The Neural Cell Adhesion Molecule (NCAM) Promotes Clustering and Activation of EphA3 Receptors in GABAergic Interneurons to Induce Ras Homolog Gene Family, Member A (RhoA)/Rho-associated protein kinase (ROCK)-mediated Growth Cone Collapse*

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Neural cell adhesion molecule (NCAM)2 is a key regulator of cell adhesion, axon growth, and fascilitation (1). NCAM also plays a critical role in the regulation of perisomatic synapses in the developing prefrontal cortex (2). For information to be transmitted effectively within the brain, an intricate balance of excitation and inhibition in the cortical circuitry must be established. Because inhibitory synapses perform essential roles in network function (3–7), the elucidation of how they are produced and refined is of vital importance. Inhibitory GABAergic synapses make and break connections in response to activity-dependent and -independent stimuli throughout development and into adulthood in a dynamic manner (6–9). Alterations of inhibition disrupt the excitation and inhibition balance and are associated with cognitive deficits in schizophrenia (10–12) and autism (13–16). Although the mechanisms regulating formation and fine-tuning of excitatory synapses have been extensively studied (17, 18), the molecular pathways underlying inhibitory synapse formation and refinement are poorly understood.

Previous work identified a novel role for the neural adhesion molecule NCAM as an obligate component of a receptor complex that acts in the early postnatal mouse medial frontal cortex to constrain basket interneuron connectivity with pyramidal neuron soma (2). Deletion of NCAM, EphA3, or ephrin-A2/A3/A5 in mice increases the number of perisomatic basket cell synaptic terminals in the mPFC (2). NCAM associates with EphA3 tyrosine kinase, an ephrin-A5 receptor, and mediates growth cone collapse of GABAergic interneuron terminals in cortical neuron cultures (2). Furthermore, ephrin-A5 treatment of cortical slices promotes loss of perisomatic basket cell synaptic puncta, and this loss is NCAM-dependent (2). Although NCAM and EphA3 co-localize in vivo at perisomatic synapses (2), the significance and mechanism of NCAM/EphA3 binding and clustering are not understood. Highlighting the importance of NCAM and ephrinA/EphA signaling in human...
cortical circuitry, genetic polymorphisms and dysregulation of NCAM (19–25) or EphA/ephrinA (26–28) are associated with schizophrenia, autism, and bipolar disorder.

EphA3 kinase activation and signaling depend on receptor clustering and are important for growth cone collapse, axon repulsion, and synaptic plasticity (29). The extracellular domain of EphA receptors consists of a ligand-binding domain (LBD), a cysteine-rich domain (CRD), and two fibronectin III (FN) domains (30). Crystallographic studies of ephrin-A5 or ephrin-A2 bound EphA2 demonstrated that ephrinA-EphA interactions are mediated by the LBD and an additional surface in the CRD, enabling formation of large multimeric receptor/ligand clusters. Studies of EphA4 clustering suggested that only the CRD interface is important for clustering of EphA4, rather than the additional LBD interface used by EphA2 (31). Together, these studies indicate that even Eph receptors in the same family can have varying clustering properties based on subtle differences in their ectodomains. The EphA3 structure has not been solved, but its overall sequence is more similar to EphA4 than to EphA2.

How NCAM engages EphA3 to promote repellent responses is unclear. NCAM has five Ig-class and two FN domains in its extracellular region and a cytoplasmic domain that differs in size among three principal isoforms (32). The NCAM extracellular region can be modified by polysialylation (PSA-NCAM), which is implicated in mediating synaptic plasticity (33). PSA-NCAM is highly expressed in the embryonic and early postnatal stages and in interneuron populations in the mature cortex (33–36). The 140-kDa isoform NCAM140 interacts physically and functionally with EphA3 to a greater extent than other isoforms (2) and is most prominent in axonal growth cones of developing neuronal cells (37). NCAM forms trans and cis homodimers in the plasma membrane, and these interactions are mediated by the Ig1, Ig2, and Ig3 domains as well as the first FN domain (32, 38). Based on the knowledge that NCAM and EphA3 colocalize at perisomatic synapses in vivo, interact biochemically, and promote remodeling of GABAergic interneuron terminals (2), we hypothesized that NCAM may promote EphA3 receptor clustering and signaling to bring about growth cone collapse and axon terminal retraction.

In this study, we utilized an approach combining molecular modeling based on crystal structures of rat NCAM Ig1–3 (PDB code 1QZ1) and the LBD/CRD regions of human EphA4 (PDB code 4M4P), analysis of receptor clustering, and functional assays to define the molecular and cellular mechanism by which NCAM promotes EphA3 signaling required for growth cone collapse of GABAergic interneurons. It was demonstrated that NCAM binding to EphA3 promotes ephrin-A5-dependent receptor clustering to activate EphA3 kinase signaling, necessary for EphA3 autophosphorylation, RhoA-GTPase activation, and growth cone collapse of cortical GABAergic interneurons.

Results

The Ig2 Domain of NCAM Binds the Cysteine-rich Domain of EphA3 through a Charged Interface—Previous studies identified an interaction between NCAM and EphA3 in the mouse forebrain, and the interaction site on NCAM was mapped to the extracellular region (2). We hypothesized that NCAM may promote receptor clustering and signaling by binding EphA3 through association with specific extracellular domains. To identify the binding determinants on EphA3 and NCAM, pull-down experiments were performed using domain deletions of each molecule. HEK293T cells were transfected with WT EphA3, EphA3 lacking the LBD (EphA3ΔLBD), and EphA3 lacking both the LBD and CRD (EphA3ΔLBD/CRD) (39). Transfected lysates were incubated with purified fusion proteins consisting of the entire extracellular region of NCAM, Ig1–5/FN1–2 with a C-terminal Fc tag (NCAM-EC), Protein A/G-Sepharose pulldown of NCAM-EC showed that the extracellular domain of NCAM bound WT EphA3 and EphA3ΔLBD, indicating that the ligand-binding domain of EphA3 was not required for interaction with NCAM. However, EphA3ΔLBD/CRD was unable to bind NCAM-EC (Fig. 1B), identifying the CRD as the critical binding domain. To delineate the EphA3 binding domain on NCAM, full-length NCAM-EC or NCAM truncations containing Ig1–3, Ig1–2, Ig2, or Ig1 as Fc fusion proteins were incubated with lysates of HEK293T cells transfected with WT EphA3. Fc pulldown assays showed that NCAM Ig1–3, Ig1–2, and the Ig2 domain alone bound EphA3, whereas NCAM Ig1 was not sufficient for binding (Fig. 1, C and D). These experiments demonstrated that the Ig2 domain of NCAM contains the required binding site for EphA3.

To more finely map the interaction determinants at the amino acid level, molecular modeling was performed using the ClusPro server (40) to analyze docking between rat NCAM Ig1–3 (PDB code 1QZ1) (38) and mouse EphA3 LBD/CRD modeled on human EphA4 (PDB code 4M4P) (41). Docking poses were screened for compatibility with interaction on the surface of a membrane in a cis conformation, lack of steric hindrance between additional domains of the molecules, and the number of hydrogen bonds within the interaction interface. Structural modeling suggested that NCAM and EphA3 bind through a charged interface involving basic residues in the NCAM Ig2 domain (Arg-156, Lys-162, and Arg-192) engaging acidic residues of the EphA3 CRD (Glu-246, Glu-248, and Glu-264) (Fig. 1A). To test this model, site-directed mutagenesis was used to generate charge reversal mutants of NCAM and EphA3 at the predicted sites of interaction. The mutations NCAM R156E and K162D decreased binding to EphA3 to levels of 11% and 10% of the WT, respectively (Fig. 1, E and F; p < 0.05). Interestingly, these two basic residues were located in the heparin binding site of the Ig2 domain, KHKGRDVILKKDVRFI (42). The heparin binding sites are solvent-exposed in NCAM dimers, suggesting that NCAM may engage in homophilic and heterophilic binding concurrently. This motif is also important for binding to extracellular matrix proteoglycans (43). NCAM R192E exhibited diminished binding to EphA3, although the decrease did not reach significance (p = 0.065). Arg-192 is contained in the sequence GRILARGEINFK of the NCAM Ig2 domain that is required for binding to Ig1 necessary for NCAM-NCAM homodimerization (44). Charge reversal mutations in predicted interaction sites of EphA3 (E248R or E264K) significantly inhibited co-immunoprecipitation of EphA3 and NCAM (~2% of the WT) (Fig. 1, G and H). Together, these mutagenesis
experiments demonstrate that the Ig2 domain of NCAM and the CRD domain of EphA3 comprise a charged interface for interaction.

NCAM Binding EphA3 Promotes Ephrin-A5-dependent Clustering of Receptors in Cortical GABAergic Interneurons—EphA3 signaling depends on ligand-induced receptor clustering and is important for growth cone collapse, axon repulsion, and synaptic plasticity (45, 46). Studies of EphA family members demonstrated that EphA2 forms extended arrays through two distinct interfaces (47) in the LBD and CRD, whereas EphA4 clusters are typically smaller and oligomeric because of their reliance on only one of these two corresponding interfaces, the CRD interface, suggesting that EphA receptors can have different clustering tendencies based on their ectodomains (31). However, the extent to which these clusters form in the absence of NCAM is not known.

The importance of NCAM/EphA3 binding for receptor clustering was assessed in cortical neuron cultures from NCAM KO mice co-transfected with WT or mutant NCAM140 together with pCMV-IREs-eGFP (Fig. 2A). We focused on GABAergic (GABA-immunopositive) interneurons in these cultures based on the identified role of NCAM at interneuron perisomatic puncta. After treatment with preclustered Fc or ephrin-A5-Fc for 30 min, cells were immunostained for GABA, NCAM, and EphA3 and imaged confocally. GFP/GABA double-positive cells were selected in single optical sections, andocolocalization of NCAM and EphA3 was assessed. GABA staining allowed the identification of individual axons to ensure that clustering was observed in single neurites and not bundles.

The proportion of colocalized signal for each channel was evaluated by colocalization analysis yielding thresholded Manders coefficients (Fig. 2, B and C; tM1, proportion of red pixels colocalizing with green pixels; tM2, proportion of green pixels colocalizing with red pixels). As a further measure of correlation of the two fluorescent signals, Pearson correlation coefficients (R > 0 indicates positive correlation, R < 0 indicates negative correlation) were generated (Fig. 2D). Ephrin-A5-Fc strongly promoted colocalization/clustering of WT NCAM and EphA3 compared with control Fc, as shown by merged fluorescent images, heat maps of colocalization, and colocalization coefficients (Fig. 2). Ephrin-A5-Fc also promoted EphA3 colocalization with NCAM R192E, a mutant that did not disrupt association of NCAM and EphA3. On the other hand, ephrin-A5-Fc did not promote colocalization of EphA3 with NCAM R156E. These colocalization analyses were consistent with co-immunoprecipitation studies that showed interaction of EphA3 with WT NCAM and NCAM R192E but not NCAM R156E (Fig. 1, E and F). The results demonstrate that binding of NCAM to EphA3 promotes ephrin-A5-dependent clustering of receptors in cortical GABAergic interneurons.

NCAM Binding EphA3 Is Required for Ephrin-A5-induced Growth Cone Collapse—Growth cone collapse is a widely used paradigm for assessment of ephrin-A5-repellent responses in developing axons and for identifying modulators of ephrin-A5 signaling in vitro (48–50). To assess the function of NCAM Ig2 interaction with the EphA3 CRD in growth cone collapse, NCAM KO cortical neuron cultures were co-transfected with empty vector (pCDNA3.1), WT NCAM, or NCAM mutants (R192E and R156E) together with pCMV-IREs-eGFP. After 48 h, cells were treated with preclustered Fc or ephrin-A5-Fc for 30 min. Cultures were fixed and immunostained for GABA and GFP to identify transfected interneurons. Growth cones were visualized by GABA staining and scored as collapsed (Fig. 3A, arrowheads) or spread (Fig. 3A, arrows) as described

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**FIGURE 1.** The EphA3 CRD binds the NCAM Ig2 domain. A, a candidate pose of Ig1-Ig3 of NCAM docked with LBD-CRD of EphA3. The predicted interface is labeled with arrows. B, Fc pulldowns of NCAM-EC and EphA3 or EphA3 deletion mutants. C, Fc pulldowns of NCAM-EC or truncation mutants and EphA3. D, densitometry of C. The amount of bound EphA3 for each Fc protein was normalized to control NCAM-EC-bound EphA3 (n = 3; p < 0.05). E, co-immunoprecipitation (IP) of WT NCAM or mutants of NCAM and EphA3 from transfected HEK293T cells. IB, immunoblot, F, densitometry of E. The amount of co-immunoprecipitated EphA3 for each NCAM immunoprecipitation was normalized to control WT NCAM-bound EphA3 (n = 3; p < 0.05). G, co-immunoprecipitation of NCAM and WT EphA3 or EphA3 mutants from transfected HEK293T cells. H, densitometry of G. The amount of co-immunoprecipitated EphA3 for each NCAM immunoprecipitation was normalized to control WT EphA3 (n = 3; p < 0.05). Student’s t test was performed for each of the binding experiments.
previously (2), and only GFP-positive (transfected) cells were included in the analysis.

Neurons expressing WT-NCAM exhibited robust growth cone collapse in response to ephrin-A5-Fc, consistent with a requirement for NCAM in ephrin-A5-mediated growth cone collapse (Fig. 3) as observed previously (2). NCAM KO neurons transfected with empty vector did not undergo significant growth cone collapse after ephrin-A5-Fc treatment. In contrast, the R156E mutant of NCAM that was unable to bind or cluster EphA3 was not effective in promoting growth cone collapse in response to ephrin-A5-Fc (Fig. 3). The NCAM R192E mutant, which did not show decreased binding to EphA3, effectively promoted growth cone collapse. These functional studies support a role for EphA3 and NCAM binding in ephrin-A5-induced growth cone collapse of GABAergic interneurons.

**NCAM Enhances EphA3 Autophosphorylation in Response to Ephrin-A5 in Cortical Neurons**—Ligand-dependent EphA3 oligomerization promotes activation of its kinase domain and induces autophosphorylation of EphA3 and transphosphorylation of tyrosine residues in the cytoplasmic domain (51, 52). Because NCAM binding to EphA3 enhanced clustering in cortical neurons, we hypothesized that NCAM promotes EphA3 kinase activation and downstream signaling. EphA3 autophosphorylation was assessed in cortical neuron cultures from WT

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**FIGURE 2. Ephrin-A5 induces clustering of NCAM and EphA3 in cortical interneurons.** A, NCAM KO cortical neurons were co-transfected with WT NCAM or NCAM mutants and pCAG-IRES-EGFP. Cells were treated with preclustered Fc or ephrin-A5-Fc, and localization of NCAM (pseudocolored green) and EphA3 (red) was assessed by confocal microscopy. Axons of GABA-immunoreactive interneurons were imaged, and colocalization of NCAM and EphA3 was assessed using ImageJ to generate a heat map of colocalization (fourth column). Scale bar = 5 μm. Thresholded Manders coefficients (tM1, B; tM2, C) and Pearson correlation coefficients (R-Total, D) were generated using ImageJ colocalization software (n = 3; *, p < 0.05; n.s., not significant; Student’s t test was performed between Fc and ephrinA5-Fc treated cells for each vector).
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A. Scale bars cone collapse in GABAergic interneurons. JOURNAL OF BIOLOGICAL CHEMISTRY

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FIGURE 3. NCAM binding to EphA3 promotes ephrin-A5-induced growth cone collapse in GABAergic interneurons. A, NCAM KO neurons were co-transfected with empty vector, WT NCAM, or mutant NCAM together with pCAG-IRES-EGFP and stained for GFP and GABA. Representative images of GABA-positive interneurons (DIV 12) treated with preclustered control Fc or ephrin-A5-Fc and stained for GABA are shown. Spread growth cones are indicated with arrows, and collapsed growth cones are marked with arrowheads. Scale bars = 10 μm and 2.5 μm in the magnified insets. B, the percentage of collapsed growth cones was determined (300 growth cones/condition; n = 3 experiments; *, p < 0.05; n.s., not significant). Student’s t test was performed between Fc- and ephrin-A5-Fc-treated cells for each vector.

and NCAM KO mice after treatment with preclustered control Fc or ephrin-A5-Fc. EphA3 was immunoprecipitated and subjected to immunoblotting with phosphotyrosine antibody (Tyr(P)-99), followed by stripping and reprobing for total EphA3. The ratio of phospho-EphA3 to total EphA3 was quantified by densitometry and compared for significant differences. In WT cultures, ephrin-A5-Fc promoted robust tyrosine phosphorylation of EphA3 (Fig. 4A). In contrast, NCAM KO cultures treated with ephrin-A5-Fc exhibited no increased phosphorylation compared with Fc controls, indicating that NCAM was required for activation and autophosphorylation of EphA3 kinase.

NCAM-dependent Activation of RhoA GTPase and ROCK Is Required for Ephrin-A5-dependent Growth Cone Collapse—To assess the role of Rho family GTPases in NCAM/EphA3 signaling, the activation of RhoA, Cdc42, and Rac1 in cortical neurons was determined by measuring GTP-bound GTPase levels in pulldown assays. Cortical neurons were stimulated with Fc or ephrin-A5-Fc and lysed, and GTP-bound GTPases were pulled down using GST-tagged effector proteins conjugated to glutathione-Sepharose beads (GST-Pak binding domain (PBD) for Rac1 and Cdc42, GST-Rho binding domain of Rhotekin (RBD) for RhoA). The ratio of active (GTP-bound) RhoA, Rac1, or Cdc-42 to input was determined for each condition (Fc- or ephrin-A5-Fc-treated) and normalized to Fc-treated cells (n = 3 for each GTPase). In WT neurons, GTP-bound RhoA increased 5-fold in response to ephrin-A5-Fc, whereas the levels of GTP-bound Rac1 and Cdc-42 were not significantly altered upon treatment (Fig. 5, A and B). In contrast, NCAM KO neurons showed no increase in GTP-bound RhoA upon stimulation with ephrin-A5-Fc, indicating a requirement for NCAM in ephrin-A5-dependent RhoA activation (Fig. 5, A and B).

To determine whether RhoA activation promoted growth cone collapse in cortical GABAergic interneurons treated with ephrin-A5-Fc, cortical neuron cultures were pretreated with the RhoA inhibitor exoenzyme C3 transferase prior to stimulation with preclustered Fc or ephrin-A5-Fc. Pretreatment of WT neurons with C3 toxin strongly inhibited ephrin-A5-dependent growth cone collapse in comparison with ephrin-A5-treated cells without C3 pretreatment (two-way ANOVA with Bonferroni post hoc testing, p < 0.001).

The role of RhoA signaling in ephrin-A5-dependent growth cone collapse was further evaluated by inhibition of ROCK1/2, a key downstream effector of RhoA. Cortical neurons were pretreated with Y-27632, a selective inhibitor of ROCK1/2, prior to stimulation with preclustered Fc or ephrin-A5-Fc, and GABA-positive interneurons were assayed for growth cone collapse. ROCK inhibition significantly decreased the percent of GABAergic cells exhibiting growth cone collapse upon ephrin-A5-Fc treatment compared with cells without Y-27632 pretreatment (two-way ANOVA with Bonferroni post hoc testing, p < 0.001). These results supported the conclusion that ephrin-A5-induced growth cone collapse in GABAergic interneurons is dependent on RhoA and its effector ROCK1/2.

Discussion

A novel role for NCAM in clustering and activation of EphA3 receptors necessary for axonal repellent responses in cortical neurons was identified using a combination of molecular modeling, mutagenesis, and functional assays. These studies revealed for the first time that NCAM promotes EphA3 receptor clustering in response to ephrin-A5 stimulation through binding between critical determinants in the NCAM Ig2 and EphA3 CRD domains. Although direct binding of NCAM and EphA3 was not demonstrated, molecular modeling and the effects of charge reversal mutations suggested that the interaction was likely to be direct. This interaction induced NCAM/EphA3 clustering, EphA3 kinase activation, and growth cone collapse of GABAergic interneurons stimulated by the ligand ephrin-A5. Downstream signaling through RhoA GTPase and one of its principal effectors, ROCK1/2, mediated the collapse response (Fig. 6). This molecular mechanism, as delineated in cortical neuron cultures, may also be important in regulating the density of axonal terminals of GABAergic interneurons in the developing neocortex.

Domain truncation analysis demonstrated that the association between NCAM and EphA3 was mediated by the NCAM Ig2 domain and EphA3 CRD. An interface involving complementary charged residues in the interacting domains was predicted from molecular modeling and shown by mutagenesis to be critical for association. Charge reversal mutations in either
of two basic residues of NCAM Ig2 (R156E and K162D) located in the predicted interface disrupted EphA3 binding. Mutation of either corresponding acidic residue in the EphA3 CRD (Glu-248 and Glu-264) predicted to bind the basic residues blocked the NCAM interaction. In contrast, mutation of R192E in the NCAM Ig2 domain did not significantly perturb EphA3 association. This residue was located within the amino acid sequence GRILARGE, a motif required for NCAM-NCAM dimerization. Interestingly, the basic residues in NCAM Ig2 (R156E and K162D) mediating EphA3 association were positioned within the heparin-binding site, which is essential for interaction of NCAM with extracellular matrix proteoglycans. It is possible that EphA3 binding to NCAM at the heparin-binding site may disrupt cell matrix adhesion as a necessary step for retraction of axon terminals.

Crystallographic studies of closely related EphA2 and EphA4 receptors identified a leucine zipper-like interface in the CRD comprised of hydrophobic residues that mediates EphA clustering in the plasma membrane (31, 41, 47, 53) and an additional LBD interface that promotes large-scale multimerization of EphA2 (31, 47, 53). EphA3 has not been crystallized, but the LBD interface involved in EphA2 dimerization is highly conserved in EphA3 (31). The predicted NCAM Ig2/EphA3 interface is sterically compatible with NCAM binding to the CRD of a dimer of EphA3 interacting through the LBD/LBD interface. It can be speculated that NCAM further enhances the higher-order clustering of EphA3 receptors by side-to-side interactions between the NCAM Ig2 domain and EphA3 CRD. This interaction may involve NCAM dimers, which likely form through Ig1-Ig2 cis interactions implicated in the crystal structure of NCAM Ig1–3 (38) (Fig. 6). NCAM forms cis and trans homodimers (32), but results strongly suggest that trans interactions of NCAM are not essential for EphA3 clustering. Functional assays of receptor clustering and growth cone collapse were done in NCAM-null cells transfected with NCAM. As individual transfected cells were analyzed, there would be few, if any, NCAM trans homodimers formed through contact with adjacent untransfected cells (which lack NCAM). Molecular modeling studies of NCAM/EphA3 were consistent with cis interactions, and trans interactions would likely occur through a different interface because of the altered orientation of the N and C termini of receptors on opposing cells.

In addition, the NCAM cytoplasmic domain directly engages the actin cytoskeleton through spectrin binding (54), which
may stabilize clusters of EphA receptors that are sensitive to adhesive force (55). Clustering of Eph receptors is critical for activating ephrin-dependent signaling (56), and additional factors may contribute, including clustering of ephrin ligands (57), concentration of Eph receptors at the cell surface (58), and segregation of ligands into discrete cell surface areas such as lipid rafts (59). Although these molecular modeling and mutagenesis experiments enabled the identification of important interaction domains of EphA3 and NCAM, crystal structures of the receptor complex would confirm and extend knowledge of the mechanism of oligomerization. Activation of RhoA downstream of EphA3/ephrin-A5 was found to be NCAM-dependent and required for growth cone collapse. The observed collapse of growth cones of axons and dendrites suggested that NCAM/EphA3 signaling may regulate the retraction of both. NCAM and EphA3 are enriched at perisomatic synapses in vivo (2), supporting a role for this pathway in axons of basket interneurons. Previous studies identified a mechanism of ephrinA-mediated RhoA activation through the guanine nucleotide exchange factor Ephexin (60, 61), but this is the first study to show that NCAM facilitates ephrin-A5-dependent activation of RhoA in cortical neurons. Selective inhibitors of RhoA or its effector ROCK1/2 substantially decreased the collapse response in GABAergic interneurons in culture. The residual collapse observed in RhoA or ROCK inhibitor-treated cells suggested that other signaling effectors may contribute to the repellent response. A limitation of this work is that long-term (DIV 10–14) cortical neuron cultures were used to study NCAM/EphA3 signaling important for growth cone collapse in GABAergic neurons. Although growth cone collapse responses in vitro correlate with inhibitory synapse densities observed in vivo in WT and NCAM-null mice, the culture assay does not distinguish ephrin-A5-induced growth cone repulsion from elimination of nascent synapses. To address this question, live imaging of perisomatic synapse formation and elimination in vivo in mice lacking NCAM and/or EphA3 would be informative.

NCAM and ephrin-A5 are cleaved by EphA3-dependent ADAM10 protease, a necessary step for growth cone collapse (62–64). This study indicates that NCAM binding to EphA3 facilitates EphA3 receptor clustering and intracellular signaling through RhoA/ROCK; thus, subsequent ADAM10-mediated
cleavage of NCAM may enable termini to retract from the opposing cell or adhesive substrate. EphA3 kinase activity is required for ADAM10-mediated NCAM cleavage (62); therefore, NCAM-mediated clustering and activation of EphA3 are likely upstream of NCAM cleavage. Notably, the interaction interfaces used by ADAM10 and NCAM for binding EphA3 are not overlapping. The EphA3 LBD interacts directly with ADAM10 in a favorable conformation or optimal proximity to cleave NCAM at perisomatic synapses that are eliminated during development.

These findings support a mechanism in which NCAM stimulates ephrin-A5-induced clustering of EphA3 receptors through interactions of NCAM with EphA3 receptors essential for growth cone collapse through RhoA/ROCK activation in cortical GABAergic interneurons (Fig. 6). As clustering of EphA receptors is essential for downstream signaling at localized sites in the neuronal membrane, the finding that NCAM can enhance or stabilize the formation of EphA3 clusters reveals an additional level of regulation in modulating growth cone guidance. NCAM and EphA3 are both prominently involved in development of the nervous system as well as in some forms of cancer (46, 56, 65); therefore, understanding how these two receptors interact functionally is relevant to both normal development and disease states.

**Experimental Procedures**

**Mice**—The NCAM-null mutant (66, 67) and WT mice were used according to the University of North Carolina Institutional Animal Care and Use Committee policies and in accordance with the National Institutes of Health guidelines. All mice were maintained on the C57BL/6 background.

**Immunochromatography and Reagents**—The monoclonal antibodies used were anti-NCAM (OB11, Sigma-Aldrich), anti-phototyrosine (Tyr(P))-99, Santa Cruz Biotechnology), anti-RhoA (610990, BD Transduction Laboratories), anti-Cdc42 (MAB2810, a gift from Dr. Patrick Brennwald, University of North Carolina Chapel Hill (68)), and anti-Rac1 (102, BD Transduction Laboratories). The polyclonal antibodies were EphA3 (C-19, Santa Cruz Biotechnology), NCAM (H300, Santa Cruz Biotechnology), GABA (A2052, Sigma; ab17413, Abcam), and anti-GFP (13970, Abcam). Normal rabbit IgG and goat anti-human IgG were purchased from Jackson ImmunoResearch Laboratories. Secondary antibodies purchased from Life Technologies included anti-mouse Alexa 488, anti-mouse Alexa 555, anti-mouse Alexa 647, anti-rabbit Alexa 555, and anti-rabbit Alexa 647. Other secondary antibodies used were anti-rabbit Alexa 405 (Abcam), anti-guinea pig Alexa 405 (Sigma-Aldrich), anti-mouse-HRP (Pierce), and donkey anti-rabbit HRP (Sigma-Aldrich). Recombinant ephrin-A5-Fc and human Fc (R&D Systems) were also used. Human NCAM-Fc proteins (69) were purified from transfected HEK293T cell conditioned media using Protein A-Sepharose (Pierce).

**Modeling of NCAM/EphA3 Binding Site**—The structure prediction server HHpred (70, 71) identified the crystal structure of the ectodomain of human EphA4 (PDB code 4M4P) (41), a closely related homolog sharing 68% sequence identity with mouse EphA3, as a template for predicting a structural model of the mouse EphA3 LBD and CRD domains. A model of the EphA3 LBD and CRD domains was built using MODELLER (72). The ClusPro protein-protein docking server (40, 73) was used to predict potential interactions between EphA3 and NCAM using the EphA3 CRD domain model and the Ig2 domain of rat NCAM from PDB code 1QZ1 (38), a crystal structure of the rat NCAM Ig1–Ig2–Ig3 domains. Docking poses were screened for compatibility with interaction on the surface of a membrane in a cis conformation, lack of steric hindrance between additional domains of the molecules, and the number of hydrogen bonds within the interaction interface. The top candidate was analyzed for residues to mutate and assayed for verification of the predicted interface.

**Site-directed Mutagenesis**—Mutations were made in the Ig2 domain of rat NCAM 140 (R192E, R156E, and K162D) in pCDNA3 and in the cysteine-rich domain of mouse EphA3 (E248R and E264K). Deletion mutagenesis was used to delete the Ig1 domain from NCAM Ig1–2-Fc cDNA while preserving the signal peptide. All mutations were generated with the Q5 site-directed mutagenesis kit (E0554S, New England Biolabs). The mutagenic primers were as follows: NCAM R192E, 5’-CTGTAAGGCGAGATCTGCGCC-3’ and 5’-CG GAAGTCCTCTAG C-3’; NCAM R156E, 5’-ACACAAAGGCGAAGATGT CATCCCC-3’ and 5’-TTCCAGATGATGGTTGGG-3’; NCAM K162D, 5’-CATCCTGAAAGGACGTGTCG-3’ and 5’-CATCTGGCCTGGTTGGTTC-3’; EphA3 E248R, 5’-CACAGAAGGAGATGGCTGGTTCC-3’; NCAM K162D, 5’-CATCCTGAAAGGACGTGTCG-3’ and 5’-ACATCTGGCTGGTTGGTTC-3’; EphA3 E264K, 5’-TTGGATGAAAAA GAGGTTCATCATATG-3’ and 5’-GACTTCAAGT GCATTGCAAGT-3’; and NCAMtg1-deletion, 5’-GCCA CCGTCAAGGTTGAG-3’ and 5’-CAGGAA AAAC AAAGTCCAGATGAG-3’.

**Immunoprecipitation and Pulldown Assays**—Proteins from cell lysates (1 mg) in radioimmunoprecipitation assay buffer (20 mM Tris (pH 7.0), 0.15 mM NaCl, 5 mM EDTA, 1 mM EGTA, 1% Nonidet P-40, 1% deoxycholate, 0.1% SDS, 200 μM Na3VO4, 10 mM NaF, and 1× protease inhibitor (Sigma)) were prepared as described previously (74), and proteins were precipitated using antibodies against EphA3 (C-19, Santa Cruz Biotechnology) or NCAM (H-300, Santa Cruz Biotechnology), with protein A/G-agarose beads (Thermo Fisher). For detection of EphA3 phosphorylation, WT and NCAM-null cells were treated with precluetered ephrin-A5-Fc or control Fc (75) for 10 min and then lysed in radioimmunoprecipitation assay buffer. EphA3 was immunoprecipitated, and tyrosine phosphorylation was detected with anti-Tyr(P)-99. For coimmunoprecipitation of EphA3 and NCAM140, HEK293T cells were transfected with EphA3 and WT or mutant NCAM140 cDNA using Lipofectamine 2000 (Invitrogen). For pulldown assays, HEK293T cells were transfected with WT EphA3, EphA3ΔLBD, or EphA3ΔLBD/CRD. After 48 h, proteins in cell lysates (500 μg) were incubated with NCAM-Fc proteins and protein A/G-agarose beads to detect protein interactions. Protein complexes were separated by SDS-PAGE, immunoblotted using antibodies to EphA3, NCAM, or phosphotyrosine, and detected with horseradish peroxidase-conjugated secondary antibodies.
NCAM/EphA3 Clustering Mediates Neuronal Growth Cone Collapse

Immunostaining and Colocalization Analysis—For cortical neuron cultures, embryonic day 0.5 (E0.5) was defined as the plug date, and embryonic cultures were made at E15.5. Dissociated cortical neurons from NCAM KO mice (E15.5) were plated onto poly-D-lysine- and laminin-coated Lab-Tek II chamber slides (1.5 × 10^5 cells/well) as described previously (74, 76). At 10 DIV, NCAM KO neurons were co-transfected with WT or mutant NCAM140 and pCAG-IREs-EGFP using Lipofectamine 2000, and 48 h later, cells were stimulated with 1 μg/ml preclustered Fc or ephrin-A5-Fc for 30 min. Cells were fixed in 4% paraformaldehyde and blocked and permeabilized in 10% horse serum and 0.5% Triton X-100/PBS. Slides were incubated in primary antibodies (anti-NCAM, anti-EphA3, and anti-GABA) overnight at 4 °C. Secondary antibodies (Alexa 405, Alexa 555, and Alexa 647) were added for 1 h and mounted using SlowFade (Life Technologies). Confocal images were obtained with Zeiss LSM700 and LSM710 microscopes using a Plan-Apochromat ×63 1.4 numerical aperture objective with x2 optical zoom using Zeiss Zen software. Only GFP/GABA double-positive cells were imaged for analysis. Colocalization of NCAM and EphA3 was analyzed using the ImageJ plugins Colocalization_Test and Colocalization_Threshold by T. Collins (Wright Cell Imaging Facility, Toronto Western Research Institute, Toronto, ON, Canada) and W. Rasband (Research Services Branch, NIMH, National Institutes of Health, Baltimore, MD). Colocalization was expressed as three parameters: R-coloc (the Pearson correlation coefficient, which varies between −1 and 1) and thresholded Manders coefficients (tM1, red channel, EphA3; tM2, green channel, NCAM), both of which represent a fraction between 0 and 1. Each of these values was calculated for pixels above a threshold level determined by the regression algorithm contained in the Colocalization_Threshold macro. For each condition, an average from measurements of at least 45 images, in which individual GFP/GABA-positive cells had been selected as regions of interest, was reported. Images of colocalized pixels generated by the Colocalization_Threshold plugin were converted to 8-bit images, and the “Fire” lookup table of ImageJ was applied to generate heat maps of colocalization.

RhoA, Rac1, and Cdc-42 GTase Assays—To measure RhoA, Cdc42, and Rac1 activation, neurons from WT or NCAM-null E15.5 mice were cultured as described previously (76) for 10 days and then treated with preclustered Fc or ephrin-A5-Fc (1 μg/ml) (75) for 30 min. The assay of Rac1 activity was performed as described previously (77) with modifications. Cells were first rinsed in chilled PBS and lysed in 50 mM Tris (pH 7.6), 150 mM NaCl, 1% Triton X-100, 0.5 mM MgCl_2, and 1× protease inhibitor mixture (Sigma). GTp-bound activated Rac1 was affinity-purified from cell lysates using an immobilized GST fusion of the Rac1 binding domain of murine p65Pak (the PBD) that binds Rac1-GTP but not Rac1-GDP (78). Rac1 that sedimented with the GST-PBD beads was separated using SDS-PAGE, transferred to a polyvinylidenefluoride membrane, and blotted with an antibody against Rac1. Cdc42-GTP binds to the same PBD construct (78), and so the same assay was used to measure Cdc42 activity, except that the blots were probed with an antibody against Cdc42 (MAB28–10) (68). Essentially the same assay was used to measure RhoA-GTP, except the RBD of Rhotekin (79) was used as the GST construct. RhoA that sedimented with the GST-RBD beads was detected with an antibody against RhoA. The GST-PBD and GST-RBD beads were kindly provided by the laboratory of Dr. Keith Burridge (University of North Carolina Chapel Hill).

Growth Cone Collapse Assay—Dissociated cortical neuron cultures were generated from NCAM-null mice as described above. At 10 DIV, WT or mutant NCAM140 plasmids were transfected along with pCAG-IREs-EGFP using Lipofectamine 2000. After 48 h, cells were treated with preclustered ephrin-A5-Fc or human Fc (1 μg/ml) for 30 min and fixed, and growth cones were visualized by immunofluorescence of staining for GFP and GABA (2). Growth cones were scored as collapsed by bullet-shaped morphology and non-collapsed by spread morphology, and the percentage of collapsed growth cones of GFP/GABA-positive neurons was compared (10 fields/well, ≥2 wells/experiment, ≥300 growth cones/condition). For Rock inhibition, neurons were pretreated with 1 μg/ml C3 (Cytoskeletal, Inc.) for 4 h prior to treatment with preclustered Fc or ephrin-A5-Fc. For ROCK inhibition, neurons were pretreated with 10 μM Y-27632 for 2 h prior to stimulation with preclustered ephrin-A5-Fc or control Fc.

Author Contributions—C. S. S. conducted most of the experiments, analyzed the results, and wrote the manuscript. M. K. made the EphA3 point mutants and performed co-immunoprecipitation experiments with the NCAM point mutants. B. S. T. performed molecular modeling of the NCAM/EphA3 binding site, predicted residues for mutagenesis, and provided advice on manuscript preparation. P. F. M. provided advice on experiments and data analysis and edited the manuscript.

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