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EphrinA/EphA-induced ectodomain shedding of neural cell adhesion molecule regulates growth cone repulsion through ADAM10 metalloprotease

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Abstract

EphrinA/EphA-dependent axon repulsion is crucial for synaptic targeting in developing neurons but downstream molecular mechanisms remain obscure. Here, it is shown that ephrinA5/EphA3 triggers proteolysis of the neural cell adhesion molecule (NCAM) by the metalloprotease a disintegrin and metalloprotease (ADAM)10 to promote growth cone collapse in neurons from mouse neocortex. EphrinA5 induced ADAM10 activity to promote ectodomain shedding of polysialic acid-NCAM in cortical neuron cultures, releasing a ~ 250 kDa soluble fragment consisting of most of its extracellular region. NCAM shedding was dependent on ADAM10 and EphA3 kinase activity as shown in HEK293T cells transfected with dominant negative ADAM10 and kinase-inactive EphA3 (K653R) mutants. Purified ADAM10 cleaved NCAM at a sequence within the E-F loop of the second fibronectin type III domain (Leu^{671}-Lys^{672}/Ser^{673}-Leu^{674}) identified by mass spectrometry. Mutations of NCAM within the ADAM10 cleavage sequence prevented EphA3-induced shedding of NCAM in HEK293T cells. EphrinA5-induced growth cone collapse was dependent on ADAM10 activity, was inhibited in cortical cultures from NCAM null mice, and was rescued by WT but not ADAM10 cleavage site mutants of NCAM. Regulated proteolysis of NCAM through the ephrin5/EphA3/ADAM10 mechanism likely impacts synapse development, and may lead to excess NCAM shedding when disrupted, as implicated in neurodevelopmental disorders such as schizophrenia.

Keywords

ADAM10; ectodomain shedding; ephrinA; growth cone collapse; NCAM

The neural cell adhesion molecule (NCAM) is a potent promoter of axon growth and synaptic plasticity (Brennaman and Maness 2010; Bukalo and Dityatev 2012), and regulates GABAergic interneuron arborization and connectivity (Pillai-Nair et al. 2005; Brennaman

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Author contributions

LHB was involved in experiment design, data acquisition and analysis/interpretation, wrote and revised the manuscript. MLM provided the reagents, designed the peptide experiment, and provided advice on ADAM10. PFM designed the experiment and revised the manuscript.

and Maness 2008; Brennaman et al. 2013). The three principal isoforms of NCAM (NCAM120, 140, and 180) arise from alternative mRNA splicing, and have overlapping and distinct roles in neural development. NCAM180 differs from NCAM140 by an insert in the cytoplasmic domain, while NCAM120 is glycophosphatidylinositol-linked. NCAM140 is a strong promoter of axon growth, NCAM180 modulates synaptic plasticity, and NCAM120 functions in glia (Maness and Schachner 2007). Mutation, altered expression, and impaired modification of NCAM have been linked to schizophrenia (Barbeau et al. 1995; Lewis et al. 2003; Atz et al. 2007; Sullivan et al. 2007; Gray et al. 2010; Ayalew et al. 2012; Hill et al. 2012; Varea et al. 2012). Notably, a soluble cleavage fragment consisting of most of the extracellular region of NCAM (NCAM-EC) is over-expressed in affected brain regions in schizophrenia (Van Aelst and D'Souza-Schorey 1997; van Kammen et al. 1998; Vawter et al. 2001; Tanaka et al. 2007). NCAM-EC normally arises during maturation of the frontal cortex in mouse (Brennaman and Maness 2008) and human (Cox et al. 2009), peaking at adolescence, a critical developmental period associated with remodeling of neural networks (Uhlhaas et al. 2010). Interestingly, a transgenic mouse model of NCAM-EC over-expression in brain (Pillai-Nair et al. 2005) displays schizophrenia-related behaviors, including impaired working memory and long-term potentiation (Brennaman et al. 2011). NCAM-EC mice also exhibit diminished synaptic contacts and axonal arborization made by GABAergic interneurons in the prefrontal cortex (Brennaman and Maness 2008), suggesting that aberrant proteolytic cleavage of NCAM may perturb interneuron connectivity. Dysregulation of NCAM is also associated with other neuropsychiatric disorders (Lewis et al. 2003; Arai et al. 2004; Atz et al. 2007; Anney et al. 2010; Gray et al. 2010; Varea et al. 2012), in which impaired function of interneurons has been implicated (Rubenstein and Merzenich 2003; Dani et al. 2005; Lewis and Gonzalez-Burgos 2008).

NCAM was recently shown to interact with the receptor tyrosine kinase EphA3 to promote growth cone collapse, reduce arborization, and limit the number of synapses of cortical GABAergic interneurons (Brennaman et al. 2013). EphrinA/EphA3 signaling has a widespread effect on axon guidance among neuronal populations, repelling axons from inappropriate areas and constraining arborization (Davy and Soriano 2005; Chen et al. 2012; North et al. 2013). EphA3 has been shown to induce a disintegrin and metalloprotease (ADAM)10 to cleave ephrinAs from opposing cells for growth cone collapse (Hattori et al. 2000; Janes et al. 2005, 2009). Although pervanadate-induced NCAM cleavage was mediated by ADAM10 metalloprotease in transfected L-fibroblasts (Hinkle et al. 2006), the physiological mechanism of NCAM shedding has not been examined. Therefore, we investigated the potential involvement of ephrinA5/EphA3 and ADAM10 in inducing NCAM shedding during growth cone repulsion.

Here, we report that NCAM is proteolytically cleaved by ADAM10 in response to ephrinA5/EphA3 at a specific sequence in the second fibronectin III (FNIII) domain of the NCAM extracellular region. Furthermore, NCAM, ADAM10, and EphA3 co-localize in processes and soma of cortical neurons in culture, and mutation of the ADAM10 cleavage site in NCAM or inhibition of ADAM10 blocks ephrinA5-induced growth cone collapse. These results implicate ectodomain shedding of NCAM by ADAM10 in response to ephrinA5/

EphA3 as a key determinant in growth cone repulsion in cortical GABAergic and non-GABAergic neurons.

Experimental procedures

Mice

NCAM null mutant [from Lynn Landmesser, Case Western Reserve University (Cremer et al. 1994; Hata et al. 2007)] and WT (C57Bl/6) male and female mice were used for these studies. Embryonic day 0.5 (E0.5) was defined as the plug date and the day of birth as postnatal day 0 (P0). All animals were used according to the University of North Carolina Institutional Animal Care and Use Committee policies and in accordance with NIH guidelines.

Immunochemicals and reagents

Monoclonal antibodies were: anti-NCAM clone OB11 (Sigma, St. Louis, MO, USA) and polysialic acid (PSA) (MAB5324; EMD Millipore Corp., Billerica, MA, USA). Polyclonal antibodies were: EphA3 (C-19), NCAM (H300; Santa Cruz Biotechnology, Santa Cruz, CA, USA or AB5032; EMD Millipore), GABA (Sigma), and ADAM10 (422751, EMD Millipore; AB946, R&D Systems, Inc., Minneapolis, MN). AlexaFluor-488 conjugated anti-green fluorescent protein (GFP) (Life Technologies, Grand Island, NY, USA), anti-Fc, secondary antibodies and normal human, rabbit, goat and mouse IgG were from Jackson ImmunoResearch Laboratories (West Grove, PA). AlexaFluor-568 conjugated phalloidin (Life Technologies), recombinant mouse ADAM10 and ephrinA5-Fc (R&D Systems) were also used. A peptide comprising the prodomain of ADAM10 was from Biozyme Inc. (Apex, NC) (Moss et al. 2007). Endoneuraminidase-N (endo-N), which removes α-2,8 sialic acid chains (El Maarouf and Rutishauser 2003), was a gift of Urs Rutishauser (Memorial Sloan-Kettering Cancer Center).

Site-directed mutagenesis

Three mutations were made in the E-F loop of the second FNIII domain of mouse NCAM140 (VMLK to VGDK, LKSL to LGDL, and VMLKSL to VGDGDL) using the QuikChange XL Site-Directed Mutagenesis kit (Agilent Technologies, Santa Clara, CA, USA). The mutagenic primers were: VGDK 5′-GGCAGTGACCACGTCGGGGACAAGTCCCTAGACTGG-3′; LGDL 5′-GCAGTGACCACGTCATGCTCGGGGACCTAGACTGGAACGCCG-3′; VGDGDL 5′-CCGTCCGGCAGTGACCACGTCGGGGACGGGGACCTAGACTGGAACGCCGAGTACG-3′.

Cellular NCAM cleavage assay

Dissociated cortical neurons (E16.5) were plated onto poly-D-lysine-coated dishes (60 mm, 2.5–3 × 10^6 cells/dish) and maintained as described (Hinkle et al. 2006; Brennaman and Maness 2008). After 24 h, media were exchanged for OptiMemI following rinses with Hank's Balanced Salt Solution (137 mM NaCl, 5.4 mM KCl, 0.34 mM Na_2HPO_4, 0.4 mM KH_2PO_4), and in some experiments ADAM10 prodomain peptide (10 μM; Moss et al. 2007) or endo-N (20 U; El Maarouf and Rutishauser 2003) was added. EphrinA5-Fc or human

IgG (1.5 μg/mL) was pre-clustered as described (Janes et al. 2005) and added to cells for 10 or 30 min. Cells were lysed in radioimmunoprecipitation assay (RIPA) lysis buffer (20 mM Tris pH 7.0, 0.15 M NaCl, 5 mM EDTA, 1 mM EGTA, 1% NP-40, 1% deoxycholate, 0.1% sodium dodecyl sulfate, 200 μm Na_3VO_4, 10 mM NaF, 1X protease inhibitors; Sigma), and protein concentration determined by bicinchoninic acid (BCA; Thermo Fisher Scientific, Pittsburgh, PA, USA). Conditioned media were harvested, treated with protease inhibitors and concentrated on Millipore centrifugal concentrators according to the manufacturer's instructions (30 000 MW cutoff; ~ 60–100 fold concentration; Millipore).

Human embryonic kidney HEK293T cells (Dulbecco's modified Eagle's medium-H, 10% bovine calf serum) were transfected with plasmid (8–9 μg) in OptiMemI using Lipofectamine2000 (Life Technologies) for 12–16 h, after which media were removed and fresh OptiMemI was added. For double transfections, 4 μg of each plasmid was used per transfection. For triple transfections, 3 μg of each plasmid was used per transfection. Transfection efficiency was ~ 90%. After 10 min, conditioned media were harvested and concentrated using Millipore centrifugal concentrators, and cells were lysed in RIPA buffer as above.

Cell lysates (75 μg) or concentrated conditioned media (values were normalized to the sample with the lowest protein concentration and equivalent amounts loaded) were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and transferred to nitrocellulose. To examine NCAM cleavage fragments, western blots of lysates were probed with antibodies to a common determinant in the NCAM140 and NCAM180 cytoplasmic domains (clone OB11), while media were probed with antibodies to the extracellular domain (H300). Proteins were detected using enhanced chemiluminescence (Thermo Fisher Scientific) and exposed to film. Blots were scanned and analyzed for levels of NCAM-EC cleavage fragments and full length NCAM using the thresholding function of ImageJ software (National Institutes of Health, Bethesda, MD, USA). The pixel density value of NCAM-EC for each sample was divided by the pixel density value of full length NCAM for the same sample to yield the amount of NCAM-EC cleavage fragment relative to full length NCAM (n = 3 for cortical neuron samples, n = 5–10 for transfected HEK293T samples).

Cell surface biotinylation

Forty-eight hours after transfection, HEK293T cells were washed in ice-cold phosphate-buffered saline (PBS) and incubated in 0.5 mg/mL sulfo-NHS-SS-biotin (Thermo Fisher Scientific) in PBS for 15 min at 4°C to label cell-surface proteins. The biotinylation reaction was neutralized with ice-cold 25 mM Tris in PBS. Cells were rinsed in ice-cold PBS and lysed in complete RIPA buffer. Biotinylated proteins (1 mg) were bound to Neutravidin UltraLink resin (Thermo Fisher Scientific) for 2 h at 4°C before separation by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and western blotting using antibodies to the NCAM cytoplasmic region (clone OB11), EphA3, or actin.

Peptide cleavage

The peptide, acetyl-RLPSGSDHVMLKSLDWNAEY-amide, was synthesized by the UNC Microprotein Sequencing and Peptide Synthesis Facility (Dr David Klapper, director). For

peptide cleavage, peptide (50 μM) was incubated with 1 μg recombinant mouse ADAM10 (rmADAM10) in 10 mM Tris pH 7.5 at 37°C for 4 h. Peptides were also incubated in the absence of rmADAM10 as a control. The reactions were stopped by adding EDTA to a final concentration of 0.05 mM. One microliter of each reaction was mixed with 49 μL of alpha-cyano-4-hydroxycinnamic acid matrix and spotted on a matrix-assisted laser desorption/ionization (MALDI) plate. MS1 spectra were obtained on an AB Sciex 4800 Plus MALDI-TOF/TOF at the UNC Michael Hooker Proteomics Facility (Dr David Smalley, director). Spectra in the range of 400–3000 Da were examined.

Growth cone collapse assay

Dissociated cortical neuron cultures were generated from NCAM null mice (P0, Hinkle et al. 2006; Brennaman and Maness 2008). WT or mutant NCAM140 plasmids were introduced along with pmax-GFP using the Amaxa nucleofector (Lonza, Germany), as previously described (Wright et al. 2007). GFP was used as an internal transfection efficiency control; ~ 60–70% of neurons were transfected. After 48-72 h, ephrinA5-Fc or human IgG (3 μg/mL) was added for 30 minutes. Neurons were fixed and growth cones visualized by immunofluorescence for GFP (1 : 400, total neurons) or GFP and GABA (interneurons; Brennaman et al. 2013). In separate experiments, ADAM10 prodomain (10 μM; Moss et al. 2007) was added to the cultures 24 h prior to induction of collapse. Cultures were stained for AlexaFluor-568 phalloidin (Wright et al. 2007; Schlatter et al. 2008) and GABA to identify total neurons and interneurons, respectively. Growth cones were scored as collapsed by bullet-shaped morphology, and non-collapsed by spread morphology. The percentage of GFP-positive neurons or GFP/GABA-positive neurons with collapsed growth cones was reported (10 fields/well; ≥ 2 wells/experiment; ≥ 300 growth cones). Similar numbers of neurites were extended from the neurons in each case.

Immunofluorescence staining

Dissociated cortical neurons (E16.5) were cultured as above. Neurons were stained for immunofluorescence using the following antibodies: ADAM10 (AB946, 1 : 100), EphA3 (C-19, 1 : 50), TUJ1 (1 : 400), NCAM (AB5032, 1 : 100). AlexaFluor-488 (1 : 400) or TRITC (1 : 150) labeled secondary antibodies were used. Images were captured on an Olympus Fluoview FV500 (Olympus Corp., Center Valley, PA, USA) using a 60× objective and 2.0× optical zoom at the UNC Microscopy Services Laboratory (Dr Robert Bagnell, Director).

Statistical analysis

Error bars on graphs indicate SEM. Significant differences between samples were calculated using the Student's *t*-test, $p < 0.05$.

Results

EphrinA5/EphA stimulates ADAM10-induced cleavage of NCAM

We demonstrated that ADAM10 cleaves NCAM in transfected cells (Hinkle et al. 2006), and that NCAM/EphA3 interaction regulates GABAergic interneuron development (Brennaman et al. 2013). Previous studies have demonstrated that ephrinA/EphA3 association stimulates

binding to and activation of ADAM10 in HEK293T (Janes et al. 2005) and NIH3T3 (Hattori et al. 2000) cells to promote ephrinA cleavage, leading us to hypothesize that NCAM may be cleaved in response to ephrinA/EphA3 activation of ADAM10. HEK293T cells were transfected with NCAM140 and EphA3, starved overnight and incubated for 10 min to induce NCAM cleavage. Cleavage fragments were examined by western blotting. In cells transfected with NCAM140 and EphA3, NCAM cleavage was strongly elevated compared to cells expressing NCAM140 alone, and produced a ~ 115 kDa soluble NCAM extracellular domain fragment (NCAM-EC) in conditioned media and a ~ 30 kDa residual cytoplasmic fragment (NCAM-IC) in cell lysates (Fig. 1a). A small amount of endogenous NCAM cleavage was observed in the absence of transfected EphA3. Treatment with ephrinA5-Fc caused little increase in cleavage (not shown), likely because of ligand-independent clustering and activation of over-expressed EphA receptors in HEK293T cells (Noren et al. 2009). EphrinA/EphA signaling has kinase-dependent and kinase-independent effects on repellent guidance (Davy and Soriano 2005; Chen et al. 2012; North et al. 2013). To determine if NCAM cleavage was kinase-dependent, HEK293T cells were co-transfected with NCAM140 and a kinase-inactive mutant EphA3 (EphA3-K653R) (Hu et al. 2009). Mutant EphA3-K653R was ineffective in inducing the ~ 30 kDa NCAM-IC and ~ 115 kDa NCAM-EC fragments (Fig. 1a). We next assessed whether endogenously expressed ADAM10 (Janes et al. 2005) was required for EphA3-mediated NCAM140 cleavage. HEK293T cells were co-transfected with NCAM140, EphA3 and a dominant negative ADAM10, which lacks the metalloprotease domain (Yan et al. 2002). There was a striking reduction of the ~ 30 kDa NCAM-IC and ~ 115 kDa NCAM-EC cleavage fragments in cells expressing dominant negative ADAM10 (Fig. 1a). We then examined cleavage of NCAM180 in response to EphA3. NCAM180 shedding was also elevated in response to EphA3, producing a ~ 115 kDa soluble NCAM-EC fragment in conditioned media and a ~ 70 kDa cytoplasmic fragment in cell lysates (Fig. 1a). Actin levels were similar across lanes, indicating that changes in cleavage were not because of differences in protein loading. We quantified the relative levels of NCAM-EC cleavage fragments produced by proteolysis of NCAM140 and NCAM180 in western blots of multiple experiments as this is conventional for assessing the amount of target protein cleavage (Perez et al. 1990; Brakebusch et al. 1994; Mullberg et al. 1994; Migaki et al. 1995; Hinkle et al. 2004). Statistical analysis indicated that co-transfection with EphA3 significantly increased the levels of NCAM-EC fragments, while loss of EphA3 kinase or ADAM10 metalloprotease activity inhibited shedding (Fig. 1b; $*p < 0.05$, $**p < 0.001$). The levels of NCAM-IC were not quantified as they did not acccurately reflect proteolytic cleavage of NCAM because of trafficking and turnover of the fragment.

To investigate whether ephrinA/EphA could induce NCAM cleavage in neurons, dissociated cultures of embryonic mouse cortical neurons (E16.5) were treated for 10-30 min with clustered ephrinA5-Fc or IgG as control, and analyzed for NCAM cleavage fragments by immuno-blotting. In cultures treated with IgG, a small amount of the ~ 250 kDa soluble NCAM-EC fragment was present in conditioned media, indicating some endogenous shedding that released the polysialylated extracellular domain (Fig 2a; Ulm et al. 2013). In cell lysates, uncleaved transmembrane NCAM isoforms migrated as a broad band at ~ 250 kDa because of the presence of PSA, and the NCAM-IC fragment migrated at ~ 30 kDa,

which corresponds in size to cleavage of NCAM140 (Hinkle et al. 2006). Small amounts of uncleaved non-PSA NCAM140 were observed in the cell lysate with very long exposure times (data not shown). Treatment of neuronal cultures with ephrinA5-Fc for 10 or 30 min stimulated cleavage of NCAM, as seen by increased levels of the NCAM-IC fragment in the cell fraction and ~ 250 kDa NCAM-EC in the media (Fig. 2a). Quantification of NCAM-EC relative to total NCAM indicated that ephrinA5 stimulated shedding 3-fold after 30 min. Numbers under the blots indicate the quantification of NCAM-EC relative to full length NCAM in the representative experiment shown ($n = 3$ samples from three separate experiments; 250 kDa NCAM-EC/total PSA-NCAM for all experiments: IgG 10′ = 0.41 ± 0.05, ephrinA5-Fc 10′ = 0.49 ± 0.05, IgG 30′ = 0.31 ± 0.15, ephrinA5-Fc 30′ = 0.49 ± 0.08). The range in the absolute values for NCAM-EC/full length NCAM between different experiments may result from some variability in the ratios of pyramidal neurons, GABAergic interneurons, and glia between the cortical neuron preparations. Actin levels remained constant in the cultures, indicating that similar levels of protein were compared.

We treated cultures with endoneuraminidase-N (endo-N, 20 U; El Maarouf and Rutishauser 2003) for 24 h prior to ephrinA5 stimulation (30 min) to remove PSA and identify which NCAM isoforms were present. NCAM140 and NCAM180 were evident in endo-N treated cultures, with NCAM140 as the predominant isoform (Fig. 2b; $n = 3$ samples from three separate experiments; NCAM-EC/relative to total NCAM for all experiments: 0 ± 0). The ~ 250 kDa NCAM-EC fragment was absent from media, consistent with identification of the media fragment shown in Fig. 2a, as PSA-NCAM. We did not observe ~ 115 kDa non-PSA NCAM-EC in conditioned media upon endoN treatment. It is possible that PSA-NCAM was preferentially cleaved in neuronal cultures or that the non-PSA NCAM-EC cleavage fragment is degraded in the media.

Western blotting with PSA-specific antibodies were also performed on cortical neuron cultures stimulated with ephrinA5. The ~ 250 kDa NCAM-EC fragment in media and full length NCAM in the lysate were detected by PSA antibodies (Fig. 2c). The ~ 250 kDa PSA-NCAM-EC fragment was increased in media with ephrinA5 treatment. Thus, PSA-NCAM appears to be cleaved in response to ephrinA5 in embryonic cortical neurons.

To determine whether ADAM10 mediated NCAM cleavage in cortical neurons, cultures were pre-incubated with an ADAM10 prodomain peptide for 24 h prior to ephrinA5-Fc stimulation (10 or 30 min). The ADAM10 prodomain is comprised of a peptide from within the ADAM10 prodomain (amino acids 23–213) and acts as a specific ADAM10 inhibitor at nanomolar concentrations, as it does not bind to other ADAMs or matrix metalloproteases (Lemieux et al. 2007; Moss et al. 2007). Cultures treated with the ADAM10 prodomain exhibited partially reduced levels (~ 40%) of the soluble ~ 250 kDa NCAM-EC fragment in media and the ~ 30 kDa NCAM-IC fragment in cells (Fig. 2d, 30 min treatment shown; 250 kDa NCAM-EC/total PSA-NCAM: 10′ = 0.3, 30′ = 0.2), supporting a role for ADAM10 in ephrinA5-induced NCAM shedding in cortical neurons. The partial reduction in levels of cleaved fragments suggests that other proteases may contribute to NCAM shedding.

To determine if EphA3, NCAM, and ADAM10 were co-expressed in cortical neuron cultures, double immunofluorescence staining was performed for the each of three proteins

using antibodies demonstrated to be specific for each protein (NCAM, EphA3: Brennaman et al. 2013; ADAM10: Glomski et al. 2011; Weber et al. 2011). NCAM exhibited strong labeling along the membrane of the soma and processes of neurons (Fig. 2e), while ADAM10 and EphA3 displayed a diffuse, punctate pattern throughout the soma and neurites (Fig. 2f-g). NCAM showed colocalization with some EphA3 and ADAM10 at the membrane (Fig. 2e, f). Double immunofluorescence labeling for EphA3 and ADAM10 showed overlap at the membrane and puncta with the appearance of internalized vesicles (Fig. 2g). Additional experiments using TUJ1 immunofluorescence confirmed that the labeled cells were neurons (data not shown). The punctate labeling of ADAM10 likely represents immature, inactive protein, while the ADAM10 localized to the cell surface represents mature enzyme (Marcello et al. 2010). Eph receptors are subject to endocytosis and recycling upon ephrin binding (reviewed in; Andersson 2012). Cortical neurons express ephrinA2, −3, and −5 (Mackarehtschian et al. 1999; Yun et al. 2003; Depaepe et al. 2005; Torii et al. 2009), which may bind to EphA3 and promote internalization, accounting for the punctate staining in these cultures.

NCAM mutations are impaired for EphA3-induced ADAM10-dependent cleavage

Many cell surface proteins are cleaved in the juxtamembrane stalk, which lies between the last structured domain and the cell membrane (Arribas and Borroto 2002). However, deletion of the juxtamembrane stalk in NCAM140 did not inhibit EphA3-induced NCAM cleavage in HEK293T cells (data not shown), suggesting that the cleavage site may reside in the second FNIII domain, based on the size of the NCAM-EC fragment. A previous report on ADAM10 substrate specificity indicated that Leu at the P1’ position C-terminal to the cleavage site in a peptide library was more commonly present than other amino acids in peptides cleaved by ADAM10 (Caescu et al. 2009). Upon examination of the NCAM sequence and comparison to other known ADAM10 targets (reviewed in; Saftig and Reiss 2011), a sequence in the E-F loop (Kiselyov et al. 2003; Carafoli et al. 2008) of the second FNIII domain, ^{661}RLPSGSDHVMLKSLDWNAEY^{680}, was identified as a likely candidate. This sequence is conserved among *Xenopus*, rodent and human NCAMs, and is located adjacent to the N-terminus of the F-G loop peptide sequence (amino acids 686–693) that binds and activates the fibroblast growth factor receptor (Kiselyov et al. 2003). We incubated a synthetic peptide encompassing this sequence with recombinant mouse ADAM10 for 4 h prior to obtaining mass spectra (Fig. 3a). Incubation with ADAM10 produced two peaks corresponding to the N-terminal cleavage fragment (1381.99 *m/z*) and the full length peptide (2359.67 *m/z*). The 1381.99 *m/z* peak indicated cleavage between Lys^{672} and Ser^{673} (LK^{672}SL; Fig. 3b). The C-terminal cleavage product was not identified by mass spectrometry because of high charge content.

ADAM10 has been reported to be unable to cleave proteins at Gly-Asp sequences (Malinverno et al. 2010). To assess the NCAM140 cleavage site in a cellular context, we created a series of three Gly-Asp mutations: one at the presumed scissile bond (P1-P1’; LK^{672}SL to LG^{672}DL), one at P2-P3 amino acids (VMLK^{672} to VGDK^{672}), and a double mutant at both sites (VGDG^{672}DL; Fig. 4a). WT and mutant NCAM constructs were co-transfected with EphA3 to evaluate whether these mutations impaired EphA3-induced cleavage. The LGDL mutant showed a decrease in the amount of NCAM-EC in media

in response to EphA3 (Fig. 4b,c, *$p < 0.05$), and the VGDK and VGDGDL mutants were more progressively impaired (Fig. 4b, c, *$p < 0.05$, **$p < 0.001$). Additional minor cleavage fragments of the NCAM cytoplasmic domain were observed in mutant lysates, which were not present in WT lysates. These likely represented cleavage at secondary, non-physiological sites, a phenomenon which has been observed in mutant forms of other cell surface proteins subject to ectodomain shedding (Perez et al. 1990; Brakebusch et al. 1994). Similar actin levels (Fig. 4b) and surface expression of WT and mutant NCAM isoforms (Fig. 4d) indicated that cleavage changes were not because of differences in protein loading or trafficking. Actin and EphA3 blotting were used as cytoplasmic and surface protein controls, respectively (Fig. 4d). Together, these results support the conclusion that Met^{670}-Leu^{671}-Lys^{672}-Ser^{673} in the second FNIII domain of NCAM140 is the site of ADAM10-mediated cleavage induced by EphA3.

ADAM10 cleavage site mutants in NCAM are impaired for ephrinA5-induced growth cone collapse

Since NCAM, ADAM10, and EphA3 were co-expressed in neurons, we wished to assess whether NCAM cleavage was required for ephrinA5-induced growth cone collapse in these cells. Cortical neuronal cultures from homozygous NCAM mutant mice (P0) were electroporated with either WT or cleavage site mutants (LGDL, VGDK, VGDGDL) in NCAM140 along with the pmax- enhanced green fluorescent protein plasmid to identify transfected cells. After 72 h, neurons were treated with ephrinA5-Fc or IgG control for 30 min to promote growth cone collapse, and electroporated cells were visualized by immunostaining with antibodies to GFP. Neurons generally displayed one to three processes, regardless of treatment, and only neurons with processes were analyzed. NCAM null neurons exhibited populations of non-collapsed (fan-shaped; Fig. 5a) and collapsed (bullet-shaped; Fig. 5A′) growth cones, but did not respond to ephrinA-Fc (Fig. 5c). However, expression of WT NCAM140 in NCAM null neurons rescued ephrinA5-dependent growth cone collapse (Fig. 5c; *$p < 0.05$). In contrast, expression of LGDL, VGDK, or VGDGDL NCAM mutants did not rescue ephri-nA5-induced growth cone collapse (Fig. 5c). Based on our findings that NCAM is required for growth cone collapse in GABAergic interneurons (Brennaman et al. 2013), these experiments were also analyzed using immunofluorescent labeling for GABA to select interneurons (Fig. 5b, B′). WT NCAM140, but not the three cleavage site mutants, rescued growth cone collapse to ephrinA5 in GABAergic interneurons (Fig. 5d, *$p < 0.05$). Because GABA-positive cells represented ~ 20% of the neurons in these cultures (Waagepetersen et al. 2002), these results suggested that NCAM cleavage at the identified site in the second FNIII domain mediated ephrinA5-induced growth cone collapse in both GABAergic and non-GABAergic neurons.

To demonstrate a role for ADAM10-mediated cleavage in ephrinA5-induced growth cone collapse, cultures were treated with the ADAM10 prodomain (10 μm; Moss et al. 2007) for 24 h prior to ephrinA5-Fc stimulation. Pre-treatment with the ADAM10 prodomain reduced ephrinA5-induced growth cone collapse in the total neuronal population, and to a lesser extent in GABAergic neurons (Fig. 5e, f, *$p < 0.05$).

Discussion

Here, we demonstrated that NCAM is subject to ectodomain shedding by the ADAM10 metalloprotease in response to ephrinA5/EphA3 signaling. The ADAM10-dependent cleavage site was identified within the second FNIII domain of the NCAM extracellular region. Mutations within this site of NCAM were not able to be cleaved by ADAM10 and, unlike WT NCAM, did not promote ephrinA5-induced growth cone collapse of cortical GABAergic or non-GABAergic neurons. NCAM is a structural cell adhesion molecule with clearly defined roles in promoting axon growth (Maness and Schachner 2007). New findings (Brennaman et al. 2013), including those presented here, extend the functional capabilities of NCAM to modulating repellent responses in neurons, and identify NCAM as a new substrate for ADAM10 that can contribute to growth cone collapse downstream of ephrinA/EphA3.

The demonstration that ADAM10 cleaves NCAM in a surface exposed loop within the second FNIII domain (Lys^{672}-Ser^{673}) is different from many cell surface proteins, which undergo ectodomain cleavage in the juxtamembrane stalk (Arribas and Borroto 2002). It was recently reported that ADAM10 prefers small amino acids at the P1-P3 positions and hydrophobic residues at the P1’ position, but can accommodate larger residues such as Gln at P1’ (Caescu et al. 2009). However, the NCAM cleavage site, Lys^{672}-Ser^{673}, is similar to the ADAM10-dependent cleavage site on FasL (Lys-Gln; Schulte et al. 2007), and identical to the ADAM17/tumor necrosis factor alpha converting enzyme-dependent cleavage site in Amphiregulin (Lys-Ser; Hinkle et al. 2004). Our finding that P2 and P3 amino acids were important for NCAM cleavage by ADAM10 is in accord with studies reporting that additional N- and C-terminal amino acids contribute to the cleavage site specificity for ADAM10 substrates ephrinA5 (Janes et al. 2005), Betacellulin (Sahin et al. 2004; Sanderson et al. 2005), and epidermal growth factor (Horiuchi et al. 2007). It is important to note that cleavage at this site in the second FNIII repeat should not interfere with polysialylation, as ST8SiaII and ST8SiaIV bind to the first FNIII repeat, and PSA is added to the fifth Ig-like domain (Rutishauser 2008). However, we cannot exclude the possibility that the mutants are differentially polysialylated compared to WT NCAM, which may alter their function.

Previous findings showed that ADAM10 associates with activated EphA3 kinase to mediate cleavage of ephrinA5 during axon repulsion (Hattori et al. 2000; Janes et al. 2005, 2009). Our results suggest that NCAM is required for repulsion, and, like ephrinA5, must be cleaved for growth cone collapse. NCAM binding to EphA3 (Brennaman et al. 2013) may facilitate EphA3 receptor clustering and intracellular signaling leading to cytoskeletal contraction. Subsequent cleavage of NCAM and ephrinA5 by ADAM10 may be necessary to release adhesive interactions enabling growth cones to collapse and retract from the opposing cell surface or adhesive substrate. PSA-NCAM may be preferentially cleaved in embryonic cortical neurons. However, non-PSA-NCAM may be cleaved to some extent in response to ephrinA5/EphA3 in adolescent or mature neurons because non-PSA-NCAM was cleaved in an EphA3-dependent manner in HEK293T cells. Cleavage of a portion of total NCAM appeared sufficient to achieve high levels of growth cone collapse, possibly reflecting NCAM shedding restricted to the growth cone. Similarly, stimulus-driven

cleavage affects only a fraction of other cell surface proteins in cultured cells (Perez et al. 1990; Brakebusch et al. 1994; Mullberg et al. 1994; Migaki et al. 1995; Hinkle et al. 2004).

The partial inhibitory of the ADAM10 prodomain on NCAM cleavage suggests that other ADAM proteases and signaling pathways may regulate NCAM shedding in neuron populations. ADAM17 induces NCAM shedding in hippocampal neurons, but the cleavage site was not identified (Kalus et al. 2006). Different types of stimuli may selectively drive ADAM10- or ADAM17-induced cleavage of NCAM, as phorbol esters promote ADAM17-dependent cleavage, while calcium influx (Horiuchi et al. 2007) and ephrinA/EphA signaling, as shown here, promote ADAM10 shedding. Furthermore, ADAM10 induces cleavage of the cell adhesion proteins L1 (Maretzky et al. 2005), protocadherins (Reiss et al. 2006), and Neuroligin-1 (Suzuki et al. 2012) in response to NMDA receptor activation in neurons. EphrinA/EphA3 activation of ADAM10 and interaction of ADAM10 with additional NCAM sequences may allow preferential cleavage of NCAM.

Deletion of ephrinA5, EphA3, or NCAM in null mutant mice results in increased numbers of perisomatic synapses made by GABAergic basket interneurons in the prefrontal cortex (Brennaman et al. 2013). The ADAM10 shedding mechanism observed here may limit the extent of interneuron axon arborization and restrict the number of inhibitory synapses important for excitatory/inhibitory balance *in vivo*. Supporting this hypothesis, over-expression of NCAM-EC in transgenic mice causes excess elimination of perisomatic inhibitory synapses (Pillai-Nair et al. 2005; Brennaman and Maness 2008). We believe that NCAM-EC is predominantly responsible for the effects on growth cone collapse, but the cell associated fragment may also contribute as expression of the NCAM140 cytoplasmic domain decreases NCAM-dependent neurite outgrowth (Buttner et al. 2004). Together, these results raise the possibility that EphA3/ADAM10-induced NCAM cleavage might be altered in disorders where cortical excitatory/inhibitory balance is disrupted.

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Abbreviations used:

ADAM	a disintegrin and metalloprotease
AP	alkaline phosphatase
DN	dominant negative
EGFP	enhanced green fluorescent protein
FNIII	fibronectin type III domain
NCAM-EC	extracellular domain of NCAM

NCAM-IC	cytoplasmic domain of NCAM
NCAM	neural cell adhesion molecule
PSA	polysialic acid

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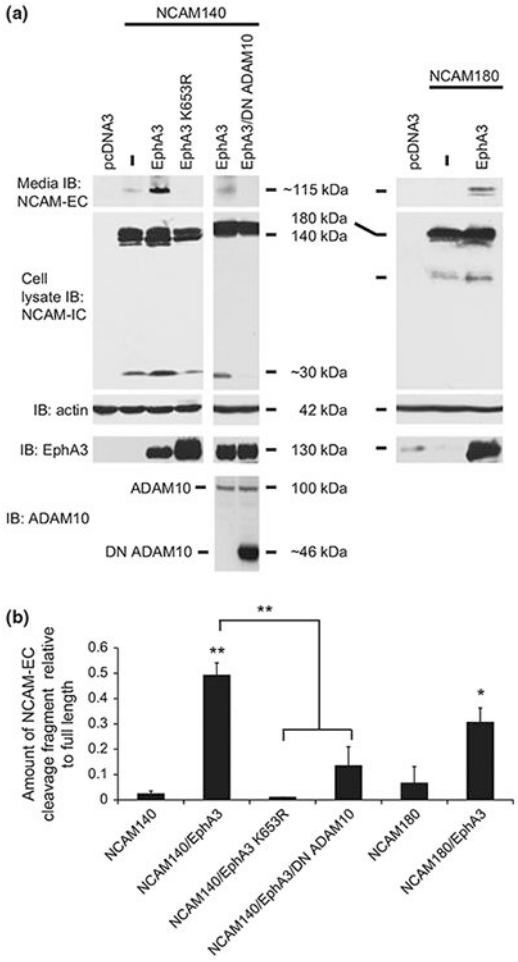


Fig. 1.

EphA3 kinase and ADAM10 are required for NCAM cleavage. (a) HEK293T cells were transfected with pcDNA3, NCAM140, NCAM180, WT EphA3, K653R EphA3, and dominant negative a disintegrin and metalloprotease (ADAM)10 plasmids [DN ADAM10 (53)]. Full length NCAM (140 or 180 kDa) and NCAM-IC fragments (NCAM140: ~ 30 kDa and NCAM180: ~ 70 kDa) were detected in cell-associated fractions by immunoblotting with antibody to the NCAM cytoplasmic domain, while the soluble extracellular region of NCAM (NCAM-EC) fragment (~ 115 kDa) was detected in conditioned media using an antibody to the NCAM extracellular domain. Actin, ADAM10, and EphA3 blots are also included. Lanes were cropped from the same gel. (b) Quantification of the levels of NCAM-EC cleavage fragment relative to full length NCAM from multiple experiments was determined using pixel densities of the NCAM-EC cleavage fragment divided by the pixel density of full length NCAM for each lane of each blot using ImageJ. Data represent ≥ 10 different samples from ≥ 4 experiments for NCAM140, and ≥ 4 samples from three experiments for NCAM180. NCAM140 and NCAM140/EphA3 data were combined from all experiments. Student's *t*-test: $*p < 0.05$, $**p < 0.001$.

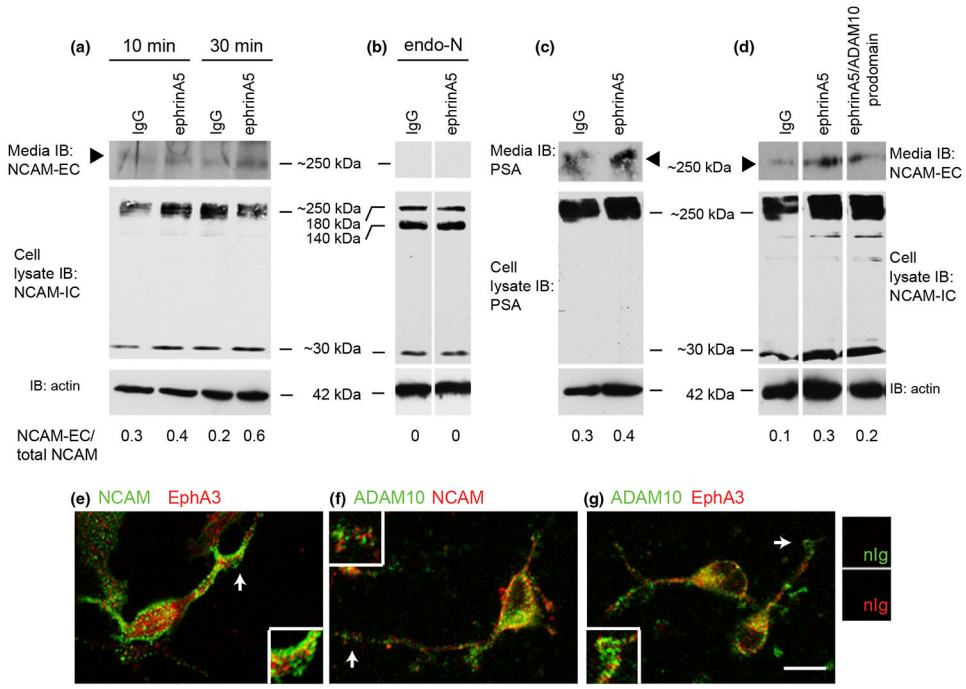
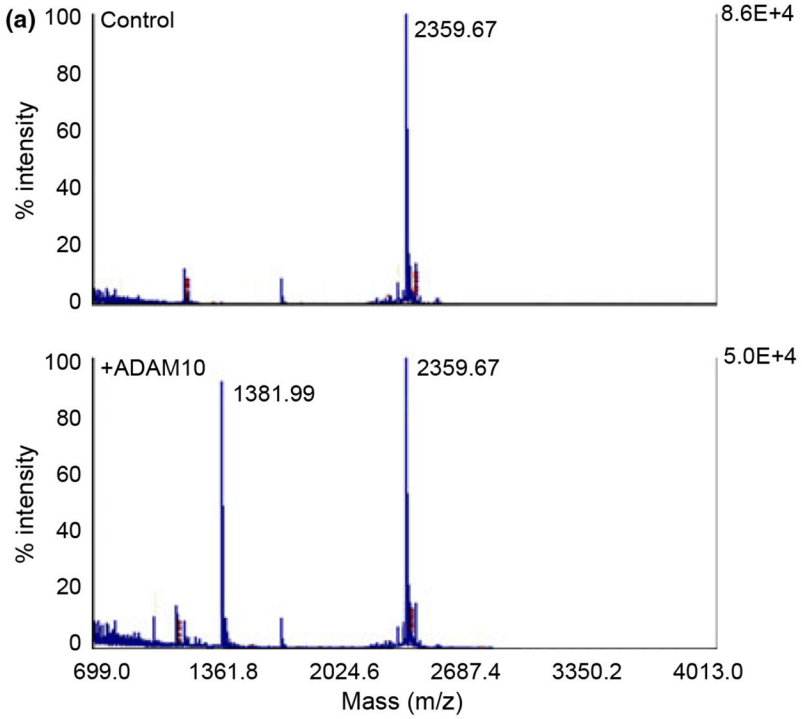


Fig. 2.

EphrinA5 induces proteolytic cleavage of NCAM in cortical neurons. (a) Dissociated cortical neuron cultures (E16.5) were grown for 48 h, then stimulated with preclustered ephrinA5-Fc (1.5 μg/mL) or non-immune IgG for 10 or 30 min. Proteins in the cell associated fraction (bottom panel) or conditioned media (top panel) were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), and subjected to immunoblotting with antibody to the NCAM cytoplasmic domain to detect the ~ 30 kDa NCAM-IC fragment or to the NCAM extracellular domain to detect the soluble ~ 250 kDa extracellular region of NCAM (NCAM-EC) fragment. Full-length polysialic acid (PSA)-NCAM migrated at ~ 250 kDa. Cultures were treated with endo-N (b; El Maarouf and Rutishauser 2003) or a disintegrin and metalloprotease (ADAM)10 prodomain peptide (c; Moss et al. 2007) for 24 h prior to ephrinA5-Fc stimulation (30 min) and proteins were western blotted as in (a). (b) Endo-N treated cells express NCAM140, NCAM180 and the ~ 30 kDa fragment in cell lysates. No NCAM-EC protein was observed in media. All lanes are from the same gel. Spaces between lanes indicate where unnecessary or duplicate lanes were cropped. (c) Western blots using antibodies to PSA. Full length PSA-NCAM was detected in lysate and the ~ 250 kDa NCAM-EC fragment was detected in media. (d) Pre-treatment with the ADAM10 prodomain reduced the ~ 250 kDa NCAM-EC fragment in media and the ~ 30 kDa fragment in cell lysates. Representative experiments are shown in a–d ($n = 3$ samples from three separate experiments). E-G) Cortical neurons (E16.5) were cultured for 72 h and immunofluorescently labeled for ADAM10, EphA3, and NCAM. Insets represent higher magnification of arrows. Representative images IgG controls for staining are shown to the left of panel (e). Scale bars = 10 μm. NCAM and EphA3 (e) or ADAM10 (f) showed colocalization at the plasma membrane. ADAM10 and EphA3 (g) showed overlap throughout the cell body and processes.



(b)

Peptide	Calc. Mass (Da)	Det. Mass (Da)	Error (Da)
acetyl-RLPSGSDHVMLKSLDWNAEY-amide	2359.62	2359.67	0.05
acetyl-RLPSGSDHVMLK	1381.60	1381.99	0.39

Fig. 3.

Identification of the NCAM cleavage site. A peptide (50 μM) comprising a putative NCAM cleavage site, acetyl-RLPSGSDHVMLKSLDWNAEY-amide, was incubated with recombinant mouse a disintegrin and metalloprotease (ADAM)10 (1 μg) or 10 mM Tris, pH 8.0 buffer for 4 h at 37°C. The reaction was stopped by the addition of 50 mM EDTA, mixed with alpha-cyano-4-hydroxycinnamic acid matrix and spotted on a MALDI plate. MS1 spectra (400–3000 Da) were obtained on an AB Sciex 4800 Plus MALDI-TOF/TOF. (a) The mass spectra for the control (top) and ADAM10 (bottom) peptide cleavage reactions. (b) Table of the calculated cleavage site based on calculated (Calc. Mass) and determined mass (Det. Mass), error between the two mass measurements is included.

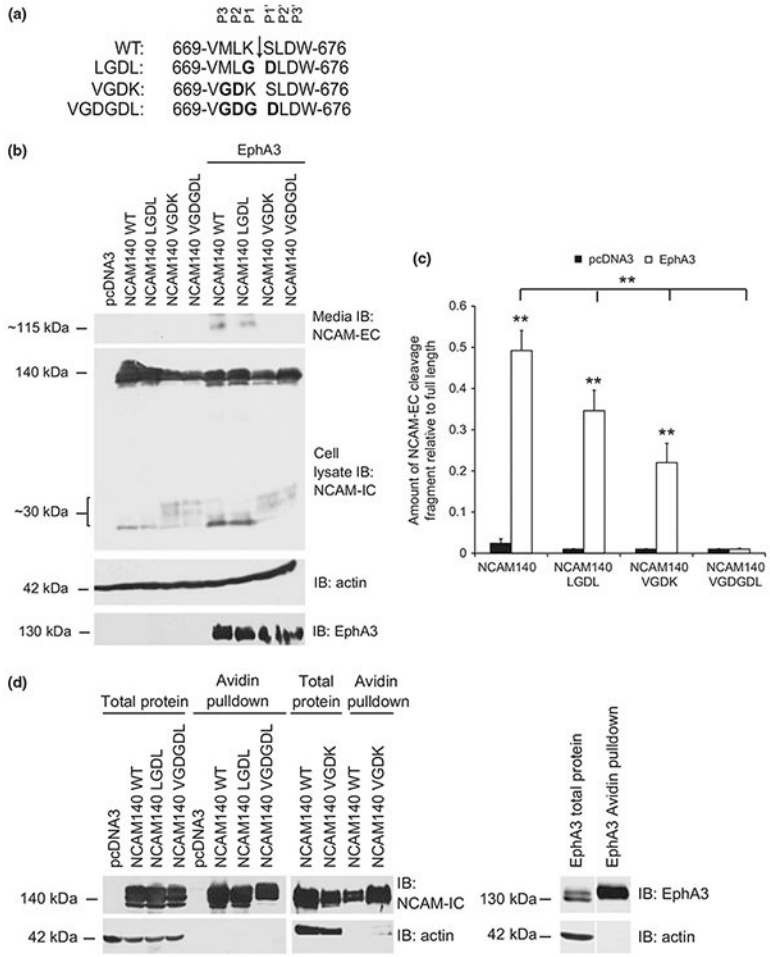


Fig. 4.

NCAM mutants are resistant to ephrinA5-induced shedding. (a) Amino acid sequence of the E-F loop in the second fibronectin type III domain (FNIII) domain of NCAM (amino acids 669–676). The NCAM cleavage site is denoted by an arrow depicting the presumed scissile bond (P1-P1'). Mutations in the P1-P1' (Leu^{671}-Gly^{672}/Asp^{673}-Leu^{674}; LGDL), P2/P3 (Val^{669}-Gly^{670}-Asp^{671}-Lys^{672}; VGDK), and P3–P1' (Val^{669}-Gly^{670}-Asp^{671}-Gly^{672}-Asp^{673}-Leu^{674}; VGDGDL) amino acids are indicated. b) HEK293T cells were transfected with WT or mutant NCAM140 and EphA3. Full length NCAM and NCAM-IC fragments (~ 30 kDa) were detected in cell-associated fractions by immunoblotting with antibody to the NCAM cytoplasmic domain, while the soluble extracellular region of NCAM (NCAM-EC) fragment (~ 115 kDa) was detected in conditioned media using an antibody to the NCAM extracellular domain. (c) Quantification of the relative level of NCAM-EC cleavage fragments was performed as in Fig. 1. Data represent ≥ 5 different samples from ≥ 3 separate experiments. NCAM140 and NCAM140/EphA3 data were combined from all experiments. Student's *t*-test: $^{**}p < 0.001$. (d) HEK293T cells transfected with WT NCAM140, mutant NCAM140, or EphA3 were subjected to cell-surface biotinylation and labeled proteins were bound to Neutravidin Ultralink resin. Bound proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and blotted with antibodies to the NCAM cytoplasmic domain, EphA3, or actin.

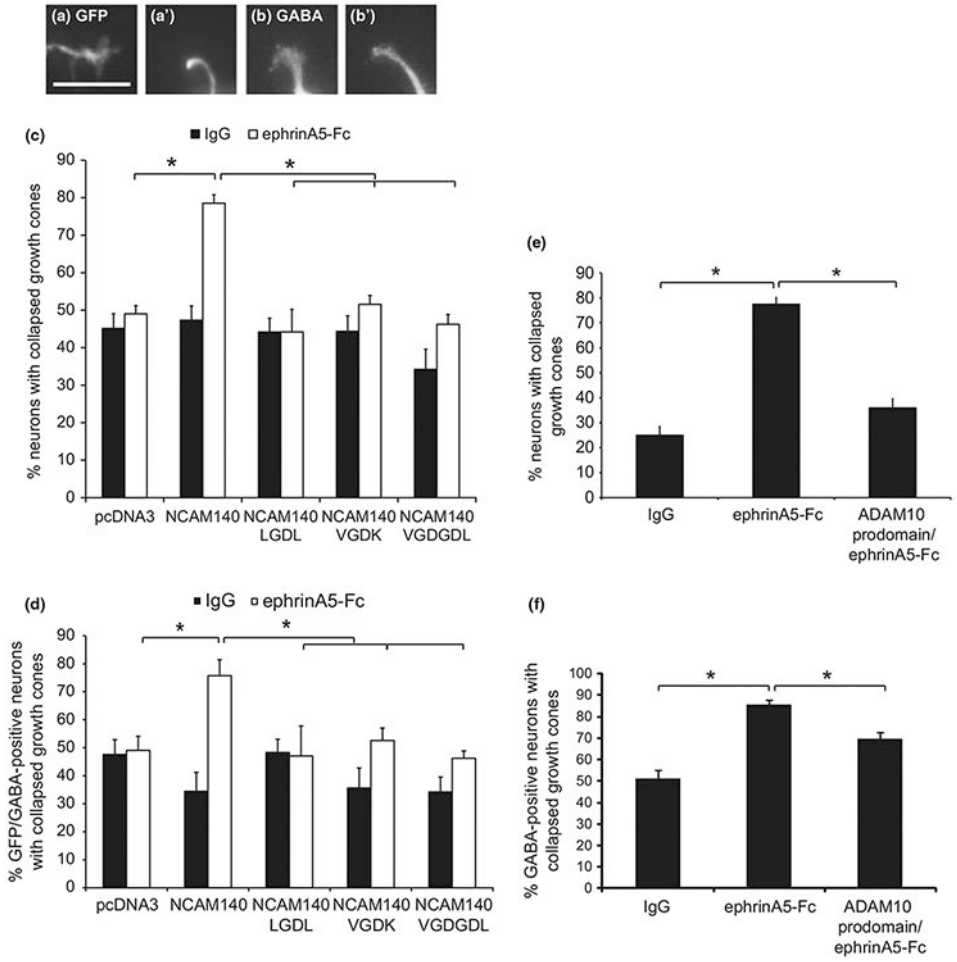


Fig. 5.

NCAM cleavage and ADAM10 activity are required for ephrinA5-dependent growth cone collapse in cortical neuronal cultures. Dissociated cortical neurons (E16.5) from NCAM null mice were electroporated with plasmids for WT and mutant NCAM140 (pcDNA3 control) along with pmax-GFP to visualize electroporated neurons. Neurons were cultured for 72 h prior to treatment with ephrinA5-Fc or normal IgG control (3 μg/mL) for 30 min to collapse neuronal growth cones. Representative images of GFP-positive (a) or GABA-positive (b) neurons with spread (a, b) or collapsed (A′, B′) growth cones. Scale bar = 5 μm. Quantification of the percent of GFP-positive (c) or GFP- and GABA-positive (d) neurons with collapsed growth cones in response to IgG or ephrinA5-Fc for each transfected cDNA. In separate experiments, neurons were treated with the a disintegrin and metalloprotease (ADAM)10 prodomain peptide (Moss et al. 2007) at 48 h, and stimulated with ephrinA5-Fc or IgG at 72 h for 30 min prior to phalloidin staining. Quantification of the percent of total (e) or GABA-positive (f) neurons with collapsed growth cones in response to IgG, ephrinA5-Fc, or ephrinA5-Fc pre-treated with ADAM10 prodomain peptide. $n \geq 300$ growth cones/condition from three to five separate animals; t-test $^{*}p < 0.05$.