM and FGF receptor

During nervous system development, short- and long-range signals cooperate to promote axonal growth, guidance, and target innervation. Whereas short-range signals act via direct contact between cells or the extracellular matrix, long-range signals require diffusion and concentration gradients of messenger molecules, which are then integrated by receptors on distant target cells. These short- and long-range intercellular signaling pathways involve distinct sets of cell surface molecules coupled to different intracellular signaling cascades. The two signaling pathways may be interconnected, because the short-range receptor NCAM directly interacts with the long-range receptor for FGF (Saffell et al., 1997; Cavallaro et al., 2001; Anderson et al., 2005). Additionally, polysialylated isoform of NCAM may form a complex with heparan sulfate proteoglycans (Storms and Rutishauser, 1998), which have been suggested to potentiate FGF receptor signaling. The functional interplay between NCAM and the FGF receptor has been shown to be critical for NCAM-dependent neurite outgrowth and synaptogenesis (Saffell et al.,

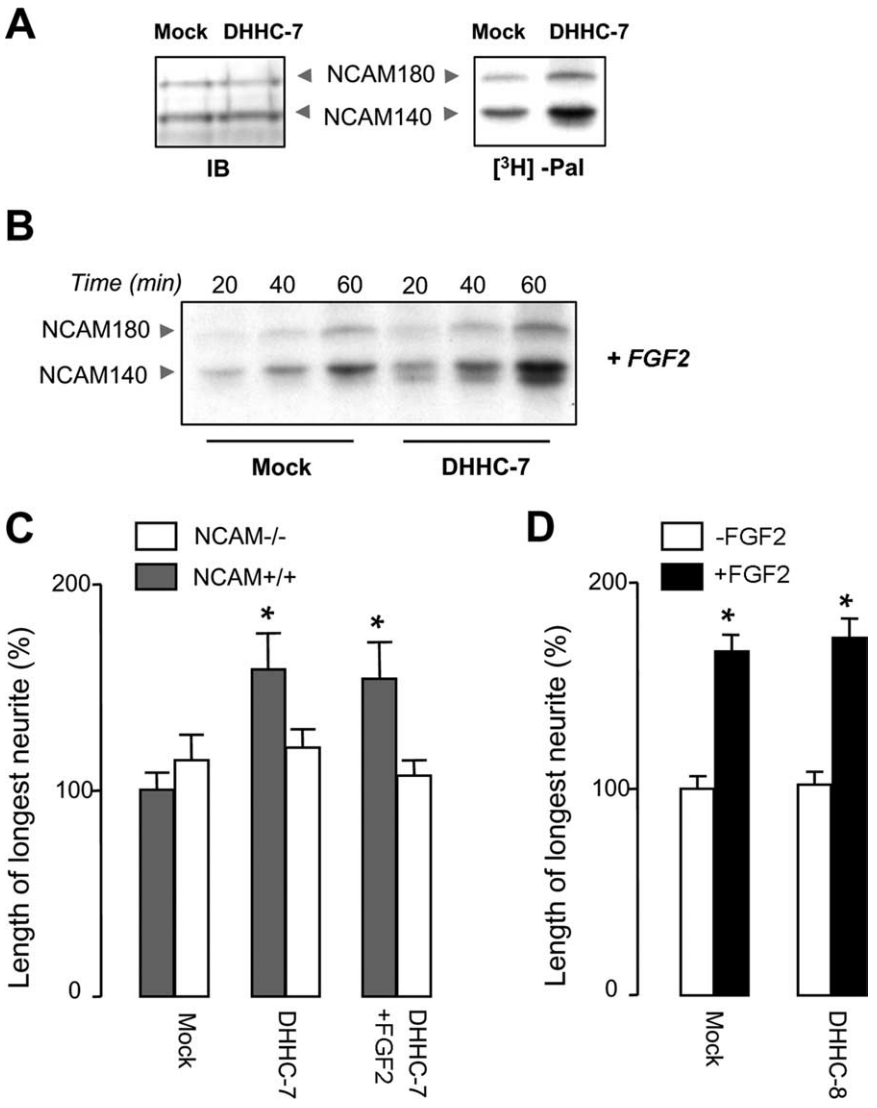

Figure 8. DHHC palmitoyltransferase 7 (DHHC-7) palmitoylates NCAM140 and NCAM180 and also stimulates neurite outgrowth in an NCAM-dependent manner. ***A***, N2A cells were transfected with DHHC-7 or mock-transfected, labeled with [^{3}H]palmitate in the presence of FGF2 (50 ng/ml) as indicated and then subjected to immunoprecipitation with anti-NCAM antibody, SDS-PAGE, and fluorography. Fluorogram (6 weeks exposure) is representative of three experiments. Expression of NCAM140 and NCAM180 was documented by immunoblotting (IB). ***B***, N2A cells transfected with DHHC-7 were labeled with [^{3}H]palmitate in the presence of either vehicle or FGF2 (50 ng/ml) and subjected to immunoprecipitation with anti-NCAM antibody, SDS-PAGE, and fluorography. Representative fluorogram (6 weeks exposure) of three experiments is shown. ***C***, ***D***, The lengths of longest neurites were measured in hippocampal neurons transfected with either DHHC-7 (***C***) or DHHC-8 (***D***) and maintained in the absence or presence of 12.5 ng/ml FGF2. Bars represent means + SEM ($n = 3$). Note that DHHC-8 does not stimulate neurite outgrowth of NCAM $^{+/+}$ neurons, whereas DHHC-7 stimulates neurite outgrowth of NCAM $^{+/+}$ but not NCAM $^{-/-}$ neurons (*$p < 0.05$, *t* test).

1997; Niethammer et al., 2002; Dityatev et al., 2004). Here, we show that the functional influences are reciprocal, because FGF receptors regulate palmitoylation of NCAM and palmitoylated NCAM is necessary for FGF2-stimulated outgrowth. The synergistic actions of NCAM and FGF receptors may be important for integration of signaling via short-distance and long-distance navigational cues by growing neurites. It is noteworthy in this respect that glial cell line-derived neurotrophic factor (GDNF) can directly bind to NCAM140 and GDNF-mediated neurite outgrowth of hippocampal neurons is impaired either by antibodies binding to the extracellular domain of NCAM or in neurons isolated from NCAM deficient mice (Paratcha et al., 2003). Furthermore, GDNF-stimulated synaptogenesis is partially reduced in cultured hippocampal neurons from NCAM deficient mice (Ledda et al., 2007) and signaling via receptors of brain- and platelet-derived neurotrophic factors, BDNF and PDGF, depend on polysialylated NCAM (Muller et al., 2000; Zhang et al., 2004). Thus, NCAM is at the crossroad of several signaling cascades activated by long-distance cues. The novelty of our present findings is that the interplay between a long-range messenger, FGF2, and the short-range signal transducers NCAM140 and NCAM180 is mediated by FGF2-regulated palmitoylation of these molecules.

FGF2 activates FGFR1–4 receptors (Mason, 2007). Analysis of the crystal structure of the FGFR1 tyrosine kinase domain revealed that PD173074, used in the present study to verify involvement of FGF receptors, fits the hydrophobic, ATP-binding pocket of the receptor (Mohammadi et al., 1998). Several studies indicate that PD172074 inhibits multiple FGF2-mediated responses via all major subtypes of FGFRs (Bansal et al., 2003; Grand et al., 2004; Ezzat et al., 2005). Thus, potentially any of FGFR1–4 may mediate the studied effects of FGF2 and it remains to be identified which FGFR subtypes signal to activate palmitoylation of NCAM.

NCAM140 and NCAM180 cooperate in FGF2-stimulated neurite outgrowth

Previous studies revealed that although both NCAM140 and NCAM180 can interact with FGF receptors, only NCAM140 signals independently of FGF receptors by activating fyn/FAK signaling. A possible explanation for this specificity could be that some conformational constraints in NCAM180 prevent its interaction with fyn (Beggs et al., 1997; Kolkova et al., 2000; Niethammer et al., 2002). Because NCAM140 may activate both FGF receptor and fyn signaling pathways, it alone is sufficient to stimulate NCAM-mediated neurite outgrowth (Niethammer et al., 2002). In contrast, here we find that FGF2-stimulated neurite outgrowth requires both NCAM140 and NCAM180 (at least in the range of protein concentrations studied), which both must be palmitoylated. The need for palmitoylation of NCAM180 may be related to NCAM180-dependent accumulation of active PKC/β1 spectrin complexes in lipid rafts. This notion is supported by two studies demonstrating that NCAM180 coimmunoprecipitated with spectrin with higher efficiency than NCAM140 or NCAM120 (Leshchyns'ka et al., 2003) and that the association between NCAM180, spectrin, PKC, and GAP-43 is important for neurite outgrowth of hippocampal neurons (Korshunova et al., 2007). Thus, the cytoskeletal protein spectrin may be an additional partner in association with NCAM180 to support FGF2-stimulated neurite outgrowth.

In light of data showing that NCAM signals via FGFRs, it is noteworthy that NCAM-Fc stimulation did not upregulate palmitoylation of NCAM, suggesting that either the magnitude of signals or signaling cascades triggered by FGF2 versus NCAM-Fc are not identical. This is not surprising in the view of much lower affinity of NCAM binding to FGFR as compared with FGF (Kiselyov et al., 2005). Our experiments showing that NCAM140 is sufficient for NCAM-stimulated outgrowth, whereas FGF2-stimulated outgrowth requires both NCAM140 and NCAM180, also support this view. More information about FGF2- versus NCAM-induced patterns of FGFR phosphorylation and recruitment of adapter proteins is necessary to understand the specific consequences of FGF2 and NCAM signaling.

FGF2 regulates palmitoyl transferase activity

Because of the importance of palmitoylation for neurite outgrowth and other neuronal functions, the regulatory mechanisms underlying palmitoylation and depalmitoylation are of major interest. Palmitate turnover on Gα subunits is stimulated by activation of their associated G-protein coupled receptors (Chen and Manning, 2000; Barclay et al., 2005). Palmitate cycling on PSD-95 is regulated by Ca^{2+} influx through NMDA receptors (El-Husseini et al., 2002). However, it has remained largely unknown whether and how DHHC palmitoyl transferase activity is regulated by extracellular signals. Here, we show for the first time that FGF2 enhances DHHC-7 activity and palmitoylation of NCAM through FGF receptors. Because we found a similar increase in NCAM palmitoylation by DHHC-7 in nonneural HEK293 and neural N2A cells, it is likely that our major conclusion regarding the role of DHHC-7 in NCAM palmitoylation is valid for different cell types and for neurons. Furthermore, our experiments in neurons directly showed that overexpression of DHHC-7 stimulates neurite outgrowth regardless of whether FGFRs are stimulated with FGF2 (Fig. 8). Our interpretation of these findings is the following: (1) overexpression of DHHC-7 may substitute for FGF2-stimulated increase in activity of endogenous DHHC-7 in terms of increasing palmitoylation of NCAM, and (2) the mechanisms promoting neurite outgrowth via NCAM palmitoylation are saturated in FGF2-stimulated DHHC-7 overexpressing neurons.

We now demonstrate that in the presence of cycloheximide palmitoylation of NCAM is strongly attenuated and FGF2 no more enhances palmitoylation of NCAM, suggesting that either predominantly newly synthesized NCAM is palmitoylated or synthesis of new molecules involved in palmitoylation of NCAM, for instance DHHC-7, is required during FGF2 stimulation. Because endogenous DHHC-17/HIP14 is associated with several vesicular structures, including the Golgi apparatus as well as sorting/recycling and late endosomal structures (Huang et al., 2004), it is conceivable that endogenous DHHC-7 is associated with such structures. Because there is good evidence that NCAM is endocytosed and recycled to the plasma membrane (Diestel et al., 2007), NCAM palmitoylation could take place in these recycling endosomes. Furthermore, FGFRs cooperate with GTPases implicated in endocytosis and vesicular trafficking (Vecchione et al., 2007), and thus it is plausible that FGFR activation triggers endocytosis of NCAM, which may be then palmitoylated in endosomes and delivered to rafts on fusion with the plasma membrane. Considering how DHHC-7 may be regulated by FGF2, it is noteworthy that activation of FGFRs triggers several intracellular signaling cascades. These include phosphorylation of nonreceptor tyrosine kinase src and phospholipase Cγ, leading to activation of PKC (Kolkova et al., 2000; Reuss and von Bohlen und Halbach, 2003). Because DHHC-7 has several potential src and PKC phosphorylation sites (http://www.hprd.org/PhosphoMotif_finder), it is plausible to assume that activation of src and/or PKC in response to activation of FGF receptors by FGF2 may lead to phosphorylation of DHHC-7 and enhancement of the DHHC-7-mediated acylation of NCAM (supplemental Fig. 1, available at www.jneurosci.org as supplemental material). In line with this hypothesis, activation of FGF receptors enhances the formation of NCAM140/NCAM180-spectrin-PKCβ2 complexes (Leshchyns'ka et al., 2003) and FGF-stimulated neurite outgrowth is blocked by a PKC inhibitor (this study). It remains, however, to be investigated whether PKC may promote palmitoylation of NCAM and/or redistribution of vesicular structures containing DHHC-7 and NCAM.

A recent study reports that a kinase activity-regulated palmitoylation of the membrane-anchored Ca^{2+}/calmodulin-dependent kinase CL3 targets it to lipid rafts and stimulates dendritogenesis (Takemoto-Kimura et al., 2007), supporting the view that regulated palmitoylation is essential for early development of neurons. Furthermore, the CL3 kinase is essential for dendritic growth stimulated by BDNF (Takemoto-Kimura et al., 2007). Although changes in palmitoylation of CL3 in response to BDNF have not been directly examined yet, all available data point to this possibility. Thus, it is likely that multiple long-range signaling molecules act via regulation of palmitoylation of short-distance signal transducers associated with the plasma membrane.

References

Anderson AA, Kendal CE, Garcia-Maya M, Kenny AV, Morris-Triggs SA, Wu T, Reynolds R, Hohenester E, Saffell JL (2005) A peptide from the first fibronectin domain of NCAM acts as an inverse agonist and stimulates FGF receptor activation, neurite outgrowth and survival. J Neurochem 95:570–583.

Bansal R, Magge S, Winkler S (2003) Specific inhibitor of FGF receptor signaling: FGF-2-mediated effects on proliferation, differentiation, and MAPK activation are inhibited by PD173074 in oligodendrocyte-lineage cells. J Neurosci Res 74:486–493.

Barclay E, O'Reilly M, Milligan G (2005) Activation of an alpha2A-adrenoceptor-Galphao1 fusion protein dynamically regulates the palmitoylation status of the G protein but not of the receptor. Biochem J 385:197–206.

Beggs HE, Baragona SC, Hemperly JJ, Maness PF (1997) NCAM140 interacts with the focal adhesion kinase p125(fak) and the SRC-related tyrosine kinase p59(fyn). J Biol Chem 272:8310–8319.

Bodrikov V, Leshchyns'ka I, Sytnyk V, Overvoorde J, den Hertog J, Schachner M (2005) RPTPalpha is essential for NCAM-mediated p59fyn activation and neurite elongation. J Cell Biol 168:127–139.

Cavallaro U, Niedermeyer J, Fuxa M, Christofori G (2001) N-CAM modulates tumour-cell adhesion to matrix by inducing FGF-receptor signalling. Nat Cell Biol 3:650–657.

Chen CA, Manning DR (2000) Regulation of galpha i palmitoylation by activation of the 5-hydroxytryptamine-1A receptor. J Biol Chem 275:23516–23522.

Diestel S, Schaefer D, Cremer H, Schmitz B (2007) NCAM is ubiquitylated, endocytosed and recycled in neurons. J Cell Sci 120:4035–4049.

Dityatev A, Dityateva G, Schachner M (2000) Synaptic strength as a function of post- versus presynaptic expression of the neural cell adhesion molecule NCAM. Neuron 26:207–217.

Dityatev A, Dityateva G, Sytnyk V, Delling M, Toni N, Nikonenko I, Muller D, Schachner M (2004) Polysialylated neural cell adhesion molecule promotes remodeling and formation of hippocampal synapses. J Neurosci 24:9372–9382.

Dityateva G, Hammond M, Thiel C, Ruonala MO, Delling M, Siebenkotten G, Nix M, Dityatev A (2003) Rapid and efficient electroporation-based gene transfer into primary dissociated neurons. J Neurosci Methods 130:65–73.

El-Husseini Ael-D, Schnell E, Dakoji S, Sweeney N, Zhou Q, Prange O,

Gauthier-Campbell C, Aguilera-Moreno A, Nicoll RA, Bredt DS (2002) Synaptic strength regulated by palmitate cycling on PSD-95. Cell 108:849–863.

Esposito A, Gerritsen HC, Wouters FS (2005) Fluorescence lifetime heterogeneity resolution in the frequency domain by lifetime moments analysis. Biophys J 89:4286–4299.

Esposito A, Dohm CP, Kermer P, Bähr M, Wouters FS (2007) alpha-Synuclein and its disease-related mutants interact differentially with the microtubule protein tau and associate with the actin cytoskeleton. Neurobiol Dis 26:521–531.

Ezzat S, Huang P, Dackiw A, Asa SL (2005) Dual inhibition of RET and FGFR4 restrains medullary thyroid cancer cell growth. Clin Cancer Res 11:1336–1341.

Ford-Perriss M, Abud H, Murphy M (2001) Fibroblast growth factors in the developing central nervous system. Clin Exp Pharmacol Physiol 28:493–503.

Fukata M, Fukata Y, Adesnik H, Nicoll RA, Bredt DS (2004) Identification of PSD-95 palmitoylating enzymes. Neuron 44:987–996.

Gill JC, Tsai PS (2006) Expression of a dominant negative FGF receptor in developing GNRH1 neurons disrupts axon outgrowth and targeting to the median eminence. Biol Reprod 74:463–472.

Grand EK, Chase AJ, Heath C, Rahemtulla A, Cross NC (2004) Targeting FGFR3 in multiple myeloma: inhibition of t(4;14)-positive cells by SU5402 and PD173074. Leukemia 18:962–966.

Hérincs Z, Corset V, Cahuzac N, Furne C, Castellani V, Hueber AO, Mehlen P (2005) DCC association with lipid rafts is required for netrin-1-mediated axon guidance. J Cell Sci 118:1687–1692.

Huang K, El-Husseini A (2005) Modulation of neuronal protein trafficking and function by palmitoylation. Curr Opin Neurobiol 15:527–535.

Huang K, Yanai A, Kang R, Arstikaitis P, Singaraja RR, Metzler M, Mullard A, Haigh B, Gauthier-Campbell C, Gutekunst CA, Hayden MR, El-Husseini A (2004) Huntingtin-interacting protein HIP14 is a palmitoyl transferase involved in palmitoylation and trafficking of multiple neuronal proteins. Neuron 44:977–986.

Kang R, Swayze R, Lise MF, Gerrow K, Mullard A, Honer WG, El-Husseini A (2004) Presynaptic trafficking of synaptotagmin I is regulated by protein palmitoylation. J Biol Chem 279:50524–50536.

Kiselyov VV, Soroka V, Berezin V, Bock E (2005) Structural biology of NCAM homophilic binding and activation of FGFR. J Neurochem 94:1169–1179.

Kolkova K, Novitskaya V, Pedersen N, Berezin V, Bock E (2000) Neural cell adhesion molecule-stimulated neurite outgrowth depends on activation of protein kinase C and the Ras-mitogen-activated protein kinase pathway. J Neurosci 20:2238–2246.

Korshunova I, Novitskaya V, Kiryushko D, Pedersen N, Kolkova K, Kropotova E, Mosevitsky M, Rayko M, Morrow JS, Ginzburg I, Berezin V, Bock E (2007) GAP-43 regulates NCAM-180-mediated neurite outgrowth. J Neurochem 100:1599–1612.

Kvachnina E, Liu G, Dityatev A, Renner U, Dumuis A, Richter DW, Dityateva G, Schachner M, Voyno-Yasenetskaya TA, Ponimaskin EG (2005) 5-HT7 receptor is coupled to G alpha subunits of heterotrimeric G12-protein to regulate gene transcription and neuronal morphology. J Neurosci 25:7821–7830.

Ledda F, Paratcha G, Sandoval-Guzmán T, Ibáñez CF (2007) GDNF and GFRalpha1 promote formation of neuronal synapses by ligand-induced cell adhesion. Nat Neurosci 10:293–300.

Leshchyns'ka I, Sytnyk V, Morrow JS, Schachner M (2003) Neural cell adhesion molecule (NCAM) association with PKCbeta2 via betaI spectrin is implicated in NCAM-mediated neurite outgrowth. J Cell Biol 161:625–639.

Linder ME, Deschenes RJ (2007) Palmitoylation: policing protein stability and traffic. Nat Rev Mol Cell Biol 8:74–84.

Little EB, Edelman GM, Cunningham BA (1998) Palmitoylation of the cytoplasmic domain of the neural cell adhesion molecule N-CAM serves as an anchor to cellular membranes. Cell Adhes Commun 6:415–430.

Mason I (2007) Initiation to end point: the multiple roles of fibroblast growth factors in neural development. Nat Rev Neurosci 8:583–596.

Mohammadi M, Froum S, Hamby JM, Schroeder MC, Panek RL, Lu GH, Eliseenkova AV, Green D, Schlessinger J, Hubbard SR (1998) Crystal structure of an angiogenesis inhibitor bound to the FGF receptor tyrosine kinase domain. EMBO J 17:5896–5904.

Muller D, Djebbara-Hannas Z, Jourdain P, Vutskits L, Durbec P, Rougon G, Kiss JZ (2000) Brain-derived neurotrophic factor restores long-term potentiation in polysialic acid-neural cell adhesion molecule-deficient hippocampus. Proc Natl Acad Sci U S A 97:4315–4320.

Niethammer P, Delling M, Sytnyk V, Dityatev A, Fukami K, Schachner M (2002) Cosignaling of NCAM via lipid rafts and the FGF receptor is required for neuritogenesis. J Cell Biol 157:521–532.

Paratcha G, Ledda F, Ibáñez CF (2003) The neural cell adhesion molecule NCAM is an alternative signaling receptor for GDNF family ligands. Cell 113:867–879.

Ponimaskin EG, Schmidt MF, Heine M, Bickmeyer U, Richter DW (2001) 5-Hydroxytryptamine 4(a) receptor expressed in Sf9 cells is palmitoylated in an agonist-dependent manner. Biochem J 353:627–634.

Ren Q, Bennett V (1998) Palmitoylation of neurofascin at a site in the membrane-spanning domain highly conserved among the L1 family of cell adhesion molecules. J Neurochem 70:1839–1849.

Reuss B, von Bohlen und Halbach O (2003) Fibroblast growth factors and their receptors in the central nervous system. Cell Tissue Res 313:139–157.

Saffell JL, Williams EJ, Mason IJ, Walsh FS, Doherty P (1997) Expression of a dominant negative FGF receptor inhibits axonal growth and FGF receptor phosphorylation stimulated by CAMs. Neuron 18:231–242.

Smotrys JE, Linder ME (2004) Palmitoylation of intracellular signaling proteins: regulation and function. Annu Rev Biochem 73:559–587.

Storms SD, Rutishauser U (1998) A role for polysialic acid in neural cell adhesion molecule heterophilic binding to proteoglycans. J Biol Chem 273:27124–27129.

Takemoto-Kimura S, Ageta-Ishihara N, Nonaka M, Adachi-Morishima A, Mano T, Okamura M, Fujii H, Fuse T, Hoshino M, Suzuki S, Kojima M, Mishina M, Okuno H, Bito H (2007) Regulation of dendritogenesis via a lipid-raft-associated Ca2+/calmodulin-dependent protein kinase CLICK-III/CaMKIgamma. Neuron 54:755–770.

Vecchione A, Cooper HJ, Trim KJ, Akbarzadeh S, Heath JK, Wheldon LM (2007) Protein partners in the life history of activated fibroblast growth factor receptors. Proteomics 7:4565–4578.

Williams EJ, Walsh FS, Doherty P (1994) The production of arachidonic acid can account for calcium channel activation in the second messenger pathway underlying neurite outgrowth stimulated by NCAM, N-cadherin, and L1. J Neurochem 62:1231–1234.

Yang X, Kovalenko OV, Tang W, Claas C, Stipp CS, Hemler ME (2004) Palmitoylation supports assembly and function of integrin-tetraspanin complexes. J Cell Biol 167:1231–1240.

Zhang H, Vutskits L, Calaora V, Durbec P, Kiss JZ (2004) A role for the polysialic acid-neural cell adhesion molecule in PDGF-induced chemotaxis of oligodendrocyte precursor cells. J Cell Sci 117:93–103.