NCAM is ubiquitylated, endocytosed and recycled in neurons

Simone Diestel[1,*], Daniel Schaefer[1], Harold Cremer[2,‡] and Brigitte Schmitz[1,‡,§]

[1]Institute of Animal Sciences, Department of Biochemistry, University of Bonn, Katzenburgweg 9a, 53115 Bonn, Germany
[2]Institut de Biologie du Développement de Marseille-Luminy, UMR 6216, CNRS/Université de la Méditeranée, Campus de Luminy-case 907, 13288 Marseille cedex 9, France

*Present address: Institut de Biologie du Développement de Marseille-Luminy, UMR 6216, CNRS/ Université de la Méditeranée, Campus de Luminy-case 907, 13288 Marseille cedex 9, France
‡Both last authors contributed equally to this work
§Author for correspondence (e-mail: schmitz@uni-bonn.de)

Accepted 12 September 2007
Journal of Cell Science 120, 4035-4049 Published by The Company of Biologists 2007
doi:10.1242/jcs.019729

Summary

The neural cell adhesion molecule NCAM plays an important role during neural development and in the adult brain. To study the intracellular trafficking of NCAM in neurons, two major isoforms, NCAM140 or NCAM180, were expressed in primary cortical neurons and in the rat B35 neuroblastoma cell line. NCAM was endocytosed and subsequently recycled to the plasma membrane, whereas only a minor fraction was degraded in lysosomes. In cortical neurons, endocytosis of NCAM was detected in the soma, neurites and growth cones in a developmentally regulated fashion. Furthermore, we found that NCAM is mono-ubiquitylated at the plasma membrane and endocytosis was significantly increased in cells overexpressing ubiquitin. Therefore, we propose that ubiquitylation represents an endocytosis signal for NCAM.

Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/120/22/4035/DC1

Key words: Neural cell adhesion molecule NCAM, Intracellular trafficking, Endocytosis, Recycling, Ubiquitylation, Primary neurons

Introduction

The neural cell adhesion molecule NCAM belongs to the superfamily of immunoglobulin-like surface molecules. The three major isoforms NCAM120, NCAM140 and NCAM180 have identical extracellular domains. NCAM140 and NCAM180 are both transmembrane glycoproteins, their only difference being that they are alternatively spliced in exon 18, leading to additional 261 amino acids in the cytosolic domain of NCAM180. In addition to several extracellular interactions, NCAM can undergo interactions with various cytosolic binding partners and activates several signal transduction pathways (Povlsen et al., 2003; Walmod et al., 2004; Büttner et al., 2003; Büttner et al., 2005). A variety of in vitro and in vivo studies provided evidence that NCAM is implicated in processes such as cell migration, axon growth and fasciculation through the promotion of cell-cell or cell-extracellular matrix interactions. Recent evidence suggests that NCAM deregulation is involved in the development of schizophrenia (Panicker et al., 2003; Pillai-Nair et al., 2005). However, despite the fact that considerable information about the structure, expression and developmental function of NCAM is available, the molecular mechanisms underlying the cellular functions of NCAM are still not well understood.

Endocytosis is generally important in the downregulation of cell surface molecules and for signal transduction. The best characterized endocytic pathway depends on clathrin although other clathrin-independent pathways exist as well, such as caveolae- or lipid raft-dependent endocytosis (Johannes and Lamaze, 2002). In neurons, endocytosis is important for correct synaptic function. For example, the stability and/or plasticity of synaptic contacts appear to be regulated by endocytosis of cytoskeletally associated proteins (Dell'Acqua et al., 2006). So far, internalization of NCAM through the clathrin-dependent pathway has been shown in astrocytes and in rat B35 cells (Minana et al., 2001) (T. Goschzik, H.C., B.S. and S.D., unpublished). Nothing is known about NCAM endocytosis in neurons.

Recently it became obvious that ubiquitin, besides its role as a proteasomal degradation signal (Hershko and Ciechanover, 1998), can also regulate endocytosis. Further studies suggest more complex roles of ubiquitylation, depending on how many ubiquitin molecules are covalently attached the substrate protein. The different types of ubiquitylation – mono-, multiple mono- or poly-ubiquitylation – are involved in different cellular processes including endocytosis of several proteins (Hicke, 2001; Haglund et al., 2003a; Mosesson et al., 2003; Pickart, 2001). Furthermore, ubiquitin can be attached to substrate proteins at different steps of endocytosis. It may, for example, regulate the first endocytic steps if it is attached to proteins that are still present at the plasma membrane, whereas it can serve as a lysosomal degradation signal if attached in the endosomes (Umebayashi, 2003).

In this study we demonstrate that in cortical neurons NCAM can be endocytosed and this process is developmentally regulated. The bulk of internalized NCAM is recycled to the plasma membrane, whereas only a small fraction is degraded in lysosomes. We furthermore show that NCAM is ubiquitylated while it is present at the cell surface, indicating that ubiquitin represents an endocytosis signal for NCAM.

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Results

Endocytosis of NCAM is developmentally regulated in primary cortical neurons

Cortical neurons were isolated from mice of embryonic day 15.5 (E15.5) and electroporated with human NCAM140 or human NCAM180 cDNA. We used both, cortical neurons from NCAM-deficient embryos ($NCAM^{-/-}$) or heterozygous littermates ($NCAM^{+/-}$) to control for possible effects of endogenous NCAM in the experiments. Cells were cultured for 1, 3 or 5 days before induction of endocytosis with antibodies specific for human NCAM. This method of NCAM clustering has been shown to mimic homophilic or heterophilic binding of NCAM and to have very similar effects on NCAM-dependent signal transduction and neurite outgrowth as recombinant NCAM-fragments (Schmid et al., 1999). We induced internalization of NCAM140 from the surface of cell somata, with the highest endocytosis rate in neurons that had been cultured for 3 days (DIV3; Fig. 1A,B, green vesicles). Endocytosis of NCAM140 was significantly decreased after DIV5. By contrast, endocytosis of NCAM180, whose internalization from the surface could also be induced, was highest after DIV5. This coincides with the time point when axon-dendrite interactions become established and growth cones become less prominent (Lesuisse and Martin, 2002). The different behavior of the two isoforms suggests that endocytosis of NCAM140 plays a role in immature neurons, whereas internalization of NCAM180 is more important at later developmental stages. Furthermore, it shows that the downregulation of NCAM140 endocytosis is specific for this molecule and does not represent a general feature of cell surface proteins in cultured cortical neurons.

NCAM is, additionally to cell somata, also present in neurites and growth cones. Therefore, we investigated whether NCAM is also endocytosed in these compartments. We found endocytic vesicles containing NCAM140 or NCAM180 in neurites and growth cones of cortical neurons (Fig. 1C). Most of the observed growth cones contained NCAM-positive vesicles in the central domain. Vesicles in the peripheral domain were only rarely observed (Fig. 1C, inset). Quantification showed that 76% of the growth cones contained NCAM140- (from a total of 153 growth cones) and 72% NCAM180-positive vesicles (from a total of 111 growth cones).

In conclusion, these results provide evidence that NCAM endocytosis is not an exclusive property of cultured astrocytes and B35 neuroblastoma cells (Minana et al., 2001) (T. Goschzik, H.C., B.S. and A.S., unpublished) but can be induced in primary neurons in different cell compartments. Since none of the above experiments showed that intrinsic expression of NCAM had an effect on the endocytosis of exogenous NCAM isoforms (not shown), only NCAM-positive cortical neurons were used in this study. Furthermore, endocytosis of NCAM140 was highest at DIV3. Therefore, all

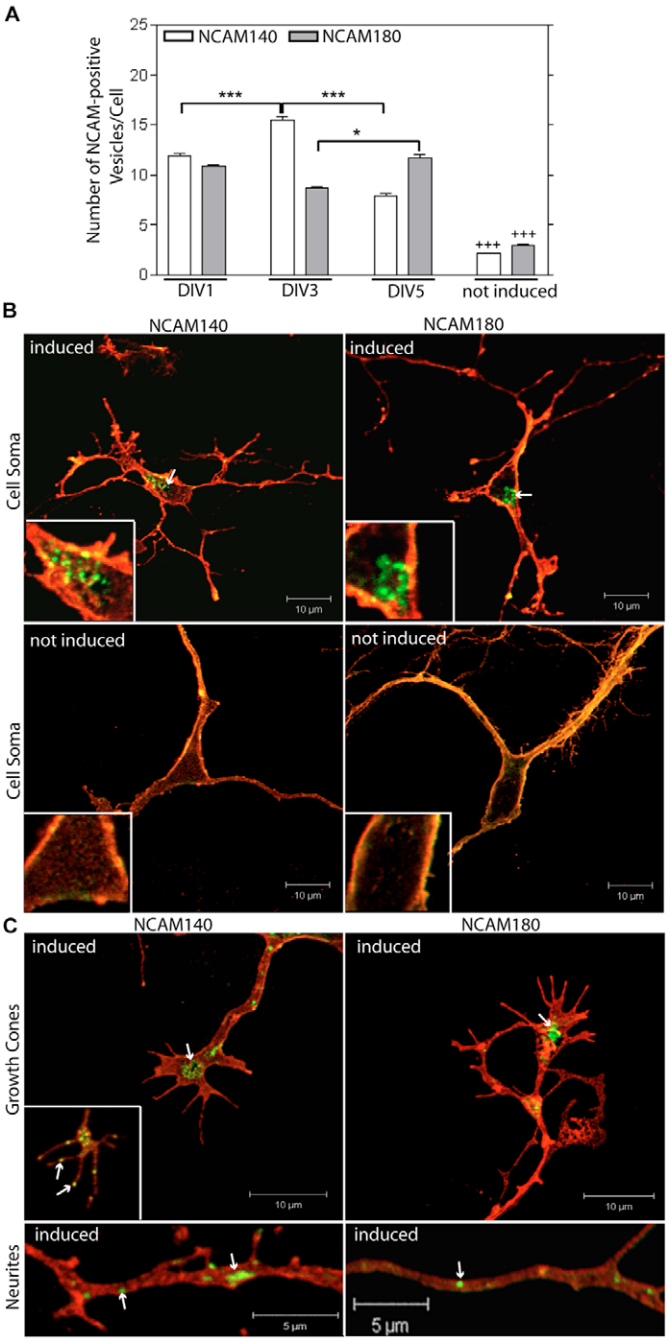

Fig. 1. Endocytosis of NCAM is developmentally regulated in cortical neurons. (A) Cortical neurons isolated from E15.5 mice ($NCAM^{+/-}$) transiently expressing either human NCAM140 or NCAM180 were cultured for 1, 3 or 5 days (DIV1, DIV3 or DIV5, respectively). Endocytosis of NCAM was not induced or induced by an antibody specific for human NCAM for 30 minutes. The numbers of NCAM-positive vesicles per cell in cell somata were counted from at least three independent experiments with approximately 15 cells analysed in each experiment. ***$P<0.0005$; *$P<0.05$ (comparison between vesicle numbers of internalized NCAM at different cultivation times); $^{+++}P<0.0001$ (comparison between vesicle numbers with or without endocytosis induction).
(B,C) Representative images of endocytosed NCAM. Cell surface associated NCAM-antibody complexes were detected with Cy3-conjugated secondary antibodies, internalized NCAM with Alexa-Fluor-488-conjugated secondary antibodies (white arrows). Images were either taken from cell somata (B) or from growth cones or neurites of cortical neurons (C) using a confocal laser scanning microscope.

following experiments described here were carried out at this time point.

NCAM is partially localized in early endosomes

To further analyse the intracellular trafficking of NCAM and to verify that the NCAM-positive vesicles observed in cell processes are not a consequence of vesicular transport but rather owing to local endocytosis, we carried out colocalization studies of NCAM with the early endosome marker Rab5, which is present exclusively in stationary organelles in neurons (Deinhardt et al., 2006). For colocalization studies we analysed NCAM endocytosis after 30 or 60 minutes because we had observed only very low amounts of internalized NCAM after 10 minutes in both cell systems. After co-expression of NCAM140 with a GFP-Rab5 construct in cortical neurons, followed by induction of endocytosis, we observed a partial overlap of NCAM140-positive vesicles with Rab5-positive vesicles in cell somata (32±4%). B35 cells showed a similar overlap (NCAM140, 46±4%; NCAM180, 41±2%; Fig. 2A, Fig. 5B). We also carried out colocalization studies of NCAM-eGFP constructs with the early endosome marker EEA-1 in B35 cells. In agreement with the results obtained with untagged NCAM and Rab5, we found partial colocalization with EEA-1 (supplementary material Fig. S1) confirming that comparable fractions of NCAM are localized in early endosomes of primary cortical neurons and B35 cells (Fig. 2A, Fig. 5C). Furthermore, the observed localization of NCAM140 in Rab5-positive vesicles in neurites and growth cones of cortical neurons indicates that at least a part of NCAM molecules is internalized in cell processes (Fig. 2B).

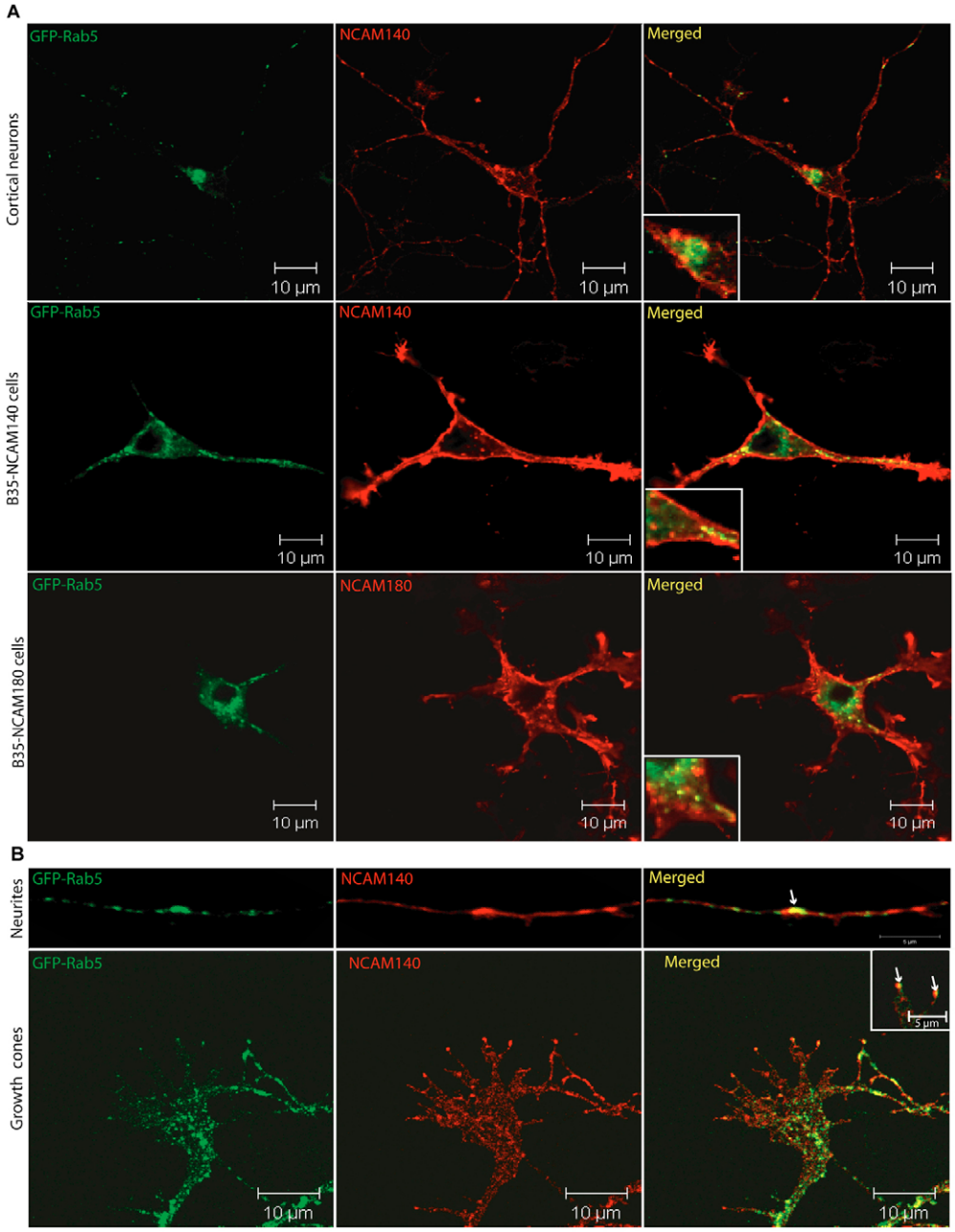

Fig. 2. NCAM is partially localized in early endosomes. Cortical neurons isolated from E15.5 mice or B35 cells were transiently co-transfected with GFP-Rab5 and human NCAM140 or NCAM180. NCAM endocytosis was induced with an NCAM-specific antibody for 30 (cortical neurons) or 60 minutes (B35 cells) and cells were directly processed for fluorescence analysis of NCAM with GFP-Rab5. Photos were taken either from entire cells of cortical neurons or B35 cells (A) or from neurites and growth cones of cortical neurons (B). The experiment was carried out three times with at least 15 cells analysed in each experiment.

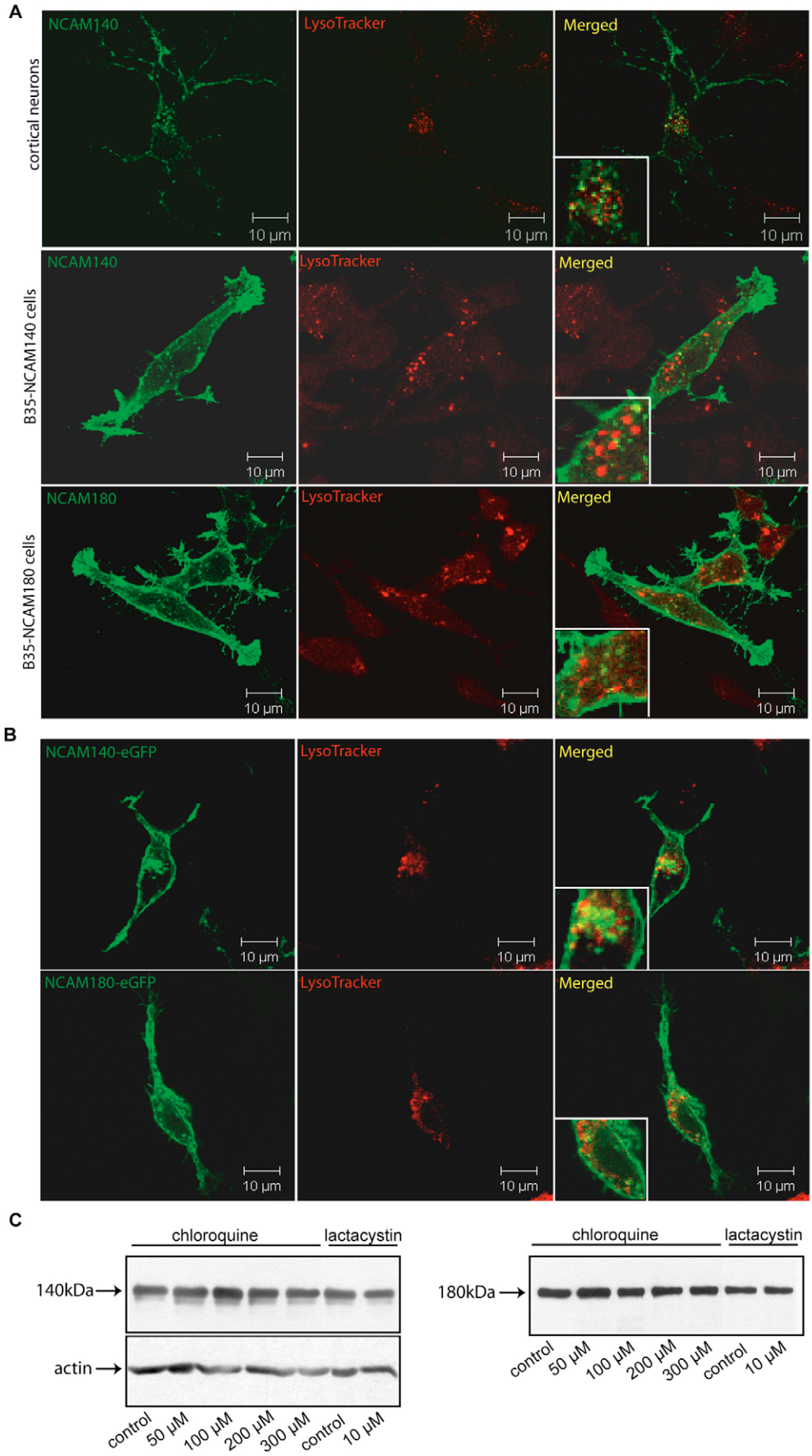

Fig. 3. NCAM is localized only to a small extent in lysosomes. (A) Cortical neurons isolated from E15.5 mice (NCAM$^{+/-}$) or B35 cells were transiently transfected with either NCAM140 or NCAM180. Endocytosis was induced for 1 hour by application of an NCAM-specific antibody in the presence of LysoTracker (200 nM). Cells were then processed for immunofluorescence analysis by visualization of NCAM with secondary Cy2-conjugated antibodies. (B) B35 cells were transiently transfected with either NCAM140-eGFP or NCAM180-eGFP. Endocytosis was induced for 1 hour by application of an NCAM-specific antibody in the presence of LysoTracker (200 nM). Cells were then directly embedded for confocal microscopy. Experiments were carried out three times with at least 15 cells analysed in each experiment. (C) B35 cells expressing either NCAM140 or NCAM180 were mock treated or treated with chloroquine at different concentrations or with 10 µM lactacystin for 4 hours. During the last hour of incubation an NCAM-specific antibody was added to induce NCAM endocytosis. After lysis, 15 µg of total protein of each sample were loaded onto an SDS gel and subjected to western blot analysis using an NCAM-specific antibody. As loading control for the NCAM140-specific blot, all lanes were blotted with an anti-actin antibody.

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NCAM is recycled to the plasma membrane

Next, we investigated whether NCAM140 becomes lysosomally degraded in cortical neurons. Therefore, colocalization experiments of endocytosed NCAM140 with the specific lysosomal dye LysoTracker were carried out (Fig. 3A, Fig. 5B). Only a very small amount of NCAM140 was present in the lysosomal compartment (13.7±2%). The lysosomal localization of NCAM140 and NCAM180 was similar in B35 cells (12±1.9% for NCAM140 and 8.8±2.5% for NCAM180). We further wanted to exclude that the low detection rate of NCAM in lysosomes is due to disassembly of the antibody-NCAM complexes in the acidic endosomal compartment and either (1) recycling of only the antibody to the cell surface is observed or (2) the antibody is degraded in lysosomes and NCAM can therefore not be detected in this compartment. For this reason, B35 cells were transiently transfected with NCAM-eGFP constructs and colocalization of NCAM with LysoTracker was investigated. As visualized through the C-terminal eGFP-tag, NCAM is – after antibody-induced endocytosis – only localized to a very small extent in lysosomes, indicating that the entire NCAM protein is also not present in lysosomes (Fig. 3B). Therefore, we can exclude the possibilities that the low amount of NCAM detected in lysosomes is due to degradation or recycling of only the NCAM antibody required for the detection by indirect immunofluorescence. Since NCAM trafficking appeared

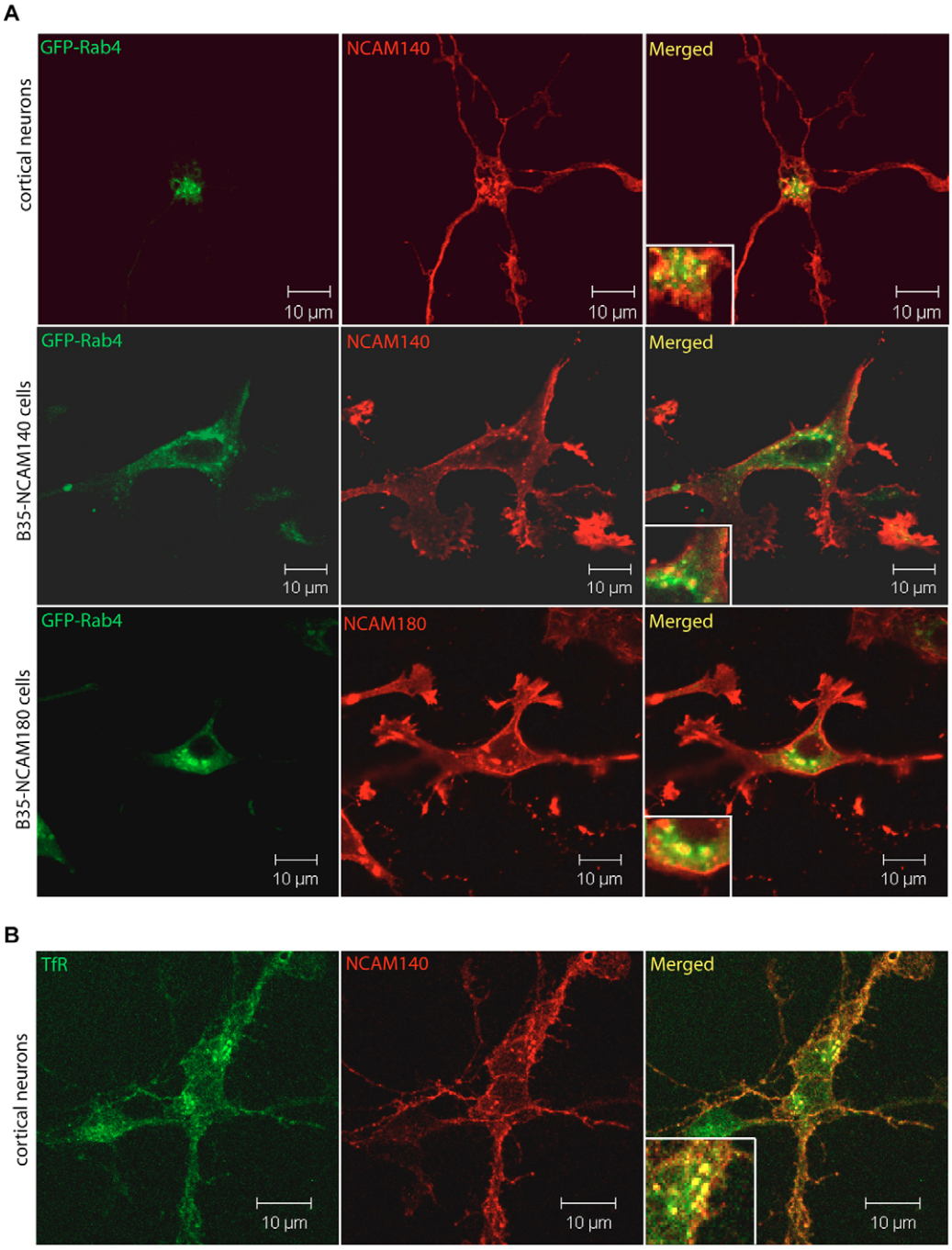


Fig. 4. NCAM is localized in Rab4- and transferrin receptor positive endosomes. (A) Cortical neurons isolated from E15.5 mice (NCAM$^{+/-}$) or B35 cells were transiently co-transfected with NCAM140 and GFP-Rab4 or B35 cells were transiently co-transfected with either NCAM140 or NCAM180 and GFP-Rab4. Endocytosis was induced for 1 hour by application of an NCAM-specific antibody. Cells were then fixed and processed for immunofluorescence analysis by visualization of NCAM with secondary Cy3-conjugated antibodies. The experiment was carried out three times with at least 15 cells analysed in each experiment. (B) Cortical neurons from E15.5 mice (NCAM$^{+/-}$) were transiently transfected with NCAM140 and endocytosis of NCAM was induced for 1 hour by application of an NCAM-specific antibody. Cells were then fixed and processed for immunofluorescence analysis using FITC-conjugated secondary antibodies for visualization of transferrin receptors (TfR) and Cy3-conjugated secondary antibodies to detect NCAM.

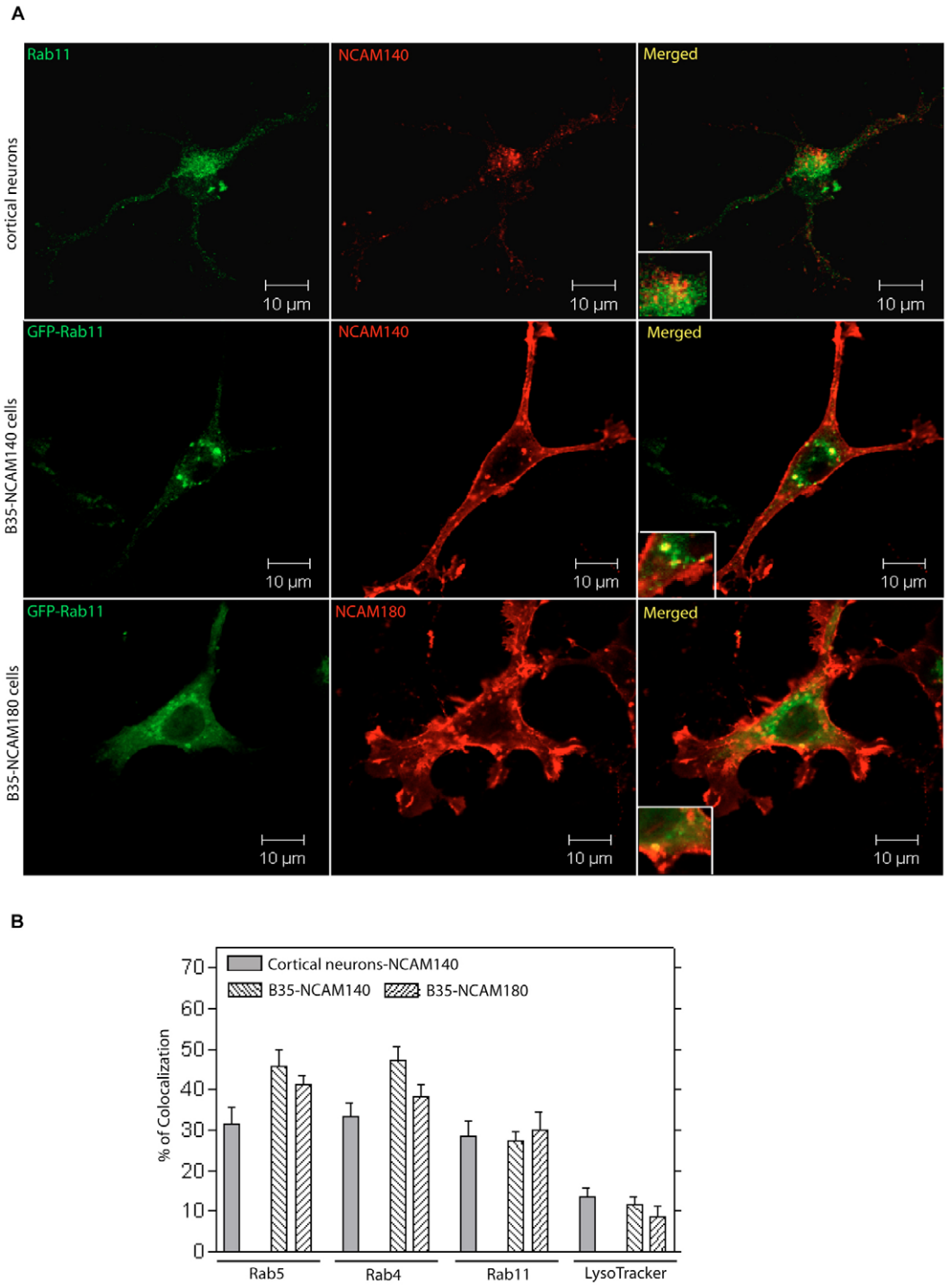


Fig. 5. NCAM is partially recycled to the plasma membrane by Rab11-positive endosomes. (A) Cortical neurons isolated from E15.5 mice ($NCAM^{+/-}$) were transiently transfected with NCAM140. B35 cells were transiently co-transfected with either NCAM140 or NCAM180 and GFP-Rab11. Endocytosis was induced for 1 hour by application of an NCAM-specific antibody. Cells were then fixed and processed for immunofluorescence analysis by visualization of NCAM with secondary Cy3-conjugated antibodies and in the case of cortical neurons with anti-Rab11-antibodies and secondary Alexa-Fluor-488-conjugated antibodies. (B) Quantification of colocalization of NCAM-positive vesicles with Rab5, Rab4, Rab11 or LysoTracker. Data are the mean ±s.e.m. of three independent experiments with at least 15 cells analysed in each experiment.

identical in both cell systems, i.e. in cortical neurons and B35 cells, all biochemical analyses were carried out using B35 cells.

To confirm the above result we investigated whether lysosomal or proteasomal degradation pathway inhibitors (chloroquine or lactacystin, respectively) affect the degradation

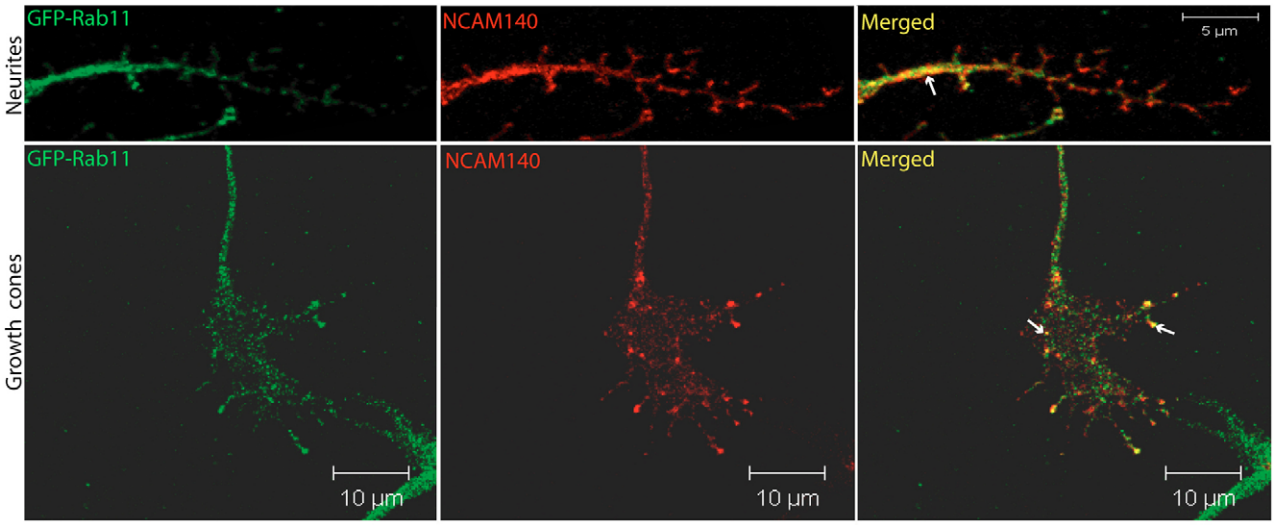


Fig. 6. NCAM140 is recycled in growth cones and neurites of cortical neurons. Cortical neurons isolated from E15.5 mice (NCAM$^{+/-}$) were transiently transfected with NCAM140. Endocytosis was induced for 1 hour by application of an NCAM-specific antibody. Cells were fixed and processed for immunofluorescence analysis. NCAM was visualized using secondary Cy3-conjugated antibodies, Rab11 was detected by Rab11-specific antibodies and Alexa-Fluor-488-conjugated secondary antibodies. Images were taken from growth cones or neurites of cortical neurons using a confocal laser scanning microscope.

of NCAM in B35 cells, cells were treated with either chloroquine or lactacystin before endocytosis induction. After cell lysis, expression of NCAM was detected by immunoblot analysis. As shown in Fig. 3C and supplementary material Fig. S2, neither increasing concentrations of chloroquine nor 10 µM lactacystin induced a significant difference in the expression levels of NCAM140 or NCAM180 compared with control cells. These results indicate that only a minority of internalized NCAM is degraded.

Consequently, we next investigated whether NCAM is recycled to the cell surface, like the related adhesion molecule L1 (Kamiguchi and Lemmon, 2000). The recycling of NCAM was first investigated by colocalization experiments with Rab4 and Rab11. Rab4 is a marker for the short recycling pathway, whereas Rab11 specifically marks the long recycling pathway (van der Sluis et al., 1992; Sheff et al., 1999; Sönnichsen et al., 2000). In primary cortical neurons considerable localization of NCAM140 in Rab4-positive vesicles (33.4±3.3%) and in Rab11-positive vesicles (28.6±3.8%) was observed (Fig. 4 and Fig. 5A). The recycling of NCAM140 was confirmed by colocalization with endogenous transferrin receptor (TfR), a well-known marker for recycling endosomes. We observed an almost complete overlap of NCAM140 and TfR (Fig. 4B).

To further analyse the intracellular trafficking in neurites and growth cones we investigated the colocalization of NCAM140 with Rab11 in these compartments. These analyses showed that NCAM140 was also present in Rab11-positive vesicles in these cell processes (Fig. 6). To verify the recycling mechanisms in B35 cells the colocalization of NCAM with Rab4 and Rab11 was also analysed in this cell system, and similar levels of colocalization were observed for Rab4-positive vesicles (47.1±3.6% of NCAM140 and 38.2±2.9% of NCAM180; Fig. 4A, Fig. 5B) and for Rab11-positive vesicles (27.2±2.2% for NCAM140; 30.2±4.4% for NCAM180; Fig. 5A,B).

For verification that NCAM140 is re-expressed at the cell surface after endocytosis, a direct recycling assay was performed. Cells were biotinylated using cleavable sulfo-NHS-SS-biotin and endocytosis was induced. After removal of biotin still present at the cell surface, recycling was allowed for different time periods. No re-expressed NCAM could be observed after 30 or 60 minutes of endocytosis without recycling (Fig. 7Ac,c′,d,d′,Ba,a′). When NCAM was allowed to recycle for 30 or 60 minutes a clear staining at the cell surface was detected (Fig. 7Ae-h′,Bb-d′). This result provides direct evidence for the re-expression of NCAM140 at the cell surface after its endocytosis in cortical neurons and B35 cells, and shows that NCAM trafficking inside the cell is a rather slow process. Taken together, these results show that the majority of the endocytosed NCAM is recycled to the cell surface.

NCAM is ubiquitylated in response to induction of endocytosis

Ubiquitylation has been shown to be involved in the regulation of endocytosis of several membrane molecules (for reviews, see Dupré et al., 2004; Haglund et al., 2003a). Furthermore, Foley et al. report that NCAM appears to become ubiquitylated in mouse brain during memory consolidation (Foley et al., 2000). Therefore, we investigated the possible link between ubiquitylation and the endocytosis of NCAM by using stably transfected B35 cells as a model system. After immunoprecipitation of NCAM and immunoblotting with antibody P4D1 that recognizes mono- and poly-ubiquitylated proteins, we observed low levels of ubiquitylated NCAM without induction of endocytosis. Ubiquitylation of NCAM140 or NCAM180, was drastically increased when endocytosis was induced by incubation with NCAM-specific antibodies (Fig. 8A,B). Absence of reactivity with the antibody FK1, which specifically recognizes poly-ubiquitylated proteins (Haglund et al., 2003b; Belouzard and Rouille, 2006) identified NCAM140

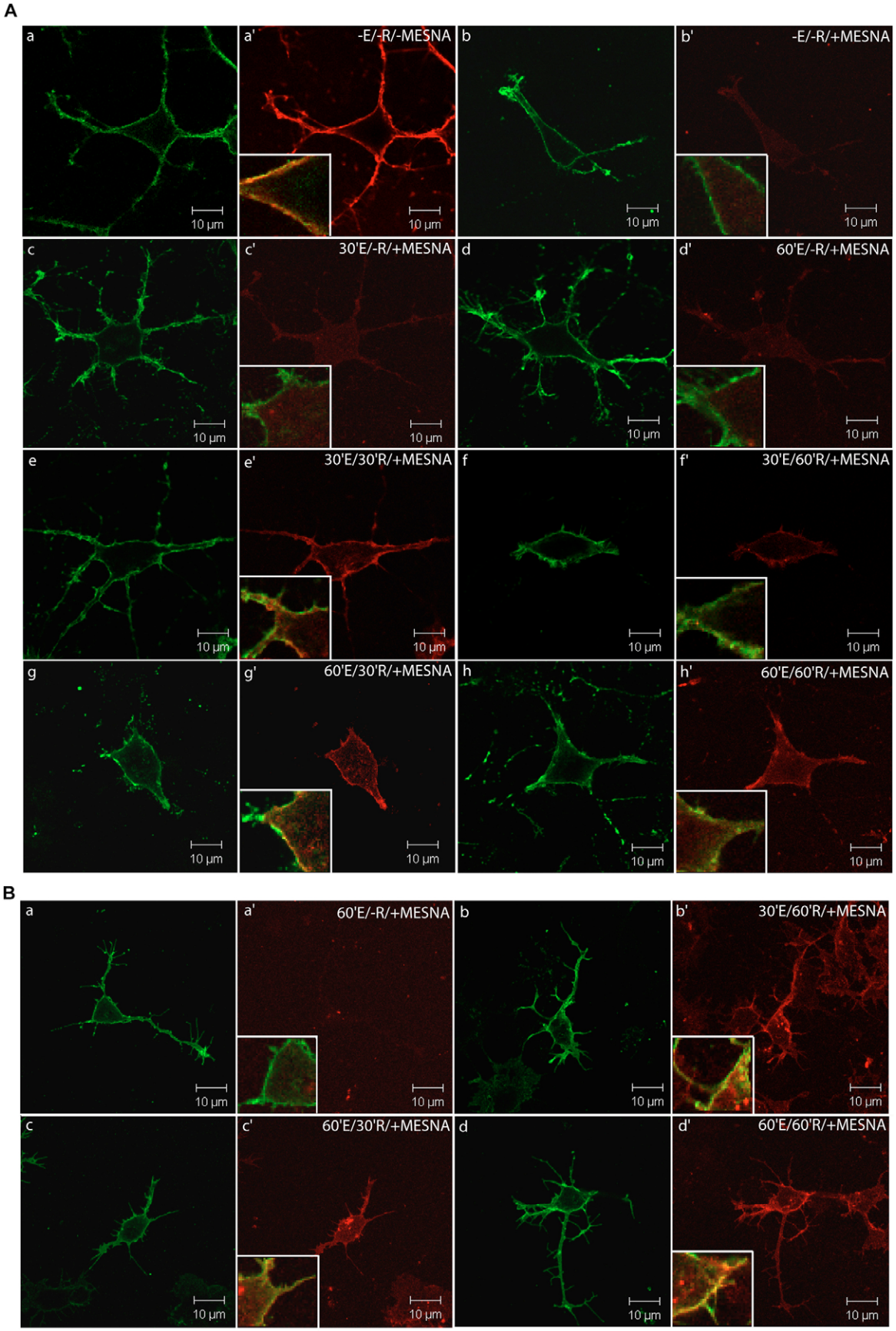


Fig. 7. See next page for legend.

as a mono-ubiquitylated protein (Fig. 8C). This result was supported by antibody removal and reprobing the same membrane with FK2 antibody, which – like the P4D1 antibody – recognizes both poly- and mono-ubiquitylated proteins. Here a band at the expected molecular mass was detected. Also, we always observed a small increase in the NCAM signal after antibody-induced endocytosis (Fig. 8, reprobed blots with 123C3), which was, however, significantly lower than the increase in ubiquitylation. We do not have an explanation for this phenomenon in the moment.

To determine whether ubiquitylation occurs at the plasma membrane, cell surface biotinylation of NCAM140-expressing B35 cells was carried out after induction of endocytosis of NCAM, which was then immunoprecipitated from cell lysates, eluted from the protein-G–sepharose beads and subjected to a second immunoprecipitation using streptavidin-agarose beads. Streptavidin-bound cell surface NCAM was separated by SDS-PAGE and analysed by immunoblot analysis. The strong signal representing ubiquitylated NCAM140 that was observed after antibody-induced endocytosis of NCAM140 provides a hint that ubiquitylation of NCAM140 might occur at the cell surface, consistent with the hypothesis that ubiquitylation of NCAM is an early step in endocytosis (Fig. 8D).

The function of ubiquitin in endocytosis is discussed controversially because it has been implicated in clathrin-dependent as well as clathrin-independent processes (Belouzard and Rouille, 2006; Sigismund et al., 2005; Chen and De Camilli, 2005). To investigate which of the pathways is relevant for NCAM, monodansylcadaverine (MDC) was used as a specific inhibitor of clathrin-mediated endocytosis. MDC is known to interfere with the polymerization of clathrin molecules leading to stabilization of the clathrin-cage assembly at the plasma membrane (Nandi et al., 1981; Phonphok and Rosenthal, 1991; Panicker et al., 2006). Furthermore, nystatin was used as an inhibitor of caveolae-dependent endocytosis. As shown in Fig. 9A,B endocytosis of NCAM140 in B35 cells was reduced to 41±2.9% by addition of MDC, to 65±4.3% in the presence of nystatin and application of both inhibitors resulted in an almost complete inhibition of endocytosis to 17±2.8% (all referred to control 100±4.6%). Similarly, NCAM180 endocytosis was reduced in the presence of MDC to 35±2.5%, to 75±6.6% with nystatin and to 11±1.9% in the presence of both inhibitors (also referrred to control 100±5.6%). In cortical neurons, similar results were obtained. Here, MDC inhibited endocytosis to 49±7.1% (NCAM140) or 52±6.4% (NCAM180), nystatin reduced levels to 81±6.3% (NCAM140) or 74±7.2% (NCAM180). The presence of both inhibitors together resulted in an inhibition of endocytosis to 31±3.8% (NCAM140) or 27±3.9% in the case of NCAM180 (Fig. 9C,D). These observations indicate that most of the endocytosis of NCAM140 and NCAM180 is mediated by clathrin-dependent and caveolae-dependent pathways in both cell systems, whereas other endocytic pathways appear to play minor roles at best. These results are in agreement with the only partial localization of NCAM in Rab5-positive or EEA-1-positive early endosomes after its endocytosis. Furthermore, additionally to its colocalization with EEA-1, we observed that endocytosed NCAM-eGFP was also partially localized in vesicles positive for caveolin-1, a marker protein for caveolae (supplementary material Fig. S1). These vesicles probably resemble caveosomes instead of early endosomes (Stenmark et al., 1994) affirming the inhibitor studies above. Induction of endocytosis for 30 and 60 minutes resulted in the same extent of colocalization (not shown).

Fig. 7. NCAM140 is re-expressed at the cell surface after endocytosis. (A) NCAM140 expressing B35 cells were biotinylated at 4°C and unreacted biotin was quenched. As positive control cells were directly fixed and stained for cell surface NCAM (a, anti-NCAM and anti-mouse Alexa-Fluor-488) and biotinylated cell surface proteins (a′, streptavidin-Cy3). Biotin was removed from the cell surface using MESNA (+MESNA). (b-h and b′-h′) Cell surface NCAM staining with anti-NCAM antibody and anti-mouse Alexa-Fluor-488-conjugated secondary antibodies (b-h) ; b′-h′ shows the same images stained with Cy3-conjugated streptavidin for the detection of cell surface biotinylated proteins without or with induction of NCAM endocytosis (–E, E) and without or with additonal time for recycling (–R, R) for 30 or 60 minutes (30′,60′); b,b′: without endocytosis and recycling (–E/–R); c,c′,d,d′: 30 or 60 minutes induction of NCAM endocytosis, no recycling time (30′E/–R or 60′E/–R, respectively); e,e′,f,f′: 30 minutes endocytosis and 30 or 60 minutes recycling time (30′E/30′R or 30′E/60′R, respectively); g,g′,h,h′: 60 minutes endocytosis and 30 or 60 minutes recycling time (60′E/30′R or 60′E/60′R, respectively). Overlays of the images are shown in insets. (B) Cortical neurons isolated from E15.5 mice ($NCAM^{+/-}$) were transiently transfected with NCAM140 cDNA. Cells were processed as described in A. In all images the cell surface biotin was removed using MESNA (+MESNA). The endocytosis was either induced for 30 or 60 minutes followed by 0, 30 or 60 minutes recycling time; a,a′: 60′E/–R; b,b′: 30′E/60′R; c,c′: 60′E/30′R; d,d′: 60′E/60′R.

Overexpression of ubiquitin regulates endocytosis of NCAM

The above results demonstrate a link between endocytosis and ubiquitylation of NCAM. Since ubiquitin has been suggested to be itself an internalization signal (Belouzard and Rouille, 2006; Sigismund et al., 2005; Chen and De Camilli, 2005) and the possibility that NCAM140 is ubiquitylated at the plasma membrane, ubiquitin may also represent an endocytosis motif for NCAM. Moreover, we did not observe an increase of NCAM140 or NCAM180 in lysosomes after overexpression of ubiquitin (not shown), indicating that it does not represent a lysosomal sorting signal for NCAM. To provide further evidence that ubiquitylation regulates internalization of NCAM, we overexpressed HA-tagged ubiquitin together with NCAM140 or NCAM180 and measured the endocytosis of NCAM. As shown in Fig. 10, endocytosis of NCAM140 was significantly increased in cells overexpressing ubiquitin (150±7.2%) compared with control cells (100±5.4%). The same effect, although less prominent as for NCAM140, was observed for NCAM180 in HA-ubiquitin overexpressing cells (130±8.2%) compared with the control (100±7.2%). To confirm that this effect is specific for NCAM, the same experminent was carried out by co-expressing HA-ubiquitin together with the NCAM-related adhesion molecule L1, which has also been shown to become inducibly endocytosed (Kamiguchi and Lemmon, 1998; Schmid et al., 2000). For L1, no difference in the rate of endocytosis was observed in the presence or absence of HA-ubiquitin (Fig. 10B). These results support the view that ubiquitylation acts as an internalization signal for cell surface NCAM.

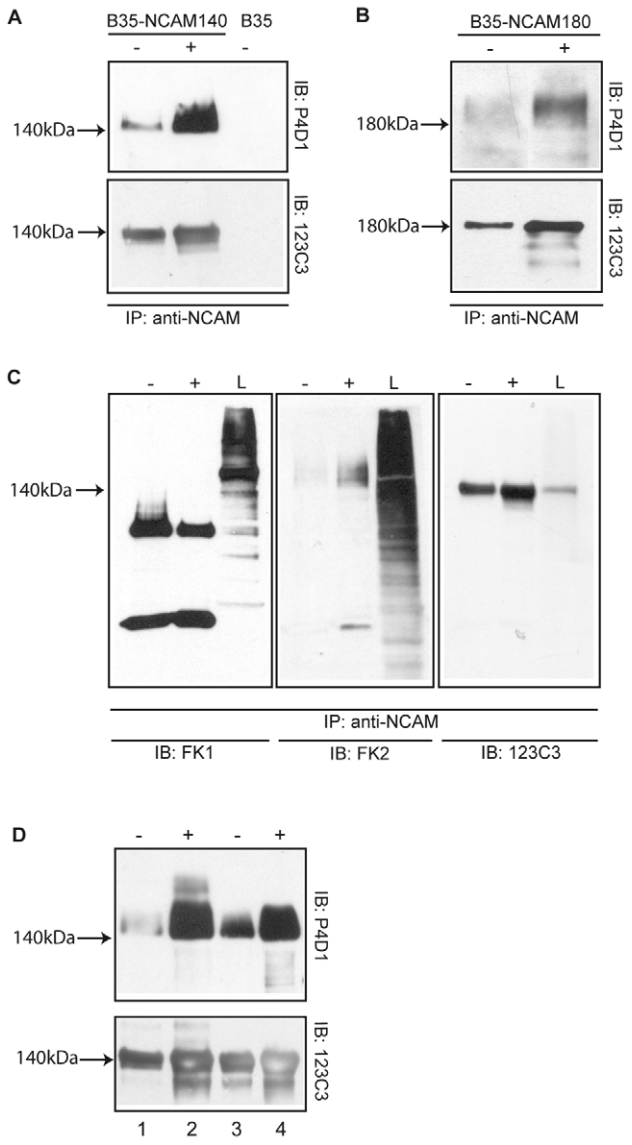


Fig. 8. NCAM is mono-ubiquitylated at the plasma membrane. (A,B) After either no induction (–) or induction of NCAM endocytosis for 60 minutes (+) in B35, NCAM140-expressing (A) or NCAM180-expressing (B) B35 cells, lysates were subjected to immunoprecipitation with an NCAM-specific antibody. Immunoblot analysis was carried out using an antibody specifically recognizing ubiquitin (P4D1, top). After antibody removal the blot was reprobed with an NCAM-specific antibody (123C3) as a control (bottom). (C) Endocytosis of NCAM140 was induced (+) for 60 minutes or not induced (–) in NCAM140-expressing B35 cells. Cell lysates were immunoprecipitated with an NCAM-specific antibody. Immunoblot analysis was carried out using an antibody recognizing specifically poly-ubiquitylated proteins (FK1). After antibody removal the blot was reprobed with an antibody recognizing mono- and poly-ubiquitylated proteins (FK2). After another antibody removal an incubation with an NCAM-specific antibody (123C3) was carried out as control. To control the functionality of the antibodies whole-cell lysates (L) were applied to the gel. (D) After no induction (–) or induction of NCAM endocytosis for 60 minutes (+) in NCAM140-expressing B35 cells, cell surface proteins were biotinylated at 4°C (lanes 1 and 2). After stopping the reaction, lysates were immunoprecipitated with an NCAM-specific antibody. Immunoprecipitated NCAM was eluted from the sepharose beads and a second precipitation was carried out using streptavidin-agarose beads to isolate cell surface NCAM proteins. In lanes 3 and 4, control immunoprecipitations were are shown with total NCAM [lanes 1 and 3 without (–) and lane 2 and 4 with (+) induction of endocytosis]. Immunoblot analysis was performed using an antibody specifically recognizing ubiquitin (P4D1, top). After antibody removal the blot was reprobed with NCAM-specific antibodies (123C3) as a control (bottom).

Discussion

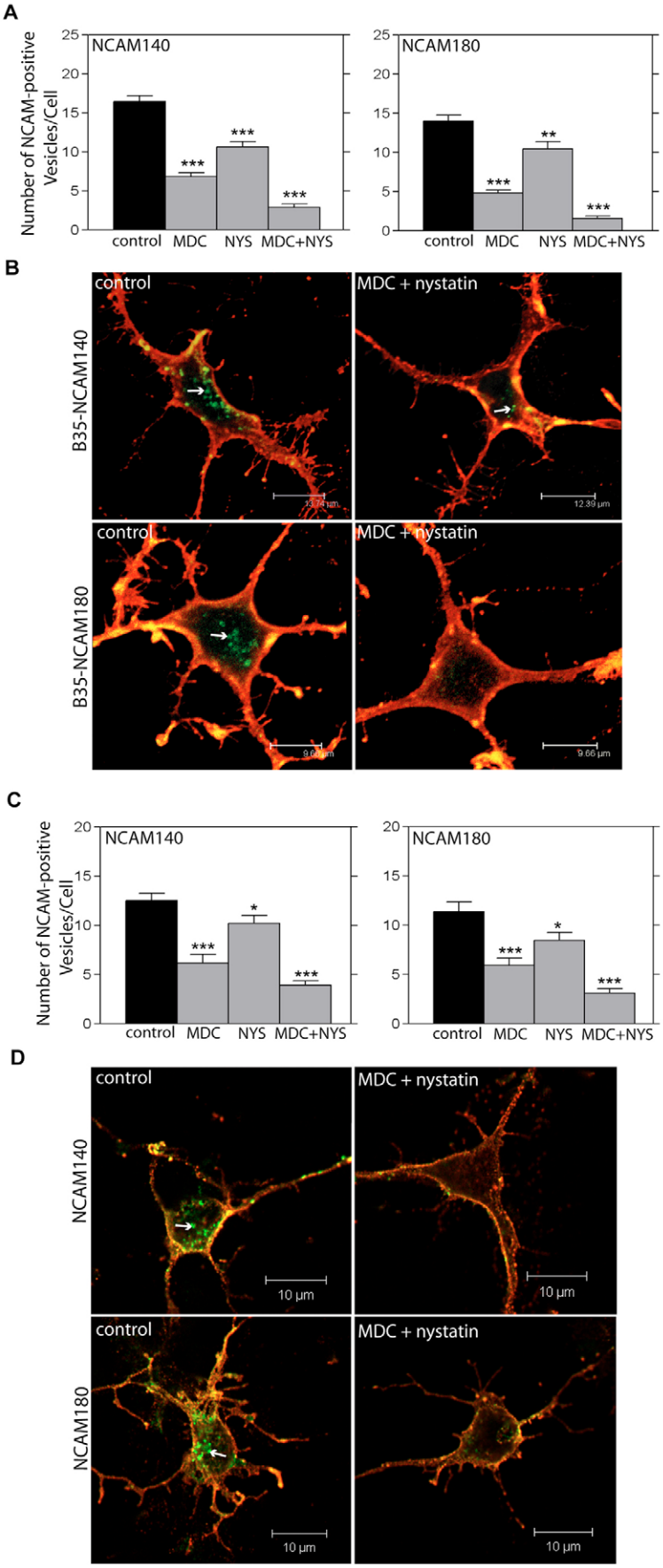
We show here that the two transmembrane NCAM isoforms NCAM140 and NCAM180 can be endocytosed in primary neurons and in neuroblastoma cells. Most of the endocytosed NCAM is recycled to the cell surface while only a minor fraction is lysosomally degraded. We furthermore demonstrate that induction of endocytosis strongly enhances mono-ubiquitylated NCAM at the cell surface thus providing evidence that this modification is involved in the regulation of endocytosis of NCAM.

Endocytosis and recycling of NCAM in neurons

In astrocytes, NCAM140 and NCAM180 have been shown to be internalized after induction of endocytosis of NCAM with NCAM-specific antibodies via the clathrin-dependent pathway (Minana et al., 2001). However, NCAM has been shown to be localized in lipid rafts and non-lipid raft fractions of neurons. Concomitant signaling from both fractions via raft-associated kinases and the non-lipid-raft-associated FGF receptor has been proposed to be essential for neuritogenesis (Niethammer et al., 2002). Our finding that NCAM is endocytosed in neurons using both, clathrin- and caveolae-dependent pathways indicates a functional role of its internalization from both membrane fractions in neuritogenesis.

Other cell adhesion molecules, such as the NCAM-related molecule L1, have also been shown to be endocytosed after induction with specific antibodies (Kamiguchi and Lemmon, 1998; Schmid et al., 2000). Endocytosis of L1 is required for activation of a MAP kinase cascade that regulates neurite outgrowth on an L1 substrate (Schmid et al., 2000). Like L1, NCAM is known to activate several signal transduction pathways, including also the MAP kinase cascade (Povlsen et al., 2003; Walmod et al., 2004). Therefore, it is possible that endocytosis of both membrane proteins is linked to complex intracellular signaling events. However, whereas endocytosis of L1 appears to be upstream of MAP kinase activation (Schmid et al., 2000), our inhibition studies using specific MAP kinase inhibitors suggest that in the case of NCAM, kinase activation is the upstream event (T. Goschzik, unpublished).

Furthermore, we demonstrate that the major part of the internalized NCAM is recycled to the plasma membrane in somata, neurites and growth cones. That NCAM is recycled rather than degraded after endocytosis is in accordance with pulse-chase experiments showing a long half-life of NCAM in different cell systems (Nybroe et al., 1989; Park et al., 1997) (our unpublished observation). Endocytosis and recycling of NCAM might be important for rapid removal of NCAM from the cell surface and/or rapid re-insertion into the plasma membrane, processes that might contribute to cell migration, growth cone motility and/or synapse plasticity.



A role of recycling in such processes has been shown for L1. Kamiguchi and Lemmon have observed that L1 is endocytosed in the central domain of the growth cone (Kamiguchi and Lemmon, 2000). After its sorting to endosomes, L1 is then recycled to the leading edge of growth cones, implicating it in growth cone motility. In addition, recycling of L1 appears also to provide a short-term modulation of presynaptic structures after LTP induction (Itoh et al., 2005).

Ubiquitin as an internalization signal for NCAM?

During the last years it became obvious that ubiquitylation is not an exclusive signal for proteasomal degradation, but that it has many more functions, such as regulating endocytosis or lysosomal sorting and Golgi trafficking (Dupré et al., 2004; Haglund et al., 2003a; Umebayashi, 2003). We demonstrate here that antibody-induced endocytosis of NCAM coincides with a most remarkable, strong increase in mono-ubiquitylation of NCAM and provides a hint that this ubiquitylation takes place at the cell surface. Furthermore, overexpression of ubiquitin increases the endocytosis rate of NCAM. Together, these results indicate that ubiquitin regulates initial rather than later steps of endocytosis and may even be an endocytosis signal for NCAM. These results are consistent with previous reports demonstrating a role of ubiquitylation in endocytosis of other molecules, such as the EGF receptor and the leptin receptor OB-Ra (Dupré et al., 2004; Haglund et al., 2003a; Belouzard and Rouille, 2006).

Several reports support the view that ubiquitylation might regulate either lipid-raft-dependent or clathrin-dependent endocytosis pathways (Chen and De Camilli, 2005; Sigismund et al., 2005; Belouzard and Rouille, 2006; Barriere et al., 2006). We show here that NCAM can be endocytosed by the clathrin-dependent, but also

Fig. 9. NCAM is internalized by clathrin-dependent and caveolae-dependent pathways. (A) NCAM140 or NCAM180-expressing B35 cells were preincubated with MDC (300 µM, 10 minutes), nystatin (NYS, 50 µg/ml, 1 hour) or both inhibitors together (MDC 10 min, NYS 1 hour). Endocytosis was induced for either 30 (NCAM140) or 60 minutes (NCAM180). Cell surface NCAM was detected using Cy3-conjugated secondary antibodies and internalized NCAM using Cy2-conjugated secondary antibodies. Data are the mean ±s.e.m. of three independent experiments with at least 15 cells analysed in each experiment. *$P<0.05$, **$P<0.005$, ***$P<0.001$. (B) Representative images of endocytosed NCAM140 or NCAM180 in B35 cells in the presence or absence of MDC and nystatin. (C) Cortical neurons isolated from E15.5 mice ($NCAM^{+/-}$) were transiently transfected with NCAM140 or NCAM180 cDNA. Cells were treated with MDC and nystatin as described in A and endocytosis was induced for 30 minutes. Cell surface and internalized NCAM was detected as described in A. Data are the mean ±s.e.m. of three independent experiments with at least 15 cells analysed in each experiment. *$P<0.05$, **$P<0.005$, ***$P<0.001$. (D) Representative images of endocytosed NCAM140 or NCAM180 in cortical neurons in the presence or absence of MDC and nystatin.

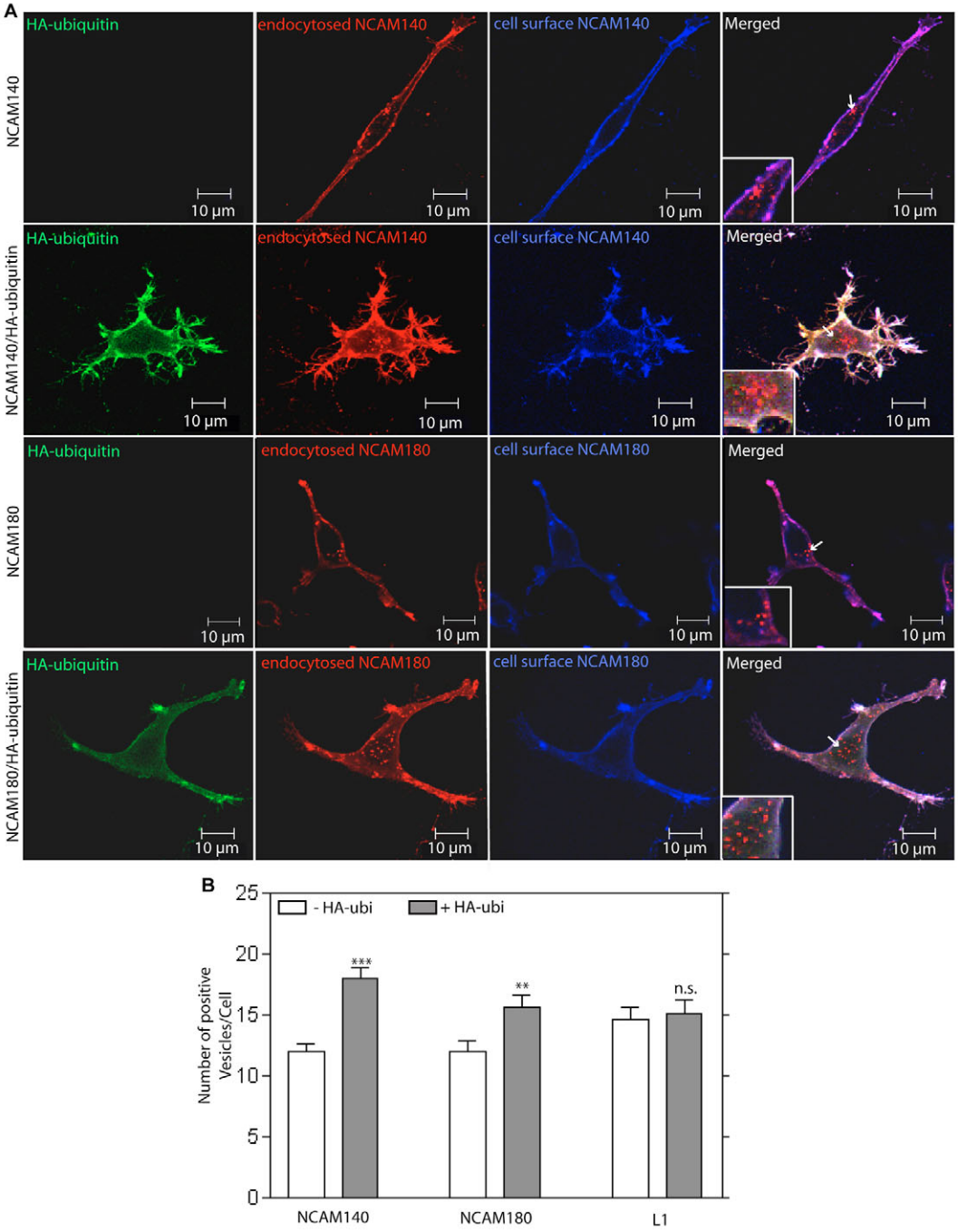


Fig. 10. Ubiquitin represents an internalization signal for NCAM. (A) Representative images of endocytosed NCAM140 or NCAM180 in the presence or absence of HA-ubiquitin (HA-ubi). B35 cells were co-transfected with HA-ubiquitin and NCAM140 or NCAM180 (second and fourth rows) or with NCAM140 or NCAM180 alone (first and third rows). Endocytosis of NCAM was induced by NCAM-specific antibodies for 60 minutes. HA-ubiquitin was visualized with FITC-conjugated secondary antibodies for identification of HA-ubiquitin-expressing cells, endocytosed NCAM with Cy3-conjugated antibodies and cell surface NCAM using Alexa-Fluor-633-conjugated secondary antibodies. (B) Quantification of endocytosed NCAM or L1 in the presence or absence of HA-ubiquitin. NCAM- or L1-positive vesicles were counted from at least three independent experiments with approximately 15 cells analysed in each experiment. Data are the mean ± s.e.m., ***P<0.0001, **P<0.01, n.s.: not significant.

the caveolae-dependent pathway. However, our analysis does not permit to conclude whether one or both pathways are regulated by ubiquitylation. Further studies will clarify this process in more detail and will also focus on the identification of the responsible ubiquitin ligase that directly binds to NCAM. Moreover, we do not yet know to which lysines in the C-terminal tail of NCAM ubiquitin is attached. Future studies will also focus on identifying which of the 14 lysines of

NCAM140 or 27 lysines of NCAM180 are responsible for their ubiquitylation.

The only known destabilization motif in the cytoplasmic tail of NCAM is the PEST sequence, which is rich in proline, glutamic acid, serine and threonine residues. It has been shown for the NCAM-related molecule ApCAM in *Aplysia californica* that phosphorylation by a MAP kinase within the PEST sequence precedes its endocytosis (Bailey et al., 1997). Analogous to ApCAM, we found that exchange of a MAP kinase consensus motif within the PEST sequence of NCAM140 or NCAM180 alters the endocytosis rate (T. Goschzik, H.C., B.S. and S.D., unpublished). For several proteins it has been demonstrated that phosphorylation precedes a modification by ubiquitin (Ewan et al., 2006; Hicke et al., 1998; Kumar et al., 2004). On the basis of these observations we propose a model in which NCAM becomes phosphorylated in response to induction of endocytosis using specific antibody. This phosphorylation might represent the signal for ubiquitylation, probably by enabling the binding of an E3 ubiquitin ligase, and consecutive endocytosis of NCAM. Inside the cell ubiquitin might be cleaved and most NCAM then be sorted to recycling vesicles (expressing Rab4 or Rab11), whereas only a small portion of NCAM is degraded in the lysosome (Fig. 11).

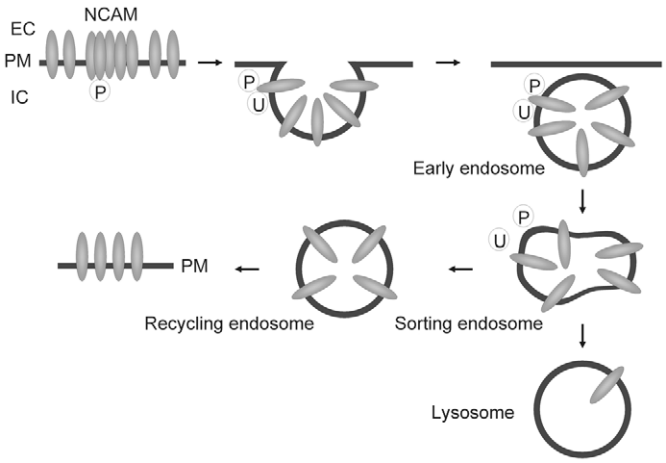


Fig. 11. Proposed model of NCAM endocytosis and trafficking. Cell surface NCAM becomes phosphorylated in response to antibody-induced clustering. This allows a subsequent ubiquitylation of NCAM leading to its internalization. Ubiquitin is cleaved after NCAM is removed from the cell surface. A small part of NCAM is degraded in lysosomes whereas the major part is recycled to the cell surface. PM, plasma membrane; EC, extracellular; IC, intracellular; P, phosphorylation; U, ubiquitylation.

What might all this mean for the in vivo function?

In the experiments described here NCAM endocytosis was induced by crosslinking NCAM with a specific antibody. There have been many discussions whether binding of an antibody to a specific NCAM epitope may mimic NCAM-NCAM interactions (Soroka et al., 2003). Since in vitro studies have shown that at least very similar effects on signal transduction or neurite outgrowth can be observed by using either recombinant NCAM constructs or mono- or bivalent NCAM antibodies (Schmid et al., 1999), it can be concluded that antibody-induced endocytosis of NCAM and its consequences described here are of physiological significance.

During development of the nervous system neurons translocate their cell soma over large distances (Marin-Padilla, 1998). Therefore, cell adhesion and detachment have to be tightly regulated. It has been shown that NCAM140 is mainly expressed in immature, migrating neurons (Kramer et al., 1997) whereas NCAM180 is expressed in synapses and has been implicated in their plasticity (Dityatev et al., 2000). This is in agreement with our observation of a higher endocytosis rate of NCAM140 in undifferentiated immature cells and a decrease of endocytosis with maturation. By contrast, NCAM180 exhibits the highest endocytosis rate later – in more differentiated neurons, indicating a possible role of its endocytosis in mature cells. It is tempting to speculate that the endocytosis of NCAM140 is of functional significance for the migration of immature neurons, whereas the endocytosis of NCAM180 is involved in regulation of synaptic plasticity.

One possibility how NCAM acts is the downregulation of NCAM-associated molecules. Since we did not observe a downregulation of surface NCAM (not shown) but the internalization of only a fraction, downregulation of NCAM itself is probably not the primary aim. Instead endocytosis might also serve to remove NCAM interacting molecules from the cell surface using NCAM as a carrier for internalization.

One such candidiate could be a member of the integrin family. Integrins play a major role in cell migration and endocytosis, and recycling of integrins has been shown to be crucial for motility of cells (Caswell and Norman, 2006). NCAM140 has been shown to regulate integrin-dependent cell migration (Diestel et al., 2005). Cell motility depends on optimal adhesiveness, whereby too much or too little adhesion reduces the process (Palecek et al., 1997). Panicker et al. demonstrated that L1 and β1-integrins are associated at the plasma membrane and that induction of L1 endocytosis leads to enhanced β1-integrin endocytosis (Panicker et al., 2006). Such a mechanism might also be active for NCAM140.

An important role of endocytosis in signaling has been described, for example, for Notch, Wnt, and the EGF and β-adrenergic receptors (Le Borgne, 2006; Blitzer and Nusse, 2006; Luttrell and Lefkowitz, 2002; Vieira et al., 1996). Since NCAM is known to activate several signal transduction pathways (Povlsen et al., 2003; Walmod et al., 2004), another possibility of the physiological role of its regulated endocytosis could be the activation of signaling events that include the above factors. Therefore, our finding that NCAM can be endocytosed in a regulated and ubiquitin-dependent manner, and is recycled to the cell surface provides new perspectives for future in vivo studies which will give new insights into its biological functions.

Materials and Methods

Antibodies

Hybridoma cells producing monoclonal antibody 123C3 against human NCAM were provided by R. Michalides (Amsterdam, The Netherlands), Antibodies: P4D1, Cell Signaling Technology (Beverley, MA); FK1 and FK2, Biomol (Pennsylvania, USA); EEA-1 and caveolin-1, BD Biosciences (Erembodegem, Belgium); HA, Roche Diagnostics (Mannheim, Germany); actin, Chemicon (Temecula, CA). Anti-Rab11- and anti-transferrin receptor antibodies were a gift from P. Pierre (CIML, Marseille, France), anti-L1 antibody (UJ 127.11) was provided by P. Altevogt (Ebeling et al., 1996). Secondary antibodies conjugated to Cy3, Cy2 or horseradish peroxidase were from Dianova (Hamburg, Germany), Alexa-Fluor-633- or Alexa-Fluor-488-conjugated

secondary antibodies, LysoTracker™ were from Molecular Probes (Eugene, OR) and Cy3-conjugated streptavidin from Immunotech (Marseille, France).

Plasmids

Expression plasmids for human NCAM140 or NCAM180 in B35 cells have already been described (Diestel et al., 2004; Diestel et al., 2005). PCDNA3-L1 cDNA was provided by P. Maness (Chapel Hill, NC, USA). For generation of NCAM-eGFP constructs the cDNA of either NCAM140 or NCAM180 was subcloned in frame into the *Bam*HI site of the pEGFP-N1 plasmid (Clontech/Takara, France). cDNA constructs of GFP-Rab4, GFP-Rab5 and GFP-Rab11 were a gift of G. Sirokmany (Semmelweis University, Budapest, Hungary) (Hunyady et al., 2002). For expression of human NCAM140, human NCAM180, GFP-Rab4 and GFP-Rab5 in cortical neurons the cDNAs were subcloned into the pCX-MCS2 plasmid (gift of Xavier Morin, IBDML, Marseille). The pCDNA3-HA-ubiquitin plasmid was provided by J. Höhfeld (Bonn, Germany).

Cell culture and expression of NCAM proteins

B35 neuroblastoma cells, B35-NCAM180 and B35-NCAM140 cells have been described earlier (Schubert et al., 1974; Diestel et al., 2004; Diestel et al., 2005). Cells were maintained in DMEM with 10% fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin and plated on 0.01% poly-L-lysine (pLL)-coated plastic dishes. For some experiments NCAM or L1 constructs were transiently co-transfected with GFP-Rab4, GFP-Rab5, GFP-Rab11 (ratio 1:1) or HA-ubiquitin (ratio 1:3) using Lipofectamine Plus™ reagent (Invitrogen, Karlsruhe, Germany).

NCAM-deficient mice have been described previously (Cremer et al., 1994). Primary cortical neurons from mice embryonic day 15.5 (E15.5) were prepared as described (David et al., 2007). Cells were then transiently transfected using the Mouse Neuron Nucleofector Kit (amaxa biosystems, Köln, Germany) slightly modified according to the manufacturer's instructions using program Q-05 and plated at a density of 300,000 cells per 24-well plate on pLL-coated coverslips. Cells were cultured for the indicated time periods in Neurobasal medium containing B27 supplement (Invitrogen), 2 mM L-glutamine, 50 U/ml penicillin and 50 μg/ml streptomycin.

Endocytosis and colocalization studies

Determination of endocytosis was carried out as described by Long et al. (Long et al., 2001). B35 cells were differentiated in OPTIMEM® containing 1 mM dbcAMP for 24 hours. To induce endocytosis, cells were incubated 30 or 60 minutes at 37°C with 25 μg NCAM-specific 123C3 antibody per ml in OPTIMEM. For L1 endocytosis, cells were incubated with 25 μg UJ 127.11 antibody per ml in OPTIMEM® (30 minutes, 37°C). Primary cortical neurons of NCAM-deficient mice or heterozygous littermates which expressed NCAM140 and in some cases additionally GFP-Rab constructs were incubated for 30 or 60 minutes with 25 μg 123C3 antibody per ml Neurobasal medium (37°C). If indicated, cells were pretreated with monodansylcadaverine (MDC; 300 μM, 10 minutes, Sigma) or nystatin (50 μg/ml, 1 hour, NYS, Sigma). Cells were rinsed with 4°C-cold medium, fixed for 30 minutes at 4°C with 8% paraformaldehyde (PFA) in PBS and were incubated 30 minutes with Cy3-conjugated anti-mouse antibodies (1:500 in 5% horse serum, in PBS blocking solution) for the detection of cell surface associated NCAM. After washing, incubation with rabbit anti-mouse immunoglobulins (0.25 mg/ml in blocking solution) followed to saturate all binding sites of the first antibody. Then cells were postfixed 5 minutes with 8% PFA at 4°C and permeabilized with 0.5% Triton X-100 (20 minutes). Internalized NCAM was visualized using Alexa-Fluor-488- or Cy2-conjugated anti-mouse antibodies (1:150). Finally cells were embedded in Permafluor (Beckman-Coulter, Marseille, France). Microscopy was carried out using a Zeiss LSM510 MetaUV confocal microscope (Oberkochen, Germany) and quantification was performed by counting NCAM-positive, green vesicles inside the cell.

For colocalization of endocytosed NCAM140 or NCAM180 with Rab4, Rab5, Rab11 or LysoTracker endocytosis was induced for 1 hour as described before. LysoTracker (200 nM) was added additionally to medium during endocytosis induction. Cells were then fixed with 8% PFA and permeabilized with 0.5% Triton X-100 (20 minutes). NCAM was visualized using either anti-mouse Cy3-, anti-mouse Cy2- or anti-mouse Alexa-Fluor-633-conjugated secondary antibodies. HA-ubiquitin was detected by anti HA-antibodies and anti-rat FITC-conjugated secondary antibodies. For colocalization of NCAM-eGFP with LysoTracker cells were directly fixed and embedded. To investigate colocalization of NCAM-eGFP with either EEA-1 or caveolin-1, cells were fixed, permeabilized and EEA-1 or caveolin-1 were visualized with Cy3-conjugated secondary antibodies.

In cortical neurons the colocalization of NCAM140 with Rab11 or the TfR was carried out by application of Rab11- or TfR-specific antibodies followed by incubation with anti-mouse Cy3- (NCAM), anti-rabbit Alexa-Fluor-488 (Rab11) or anti-rat FITC-TfR-conjugated secondary antibodies.

Recycling assay

For recycling B35 cells-NCAM140 cells or cortical neurons were treated as above described for endocytosis experiments. Cells were biotinylated with 0.2 mg/ml sulfo-NHS-SS-biotin in PBS (4°C, 20 minutes, Perbio, Brebrières, France). Unbound biotin was quenched by washing the cells three times with 50 mM glycine in TBS (4°C). As a positive control one coverslip was directly fixed (8% PFA, 4°C, 30 minutes) and stained with 123C3 and anti-mouse Alexa-Fluor-488 to stain cell surface NCAM and streptavidin-Cy3 (1:1000) to stain biotinylated cell surface proteins. Endocytosis of NCAM was induced for 30 or 60 minutes. Cells were then washed twice with TBS (4°C) and the remaining biotin at the cell surface was stripped with 150 mM MESNA (sodium 2-mercapto-ethanesulfonate) in 100 mM Tris-HCl (pH 8.6) containing 100 mM NaCl and 2.5 mM $CaCl_2$. Then cells were washed again (TBS, 4°C, three times) and were incubated for the indicated time periods at 37°C to allow recycling of internalized proteins. After fixation with 8% PFA cells were stained with anti-mouse Alexa-Fluor-488 to stain cell surface NCAM. For detection of recycled NCAM cells were labeled with streptavidin-Cy3. Images were taken using an LSM510 confocal laser scanning microscope with the same laser intensity and detector gain in all samples. Only the pictures of the positive control were taken with lower laser intensity because the signal was much stronger than in all the other conditions.

Immunoprecipitation and cell surface biotinylation

For immunoprecipitation of NCAM from B35 cells, cells were plated on plastic dishes of 10-cm diameter. Cells were differentiated, endocytosis was induced as described above and cells were lysed using RIPA buffer. Protein concentration was determined using the Biorad D_c protein assay (München, Germany). Supernatants were subjected to immunoprecipitation with 123C3 antibody and protein G sepharose (Amersham, Freiburg, Germany) overnight at 4°C. Immunoprecipitated NCAM was eluted by heating in sample buffer (5 minutes, 95°C).

For determination of cell surface NCAM, cells were treated as described before. Cell surface biotinylation was carried out 1 hour after endocytosis induction at 4°C for 30 minutes with sulfo-NHS-LC-biotin (0.2 mg/ml PBS, Molecular Biosciences, Boulder, CO). Biotinylation was stopped with TBS and cells were lysed. After immunoprecipitation of NCAM, the antibody-protein complexes were eluted from the protein-G–sepharose beads with elution buffer (10 mM diethylamine, 1 mM EDTA, 1 mM EGTA, 100 mM NaCl, pH 11.5), neutralized with 1 M Tris-HCl, pH 6.8 and a second immunoprecipitation was carried out using streptavidin-agarose beads, thereby precipitating biotinylated proteins (2 hours, 4°C). Precipitated proteins were eluted from streptavidin-agarose beads by heating in sample buffer. Supernatants were applied onto an SDS gel and subjected to immunoblot analysis.

Immunoblot analysis of NCAM and ubiquitin

Cells were pretreated 4 hours before lysis with different concentrations of chloroquine (Sigma, Taufkirchen, Germany), lactacystin (10 μM, Sigma) or control treated. Proteins were subjected to SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked in blocking solution (2 hours, 5% milk powder in TBS/0.1% Tween-20) prior to incubation with the first antibody in 5% BSA in TBS/0.1%Tween-20 (overnight, 4°C). Membranes were washed and incubated with peroxidase-conjugated secondary antibodies for 1 hour in blocking solution. Proteins were detected by enhanced chemiluminescence (Perbio Science, Bonn, Germany).

Quantification and statistical analysis

Endocytosis was quantified by counting NCAM-positive vesicles inside the cell. Quantification of colocalization was performed by counting NCAM-positive vesicles and vesicles containing both, NCAM and the specific marker (indicated in yellow in figures). These two values were set in relation to each other and the percentage of colocalization was calculated. Vesicle couting was done without observer bias by at least two different persons. Data were calculated from at least three different experiments with approximately 15 cells analysed in each experiment. Statistical analysis was carried out using the unpaired *t*-test and data are presented as the mean ±s.e.m. Densitometric analysis of the immunoblot experiments was carried out using Image J software.

This work was supported by grants from the European Community Network of Excellence 'NeuroNE', the Fondation de France and the Association Française Contre le Myopathies to H.C.

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