

NCAM induces CaMKII α -mediated RPTP α phosphorylation to enhance its catalytic activity and neurite outgrowth

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Receptor protein tyrosine phosphatase α (RPTP α) phosphatase activity is required for intracellular signaling cascades that are activated in motile cells and growing neurites. Little is known, however, about mechanisms that coordinate RPTP α activity with cell behavior. We show that clustering of neural cell adhesion molecule (NCAM) at the cell surface is coupled to an increase in serine phosphorylation and phosphatase activity of RPTP α . NCAM associates with T- and L-type voltage-dependent Ca²⁺ channels, and NCAM clustering at the cell surface results in Ca²⁺ influx via these channels and activation of

NCAM-associated calmodulin-dependent protein kinase II α (CaMKII α). Clustering of NCAM promotes its redistribution to lipid rafts and the formation of a NCAM–RPTP α –CaMKII α complex, resulting in serine phosphorylation of RPTP α by CaMKII α . Overexpression of RPTP α with mutated Ser180 and Ser204 interferes with NCAM-induced neurite outgrowth, which indicates that neurite extension depends on NCAM-induced up-regulation of RPTP α activity. Thus, we reveal a novel function for a cell adhesion molecule in coordination of cell behavior with intracellular phosphatase activity.

Introduction

Cell interactions in the nervous system depend on multiple cues acting sequentially or in parallel. Adhesion molecules initiate recognition of the extracellular matrix and other cells, and, as transmembrane receptors, activate intracellular signaling cascades fundamental to all aspects of cell behavior. This line of communication is important not only during ontogenetic development but also in the adult nervous system during functional changes, such as learning, memory, and regeneration after traumatic injury. The neural cell adhesion molecule (NCAM) has been recognized as an important mediator of cell interactions via its extracellular domain, which consists of immunoglobulin-like and fibronectin type III–homologous structures that act as ligand and receptors in homophilic and heterophilic cell interactions. Two of the major isoforms of NCAM with molecular

masses of 180 kD (NCAM180) and 140 kD (NCAM140) are transmembrane glycoproteins that trigger signaling cascades in the cell interior when clustered either by their natural ligands or by antibodies (Schuch et al., 1989; for review see Maness and Schachner, 2007). Signaling cascades triggered by NCAM have been implicated in neurite outgrowth, neuronal survival, and synaptic plasticity (Rutishauser et al., 1988; Lüthi et al., 1994; Bukalo et al., 2004; Walmod et al., 2004).

The most well-described intracellular signaling pathways activated by NCAM to induce neurite outgrowth and neuronal differentiation include activation of PKC with subsequent NCAM-dependent redistribution of the enzyme to cholesterol-enriched plasma membrane microdomains, so-called lipid rafts, where PKC activates GAP43 (Leshchyn's'ka et al., 2003; Korshunova et al., 2007). Association of PKC with NCAM depends on the FGF receptor (Leshchyn's'ka et al., 2003), which associates with NCAM and is activated in response to NCAM clustering at the cell surface (Niethammer et al., 2002; Kiselyov et al., 2005).

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Abbreviations used in this paper: Bisl, bisindolylmaleimide I; CaM, calmodulin; CaMKII α , CaM-dependent protein kinase II α ; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GPI, glycosylphosphatidylinositol; NCAM, neural cell adhesion molecule; RPTP α , receptor protein tyrosine phosphatase α ; RPTP α -ID, intracellular domains of RPTP α ; RPTP α WT, wild-type RPTP α ; VDCC, voltage-dependent Ca²⁺ channels.

The online version of this paper contains supplemental material.

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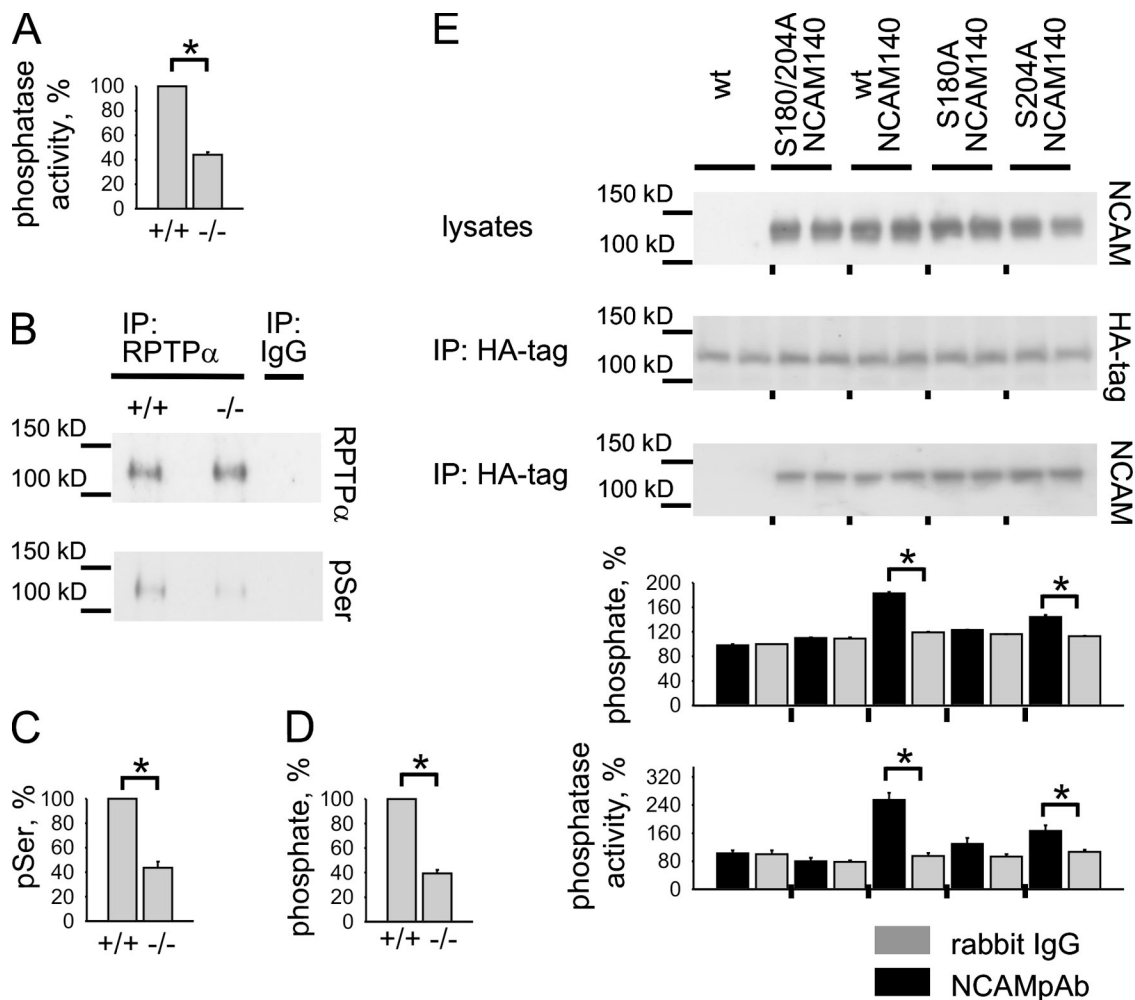


Figure 1. NCAM140 clustering induces phosphorylation of RPTP α on Ser180 and Ser204 and enhances the phosphatase activity of RPTP α . (A) Graph shows phosphatase activity of RPTP α from NCAM $^{+/+}$ and NCAM $^{-/-}$ brain lysates. (B–D) RPTP α immunoprecipitates were probed by Western blotting with antibodies against RPTP α and phospho serines (B and C) or subjected to serine phosphorylation estimation by the alkaline hydrolysis (D). Note that similar levels of RPTP α were immunoprecipitated from NCAM $^{+/+}$ and NCAM $^{-/-}$ brain lysates (B). Mock immunoprecipitation with nonspecific IgG served as a control. Phosphatase activity and serine phosphorylation of RPTP α are reduced in NCAM $^{-/-}$ brains. Graph in C shows quantification of the blots in B with optical density for NCAM $^{+/+}$ brains set to 100%. In A and D, mean values of phosphatase activity (A) or phosphate released by alkaline (D) in RPTP α immunoprecipitates from NCAM $^{+/+}$ brains were set to 100%. (E) RPTP α -negative fibroblasts transfected with RPTP α WT alone or cotransfected with NCAM140 and RPTP α WT, RPTP α S180A, RPTP α S204A, or RPTP α S180/204A were treated with NCAM polyclonal antibodies or nonspecific rabbit IgG. Note that similar levels of NCAM140 were expressed in cells (lysates) and that similar levels of RPTP α were immunoprecipitated from the lysates (IP: HA-tag). Mutation of Ser180 and/or Ser204 does not influence coimmunoprecipitation of NCAM140 with RPTP α . Immunoprecipitated RPTP α was then subjected to the serine phosphorylation estimation by the alkaline hydrolysis (top) and phosphatase activity analysis (bottom). Application of NCAM antibodies but not IgG increased serine phosphorylation and phosphatase activity of RPTP α WT in NCAM140–RPTP α WT–cotransfected cells. Phosphorylation and activation of RPTP α mutants were inhibited. Mean values of phosphate released by alkaline (top) or phosphatase activity (bottom) in RPTP α immunoprecipitates from IgG-treated NCAM140–RPTP α WT–cotransfected cells were set to 100%. Mean values \pm SEM are shown (A and D, $n \geq 8$; C, $n = 6$; E, $n \geq 6$). *, $P < 0.05$ (paired t test).

Another pathway includes activation of p59fyn (hereafter referred to as fyn)/FAK (Beggs et al., 1994, 1997) being induced in response to NCAM clustering or via binding of the glial cell line–derived neurotrophic factor (GDNF) to NCAM (Paratcha et al., 2003). Activation of this pathway depends on NCAM's association with glycosylphosphatidylinositol (GPI)-anchored proteins, such as prion protein (Santucci et al., 2005) and GFR α 1, a cognate receptor for GDNF (Paratcha et al., 2003), and palmitoylation of the intracellular domain of NCAM (Niethammer et al., 2002), linking NCAM to p59fyn enriched in lipid rafts. We have previously found that this pathway is induced by NCAM140, which associates with the receptor protein tyrosine phosphatase α

(RPTP α) by direct interaction (Bodrikov et al., 2005). When NCAM is clustered at the neuronal cell surface, the NCAM140–RPTP α complex is further stabilized by the membrane–cytoskeleton linker protein spectrin and redistributes to lipid rafts, where RPTP α binds to and activates fyn (Bodrikov et al., 2005).

We now present evidence that clustering of NCAM at the cell surface results in an enhancement of serine phosphorylation and phosphatase activity of RPTP α . By investigating the mechanisms of NCAM-dependent RPTP α activation, we found that PKC δ , which had been shown in other studies to mediate activation of RPTP α (Brandt et al., 2003), is not involved in NCAM-induced activation. Instead, we identified calmodulin

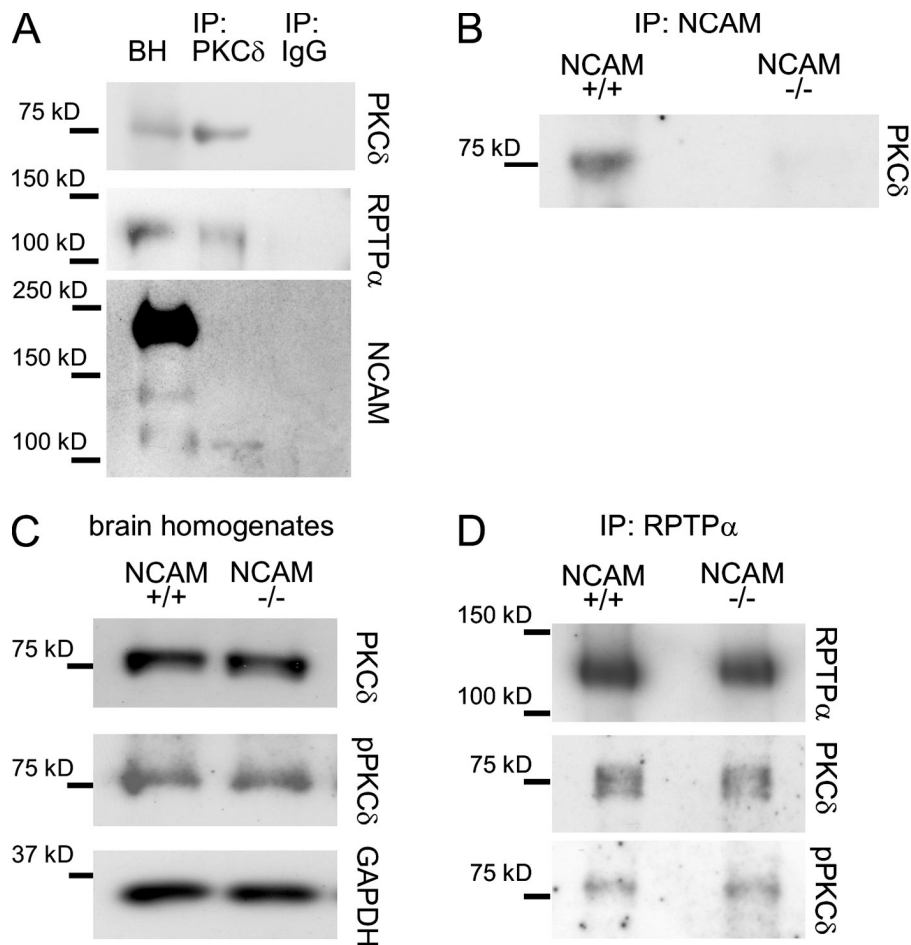


Figure 2. PKC δ associates with NCAM and RPTP α but its activity is not regulated by NCAM. (A and B) PKC δ (A) or NCAM (B) immunoprecipitates (IP) from NCAM $^{+/+}$ brain lysates were analyzed by Western blotting as indicated. Mock immunoprecipitation with nonspecific IgG (A) or from NCAM $^{-/-}$ brain lysates (B) served as a control. RPTP α and NCAM120 coimmunoprecipitate with PKC δ , and PKC δ coimmunoprecipitates with NCAM. BH, brain homogenate. (C and D) NCAM $^{+/+}$ and NCAM $^{-/-}$ brain homogenates (C) and RPTP α immunoprecipitates from NCAM $^{+/+}$ and NCAM $^{-/-}$ brain lysates (D) were probed by Western blotting with antibodies against total PKC δ and active Thr505-phosphorylated PKC δ . Note the similar loading (GAPDH labeling in C) and immunoprecipitation efficiency (RPTP α labeling in D). Total levels of active PKC δ and levels of active PKC δ associated with RPTP α are not changed in NCAM $^{-/-}$ brains.

(CaM)-dependent protein kinase II α (CaMKII α) as a previously unrecognized enzyme to bind to and phosphorylate RPTP α at two serine residues that increase the phosphatase activity of RPTP α . We show that clustering of NCAM at the cell surface induces lipid raft-dependent activation of CaMKII α , which then phosphorylates RPTP α at the two serine residues, which, in turn, leads to activation of fyn. Overexpression of RPTP α mutated in both serine residues interferes with NCAM-induced neurite outgrowth of hippocampal neurons in vitro. These observations attribute an important role in the trifunctional interaction between NCAM, CaMKII α , and RPTP α in lipid rafts and thus add a new dimension in NCAM-mediated signaling pathways.

Results

NCAM up-regulates RPTP α activity by increasing its phosphorylation on Ser180 and Ser204

To analyze the role of NCAM in regulation of the phosphatase activity of RPTP α , RPTP α was immunoprecipitated from NCAM $^{+/+}$ and NCAM $^{-/-}$ brain lysates. We then used two commercially available phosphotyrosine-containing peptides derived from the epidermal growth factor receptor, which serve as substrates for many protein tyrosine phosphatases, and estimated the efficiency of the release of phosphate from these peptides in the presence of RPTP α immunoprecipitates. This analysis showed that the

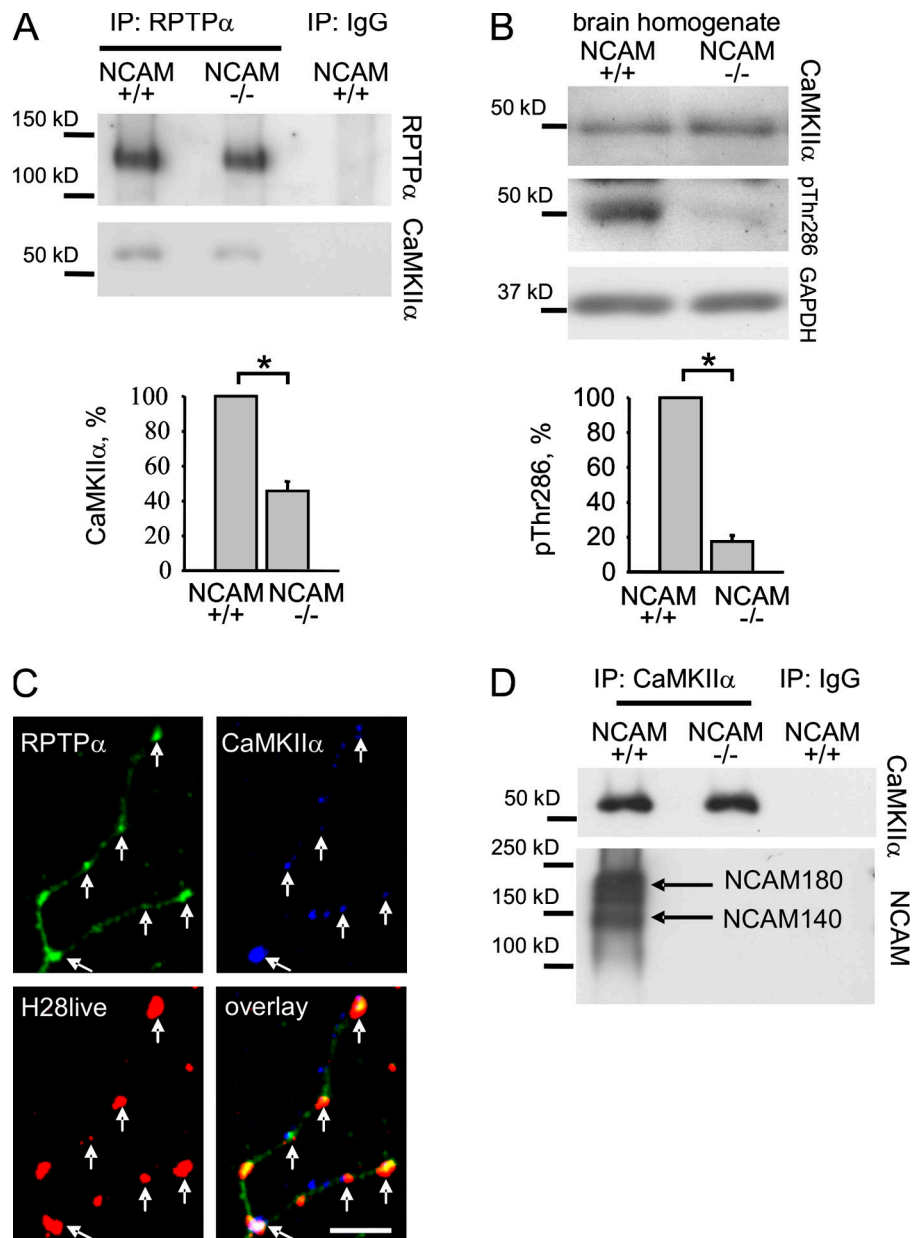
phosphatase activity of RPTP α from NCAM $^{-/-}$ brains was reduced by $\sim 55\%$ when compared with RPTP α from NCAM $^{+/+}$ brains (Fig. 1 A).

Because the phosphatase activity of RPTP α is regulated by phosphorylation, we analyzed phosphorylation of RPTP α by probing RPTP α immunoprecipitates with antibodies against phosphorylated serine residues by Western blotting. In addition, we estimated levels of phosphate released from serine residues of the immunoprecipitated RPTP α by alkaline treatment. Both methods showed that serine phosphorylation of RPTP α was reduced in NCAM $^{-/-}$ brains by $\sim 60\%$ (Fig. 1, B–D).

Phosphorylation of Ser180 and Ser204 within RPTP α intracellular domain has been shown to be critical for the induction of RPTP α phosphatase activity (Stetak et al., 2001; Zheng et al., 2002). To analyze the role of Ser180 and Ser204 in NCAM-mediated RPTP α regulation, an HA tag containing wild-type RPTP α (RPTP α WT) and RPTP α mutants with Ser180 (RPTP α S180A), Ser204 (RPTP α S204A), or both (RPTP α S180/204A) substituted with alanine were coexpressed with NCAM140 in RPTP α -deficient fibroblasts. Similar levels of NCAM140 coimmunoprecipitated with RPTP α WT and RPTP α mutants from lysates of transfected cells (Fig. 1 E). Thus, mutation of Ser180 and Ser204 does not affect the binding of RPTP α to NCAM. Clustering of NCAM140 at the surface of fibroblasts by NCAM antibodies increased levels of serine phosphorylation of RPTP α WT by $\sim 200\%$ (Fig. 1 E). In contrast, phosphorylation of RPTP α S180A

Figure 3. NCAM promotes CaMKII α activation and RPTP α -CaMKII α complex formation.

(A) RPTP α immunoprecipitates (IP) from NCAM $^{+/+}$ and NCAM $^{-/-}$ brain lysates were probed by Western blotting with antibodies against CaMKII α . Note that similar levels of RPTP α were immunoprecipitated. Mock immunoprecipitation with nonspecific IgG served as control. Coimmunoprecipitation of CaMKII α with RPTP α is reduced in NCAM $^{-/-}$ brain lysates. (B) NCAM $^{+/+}$ and NCAM $^{-/-}$ brain homogenates were probed by Western blotting with antibodies against total and active Thr286-phosphorylated CaMKII α and GAPDH (loading control). The levels of active CaMKII α were reduced in NCAM $^{-/-}$ brain homogenates. Graphs show quantitation of the blots (mean \pm SEM, $n = 6$ for A and B) with optical density for NCAM $^{+/+}$ probes set to 100%. *, $P < 0.05$, paired t test. (C) NCAM was clustered at the cell surface of cultured hippocampal neurons by NCAM antibodies (H28live). Neurons were then fixed and colabeled with antibodies against RPTP α and CaMKII α . A high-magnification image of a neurite is shown. NCAM clusters overlap with accumulations of RPTP α and CaMKII α (arrows). Bar, 5 μ m. (D) CaMKII α immunoprecipitates from NCAM $^{+/+}$ and NCAM $^{-/-}$ brain lysates were probed by Western blotting with antibodies against NCAM. NCAM140 and NCAM180 coimmunoprecipitated with CaMKII α . Mock immunoprecipitation with nonspecific IgG served as a control.



and RPTP α S180/204A in response to NCAM140 clustering was blocked, whereas phosphorylation of RPTP α S204A was partially inhibited (Fig. 1 E). Hence, Ser180 and Ser204 are phosphorylated in response to NCAM140 clustering, with Ser180 being required for NCAM-dependent RPTP α phosphorylation.

Phosphatase activity of RPTP α WT was increased after NCAM140 clustering by $\sim 250\%$ (Fig. 1 E). Phosphatase activity of RPTP α mutants, however, was reduced when compared with RPTP α WT. Mutation of Ser180 induced a stronger inhibitory effect on the catalytic activity of RPTP α when compared with Ser204 (Fig. 1 E). The catalytic activity of RPTP α was fully inhibited when both Ser180 and Ser204 were mutated (Fig. 1 E). In contrast to fibroblasts transfected with NCAM140, application of NCAM antibodies to the fibroblasts cotransfected with RPTP α WT and NCAM180 or NCAM120 did not result in phosphorylation and activation of RPTP α WT (Fig. S1, available at <http://www.jcb.org/cgi/content/full/jcb.200803045/DC1>).

Hence, NCAM140 is the major NCAM isoform involved in regulation of RPTP α activity.

NCAM does not regulate PKC δ activity and its association with RPTP α

Previous studies indicated that among all PKC isoforms, only PKC δ phosphorylates RPTP α on Ser180 and Ser204 in nonneuronal cells (den Hertog et al., 1995; Tracy et al., 1995; Zheng et al., 2002; Brandt et al., 2003). NCAM associates with and induces activation of several PKC isoforms (Leshchyn'ska et al., 2003; Kolkova et al., 2005), but its role in PKC δ activation has not been analyzed. We thus analyzed whether NCAM regulates RPTP α phosphorylation by inducing PKC δ activation. RPTP α coimmunoprecipitated with PKC δ (Fig. 2 A), and PKC δ coimmunoprecipitated with NCAM (Fig. 2 B) from brain lysates. However, levels of activated PKC δ phosphorylated at Thr505 in the activation loop of the kinase were not changed in NCAM $^{-/-}$ brain homogenates

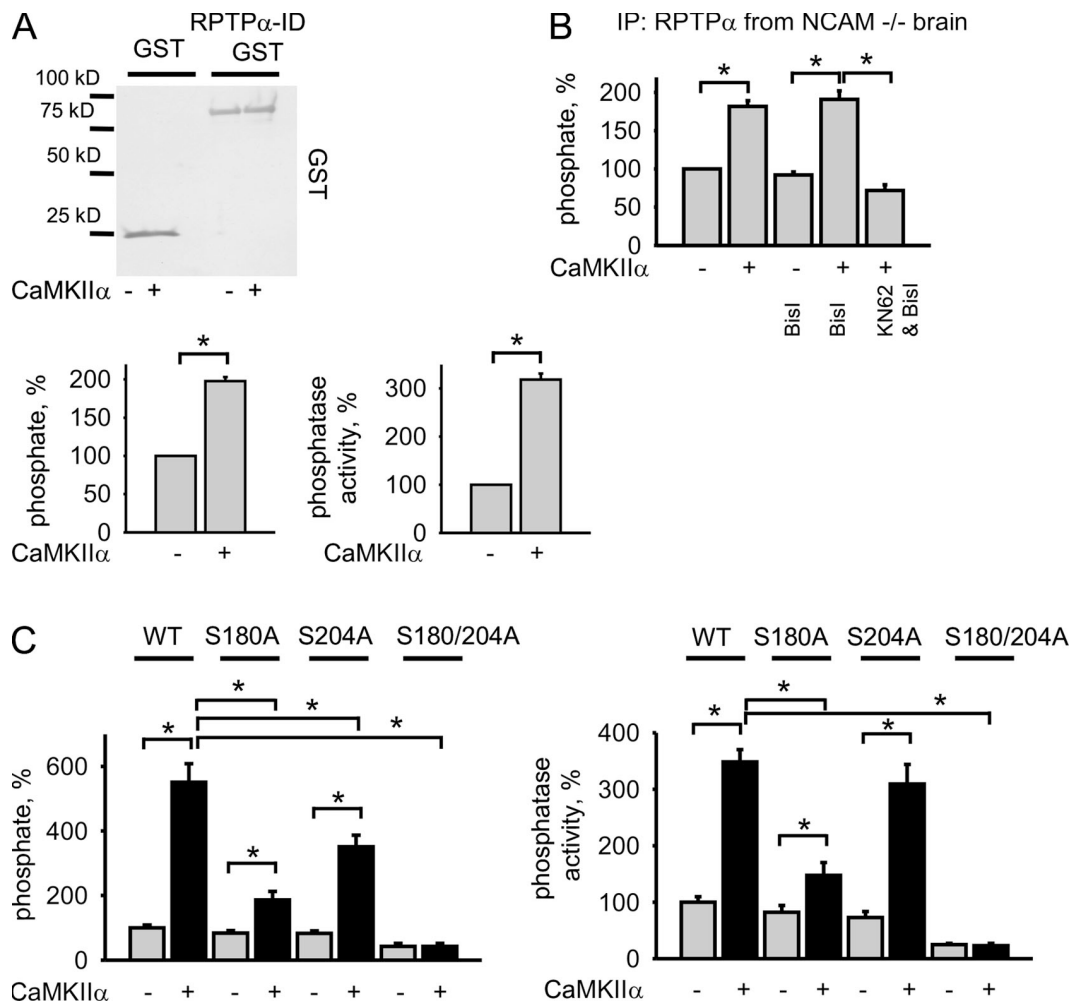


Figure 4. RPTP α is phosphorylated by CaMKII α on Ser180 and Ser204. (A) Recombinant RPTP α -ID-GST or GST coupled to beads were incubated with CaMKII α . The gel stained with Coomassie blue shows that approximately equal amounts of RPTP α -ID-GST and GST were bound to beads. The beads, treated and nontreated with CaMKII α , were then subjected to the analysis of serine phosphorylation by alkaline hydrolysis (bottom left) or phosphatase activity (bottom right). Values for GST were subtracted as background, and values of serine phosphorylation (bottom left) or phosphatase activity (bottom right) for RPTP α -ID-GST not treated with CaMKII α were set to 100%. (B) RPTP α from NCAM $^{-/-}$ brain lysates was incubated with CaMKII α in the presence or absence of KN62 and Bis1. RPTP α was then subjected to the analysis of serine phosphorylation by alkaline hydrolysis. Values of phosphate released by alkaline from RPTP α not treated with CaMKII α were set to 100%. (C) RPTP α immunoprecipitated from lysates of RPTP α -negative fibroblasts transfected with RPTP α WT, RPTP α S180A, RPTP α S204A, or RPTP α S180/204A was incubated with CaMKII α . The immunoprecipitates, treated and untreated with CaMKII α , were then subjected to the analysis of serine phosphorylation by alkaline hydrolysis (left) or phosphatase activity (right). Mean values of serine phosphorylation (left) or phosphatase activity (right) of RPTP α WT not treated with CaMKII α were set to 100%. Mean values \pm SEM are shown (A, $n = 6$; B, $n = 11$; C, $n = 9$). *, $P < 0.05$ (paired t test).

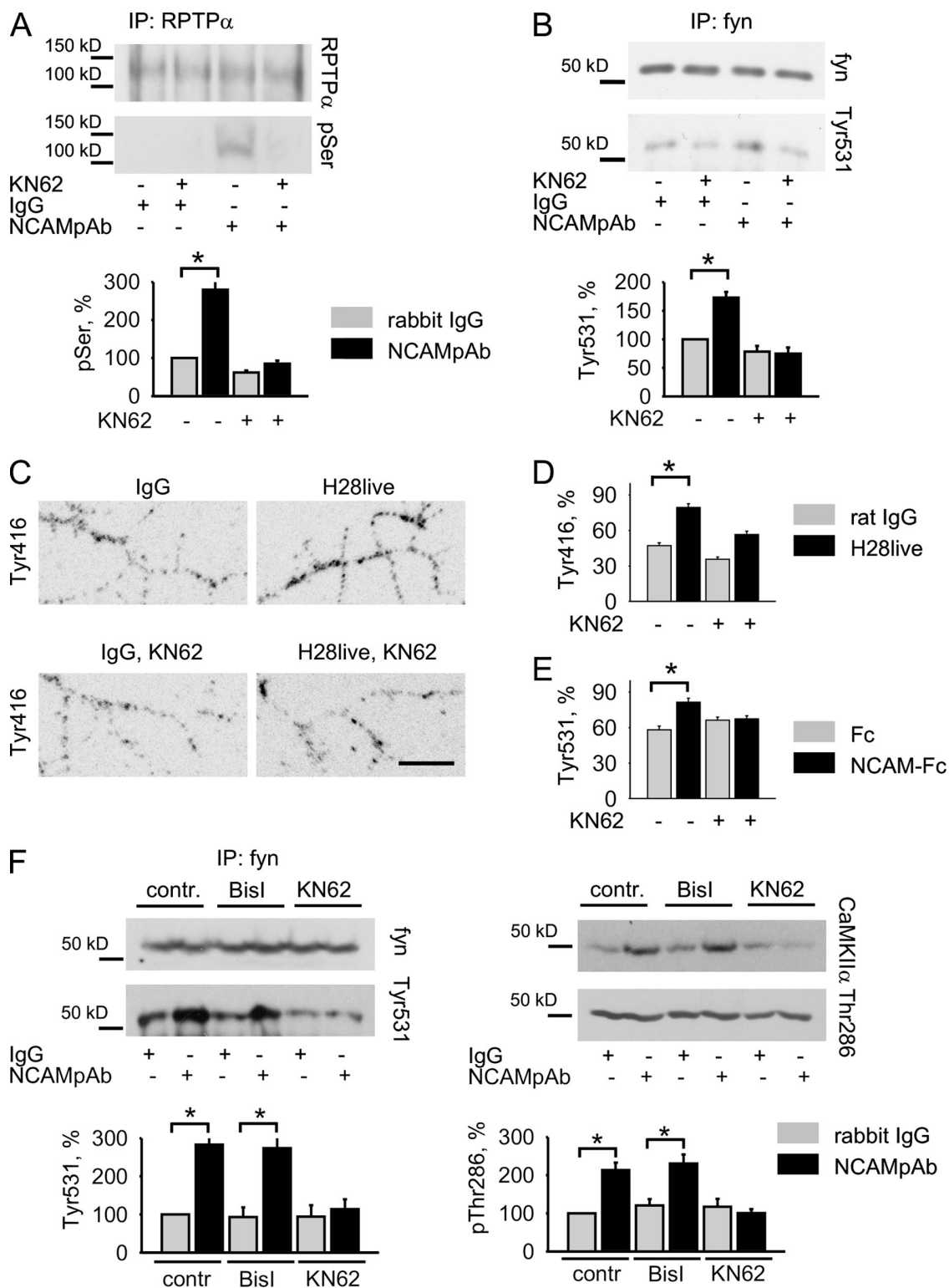
(Fig. 2 C). Furthermore, similar levels of activated PKC δ coimmunoprecipitated with RPTP α from NCAM $+/+$ and NCAM $-/-$ brain lysates (Fig. 2 D). Thus, NCAM does not regulate association of RPTP α with PKC δ , nor does it regulate PKC δ activation in the brain. In agreement, the most prominent NCAM isoform that coimmunoprecipitated with PKC δ was the smallest GPI-linked NCAM isoform with a molecular mass of 120 kD (NCAM120; Fig. 2 A). NCAM120 lacks the intracellular domain and therefore probably associates indirectly with PKC δ , as has been shown for spectrin, another intracellular binding partner of NCAM120 that associates with NCAM120 via lipids (Leshchynska et al., 2003).

NCAM induces RPTP α phosphorylation by activating CaMKII α

CaMKII α is another serine/threonine protein kinase that associates with NCAM (Sytnyk et al., 2006). CaMKII α coimmunoprecipi-

tated with RPTP α from brain lysates (Fig. 3 A). Interestingly, binding of CaMKII α to RPTP α was reduced in NCAM $-/-$ brains (Fig. 3 A). Levels of activated Thr286-phosphorylated CaMKII α were also reduced in NCAM $-/-$ brains (Fig. 3 B). Hence, NCAM regulates CaMKII α activation and RPTP α -CaMKII α complex formation.

In agreement, in cultured hippocampal neurons maintained *in vitro* for 24 h, CaMKII α accumulated in growth cones of the growing neurites, where distributions of CaMKII α and NCAM partially overlapped (see Fig. 7 B). Clustering of NCAM at the cell surface of neurites with NCAM antibodies applied to live neurons induced partial redistribution of RPTP α to NCAM clusters (Fig. 3 C; Bodrikov et al., 2005). Overlapping accumulations of NCAM and RPTP α also colocalized with CaMKII α aggregates (Fig. 3 C). Thus, CaMKII α is a likely candidate involved in NCAM140-induced RPTP α phosphorylation accompanying NCAM clustering.



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Figure 5. CaMKII α activity is required for NCAM140-induced fyn activation. (A and B) CHO cells were cotransfected with NCAM140 and RPTP α WT and treated with nonspecific rabbit IgG or NCAM polyclonal antibodies in the absence or presence of KN62. RPTP α and fyn immunoprecipitates from the lysates of these cells were then probed with phosphoserine antibodies (A) or antibodies against activated Tyr531-dephosphorylated fyn (B) by Western blotting. Note that similar levels of RPTP α and fyn were immunoprecipitated in all groups. KN62 inhibited serine phosphorylation of RPTP α and activation of fyn in response to NCAM antibodies. Graphs show quantitation of the blots (mean \pm SEM), with the levels in cells treated with nonspecific IgG without KN62 set to 100%. (C) Representative images of neurites of cultured hippocampal neurons treated with nonspecific rat IgG or NCAM monoclonal antibodies (H28live) in the presence or absence of KN62 and labeled with antibodies against activated Tyr416-phosphorylated fyn by indirect immunofluorescence. Inverted immunofluorescence images are shown to accentuate the differences in labeling intensities. Note the higher levels of activated fyn along neurites of NCAM antibody-treated neurons and the inhibition of this effect by KN62. Bar, 10 μ m. (D and E) Graphs show mean levels \pm SEM in arbitrary units of active Tyr416-phosphorylated (D) or Tyr531-dephosphorylated (E) fyn along neurites of hippocampal neurons treated with nonspecific rat IgG or NCAM monoclonal antibodies (H28live, D), or Fc or NCAM-Fc (E) in the absence or presence of KN62. $n > 90$ neurites from 45 neurons from 3 coverslips

In agreement with this idea, NCAM140 coimmunoprecipitated with CaMKII α from brain lysates (Fig. 3 D). NCAM180 also coimmunoprecipitated with CaMKII α (Fig. 3 D), in accordance with our previously published data (Sytnyk et al., 2006). It is interesting in this respect that clustering of NCAM140 induced activation of both CaMKII α and RPTP α (Figs. 1 and S2, available at <http://www.jcb.org/cgi/content/full/jcb.200803045/DC1>), whereas clustering of NCAM180 induced activation of CaMKII α but not RPTP α (Figs. S1 and S2). The intracellular domain of NCAM140 binds to the intracellular domain of RPTP α with higher affinity than the intracellular domain of NCAM180 in *in vitro* binding assays (Bodrikov et al., 2005). Furthermore, only NCAM140 but not NCAM180 associates with RPTP α in transfected CHO cells (Bodrikov et al., 2005). Collectively, previous observations and our new data suggest that the tight association of NCAM140 with RPTP α is required for CaMKII α -mediated serine phosphorylation of RPTP α .

CaMKII α phosphorylates the intracellular domain of RPTP α and increases its phosphatase activity

To directly analyze the CaMKII α -mediated serine phosphorylation of RPTP α , recombinant intracellular domains of RPTP α (RPTP α -ID) were used in an *in vitro* phosphorylation assay: incubation of RPTP α -ID with recombinant CaMKII α resulted in a pronounced increase in serine phosphorylation and phosphatase activity of RPTP α -ID (Fig. 4 A).

Next, we verified whether CaMKII α also phosphorylates endogenous full-length RPTP α , which was immunopurified from NCAM $-/-$ brain lysates to assure that serine phosphorylation of RPTP α was not saturated. Incubation of RPTP α with recombinant CaMKII α resulted in RPTP α phosphorylation (Fig. 4 B). Similarly, RPTP α was phosphorylated by CaMKII α immunopurified from rat brain and used instead of recombinant CaMKII α (unpublished data). Bisindolylmaleimide I (BisI), an inhibitor of PKC isozymes, among them PKC δ , had no effect on RPTP α phosphorylation in these experiments (Fig. 4 B), which excluded the possibility that RPTP α was phosphorylated by PKC δ copurified with RPTP α from brain lysates. Phosphorylation of RPTP α by recombinant CaMKII α was blocked in the presence of KN62, an inhibitor of CaMKII α (Fig. 4 B).

To analyze the sites of CaMKII α -mediated phosphorylation, RPTP α WT and RPTP α S180A, RPTP α S204A, and RPTP α S180/204A mutants were transfected into RPTP α -negative fibroblasts. Transfected proteins were then immunoprecipitated from cell lysates and incubated with recombinant CaMKII α . Incubation with CaMKII α resulted in an approximately sixfold increase in serine phosphorylation of RPTP α WT, whereas CaMKII α -mediated serine phosphorylation of RPTP α S180A and RPTP α S204A was reduced and serine phosphorylation of RPTP α S180/204A was fully blocked (Fig. 4 C). The levels of serine phosphorylation of RPTP α induced by CaMKII α correlated with the levels of

RPTP α phosphatase activity after incubation with CaMKII α : the highest increase in the phosphatase activity was observed for RPTP α WT, being lower for RPTP α S180A and RPTP α S204A, whereas it was not detectable for RPTP α S180/204A (Fig. 4 C). Interestingly, mutation of Ser180 had a more profound effect on CaMKII α -induced serine phosphorylation and phosphatase activity of RPTP α than the Ser204 mutation (Fig. 4 C), which correlates with the effect of these mutations on NCAM-induced RPTP α phosphorylation (Fig. 1). We conclude that CaMKII α phosphorylates RPTP α at Ser180 and Ser204.

CaMKII α is required for NCAM-induced fyn activation

To confirm that NCAM140-induced serine phosphorylation of RPTP α is mediated by CaMKII α in live cells, CHO cells were cotransfected with NCAM140 and RPTP α WT and treated with NCAM antibodies in the absence or presence of the CaMKII α inhibitor KN62. Application of NCAM antibodies strongly increased serine phosphorylation of RPTP α WT in transfected cells, as measured by Western blotting with phosphoserine-specific antibodies (Fig. 5 A). NCAM-dependent serine phosphorylation of RPTP α WT was inhibited by KN62 (Fig. 5 A), which indicates that CaMKII α activation is required for NCAM-induced RPTP α phosphorylation.

Because RPTP α is the major phosphatase that induces fyn activation in NCAM-activated signaling (Bodrikov et al., 2005), we analyzed whether CaMKII α activity is required for fyn activation in response to NCAM clustering at the cell surface. Application of NCAM antibodies to CHO cells cotransfected with NCAM140 and RPTP α WT increased levels of Tyr531-dephosphorylated and thus activated fyn in these cells, as measured by Western blot analysis (Fig. 5 B). NCAM-induced fyn activation was completely inhibited by KN62 (Fig. 5 B).

Similarly, KN62 inhibited fyn activation in response to application of NCAM antibodies or NCAM-Fc to cultured neurons as shown by immunofluorescence labeling or Western blot analysis of active fyn with antibodies specific for Tyr531-dephosphorylated or Tyr416-autophosphorylated fyn (Fig. 5, C–F). The PKC δ inhibitor BisI had no effect on NCAM-induced fyn activation (Fig. 5 F). Thus, we conclude that CaMKII α activity is required for NCAM-induced RPTP α -mediated fyn activation.

Association of NCAM with lipid rafts is required for NCAM-induced CaMKII α activation

Clustering of NCAM140 and NCAM180 induces their redistribution to lipid rafts (Niethammer et al., 2002; Leshchyns'ka et al., 2003; Bodrikov et al., 2005; Santucci et al., 2005). Therefore, we analyzed whether lipid rafts play a role in NCAM-induced CaMKII α activation. In cultured hippocampal neurons extracted with cold 1% Triton X-100 and colabeled with antibodies against Thr286-phosphorylated CaMKII α and the lipid raft marker PI(4,5)

analyzed in each group (for D and E). (F) Cultured cortical neurons were treated with nonspecific rabbit IgG or NCAM polyclonal antibodies in the absence or presence of BisI and KN62. Lysates of these cells and fyn immunoprecipitates from the lysates were then probed with the indicated antibodies by Western blotting. Note that KN62 but not BisI inhibited fyn and CaMKII α activation in response to NCAM antibodies. Graphs show quantitation of the blots (mean \pm SEM) with the levels in cells treated with nonspecific IgG without inhibitors set to 100%. *, $P < 0.05$ (paired *t* test).

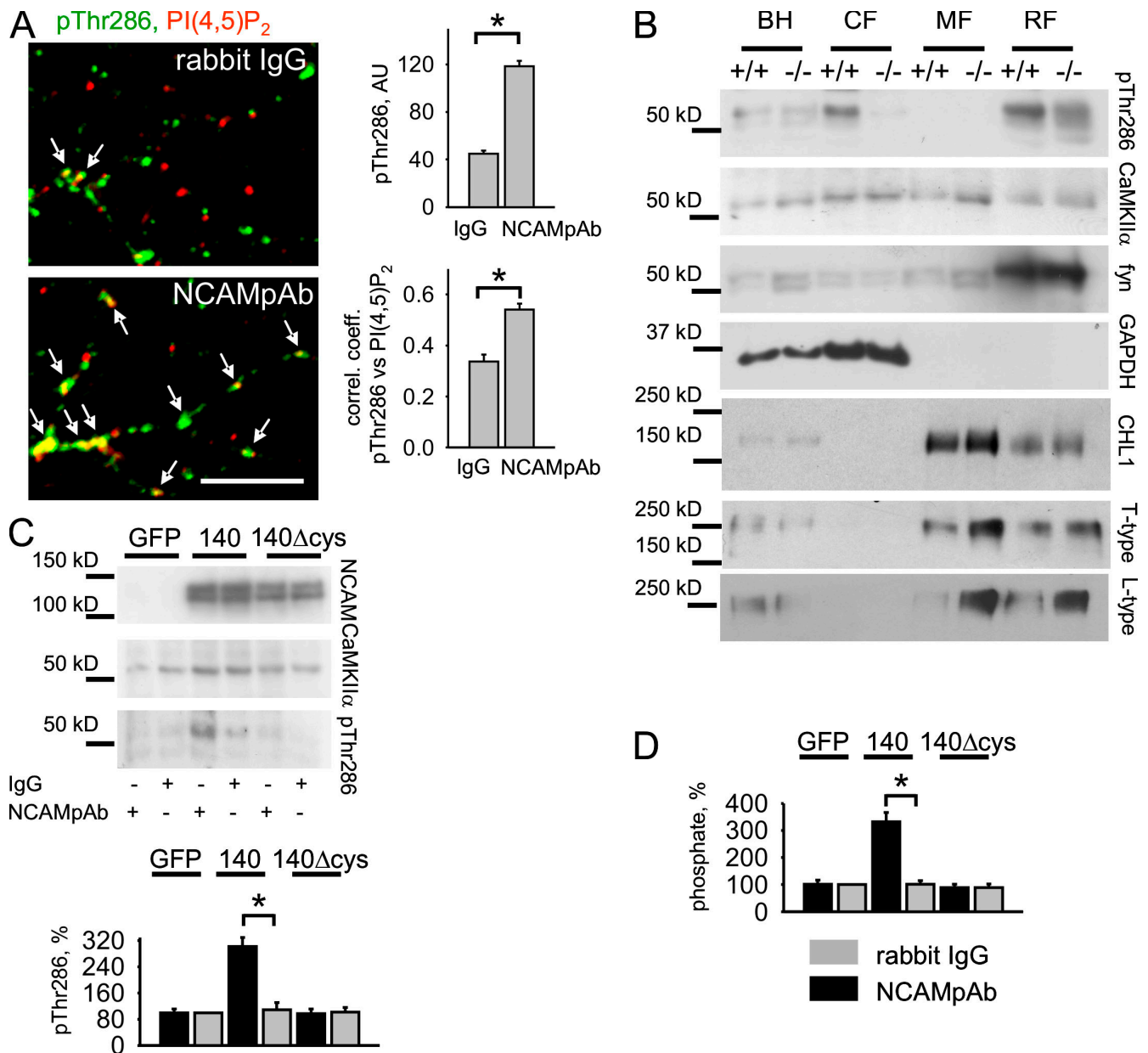


Figure 6. Association of NCAM140 with lipid rafts is required for CaMKII α activation. (A, left) Representative images of neurites of cultured hippocampal neurons treated with nonspecific rabbit IgG or NCAM polyclonal antibodies, extracted in cold 1% Triton X-100, and labeled with antibodies against activated Thr286-phosphorylated CaMKII α and PI(4,5)P₂ by indirect immunofluorescence. Note the higher levels of activated CaMKII α and the higher degree of colocalization of activated CaMKII α with PI(4,5)P₂ along neurites of NCAM antibody-treated neurons. Arrows show clusters of activated CaMKII α colocalizing with PI(4,5)P₂ accumulations. Bar, 10 μ m. (right) Graphs show mean levels \pm SEM in arbitrary units (AU) of active Thr286-phosphorylated CaMKII α (top) and coefficients of correlation between distributions of Thr286 phosphorylated CaMKII α and PI(4,5)P₂ (bottom) along neurites. $n > 90$ neurites from 45 neurons from 3 coverslips analyzed in each group. (B) Brain homogenates (BH), cytosolic fraction (CF), total membrane fraction (MF), and lipid raft fraction (RF) from NCAM^{+/+} and NCAM^{-/-} brains were probed by Western blotting with antibodies against activated Thr286 phosphorylated CaMKII α , total CaMKII α , T- and L-type VDCCs, lipid raft marker fyn, the soluble protein marker GAPDH, and the membrane protein marker CHL1. Note the accumulation of activated CaMKII α in lipid rafts. (C) Lysates of CHO cells cotransfected with RPTP α WT and NCAM140, NCAM140 Δ cys, or GFP and treated with nonspecific rabbit IgG or NCAM polyclonal antibodies were probed with antibodies against NCAM, activated Thr286-phosphorylated CaMKII α , and total CaMKII α . Note the similar levels of expression of NCAM140 and NCAM140 Δ cys but the reduced activation of CaMKII α in NCAM140 Δ cys-versus NCAM140-transfected cells. The graph shows quantitation of the blots with the levels of activated CaMKII α in GFP-transfected cells treated with nonspecific IgG set to 100%. (D) RPTP α was immunoprecipitated from cell lysates in C, and serine phosphorylation of RPTP α was analyzed by alkaline hydrolysis. The amount of phosphate released by alkaline hydrolysis from RPTP α from cells transfected with GFP instead of NCAM and treated with nonspecific IgG was set to 100%. For C and D, mean values \pm SEM are shown ($n = 6$). *, $P < 0.05$ (paired t test).

P2 (Niethammer et al., 2002), accumulations of activated CaMKII α partially overlapped with the clusters of PI(4,5)P₂ (Fig. 6A), which indicates that cytoskeleton- and/or lipid raft-associated detergent-insoluble pools of CaMKII α were present in neurites. Clustering of

NCAM at the cell surface with NCAM antibodies resulted in an approximately threefold increase in the levels of detergent-insoluble active CaMKII α and a higher overlap in distribution of CaMKII α and PI(4,5)P₂ along neurites when compared with

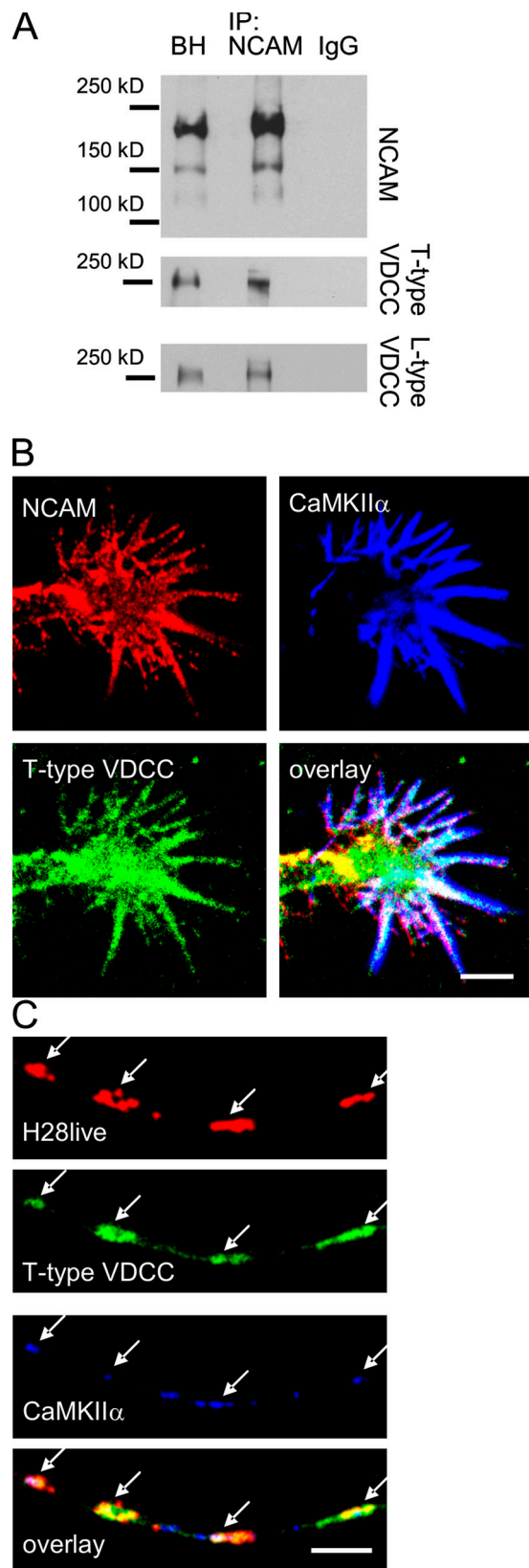


Figure 7. NCAM associates with T- and L-type VDCC. (A) NCAM immunoprecipitates (IP) from NCAM^{+/+} brain lysates were analyzed by Western blotting with antibodies against NCAM and T- and L-type VDCC. Mock immunoprecipitation with nonspecific IgG served as control. Brain homogenate (BH) is shown for comparison. T- and L-type VDCC coimmunoprecipitate with NCAM. (B) A growth cone of a cultured hippocampal neuron

neurons treated with nonspecific immunoglobulins (Fig. 6 A). This observation suggests that activation of CaMKII α in response to NCAM clustering occurs in lipid rafts.

In agreement, Western blot analysis of the cytosolic, total membrane, and lipid raft fractions isolated from the brain tissue of young mice showed that activated CaMKII α accumulated in lipid rafts (Fig. 6 B). In contrast, total CaMKII α protein was broadly distributed in all fractions (Fig. 6 B). Importantly, levels of activated CaMKII α but not total CaMKII α protein, fyn, or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were reduced in the cytosolic fraction and lipid rafts from NCAM^{-/-} versus NCAM^{+/+} brains (Figs. 6 B and S3, available at <http://www.jcb.org/cgi/content/full/jcb.200803045/DC1>), which indicates that NCAM is implicated in the activation of CaMKII α that accumulates in lipid rafts and/or redistributes to the soluble pool.

To analyze whether redistribution of NCAM to lipid rafts is required for CaMKII α activation and RPTP α phosphorylation, CHO cells were cotransfected with RPTP α WT and NCAM140 or NCAM140 Δ Cys, an NCAM140 mutant with inactivated palmitoylation sites within its intracellular domain that blocks its association with lipid rafts (Niethammer et al., 2002). Application of NCAM antibodies to NCAM140-transfected cells resulted in a pronounced increase in the levels of activated CaMKII α and serine phosphorylation of RPTP α WT in these cells (Fig. 6, C and D). Although expression levels of NCAM140 Δ Cys were similar to those of NCAM140, application of NCAM antibodies did not influence levels of activated CaMKII α and phosphorylated RPTP α WT in NCAM140 Δ Cys-transfected cells nor in GFP-transfected cells used as a negative control (Fig. 6, C and D). NCAM140-dependent CaMKII α activation and RPTP α phosphorylation and activation were also blocked in CHO cells treated with methyl- β -cyclodextrin (Fig. S3), an agent that binds to cholesterol, thereby destroying the integrity of lipid rafts (Niethammer et al., 2002). Thus, association of NCAM with lipid rafts is required for NCAM-dependent CaMKII α activation and RPTP α phosphorylation.

NCAM associates with T- and L-type voltage-dependent Ca²⁺ channels (VDCC)

CaMKII α activation depends on an increase in cytosolic Ca²⁺ that binds to CaM. Binding of Ca²⁺-CaM to the regulatory domain of the kinase activates the kinase by releasing the catalytic domain from inhibition by autoregulatory sequences proximal to the CaM binding site. NCAM clustering at the cell surface has been shown to be accompanied by an increase in Ca²⁺ concentration in the cytosol, with the VDCC of the T and, to a lower extent, L type playing a major role in NCAM-dependent Ca²⁺ influx to the cell (Schuch et al., 1989; Kiryushko et al., 2006). T- and L-type VDCC were present at high levels in lipid rafts from brain tissue (Fig. 6 B), thus they are well poised to provide Ca²⁺ for

colabeled with antibodies against NCAM, CaMKII α , and T-type VDCC is shown. Note the colocalization of these three proteins in filopodia. (C) NCAM was clustered at the surface of cultured hippocampal neurons with NCAM monoclonal antibodies (H28live), and neurons were colabeled with antibodies against CaMKII α and T-type VDCC. A representative neurite is shown. Note that clusters of NCAM overlap with accumulations of T-type VDCC and CaMKII α (arrows). Bars, 5 μ m.

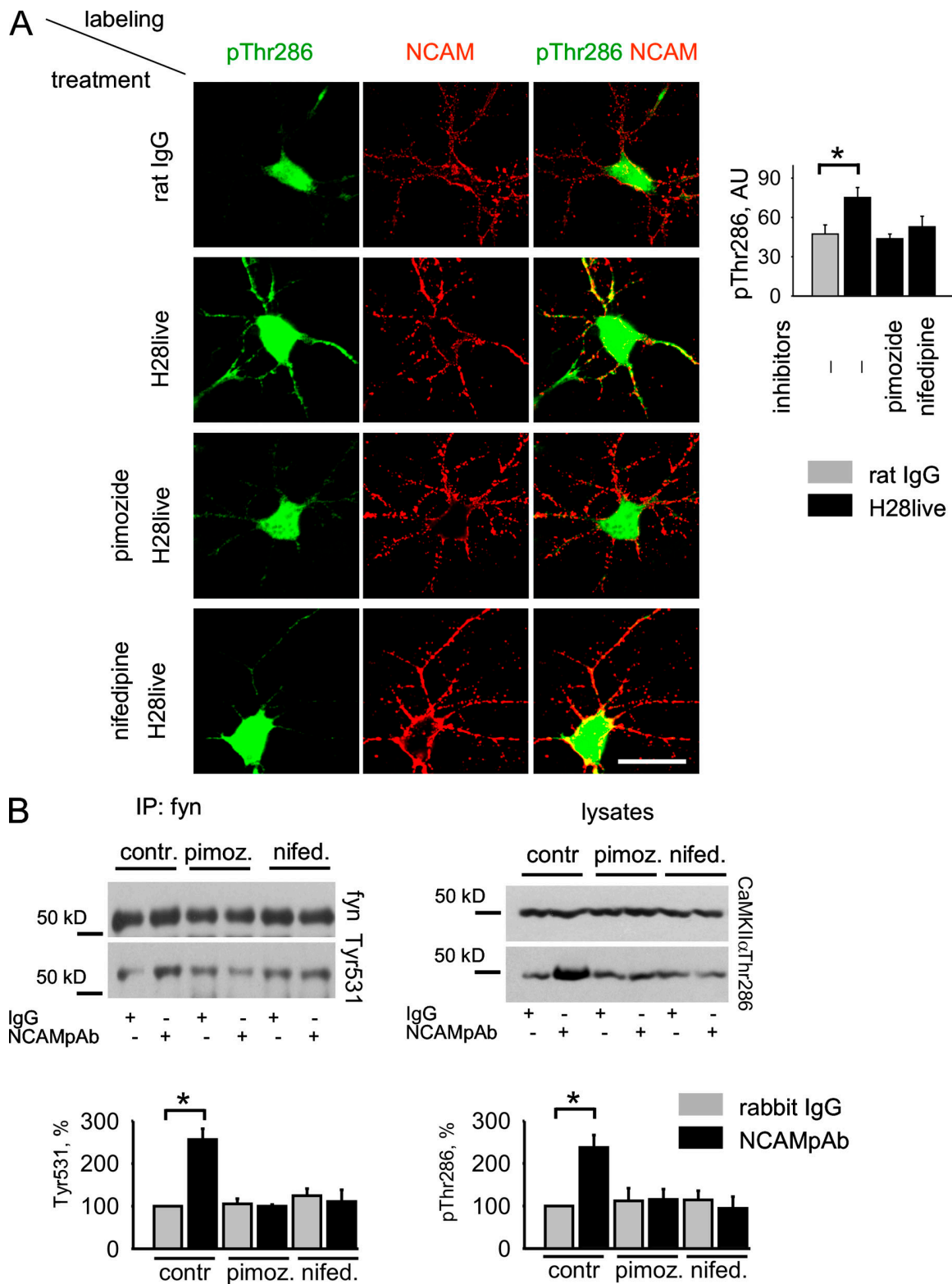


Figure 8. NCAM-induced CaMKII α activation depends on Ca²⁺ influx via T- and L-type VDCC. (A) Cultured hippocampal neurons were incubated with pimoizide and nifedipine, inhibitors of T- and L-type VDCC, respectively. Neurons were then stimulated with rat NCAM monoclonal antibodies (H28live) or nonspecific rat IgG, then fixed and colabeled with antibodies against activated Thr286-phosphorylated CaMKII α . Representative images are shown. Note that clustering of NCAM without inhibitors induces an increase in active CaMKII α levels along neurites and that active CaMKII α accumulates in NCAM clusters. Pimoizide and nifedipine inhibit CaMKII α activation. Graph shows mean levels \pm SEM in arbitrary units (AU) of active CaMKII α along neurites. $n > 45$ neurites from 30 neurons from 3 coverslips analyzed in each group. Bar, 20 μ m. (B) Cultured cortical neurons were treated with nonspecific rabbit IgG or NCAM polyclonal antibodies in the absence or presence of pimoizide and nifedipine. Lysates of these cells and fyn immunoprecipitates from the lysates were then probed with the indicated antibodies by Western blotting. Note that pimoizide and nifedipine inhibited fyn and CaMKII α activation in response to NCAM antibodies. Graphs show quantitation of the blots (mean \pm SEM), with the levels in cells treated with nonspecific IgG without inhibitors set to 100%. *, $P < 0.05$ (paired t test).

CaMKII α activation in response to NCAM redistribution to lipid rafts. Moreover, T- and L-type VDCC associated with NCAM in the brain, as shown by coimmunoprecipitation of these VDCC with NCAM from brain lysates (Fig. 7 A). Although L-type VDCC are known to accumulate in growth cones being involved in cytoskeleton rearrangements (Ohbayashi et al., 1998), much less is known about T-type VDCC. Colabeling of cultured hippocampal neurons with antibodies against NCAM, T-type VDCC, and CaMKII α showed that all three proteins accumulated in growth cones and, in particular, growth cone filopodia (Fig. 7 B). When NCAM was clustered at the neuronal surface with NCAM antibodies, T-type VDCC and CaMKII α accumulated in NCAM clusters along neurites (Fig. 7 C). Thus, we hypothesize that clustering of NCAM at the growth cones induces formation of the NCAM–T- and L-type VDCC–CaMKII α complex, thereby linking CaMKII α to the Ca²⁺ source.

NCAM-induced CaMKII α activation depends on Ca²⁺ influx via T- and L-type VDCC

Next, we verified the role of T- and L-type VDCC in NCAM-dependent CaMKII α activation. In cultured hippocampal neurons, clustering of NCAM at the neuronal surface induced an ~80% increase in the levels of activated Thr286-phosphorylated CaMKII α along neurites, as shown by immunocytochemical analysis (Fig. 8 A). Pimozide, a T-type VDCC inhibitor, completely blocked this effect (Fig. 8 A). Nifedipine, an L-type VDCC inhibitor, also inhibited NCAM-dependent CaMKII α activation, although with a slightly lower efficiency than pimozide (Fig. 8 A).

Similarly, pimozide and nifedipine inhibited NCAM-dependent CaMKII α activation in cultured cortical neurons analyzed by Western blotting (Fig. 8 B). Importantly, pimozide and nifedipine also blocked NCAM-dependent dephosphorylation of fyn at Tyr531 (Fig. 8 B). Hence, we conclude that NCAM induces Ca²⁺ influx via T- and L-type VDCC for CaMKII α activation, which results in up-regulation of the phosphatase activity of RPTP α and fyn dephosphorylation and activation.

Phosphorylation of RPTP α on Ser180 and Ser204 is required for NCAM-mediated neurite outgrowth

In neurons exposed to NCAM-Fc in the culture medium (Fig. 9 A) or seeded on top of NCAM-transfected fibroblasts (Williams et al., 1995), the KN62 inhibitor of CaMKII α abolished the NCAM-dependent increase in neurite outgrowth over the baseline level of nonstimulated neurons, which indicates that CaMKII α activity is required for NCAM-mediated neurite outgrowth.

To analyze whether CaMKII α -dependent RPTP α phosphorylation on Ser180 and/or Ser204 is required for NCAM-mediated neurite outgrowth, cultured neurons were transfected with GFP or cotransfected with GFP and RPTP α WT or the RPTP α mutants RPTP α S180A, RPTP α S204A, or RPTP α S180/204A. These mutations within RPTP α did not affect binding of RPTP α to NCAM (Fig. 1). Hence, RPTP α mutants overexpressed in neurons should act in a dominant-negative fashion by substituting for endogenous RPTP α in the NCAM–RPTP α complex. Overexpression of RPTP α WT in neurons resulted in enhanced neurite outgrowth most likely caused by the nonspecific outgrowth-

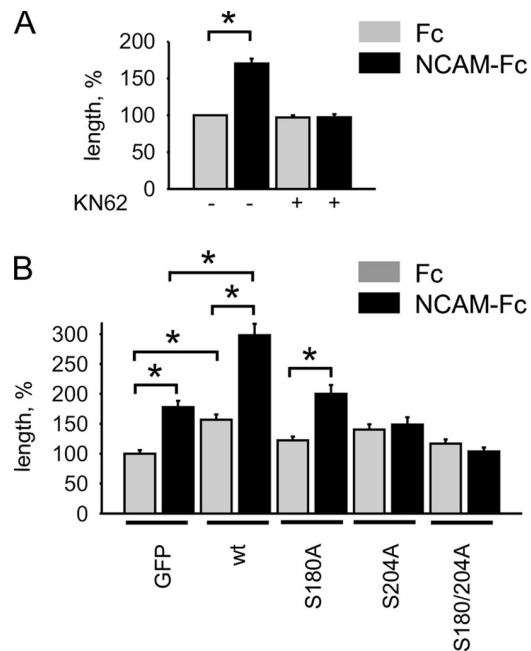
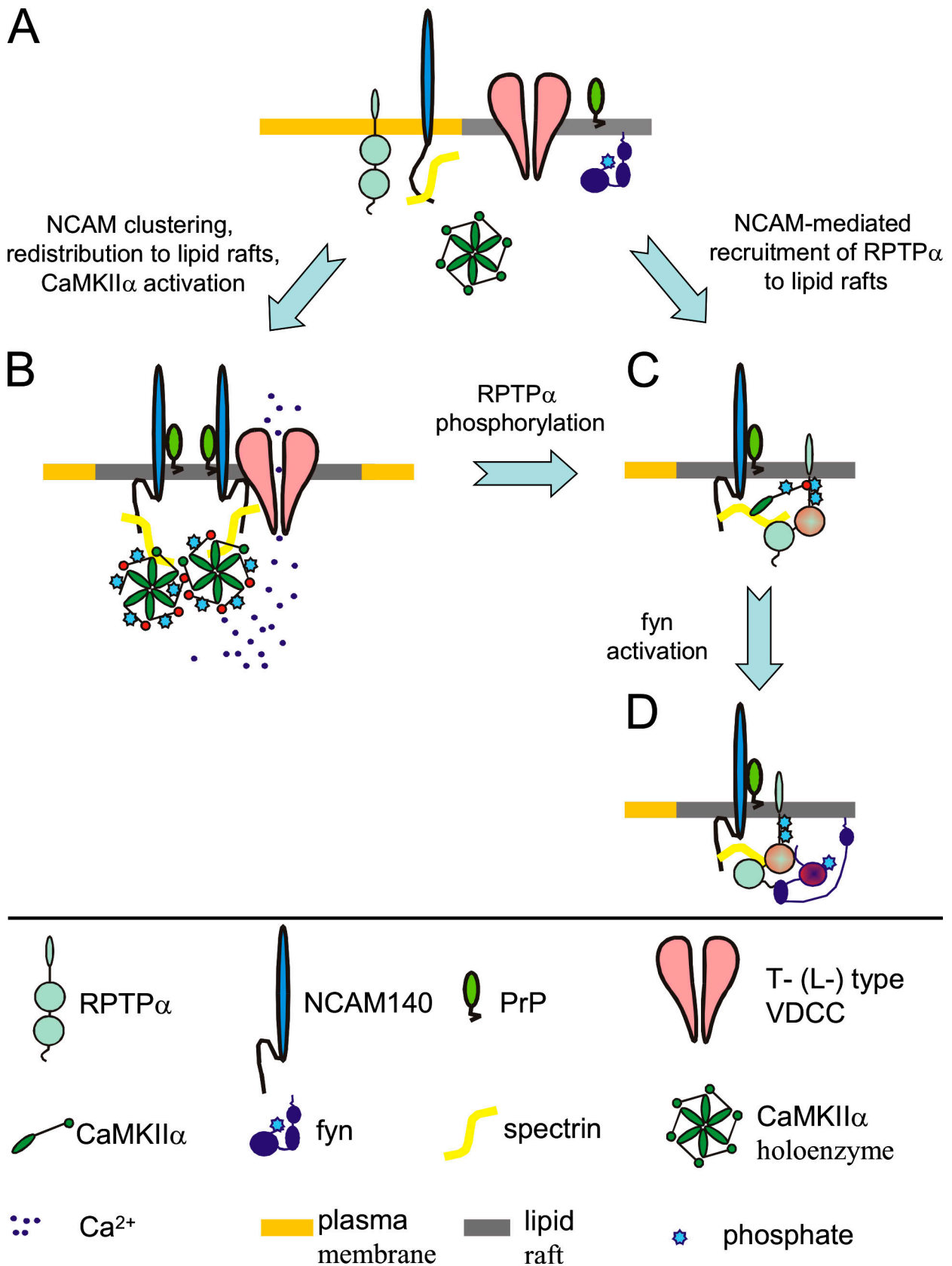


Figure 9. NCAM-induced phosphorylation of RPTP α on Ser180 and Ser204 is required for NCAM-mediated neurite outgrowth. (A) Cultured hippocampal neurons were treated with Fc or NCAM-Fc in the presence or absence of KN62. Graph shows mean lengths of neurites \pm SEM normalized to the mean neurite length in neurons treated with Fc in the absence of KN62 ($n > 200$). Note that KN62 inhibits NCAM-dependent neurite outgrowth. (B) Cultured hippocampal neurons were transfected with GFP alone or cotransfected with GFP and RPTP α WT or RPTP α mutants and treated with Fc or NCAM-Fc. Graph shows mean lengths of neurites \pm SEM normalized to the mean neurite length in Fc-treated GFP-transfected neurons ($n > 150$). Note that RPTP α WT promotes neurite outgrowth and potentiates the response to NCAM-Fc. Mutation in either Ser180 or Ser204 inhibits the outgrowth-promoting effect of RPTP α , whereas the RPTP α S180/204A mutant functions as a dominant-negative construct that fully blocks NCAM-dependent neurite outgrowth. *, $P < 0.05$ (t test).

promoting activation of Src-family kinases by RPTP α WT (Fig. 9 B). Exposure of GFP-transfected neurons to NCAM-Fc applied in the culture medium increased neurite lengths by ~100% when compared with Fc-treated GFP-transfected control neurons (Fig. 9 B). A similar, ~100% increase in neurite length was observed for NCAM-Fc–versus Fc-treated RPTP α WT-transfected neurons (Fig. 9 B). However, neurites in NCAM-Fc–treated RPTP α WT-transfected neurons were approximately two times longer than in NCAM-Fc–treated GFP-transfected neurons, which indicates that RPTP α WT potentiates the response to NCAM-Fc (Fig. 9 B). RPTP α S180A and RPTP α S204A mutants showed a reduced ability to promote neurite outgrowth and to enhance responsiveness to NCAM-Fc when compared with RPTP α WT (Fig. 9 B). The RPTP α S180A mutant was strongly impaired in its ability to nonspecifically promote neurite outgrowth but did not completely block the response to NCAM-Fc. In contrast, the RPTP α S204A mutant strongly interfered with the response to NCAM-Fc but was still able to nonspecifically promote neurite outgrowth at a level that was not different from that of RPTP α WT. A plausible explanation for this observation is that Ser180 and Ser204 may play slightly distinct roles in the maintenance of the unregulated basal versus the NCAM-mediated, thus



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Figure 10. **A schematic model of molecular interactions induced by NCAM140 clustering and resulting in fyn activation.** (A) Nonclustered NCAM140 resides outside of lipid rafts and does not associate with RPTP α , CaMKII α (shown as a holoenzyme), and fyn, which are present in inactive forms in the plasma membrane outside of lipid rafts, in the cytosol and in lipid rafts, respectively. (B) NCAM140 clustering induces palmitoylation of the intracellular domain of NCAM140 that promotes NCAM140 redistribution to lipid rafts, where it binds to prion protein (PrP). In parallel, NCAM140 clustering induces FGFR activation, promoting arachidonic acid production (not depicted), which results in arachidonic acid-dependent Ca $^{2+}$ influx (Williams et al., 1994)

regulated, neurite outgrowth. A double mutant RPTP α S180/204 did not promote neurite outgrowth and totally blocked the NCAM-Fc-induced response (Fig. 9 B). Thus, we conclude that CaMKII α -mediated phosphorylation of Ser180 and Ser204 within the RPTP α intracellular domain is required for NCAM-induced neurite outgrowth.

Discussion

In this study, we have further explored the cascades of molecular interactions activated by the neural cell adhesion molecule NCAM; the molecular players in the functional interconnection had been previously implicated in NCAM-dependent neurite outgrowth. The tyrosine kinase fyn was the first to be identified as an NCAM-associated signaling partner that triggers the MAP kinase/FAK-related downstream signaling of NCAM, leading to activation of transcription factors, such as CREB (Beggs et al., 1997; Schmid et al., 1999; Jessen et al., 2001). The fyn pathway then became very important as a cosignaling pathway in NCAM-induced signaling, which needed to be activated in lipid rafts in parallel with the FGF receptor located outside of lipid rafts in order to trigger converging signaling cascades to enhance neurite outgrowth (Niethammer et al., 2002). Because the intracellular domain of NCAM does not contain sequences known to induce fyn activation, we previously investigated the relationship between NCAM and the well-known activator of Src-family tyrosine kinases, of which fyn is a member, the receptor protein tyrosine phosphatase RPTP α . RPTP α that dephosphorylates Tyr-531 of fyn to activate the enzyme (Bhandari et al., 1998) is highly enriched in neurons and growth cones (Helmke et al., 1998), where it colocalizes with NCAM (Bodrikov et al., 2005). We identified RPTP α as a direct binding partner of NCAM140, linking this NCAM isoform to fyn. Interestingly, the interaction between NCAM and RPTP α was enhanced by spectrin (Bodrikov et al., 2005), which also interacts directly with the intracellular domains of the transmembrane isoforms of NCAM, NCAM140, and, in particular, NCAM180 (Pollerberg et al., 1986, 1987; Sytnyk et al., 2002; Leshchyns'ka et al., 2003) and indirectly via lipid rafts with the GPI-anchored isoform NCAM120 (Leshchyns'ka et al., 2003).

In this study, we expand our previous findings by showing that clustering of NCAM at the cell surface enhances serine phosphorylation and phosphatase activity of RPTP α , thus identifying NCAM as the first recognition molecule and surface receptor that not only associates with but also regulates the catalytic activity of RPTP α . The catalytic phosphatase activity of RPTP α can be enhanced by PKC-mediated Ser180 and/or Ser204 phosphorylation of the intracellular domain of RPTP α (den Hertog et al., 1995; Tracy et al., 1995; Stetak et al., 2001; Zheng et al., 2002), with

PKC δ but not other PKC isoforms playing the major role in the phosphorylation of RPTP α on Ser180 and Ser204 (Brandt et al., 2003). However, we found that PKC δ is not involved in NCAM-induced activation. In agreement with this notion is the observation that 2.5–10 μ M of the PKC δ inhibitor rottlerin did not inhibit RPTP α serine phosphorylation in nonstimulated NIH3T3 fibroblasts, and that very high concentrations of rottlerin (50 μ M) only mildly affected RPTP α serine phosphorylation (unpublished data), which suggests that other enzymes are involved. Furthermore, we show that BisI, a PKC δ inhibitor, does not block RPTP α -mediated fyn activation in response to NCAM clustering. Accordingly, we identified CaMKII α as a previously unrecognized enzyme that binds to and phosphorylates RPTP α at serine residues Ser180 and Ser204, increasing the phosphatase activity of RPTP α . We also show that CaMKII α , which associates with NCAM via spectrin (Sytnyk et al., 2006), is activated in response to clustering of NCAM at the cell surface. Calmodulin, which associates with RPTP α in the presence of Ca²⁺, may thus play a role in CaMKII α activation (Liang et al., 2000). NCAM-induced CaMKII α activation then leads to phosphorylation of RPTP α at Ser180 and Ser204, which, in turn, leads to activation of lipid raft-enriched fyn (Fig. 10).

Overexpression of RPTP α in neurons enhances basal neurite outgrowth rates, probably by nonspecific activation of Src-family kinases. Indeed, overexpression of RPTP α in nonneuronal cells results in persistent activation of Src, with concomitant cell transformation and tumorigenesis (Zheng et al., 1992, 2000), and mutation of Ser180 or Ser204 blocks neoplastic transformation of cells by RPTP α (Zheng et al., 2002). This suggests that phosphorylation and activity of RPTP α are tightly regulated in normal cells. Homeostatic mechanisms that coordinate changes in RPTP α phosphatase activity are therefore intimately linked with the changes in cell behavior. It is thus not surprising that disturbance of the relationship of NCAM with RPTP α may link NCAM to tumorigenesis in different cell types that express NCAM (Cavallaro and Christofori, 2004a,b).

Our study again emphasizes the importance of lipid-enriched microdomains, the so-called lipid rafts. The fact that NCAM uses this signaling platform for fyn activation has previously been shown by ablating the palmitoylation sites in the intracellular domain of NCAM, which target the two major transmembrane isoforms of NCAM to lipid rafts (Niethammer et al., 2002). NCAM-dependent fyn activation was also reduced in prion protein-deficient mice, which correlates with reduced levels of NCAM in lipid rafts isolated from these mice and implicates prion protein as a lipid raft-recruiting signal for NCAM (Santuccione et al., 2005). In this study, we provide further details on the molecular interactions induced by NCAM in lipid rafts by showing that if the targeting of NCAM140 to lipid rafts

via T- and L-type VDCC. An increase in Ca²⁺ concentration promotes binding of Ca²⁺/CaM to CaMKII α , releasing the catalytic domain of CaMKII α from inhibition by autoregulatory sequences proximal to the CaM binding site (not depicted). By associating with T- and L-type VDCC, NCAM anchors CaMKII α , which is bound to NCAM140 via spectrin, near the Ca²⁺ influx sites. NCAM-induced aggregation of CaMKII α in lipid rafts promotes transautophosphorylation of the CaMKII α holoenzymes at Thr286, resulting in the constitutive activation of CaMKII α . (C) In parallel to CaMKII α activation, NCAM140 promotes redistribution of RPTP α to lipid rafts. In lipid rafts, activated CaMKII α (only one CaMKII α molecule is shown for simplicity) phosphorylates RPTP α at Ser180 and Ser204, which changes the conformation of RPTP α , resulting in enzyme activation. (D) Activated RPTP α binds and dephosphorylates fyn at Tyr531, activating the enzyme. Downstream targets of active fyn include the Ras-MAP (MAP) kinase pathway, the sustained activity of which is required for neuronal differentiation (not depicted).

is disturbed, NCAM-induced activation of CaMKII α in lipid rafts and subsequent phosphorylation and activation of RPTP α , and thus of fyn, are reduced. Because aggregation and oligomerization of CaMKII α is necessary for full activation of the enzyme (Hudmon et al., 2005), we propose that the special environment of lipid rafts promotes oligomerization of NCAM-associated CaMKII α to induce its full activation (Fig. 10). In agreement, clustering of NCAM increases the levels of detergent-insoluble oligomerized CaMKII α in cultured hippocampal neurons (Sytnyk et al., 2006). Association of NCAM with T- and L-type VDCC accumulating in lipid rafts should then bring CaMKII α into the vicinity of the sites of Ca²⁺ influx into the cell (Fig. 10). Thus, our present study confirms the importance of lipid rafts in cell signaling for neurite outgrowth. The generation of such platforms during ontogenetic development appears to be crucial for neurite outgrowth. It will be interesting to determine whether such cell adhesion molecule-mediated platforms are required for synaptic plasticity in the adult brain, which requires functional and structural modifications of synaptic membranes and associated signaling mechanisms.

Materials and methods

Antibodies and toxins

Rabbit polyclonal antibodies against NCAM (for biochemical and immunocytochemical experiments; Niethammer et al., 2002) and rat monoclonal antibodies H28 against NCAM (for immunocytochemical experiments; Gennarini et al., 1984) were against the extracellular domain of all NCAM isoforms. Rabbit antibodies against RPTP α (den Hertog et al., 1994) and CHL1 (Leshchyn'ska et al., 2006) were generated as described previously. Mouse monoclonal antibodies against PI(4,5)P2 were obtained from K. Fukami (University of Tokyo, Tokyo, Japan). Cholera toxin B subunit tagged with biotin to label GM1, rabbit polyclonal antibodies against the HA tag, L-type VDCC (α_{1C} subunit), T-type VDCC (α_{1H} subunit), and CaMKII α were obtained from Sigma-Aldrich. Rabbit polyclonal and mouse monoclonal antibodies against p59^{l^{yn}} protein were obtained from Santa Cruz Biotechnology, Inc. Rabbit polyclonal antibodies against Tyr527-dephosphorylated or Tyr416-phosphorylated pp60^{c-Src} kinase that cross-react with Tyr531-dephosphorylated or Tyr420-phosphorylated p59^{l^{yn}}, against Thr505-phosphorylated PKC δ , and against Thr286-phosphorylated CaMKII α were obtained from Cell Signaling Technology. Mouse monoclonal antibodies recognizing phosphorylated serine residues were obtained from QIAGEN, mouse monoclonal antibodies against PKC δ were obtained from BD Biosciences, rat monoclonal antibodies against GAPDH were obtained from Millipore, and mouse monoclonal antibodies against CaMKII α were obtained from Assay Designs. Secondary antibodies against rabbit, rat, and mouse Ig coupled to HRP, Cy2, Cy3, or Cy5; and nonspecific rabbit, rat, and mouse immunoglobulins (IgG) were obtained from Dianova (Hamburg, Germany).

Animals

Wild-type 0–4-d-old C57BL/6J mice were used and compared when indicated to age-matched NCAM^{-/-} mice provided by H. Cremer (Developmental Biology Institute of Marseille, Marseille, France; Cremer et al., 1994) and inbred for at least nine generations onto the C57BL/6J background.

Cultures of hippocampal and cortical neurons

Cultures of hippocampal neurons (for immunocytochemistry, cell ELISA, and neurite outgrowth assay) and cortical neurons (for Western blot analysis) were prepared from 1–3-d-old mice. Neurons were grown in the presence of 10% horse serum on glass coverslips coated with 100 μ g/ml poly-L-lysine (Sytnyk et al., 2002, 2006).

Clustering of NCAM at the neuronal cell surface

NCAM was clustered at the neuronal surface using NCAM-Fc, rat monoclonal (clone H28), or rabbit polyclonal antibodies against the extracellular domain of NCAM. Application of these three agents produced similar levels of fyn activation in cultured neurons and fibroblasts cotransfected

with NCAM140 and RPTP α WT (Figs. 5 and S4, available at <http://www.jcb.org/cgi/content/full/jcb.200803045/DC1>). 8 μ g/ml NCAM-Fc and 8 μ g/ml of human Fc, or 10 μ g/ml NCAM polyclonal antibodies and 10 μ g/ml nonspecific rabbit IgG were applied to live neurons in the culture medium for 10 min in a CO₂ incubator. Rat monoclonal antibodies H28 or 10 μ g/ml nonimmune rat IgG were applied to live neurons for 15 min and clustered by secondary antibodies applied for 5 min, followed by washing for 5 min, all in a CO₂ incubator. We have found that with this protocol, fyn and CaMKII α were activated within 5 min after secondary antibody application, and levels of active fyn and CaMKII α reached a plateau within 10 min after secondary antibody application (Fig. S5). When indicated, 10 μ M of the CaMKII α inhibitor KN62 (Merck & Co., Inc.) or 0.5 μ M of the PKC inhibitor Bis1 was applied 1 h before stimulation with antibodies or NCAM-Fc. 10 μ M nifedipine and 5 μ M pimezide (both from Alomone Laboratories Ltd.) were applied for 10 min before stimulation.

DNA constructs

NCAM140 and NCAM180 constructs were provided by P. Maness (University of North Carolina at Chapel Hill School of Medicine, Chapel Hill, NC). NCAM120 was provided by E. Bock (Institute of Molecular Pathology, University of Copenhagen, Copenhagen, Denmark). NCAM140 Δ cys was as described previously (Niethammer et al., 2002). The plasmid encoding the intracellular domain of RPTP α was a gift from C.J. Pallen (University of British Columbia, Vancouver, Canada). N-terminally HA-tagged full length RPTP α was used as described previously (den Hertog and Hunter, 1996; Blanchetot and den Hertog, 2000; Buist et al., 2000). HA-tagged RPTP α S180A, RPTP α S204A, and RPTP α S180/204A were generated by PCR-mediated site-directed mutagenesis using the following oligonucleotides: 5'-AGTCATCCAACGCTTCCGCTGTCA-3' for S180A and 5'-GCCAGTCCCCAGCCACCAACAGGAAG-3' for S204A. The constructs were verified by sequencing.

Neurite outgrowth assay

Neurite outgrowth was assayed as described previously (Niethammer et al., 2002). When indicated, neurons were cotransfected with GFP (Clontech Laboratories, Inc.) and wild-type or mutated RPTP α constructs 6 h after plating using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. For nontransfected neurons, 8 μ g/ml NCAM-Fc (Chen et al., 1999) or 8 μ g/ml human Fc (Dianova) were applied immediately after plating together with or without 10 μ M KN62. For transfected neurons, NCAM-Fc or Fc were applied 6 h after transfection. Neurons were fixed 24 h after NCAM-Fc and Fc application. Lengths of neurites were measured using ImageJ software (National Institutes of Health).

Immunofluorescence labeling

In the indicated experiments, NCAM was clustered at the neuronal surface, with antibodies against the extracellular domain of NCAM applied to live neurons for 15 min and clustered/visualized by secondary antibodies applied for 5 min followed by washing for 5 min, all in a CO₂ incubator. In control experiments, we verified that application of nonspecific IgG does not induce NCAM clustering or redistribution of NCAM-associated proteins. When indicated, before fixation, neurons were also extracted with ice-cold 1% Triton X-100 as described previously (Bodrikov et al., 2005). Neurons were fixed in 4% formaldehyde in PBS, pH 7.3, for 15 min at room temperature, washed, permeabilized with 0.25% Triton X-100 in PBS for 5 min, and blocked in 1% BSA in PBS. For extracted neurons, the permeabilization step was omitted. Primary antibodies were applied in 1% BSA in PBS for 2 h at room temperature and detected with corresponding secondary antibodies applied for 45 min in 1% BSA in PBS at room temperature.

Image acquisition and manipulation

We used Cy2, Cy3, or Cy5 fluorochromes. Coverslips were embedded in Aqua-Poly/Mount (Polysciences, Inc.). For neurite outgrowth measurements, images of neurons were acquired at room temperature using a microscope (Axiovert 2) equipped with a digital camera (AxioCam HRC), AxioVision software (version 3.1), and a Plan-Neofluar 40x objective (numerical aperture 0.75; all from Carl Zeiss, Inc.). Immunofluorescence images were acquired at room temperature using a confocal laser scanning microscope (LSM510), LSM510 software (version 3) and an oil Plan-Neofluar 40x objective (numerical aperture 1.3; all from Carl Zeiss, Inc.) at 3x digital zoom. Contrast and brightness of the images were further adjusted in Corel Photo-Paint 9 (Corel Corporation).

Immunofluorescence quantification

Profiles of distributions of the immunofluorescence signals along neurites were obtained using ImageJ software, then used to calculate mean

immunofluorescence intensities along neurites and to analyze coefficients of correlation between distributions of the Thr286-phosphorylated CaMKII α and PI(4,5)P₂.

Cultures and transfection of CHO cells and fibroblasts

CHO cells and fibroblasts were maintained in Glasgow or Dulbecco's modified Eagle's medium, respectively, containing 10% of fetal calf serum. Cells were transfected using Lipofectamine and Plus reagent (Invitrogen) according to the manufacturer's instruction. When indicated, 10 μ g/ml NCAM polyclonal antibodies or 10 μ g/ml nonspecific rabbit IgG were applied to cells for 10 min. 10 μ M KN62 was applied 1 h before stimulation, and NCAM antibodies were applied (as described in the Clustering of NCAM at the neuronal cell surface section) but in the presence of KN62. To deplete cholesterol from lipid rafts, cultures were incubated for 15 min with 5 mM methyl- β -cyclodextrin (MCD; Sigma-Aldrich) before stimulation, and NCAM antibodies were applied (as described in the Clustering of NCAM at the neuronal cell surface section) but in the presence of MCD. All reagents were applied in culture medium at 37°C in a CO₂ incubator.

Immunoprecipitation and coimmunoprecipitation

Brain homogenates were prepared using 50 mM Tris-HCl buffer, pH 7.5, containing 1 mM CaCl₂, 1 mM MgCl₂, and 1 mM NaHCO₃. Samples containing 1 mg of total protein were lysed for 40 min on ice with RIPA buffer (50 mM Tris-HCl buffer, pH 7.5, containing 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 1 mM Na₂P₂O₇, 1 mM NaF, 2 mM NaVO₄, 0.1 mM PMSF, and EDTA-free protease inhibitor cocktail [Roche]). In experiments with transiently transfected CHO cells, cells were washed twice with ice-cold PBS and lysed 30 min on ice with RIPA buffer. After centrifugation, supernatants were cleared with protein A/G-agarose beads (Santa Cruz Biotechnology, Inc.) for 3 h at 4°C, then incubated with corresponding antibodies or control IgG for 1.5 h at 4°C followed by precipitation with protein A/G-agarose beads for 1 h at 4°C. The beads were washed three times with RIPA buffer and twice with PBS, then analyzed by immunoblotting.

Isolation of cytosolic, total membrane, and lipid raft fractions

Brain homogenates were prepared using a Potter homogenizer in HOMO buffer (1 mM MgCl₂, 1 mM CaCl₂, 1 mM NaHCO₃, and 5 mM Tris, pH 7.4) containing 0.32 M sucrose (HOMO-A) and centrifuged at 700 *g* for 10 min at 4°C to pellet nuclei and mitochondria. The resulted supernatants were centrifuged at 100,000 *g* at 4°C for 30 min. Supernatants obtained after centrifugation were used as fractions enriched in cytosolic proteins, whereas pellets were used as total membrane fractions. Rafts were isolated from total membrane fractions as described previously (Leshchyn'ska et al., 2003). In brief, membranes were resuspended in ice-cold TBS, pH 7.5, and extracted for 20 min on ice with 4 volumes of 1% Triton X-100 in TBS. Extracted membranes were mixed with equal volume of 80% sucrose in 0.2 M sodium carbonate, overlaid sequentially with 30% sucrose in TBS, 10% sucrose in TBS, and TBS, and centrifuged at 230,000 *g* for 17 h at 4°C. The lipid raft fraction was collected at the interface between 10 and 30% sucrose, pelleted by centrifugation at 100,000 *g* for 1 h at 4°C, and resuspended in TBS. Total protein concentration was measured using the BC kit (Interchim).

Recombinant proteins

Human Fc-tagged extracellular domain of mouse NCAM and GST-tagged intracellular domains of RPTP α were used as described previously (Chen et al., 1999; Bodrikov et al., 2005). Calmodulin-dependent protein kinase II α (CaMKII α) was obtained from New England Biolabs, Inc.

In vitro phosphorylation

Recombinant CaMKII α or CaMKII α immunopurified from rat brain (BIOMOL International, L.P.) was activated according to the manufacturer's instructions. For analysis of the phosphorylation in vitro, proteins were immunoprecipitated from CHO cells, fibroblasts, or brain homogenates as described in the Immunoprecipitation and coimmunoprecipitation section. Mock immunoprecipitation with nonspecific IgG served as a control of immunoprecipitation specificity, and beads with immunoprecipitated nonspecific IgG obtained after mock immunoprecipitation were used as control in phosphorylation reactions. Beads were then washed twice with RIPA buffer, three times with TBS, and diluted in TBS. To analyze phosphorylation of recombinant RPTP α -ID, they were coupled to glutathione beads. Glutathione beads with GST coupled instead of RPTP α -ID were used as control. Beads were washed three times with TBS and diluted in TBS. Equal volumes of control beads or beads with proteins of interest were incubated for 30 min at 30°C in CaMKII α reaction buffer (New England Biolabs, Inc.)

supplemented with 200 μ M ATP in the presence of activated CaMKII α , then washed with TBS, centrifuged for 1 min, and used for Western blot analysis or protein phosphorylation estimation.

Estimation of the serine phosphorylation of RPTP α

Serine/threonine phosphorylation was analyzed by the alkaline hydrolysis of phosphate from seryl and threonyl residues in phosphoproteins. Because mutations of Ser180 and Ser204 within RPTP α -ID completely inhibited serine/threonine phosphorylation of RPTP α , we refer to this method as to the analysis of serine phosphorylation of RPTP α throughout the text. In brief, beads containing phosphorylated and control proteins were treated with 1 N NaOH for 30 min at 65°C. The reaction was stopped by the addition of a similar volume of 3.1 N HCl, and released phosphate was measured using Malachite green phosphate detection kit (R&D Systems) according to manufacturer's instructions.

Analysis of the RPTP α phosphatase activity

Control beads and beads with immunoprecipitated RPTP α were washed twice with RIPA buffer, three times with TBS, and diluted in TBS. Glutathione beads with GST or recombinant RPTP α -ID were washed and diluted in TBS. Phosphatase activity was measured using a nonradioactive phosphatase assay system (Promega) according to manufacturer's instructions.

Gel electrophoresis and immunoblotting

Proteins were separated by 8% SDS-PAGE and electroblotted onto nitrocellulose transfer membrane (PROTRAN; Sigma-Aldrich) overnight at 5 mA. Immunoblots were blocked in milk and incubated with appropriate primary antibodies followed by incubation with peroxidase-labeled secondary antibodies and visualized using Super Signal West Pico reagents (Thermo Fisher Scientific) on BIOMAX film (Sigma-Aldrich). For immunoblots labeled with phospho-specific antibodies, 3% of BSA was used instead of milk. Molecular weight markers were prestained protein standards from Bio-Rad Laboratories. For quantitative comparisons of chemiluminescence between the lanes, the same amounts of total protein or equal amounts of immunoprecipitates were loaded in each lane. All preparations were performed three times and at least two Western blots were performed with an individual sample ($n \geq 6$). Values from all Western blots were used to calculate mean values and standard errors of the mean. The chemiluminescence quantification was performed using TINA 2.09 software (University of Manchester) or Scion Image for Windows (Scion Corporation). Group comparisons were made using a paired *t* test.

Online supplemental material

Fig. S1 shows the analysis of the Ser180 and Ser204 phosphorylation and phosphatase activity of RPTP α WT, expressed in RPTP α -negative fibroblasts alone or together with NCAM120 or NCAM180, after application of NCAM polyclonal antibodies or nonspecific rabbit IgG to transfected fibroblasts. Fig. S2 shows the analysis of the RPTP α WT phosphorylation and CaMKII α , PKC δ , and fyn activation in CHO cells that were cotransfected with RPTP α WT and NCAM120, NCAM140, NCAM180, or GFP and treated with NCAM antibodies or nonspecific rabbit IgG. Fig. S3 shows levels of activated CaMKII α in lipid rafts from NCAM+/+ and NCAM-/- brains, and provides the analysis of the influence of lipid raft disruption by methyl- β -cyclodextrin on CaMKII α activation and RPTP α phosphorylation and activation in NCAM140-transfected CHO cells. Fig. S4 contains a comparison of the effects of NCAM clustering by polyclonal or monoclonal antibodies against the extracellular domain of NCAM or by NCAM-Fc on fyn activation in RPTP α -negative fibroblasts cotransfected with NCAM140 and RPTP α WT. Fig. S5 shows the time course of NCAM-dependent fyn and CaMKII α activation in dissociated hippocampal neurons treated with NCAM monoclonal antibodies or nonspecific rat IgG. Online supplemental material is available at <http://www.jcb.org/cgi/content/full/jcb.200803045/DC1>.

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