HIGHLIGHTS

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TIEN-CHENG WANG, YI-HSUAN LI, KUAN-WEI CHEN, CHING-JEN CHEN, CHIA-LING WU, NAN-YUAN TENG, AND LINYI CHEN

2063 **SH2B1β Regulates N-Cadherin**

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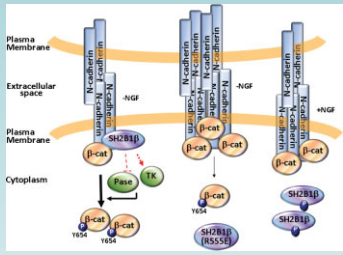
The interplay between cell-cell and cell-extracellular matrix adhesion contributes to cell fate determination during development. The cell adhesion molecule N-cadherin mediates cell-cell adhesion and regulates cell motility in various systems. Wang *et al.* demonstrate that overexpression of the signaling adaptor protein SH2B1β reduces, while the dominant negative mutant of SH2B1β increases, the expression of N-cadherin in PC12 cells, a well-established neuronal model system. Through binding to N-cadherin, SH2B1β may recruit a putative kinase to phosphorylate β-catenin, reduce N-cadherin-β-catenin complexes and thus the inter-cellular interaction. In the presence of nerve growth factor (NGF), SH2B1β leaves the N-cadherin-containing complexes, allowing N-cadherin to accumulate at the cell surface during neurite elongation. Results from this study implicate the differential requirement of N-cadherin during neurite initiation and elongation which is challenging to address using primary neural culture or *in vivo* systems. Wang *et al.* present a novel mechanism by which the adaptor protein SH2B1β reduces N-cadherin levels and cell-cell adhesion to promote earlier neurite initiation. This mechanism also explains why overexpression of the dominant negative mutant SH2B1β(R555E) blocks neurite initiation without inhibiting NGF signaling.



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B. D'ANGELO, E. BENEDETTI, S. DI LORETO, L. CRISTIANO, G. LAURENTI, M.P. CERÙ, AND A. CIMINI

2170 **PPARβ/δ-Induced Neuronal Differentiation**

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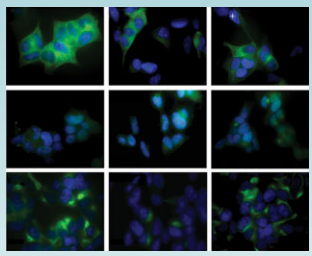
A fundamental problem in neuroblastomas is the high mortality rate and rapid tumor progression. In particular, pediatric patients whose neuroblastomas express high levels of BDNF and TrkB have an unfavourable prognosis. PPARβ/δ is a transcription factor abundantly expressed in the central nervous system, and was recently implicated in neuronal differentiation. D'Angelo *et al.* demonstrate that PPARβ/δ natural and synthetic ligands trigger neuronal differentiating activity in neuroblastoma cells. This effect relates to their ability to modulate BDNF and TrkB expression. This leads to a decrease in cell proliferation, neuronal differentiation and an increase of neuronal differentiation markers, thus suggesting a new therapeutic strategy for neuroblastoma. The authors also investigated neuroblastoma cells after PPAR β/δ silencing and the effects of natural and synthetic PPARβ/δ ligands on the BDNF signal transduction pathway. After silencing, no differentiating activity was observed, thus supporting PPARβ/δ's dependence on differentiating effects. The authors provide evidence to further support the important role of PPAR β/δ as a modulator of pathways crucial for neuronal differentiation.



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Journal of Cellular Physiology

SH2B1β Regulates N-Cadherin Levels, Cell–Cell Adhesion and Nerve Growth Factor-Induced Neurite Initiation

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Little is known regarding the role of inter-cellular interaction during neuronal differentiation. Homophilic N-cadherin engagement between cells contributes to neuronal migration. However, its function in neurite initiation is not clear. In this study, we provide the first evidence that the adaptor protein SH2B1β regulated N-cadherin levels and neurite initiation. Overexpression of SH2B1β reduces N-cadherin levels and increased phosphotyrosine 654 β-catenin, leading to increased nerve growth factor-induced neurite initiation in PC12 cells, an established model for neuronal differentiation. In contrast, overexpression of the dominant-negative mutant SH2B1β(R555E) increases N-cadherin expression, cell–cell aggregation, and reduces neurite initiation. Moreover, SH2B1β binds directly or indirectly to N-cadherin indicative of its involvement in regulating the levels of N-cadherin. Taken together, these findings provide significant new insights into how N-cadherin-mediated inter-cellular interactions may influence neurite initiation and how SH2B1β may regulate these processes.

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Morphogenesis during neuronal differentiation is regulated by a variety of processes, including signal transduction, cell adhesion, gene expression, and proliferation. The microenvironment that coordinates neuronal precursor maintenance versus differentiation is poorly understood. Cell–extracellular matrix (ECM) interactions have been shown to play instrumental role in neuronal differentiation. However, little is known regarding the role of cell–cell adhesion during neuronal differentiation. One of the main cell adhesion molecules (CAMs) in neuronal cells, N-cadherin, forms calcium-dependent, trans-homophilic dimers to mediate cell–cell interaction that serves as molecular cues for shaping both anatomically distinct brain domains and neural circuits (Redies, 2000). The expression levels of adhesion molecule N-cadherin have been shown to regulate neuronal migration during development. Reduced N-cadherin and cadherin 6B expression in neural crest cells may be a prerequisite for emigration from the neural tube (Hatta et al., 1987; Nakagawa and Takeichi, 1995, 1998). Once the neural crest cells reach their targets, N-cadherin levels are increased (Hatta et al., 1987; Akitaya and Bronner-Fraser, 1992). Overexpression of N-cadherin in neural crests inhibits neural crests delamination during development (Shoval et al., 2007). In contrast, genetic deletion of N-cadherin results in embryonic lethality due to massive cardiac and neural tube defects (Radice et al., 1997). These results indicate that regulated expression of N-cadherin is essential during development. N-Cadherin-mediated cell adhesion is augmented by N-cadherin linkage to the actin cytoskeleton through the association of its cytosolic domain to β- and then α-catenin (Kwiatkowski et al., 2007; Suzuki and Takeichi, 2008). Phosphorylation of β-catenin has also been suggested to regulate its interaction with N-cadherin and thus cell–cell adhesion (Schuman and Murase, 2003; Rhee et al., 2007).

Despite many studies implicating the role of N-cadherin in neural migration, the involvement of N-cadherin in neurite initiation has not been investigated. PC12 cells provide an excellent experimental system for studying the mechanisms that drive the initiation of neurite outgrowth. Studying neuronal development in the PC12 cell culture system has some advantages over the study of this process in primary neural culture. While primary cultures develop beautiful neuronal architectures in the 2-week post-explantation period, the changing morphology likely reflects a restorative regeneration of neuronal structure, rather than the de novo differentiation that occurs in NGF-treated PC12 cells. We and others have shown that overexpressing the signaling adaptor protein SH2B1 enhances nerve growth factor (NGF)-, glial-derived neurotrophic factor (GDNF)-, and fibroblast growth factor 1 (FGF1)-induced neuronal differentiation of PC12 cells, a well-established neuronal model system (Qian et al., 1998; Rui et al., 1999; Zhang et al., 2006; Lin et al., 2009). SH2B1 belongs to SH2B family members. Four SH2B1 splice variants (α, β, γ, and

Abbreviations: NGF, nerve growth factor; Cdk5, Cyclin-dependent-kinase 5; ECM, extracellular matrix.

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δ) have been identified so far. In response to NGF, SH2B1β (β isoform of SH2B1) is phosphorylated and recruited to the activated NGF receptor, TrkA (Rui et al., 1999). Although the SH2B1β dominant-negative mutant, SH2B1β(R555E), inhibits NGF-induced neuronal differentiation, it does not affect NGF-initiated signaling (Rui et al., 1999). These findings imply that SH2B1β may utilize other mechanisms to direct differentiation. In this study, we tested whether SH2B1β may regulate cell adhesion to affect neuronal differentiation. In addition to present a novel regulatory mechanism of neurite initiation in PC12 cells, we report that SH2B1β regulates N-cadherin expression, the formation of N-cadherin/β-catenin complexes, and thereby modulating cell–cell adhesion and NGF-induced neurite initiation.

Materials and Methods

Antibodies, plasmids, and reagents

Anti-β-catenin was from Millipore (Billerica, MA). Anti-N-cadherin was from ECM Biosciences (Versailles, KY). Anti-NCAM and anti-phosphotyrosine 654 (pY654)-β-catenin were from Developmental Studies Hybridoma Bank at the University of Iowa (DiFiglia et al., 1989; Rhee et al., 2007). Anti-ERK1/2 antibody was obtained from Sigma (St. Louis, MO). Anti-GFP antibody was obtained from Clontech (Mountain View, CA). Anti-rabbit, anti-mouse Alexa Fluor 555-conjugated antibodies and Prolong Gold were from Invitrogen (Carlsbad, CA). pEGFP-C1 vector, GFP-SH2B1β and GFP-SH2B1β(R555E) constructs were purchased and made according to Chen and Carter-Su (2004). Complete cDNAs of SH2B1β and SH2B1β(R555E) were cloned into pGEX-KT vector between *Bam*HI and *Eco*RI sites to engineer glutathione-S-transferase (GST)-SH2B1β and GST-SH2B1β(R555E) constructs. NGF, rat-tail collagen I, and growth factor-reduced Matrigel were purchased from BD Biosciences (San Jose, CA).

Cell lines and cultures

PC12 and COS-7 cells were purchased from American Type Culture Collection (Manassas, VA). PC12 cells were maintained on the collagen I-coated plates and grown in complete medium, Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat-inactivated horse serum (HS), 5% fetal bovine serum (FBS), 1 mM L-glutamine and 1 mM antibiotic-antimycotic (AA) (Invitrogen), at 37°C, 10% CO_2. COS-7 cells were cultured in DMEM containing 10% FBS, 1 mM L-glutamine and 1 mM AA, at 37°C, 5% CO_2. PC12 cells stably overexpressing GFP or GFP-SH2B1β were made and cultured as described in Chen et al. (2008). To make PC12-R555E stable cell line, PC12 cells transiently transfected with GFP-SH2B1β(R555E) (with point mutation on amino acid 555, converting Arg–Glu) were cultured in G418-containing medium for 45 days before sorting for green fluorescence-positive cells using flow cytometry. To make N-cadherin knockdown stable cell line (N-cad KD), pLKO.1 lentiviral vector that contains oligoneucleotides (CCGGGCAGTGGACATCAATGGCAATCTCGAGATTGCC-ATTGA TGTCCACTGCTTTTTG) targeting mouse Cdh2 sequences (GCAGTGGACATCAATGGCAAT), pLKO.1-shN-cadherin (Clone number TRCN0000094857), was obtained from National Core Facility at the Institute of Molecular Biology, Genomic Research Center, Academic Sinica, Taiwan. Lentivirus containing pLKO.1-shN-cadherin was prepared by the same RNAi Core Facility and used to infect PC12 cells followed by selecting for puromycin-resistant clones for at least 60 days. Pooled populations of stable clones were used to avoid clonal variation.

Neurite outgrowth and measurement of neurite outgrowth velocity

For NGF-induced neurite outgrowth, PC12 cells were seeded onto collagen I-coated six-well plates at about 30% confluency. The next day, culture medium was changed to low serum differentiation medium (DMEM containing 2% HS, 1% FBS, 1 mM L-glutamine, 1 mM AA) in the presence of 50 or 100 ng/ml NGF. For time lapse microscopy, Axio Vision "Mark & Find" software (Zeiss) was used to take live cell images of the same coordinates every 30 min for 48 h. In response to NGF, cells with neurites of which the length was at least the diameter of the cell body were counted. During early stages of neurite outgrowth, cells were often motile and thus the relative position of cells varied from time to time. Images of cells were taken using Zeiss Axiover 135 or Carl Zeiss Observer Z1 fluorescence microscope. For measuring neurite outgrowth velocity, Image J software was used to trace 50 longest neurites among 800 cells for each stable cell lines. The velocity was obtained by calculating the averaged length of neurites divided by time of NGF treatment.

Cell aggregation assay

Cells of 50% confluency were detached using PBS containing 2 mM EGTA for 10–15 min at 37°C. Detached cells were washed once with PBS and then gently re-suspended in either Ca^{2+}-free medium [Ca^{2+}-free DMEM, 0.1% bovine serum albumin (BSA), 1% AA and 1% L-glutamine] or Ca^{2+}-containing medium (DMEM, 0.1% BSA, 1% AA and 1% L-glutamine). Equal amounts of cells in six-well plates were shaken at 60 rpm in a 37°C floor shaker (to avoid any confounding effect from cell–extracellular matrix adhesion). Images were taken using Carl Zeiss Observer Z1. Cell aggregates of >30 cells per aggregation unit were counted at 0 and 60 min of shaking. The percentage of >30 cells/aggregate unit was calculated. More than 100 U per condition per cell line were counted from three independent experiments.

Immunoblotting and immunoprecipitation

Cells were harvested into RIPA buffer (50 mM Tris–HCl, pH7.5, 150 mM NaCl, 2 mM EGTA, 1% Triton X-100) containing 1 mM Na_3VO_4, 10 ng/ml aprotinin, 10 ng/ml leupeptin, and 1 mM phenylmethanesulphonyl fluoride (PMSF). Proteins were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and then immobilized to nitrocellulose membrane for Western blotting analysis using the indicated antibodies. The immunoblots were detected using either IRDye-conjugated IgG and the Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE) or horseradish peroxidase-conjugated IgG and ECL system.

For immunoprecipitation, 2 mg protein lysates were incubated with 4 μg of the corresponding antibody on ice overnight. Protein A-conjugated Sepharose beads (BD Biosciences) or Protein G agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA) were added and rotated at 4°C for 1 h. After washing with pre-chilled RIPA buffer, the immunoprecipitated proteins were analyzed using SDS–PAGE and Western blotting. Gel-Pro Analyzer 3.1 software was used to quantify results.

Transient transfection

The GFP-tagged full length of SH2B1β, SH2B1β(R555E), SH2B1β(270–670) (containing amino acids 270–670), and SH2B1β(1–260) (containing amino acids 1–260) plasmids were constructed as described in Chen and Carter-Su (2004). PC12 or COS-7 cells were plated onto 10-cm with (for PC12 cells) or without (for COS-7 cells) collagen I-coated dishes 1 day before transfected with 10 or 1 μg DNA. Cell lysates were collected for analysis 48 h after transfection. For N-cadherin transfection experiments, 5 μg N-cadherin-GFP or CTF2-GFP (containing amino acids 752–912 of N-cadherin), a gift from Dr. Chaya Kalcheim at Hebrew University-Hadassah Medical School, Jerusalem, Israel (Shoval et al., 2007), was used to transfect PC12 cells. Neurite initiation was monitored after 48 h of NGF stimulation.

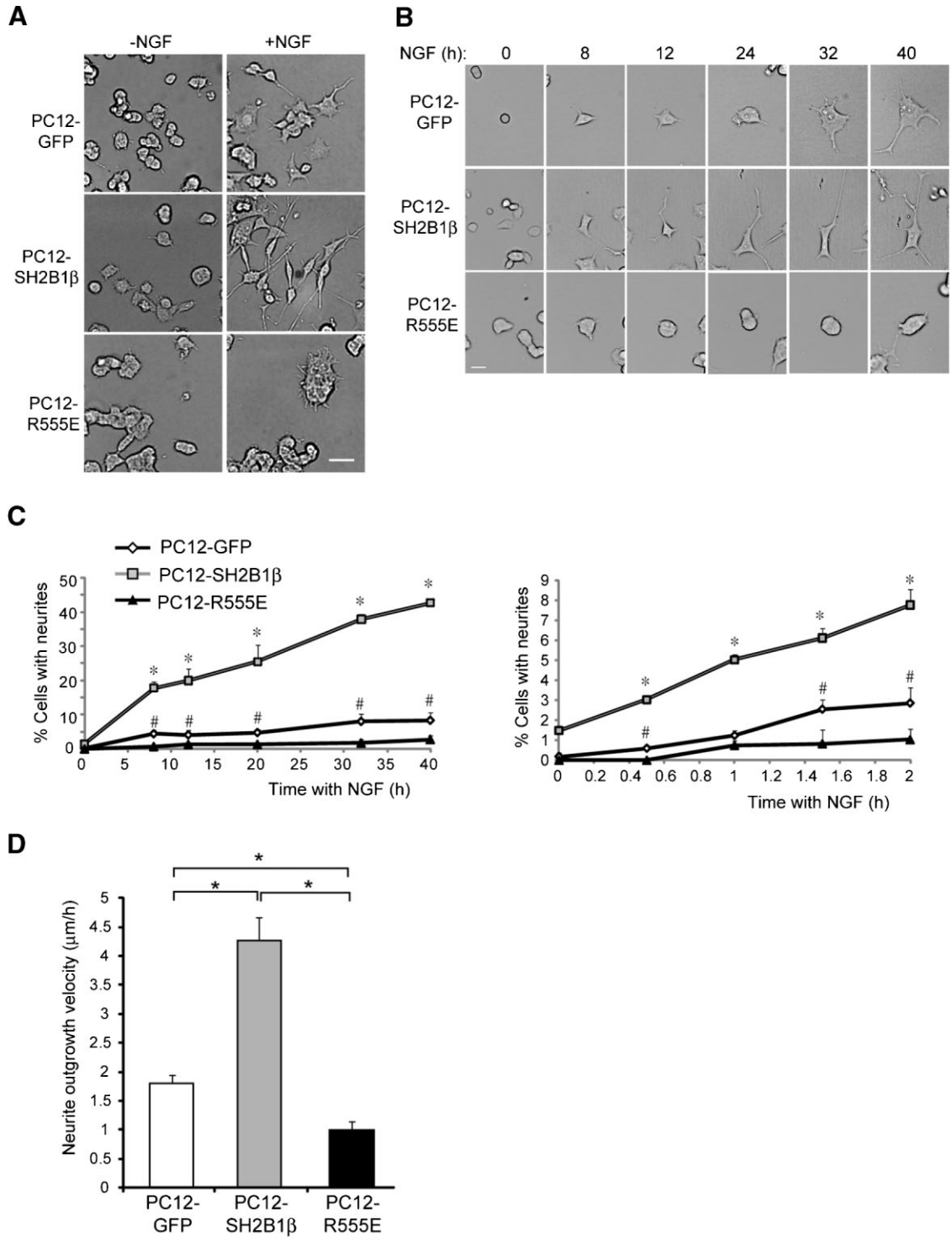

Fig. 1. NGF-induced neurite initiation and elongation in PC12-GFP, PC12-SH2B1β, and PC12-R555E cells. All live cell images were taken as described in the Materials and Methods Section, and representative images are shown. A: PC12-GFP, PC12-SH2B1β, and PC12-R555E cells were treated without (−NGF) or with 100 ng/ml NGF (+NGF) for 40 h. Scale bar: 50 μm. B: PC12-GFP, PC12-SH2B1β, and PC12-R555E cells were treated with 50 ng/ml NGF for the indicated time periods. Scale bar: 20 μm. C: PC12-GFP, PC12-SH2B1β, and PC12-R555E cells were treated as described in (B). A total of 198–266 cells per time point per cell line were counted from three independent experiments and the percentage of cells with neurites was quantified. D: PC12-GFP, PC12-SH2B1β, and PC12-R555E cells were treated with 50 ng/ml NGF for 48 h. Fifty longest neurites were measured and velocity of neurite outgrowth was calculated as described in the Materials and Methods Section (N = 3). *Significant difference between PC12-GFP and PC12-SH2B1β cells. #Significant difference between PC12-GFP and PC12-SH2B1β(R555E) cells.

GST pull down assays

pGEX-KT vector, GST-SH2B1β and GST-SH2B1β(R555E) constructs were expressed in BL21 strain and lysates were harvested in harvest buffer (40 mM Tris pH 8.0, 100 mM NaCl, 5 mM EDTA, 1% Triton X-100) containing 10 ng/ml aprotinin, 10 ng/ml leupeptin, and 1 mM PMSF. GST-fusion proteins were pulled down via glutathione Sepharose 4B (GE Healthcare, Piscataway, NJ). Sepharose beads were then incubated with COS-7 cell lysates for 2 h at 4°C. Beads were washed using RIPA buffer. Sample buffer was added to the beads and boiled to dissociate proteins from beads.

Immunofluorescence staining

PC12 cells were seeded on growth factor-reduced Matrigel-coated coverslips, then cultured in multi-well dishes and treated as indicated. Cells were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100. Cells were then pre-treated with blocking buffer, washed and then incubated with specific antibodies. Anti-N-cadherin and anti-β-catenin antibodies were used at a dilution of 1:200 and Alexa Fluor 555-conjugated IgG was used at a dilution of 1:1,000. Cells were mounted with Prolong Gold (Invitrogen). Confocal images were taken using Zeiss LSM 5 PASCAL microscope system.

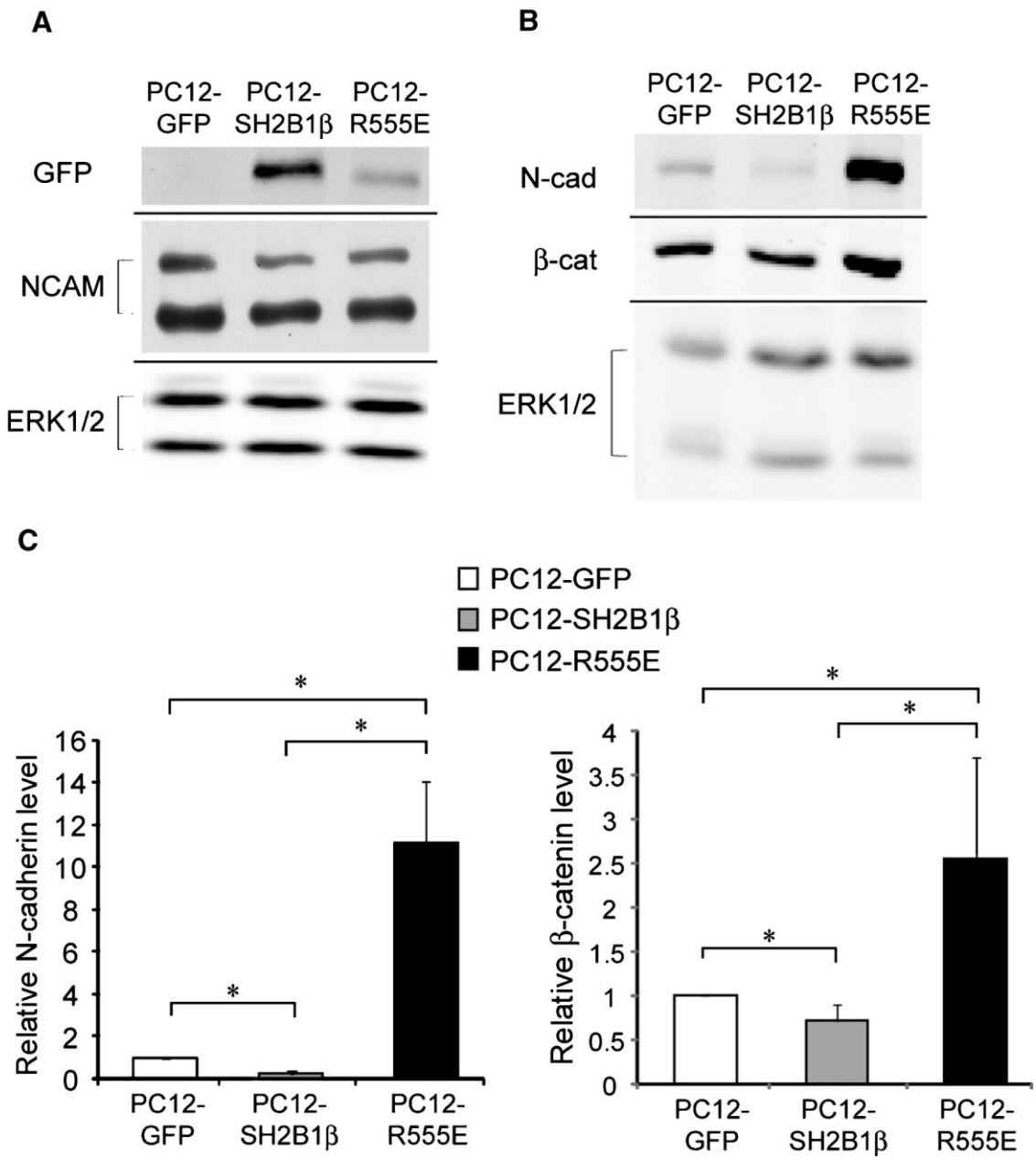

Fig. 2. The levels of NCAM and N-cadherin in PC12-GFP, PC12-SH2B1β, and PC12-R555E cells. A,B: Cell lysates from PC12-GFP, PC12-SH2B1β, and PC12-R555E cells were collected and resolved via SDS–PAGE and immunoblotted with anti-GFP, NCAM, N-cadherin (N-cad), β-catenin (β-cat), or ERK1/2 antibody. Total ERK1/2 was used as a loading control. C: Relative protein levels of N-cadherin (N = 9) and β-catenin (N = 6) were quantified, normalized to total ERK 1/2 levels, and compared among cell lines.

Statistical Analysis

All results were expressed as mean ± SE. The paired Student's *t*-test was performed. Significance (*) was set at $P < 0.05$.

Results

SH2B1β and SH2B1β(R555E) differentially regulate neurite initiation, cell–cell adhesion and N-cadherin levels

Neurite initiation is a phase of dynamic formation and retraction of the neuronal processes. Neurite elongation phase starts after initiation when cells exhibit stable elongation of the processes. To assess whether SH2B1β regulates neurite initiation or elongation, PC12 cells stably expressing GFP (PC12-GFP cell line), GFP-SH2B1β (PC12-SH2B1β cell line) or GFP-SH2B1β(R555E) (PC12-R555E cell line) were stimulated with NGF and neurite outgrowth was monitored. Consistent with previous results, the PC12-SH2B1β cell line had an increased percentage of cells bearing neurites whereas PC12-R555E cells showed reduced neurite outgrowth compared to PC12-GFP cells (Fig. 1A). Neurite initiation and elongation were assessed by time-lapse microscopy (Fig. 1B). For PC12-SH2B1β cells, neurites were more rapidly initiated and more stable with time compared to PC12-GFP cells (Fig. 1B). The percentage of cells bearing neurites was much higher in PC12-SH2B1β cells compared to PC12-GFP cells at all time points. For PC12-R555E cells, neurite outgrowth was significantly reduced with neurites detected only on a very small fraction of the cells at very late time points (Fig. 1B,C and Supplemental Fig. 1). Indeed, the vast majority of PC12-R555E cells showed no evidence of neurite initiation over the entire 48 h time course. Among cells with neurites, the neurite outgrowth velocity was measured for the top 50 longest neurites. As revealed in Figure 1D, the neurite outgrowth velocity in PC12-SH2B1β cells was significantly faster than the other two cell lines. These data suggest that overexpressing SH2B1β enhances NGF-induced neurite initiation whereas overexpressing SH2B1β(R555E) significantly delays neurite initiation.

The other notable effect seen in the above analysis (Fig. 1) is the striking cell–cell aggregation induced in PC12-R555E cells (Fig. 1A,B, lower parts). The aggregative phenotype of

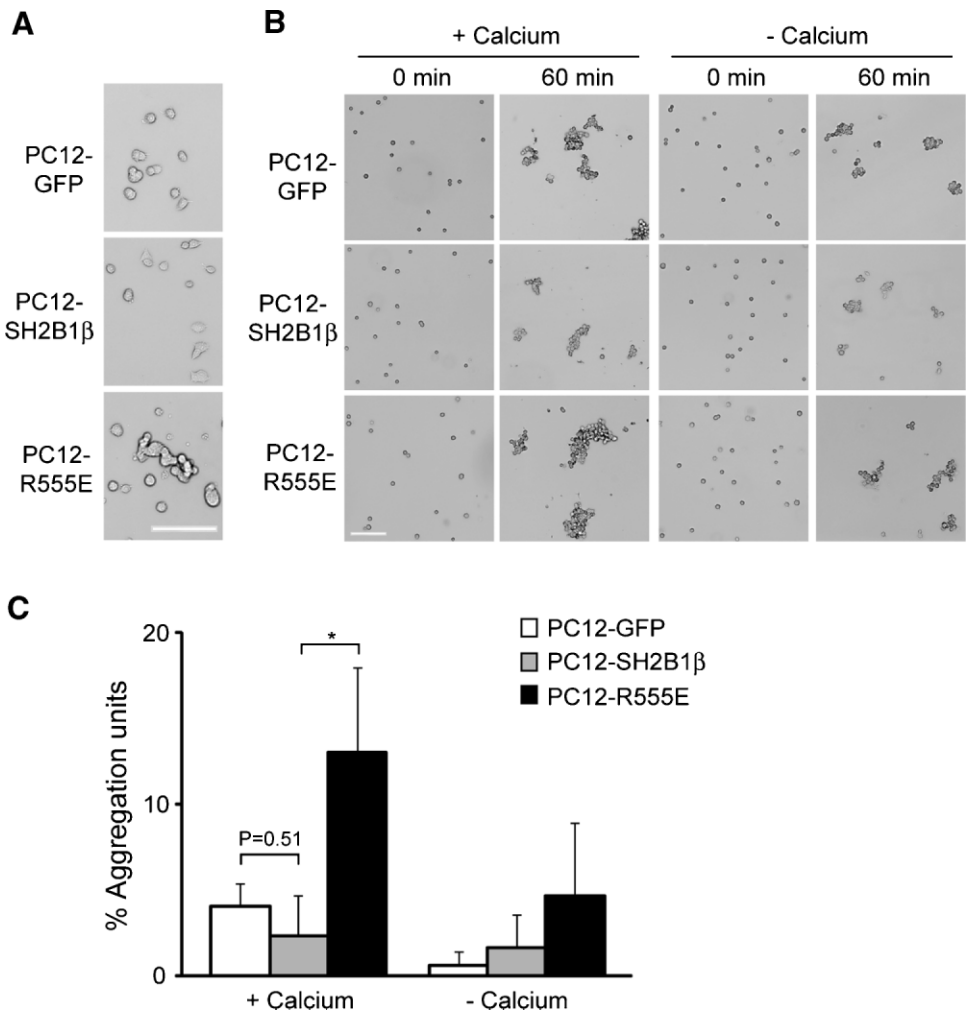

Fig. 3. Calcium-dependent cell aggregation in PC12-GFP, PC12-SH2B1β, and PC12-R555E cells. A: Equal numbers of PC12-GFP, PC12-SH2B1β, and PC12-R555E cells were seeded onto collagen I-coated plates, and live cell images were taken after 48 h. Representative images are shown. Scale bar: 100 μm. B: Equal amounts of PC12-GFP, PC12-SH2B1β, and PC12-R555E cells were added to non-coated plates in either Ca^{2+}-containing DMEM (+Calcium) or Ca^{2+}-free DMEM (−Calcium) and shaken for 0 or 60 min at 37°C as described in the Materials and Methods Section. Representative live cell images are shown. Scale bar: 100 μm. C: Cells were treated as described in (B). Percentages of aggregation units were counted. Cell aggregation was quantified as described in the Materials and Methods Section.

PC12-R555E cells and their ability to inhibit neurite initiation lead us to hypothesize that overexpressing SH2B1β(R555E) may increase cell–cell adhesion and thereby interfere with neurite initiation. In neuronal cells, neural cell adhesion molecule (NCAM) and N-cadherin are two of the major adhesion molecules (reviewed in Kiryushko et al., 2004). Expression of these adhesion molecules was examined in PC12-GFP, PC12-SH2B1β, and PC12-SH2B1β(R555E) stable cell lines (Fig. 2A). NCAM showed only minor expression-level changes, being slightly reduced in PC12-SH2B1β cells relative to levels seen in PC12-GFP and PC12-R555E cells (Fig. 2A, middle part). In contrast, significant changes of N-cadherin expression levels were seen—reduced to 0.4-fold in PC12-SH2B1β cells and increased to 11-fold in PC12-R555E cells compared with PC12-GFP control cells (Fig. 2B, top part). As β-catenin is an N-cadherin binding partner at the cell–cell junction serving to stabilize adhesion, its levels were also determined (Fig. 2B, middle part). Correlated changes in β-catenin expression were found, with significantly up-regulated expression seen in PC12-R555E cells and somewhat down-regulated expression in PC12-SH2B1β cells (Fig. 2B, middle part). Quantification of N-cadherin and β-catenin protein levels is shown in Figure 2C. Both N-cadherin and β-catenin levels were reduced in PC12-SH2B1β cells and significantly increased in PC12-R555E cells compared with PC12-GFP cells. These results suggest that the high cell–cell adhesion shown in PC12-R555E cells may result from increased level of N-cadherin.

The increased cell–cell adhesion in PC12-R555E cells is in part Ca^{2+}-dependent

The high levels of N-cadherin and β-catenin expression found in PC12-R555E cells suggest a possible explanation for aggregative behavior of these cells. To test if the high level of cell–cell adhesion seen in PC12-R555E cells might be a consequence of elevated N-cadherin expression, we test if PC12-R555E cell aggregation is Ca^{2+}-dependence, since N-cadherin-mediated cell adhesion requires Ca^{2+}. Cell aggregation assays were carried out in the presence or absence of Ca^{2+} (Fig. 3). In the presence of Ca^{2+}, the percentage of aggregation units in PC12-R555E cells was the highest (13%) compared with PC12-GFP (4%) and PC12-SH2B1β (2.3%) cells. In the absence of Ca^{2+}, cell aggregation was reduced in all three stable lines, especially in PC12-R555E cells. Although aggregation was evident in the absence of Ca^{2+}, significantly enhanced aggregation was seen for all three cell lines in the presence of Ca^{2+}, consistent with a major N-cadherin involvement in cell–cell adhesion. Furthermore, consistent with the N-cadherin expression levels documented above (Fig. 2), Ca^{2+}-induced aggregation was the greatest for PC12-R555E and the least for PC12-SH2B1β cells (Fig. 3). Thus, the differential expression levels of N-cadherin in PC12-R555E and PC12-SH2B1β cells are likely responsible for their different aggregative behaviors.

The subcellular distribution of N-cadherin and β-catenin was determined by immunofluorescence staining. N-cadherin mainly localized to the cell surface, concentrating at the cell–cell contact sites (Fig. 4A). Consistent with results from Figure 2, the relative N-cadherin level was the highest in PC12-R555E and lowest in PC12-SH2B1β cell lines. Similarly, β-catenin level at the cell surface was low in PC12-SH2B1β and high in PC12-R555E cells suggesting that N-cadherin recruits β-catenin to the cell–cell contact (Fig. 4B). These results support the notion that, in PC12-R555E cells, cell surface localized N-cadherin and β-catenin function at the cell–cell contact to increase cell–cell adhesion.

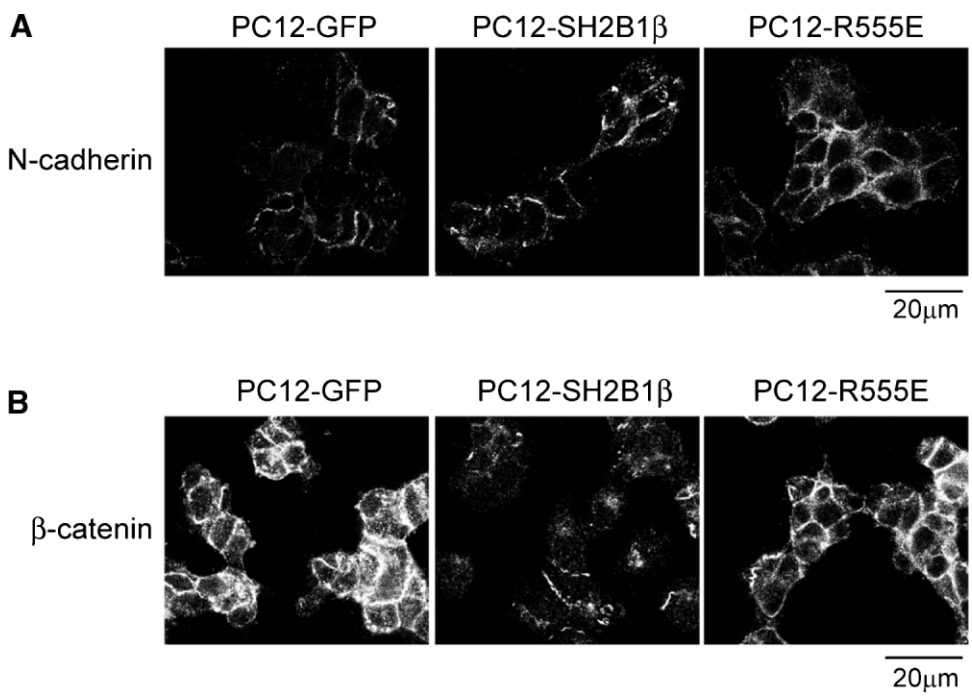

Fig. 4. N-Cadherin and β-catenin localize to the cell–cell contact sites. A: PC12-GFP, PC12-SH2B1β, and PC12-R555E cells were fixed, permeabilized and incubated with anti-N-cadherin (A) or anti-β-catenin (B) antibody followed by Alexa Fluor 555-conjugated secondary antibody. Confocal images were taken using Zeiss LSM 5 PASCAL and representative images are shown from three independent experiments. Scale bar: 20 μm.

Overexpressing N-cadherin reduces NGF-induced neurite initiation

It is possible that the observed inhibitory or stimulating effects on neurite initiation is a secondary effect of the overexpression of SH2B1β or SH2B1β(R555E) on mechanisms other than affecting N-cadherin. To further determine whether N-cadherin levels directly affect neurite initiation, GFP, N-cadherin-GFP, or CTF2-GFP were transiently transfected to PC12 cells, and NGF-induced neurite initiation was evaluated.

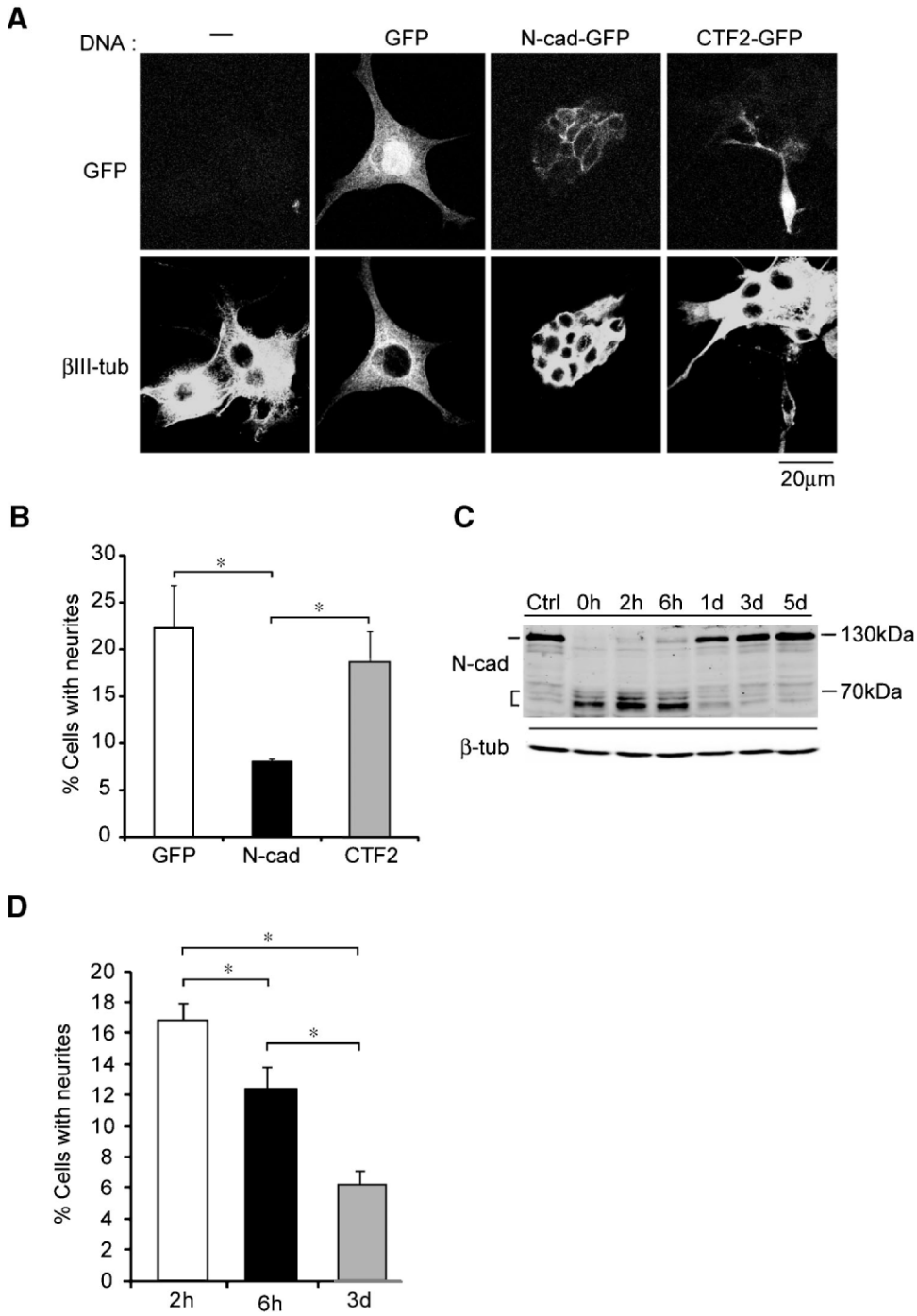

Fig. 5. Overexpressing N-cadherin inhibits NGF-induced neurite initiation. A: PC12 cells transiently transfected with GFP vector, N-cadherin-GFP (N-cad-GFP), or CTF2-GFP were treated with 100 ng/ml NGF for 48 h. Representative confocal images are shown. GFP fluorescence (top parts) showed the transfected cells whereas immunostaining with neuronal tubulin (βIII-tub) was used to show cell morphology (bottom parts). B: Neurite initiation was compared among GFP-, N-cadherin-GFP (N-cad)-, or CTF2-GFP (CTF2)-transfected cells (N = 3). C: PC12-R555E cells were treated with trypsin-EDTA to cleave cell surface N-cadherin and then re-plated for 0 h to 5 days. The amount of N-cadherin without trypsin-EDTA treatment is shown as "Ctrl." "–" Indicates the full length of N-cadherin. Bracket showed the cleaved products of N-cadherin. β-tubulin (β-tub) levels were used as loading control. D: NGF (100 ng/ml) was added to 2h, 6h, or 3d re-plated cells for 48 h and neurite initiation was quantified from three independent experiments.

CTF2-GFP is a truncated N-cadherin construct lacking the extracellular and transmembrane domains and thus cannot engage inter-cellular adhesion. Upon NGF stimulation for 48 h, most of the un-transfected, GFP- or CTF2-GFP-transfected cells have initiated short processes or neurites. However, cells overexpressing N-cadherin-GFP in an aggregate significantly reduced neurite initiation (Fig. 5A,B). These results suggest that high levels of trans-homophilic interactions of N-cadherin among cells inhibited NGF-induced neurite initiation.

If overexpression of N-cadherin is the main reason the neurite initiation is inhibited in PC12-R555E cells, reducing N-cadherin should relieve the inhibitory effect. To this end, cell surface N-cadherin in PC12-R555E cells was cleaved by trypsin and then cells were re-plated. Full length N-cadherin was re-synthesized in 2 h and the level increased over time (Fig. 5C). NGF was added to 2, 6 h or 3 days re-plated cells and neurite initiation was quantified. As shown in Figure 4D, neurite initiation from 2 and 6 h re-plated cells was much faster than that from 3 days re-plated cells of which N-cadherin levels were much higher.

Furthermore, we have established a stable cell line that expresses reduced N-cadherin levels (Fig. 6A). In response to NGF, N-cadherin knockdown cell line clearly initiated neurites faster than control cells (Fig. 6B). The percentages of cells bearing neurites and percentages of differentiated cells in N-cadherin knockdown cell line was twice of those in control cells (Fig. 6C,D). These results are in line with our hypothesis that reduced N-cadherin levels in PC12-SH2B1β cells enhances neurite initiation whereas increased N-cadherin in PC12-R555E cells contributes to the inhibitory effect on neurite initiation.

Phosphorylation of tyrosine 654 on β-catenin correlates with reduced interaction between N-cadherin and β-catenin

To further investigate the regulation of N-cadherin levels, co-immunoprecipitation experiments were carried out to assess the interaction between N-cadherin and β-catenin among the three stable cell lines. Co-immunoprecipitation of N-cadherin using anti-β-catenin antibodies revealed that the relative amount of N-cadherin bound to β-catenin was the lowest in PC12-SH2B1β and the highest in PC12-R555E cells (Fig. 7A,B). Phosphorylation of β-catenin at tyrosine 654 (pY654) was suggested to reduce cadherins/β-catenin complex (Schuman and Murase, 2003). Consistent with this, the relative amount of pY654 β-catenin was found higher in PC12-SH2B1β cells as compared to those in PC12-GFP and PC12-R555E cells (Fig. 7A,C), suggesting that higher pY654 β-catenin levels were associated with lower amounts of N-cadherin/β-catenin

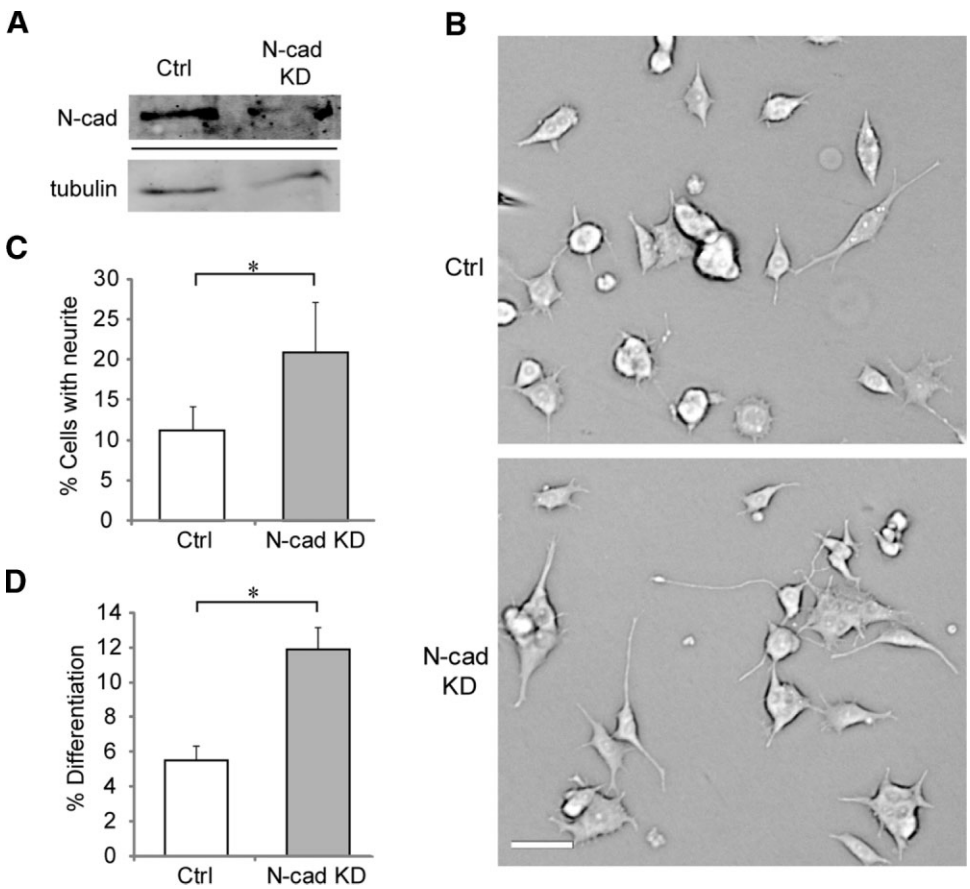

Fig. 6. Reducing N-cadherin increases neurite initiation. A: Protein levels of N-cadherin in PC12 stable cell line that expresses reduced N-cadherin (N-cad KD) and in control (Ctrl) cells were compared via Western blotting using anti-N-cadherin (N-cad) or anti-tubulin (tubulin) antibody (as loading control). Representative blots from three independent experiments are shown. B: Control and N-cad KD cells were treated with 50 ng/ml NGF for 24 h. Representative live cell images are shown. Scale bar: 20 μm. Percentages of cells bearing neurites (C) or percentages of differentiated cells (D) were determined and shown (N = 3). The definition of differentiation in PC12 cells is that the length of neurites must be at least twice of the diameter of the cell body.

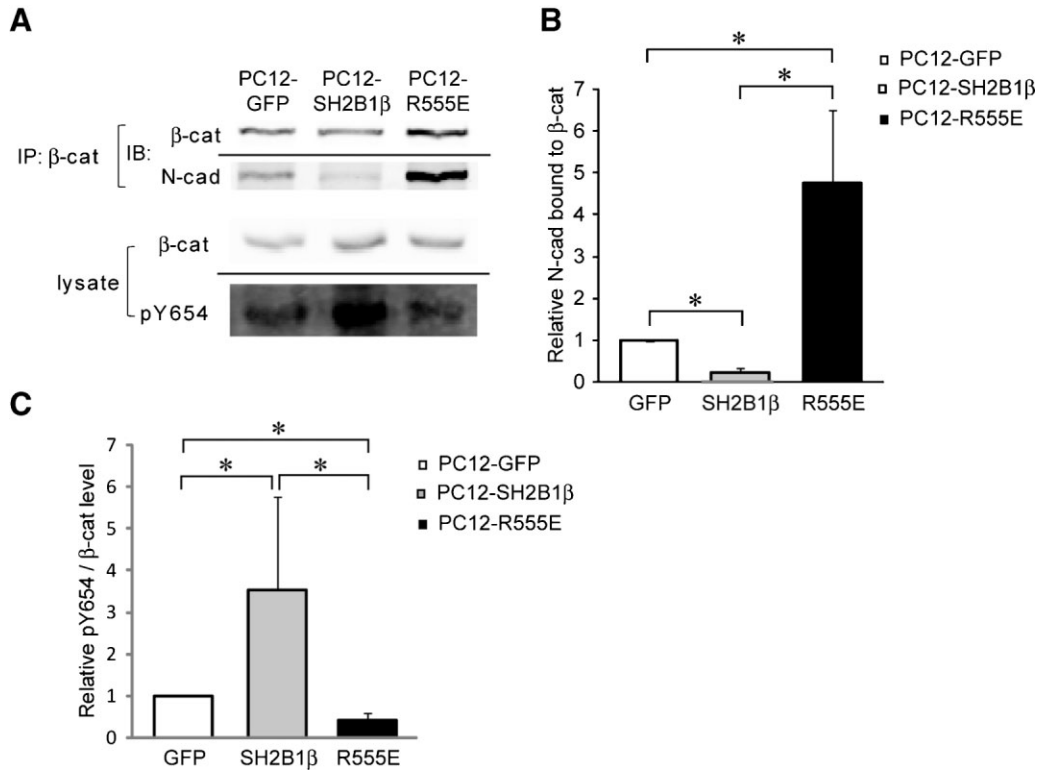

Fig. 7. Phosphorylation of tyrosine 654 correlates with reduced interaction between N-cadherin and β-catenin. A: Cell lysates from PC12-GFP, PC12-SH2B1β, and PC12-R555E cells were immunoprecipitated with anti-β-catenin (β-cat) antibody and immunoblotted with anti-β-catenin or anti-N-cadherin (N-cad) antibody. The bottom two parts show cell lysates resolved via SDS–PAGE and immunoblotted with anti-β-catenin, or anti-phosphotyrosine 654 of β-catenin (pY654) antibody. B: Relative amount of N-cadherin bound to β-catenin was quantified (N = 6) by normalizing to immunoprecipitated β-catenin levels. C: Relative amount of pY654 β-catenin in each stable cell line was quantified and normalized to β-catenin levels (N = 3). Lysate control = 5% of input for immunoprecipitation.

complexes. These results imply that SH2B1β and SH2B1β(R555E) differentially affect pY654 β-catenin and thus the interactions between N-cadherin and β-catenin.

SH2B1β interacts directly or indirectly with N-cadherin

The fact that SH2B1β regulated the levels of N-cadherin, we asked whether SH2B1β interacts with N-cadherin. To this end, GST-SH2B1β was pulled down by glutathione-S-Sepharose and then incubated with COS-7 cell lysates. As demonstrated in Figure 8A, N-cadherin was pulled down by SH2B1β but significantly less by its dominant negative mutant SH2B1β(R555E). To determine whether SH2B1β interacts with N-cadherin in PC12 cells, GFP-SH2B1β was transiently transfected to PC12 cells, treated without or with NGF, and GFP-SH2B1β was immunoprecipitated by anti-GFP antibody. Similarly, N-cadherin was co-immunoprecipitated with SH2B1β at resting state. Interestingly, the interaction between SH2B1β and N-cadherin was reduced in the presence of NGF (Fig. 8B). The lysate control on the right parts showed that SH2B1β was phosphorylated in the presence of NGF. Thus, it is possible that SH2B1β dissociates from N-cadherin once being phosphorylated. To identify which domain(s) is required for the interaction with N-cadherin, various mutants of SH2B1β were transiently transfected to COS-7 cells and immunoprecipitated. Full length SH2B1β and SH2B1β(270–670) interacted with N-cadherin whereas the interaction of SH2B1β(R555E) and SH2B1β(1–260) with N-cadherin was significantly reduced (Fig. 8C). Co-immunoprecipitation experiments using PC12 cell lysate confirmed that the interaction between SH2B1β(R555E) and N-cadherin was much reduced compared to SH2B1β (Fig. 8D). Because SH2B1β(270–670) contains both PH and SH2 domains and SH2B1β(R555E) contains a defective SH2 domain, these results suggest the importance of PH and SH2 domains in interacting with N-cadherin. Interestingly, the interaction between SH2B1β and N-cadherin was reduced in response to NGF stimulation. This result raises an intriguing possibility that SH2B1β binds directly or indirectly to N-cadherin to reduce the stability of N-cadherin thus reduces the formation of N-cadherin/β-catenin complexes at resting state, allowing for earlier neurite initiation.

Discussion

The current study demonstrates that overexpression of the adaptor protein SH2B1β reduces the expression of N-cadherin, whereas overexpression of the dominant-negative mutant SH2B1β(R555E) increases N-cadherin levels, interaction between N-cadherin and β-catenin and cell–cell adhesion. Reduced interaction between N-cadherin and β-catenin in PC12-SH2B1β cells correlates with higher pY654 β-catenin levels and earlier neurite initiation. Furthermore, our data indicate that SH2B1β binds to N-cadherin at resting state. Together, these findings reveal a novel role of the adaptor protein SH2B1β in negatively regulates the levels of N-cadherin and the formation of N-cadherin/β-catenin complexes at resting state allowing for earlier neurite initiation upon NGF stimulation. Although we cannot exclude the possibility that

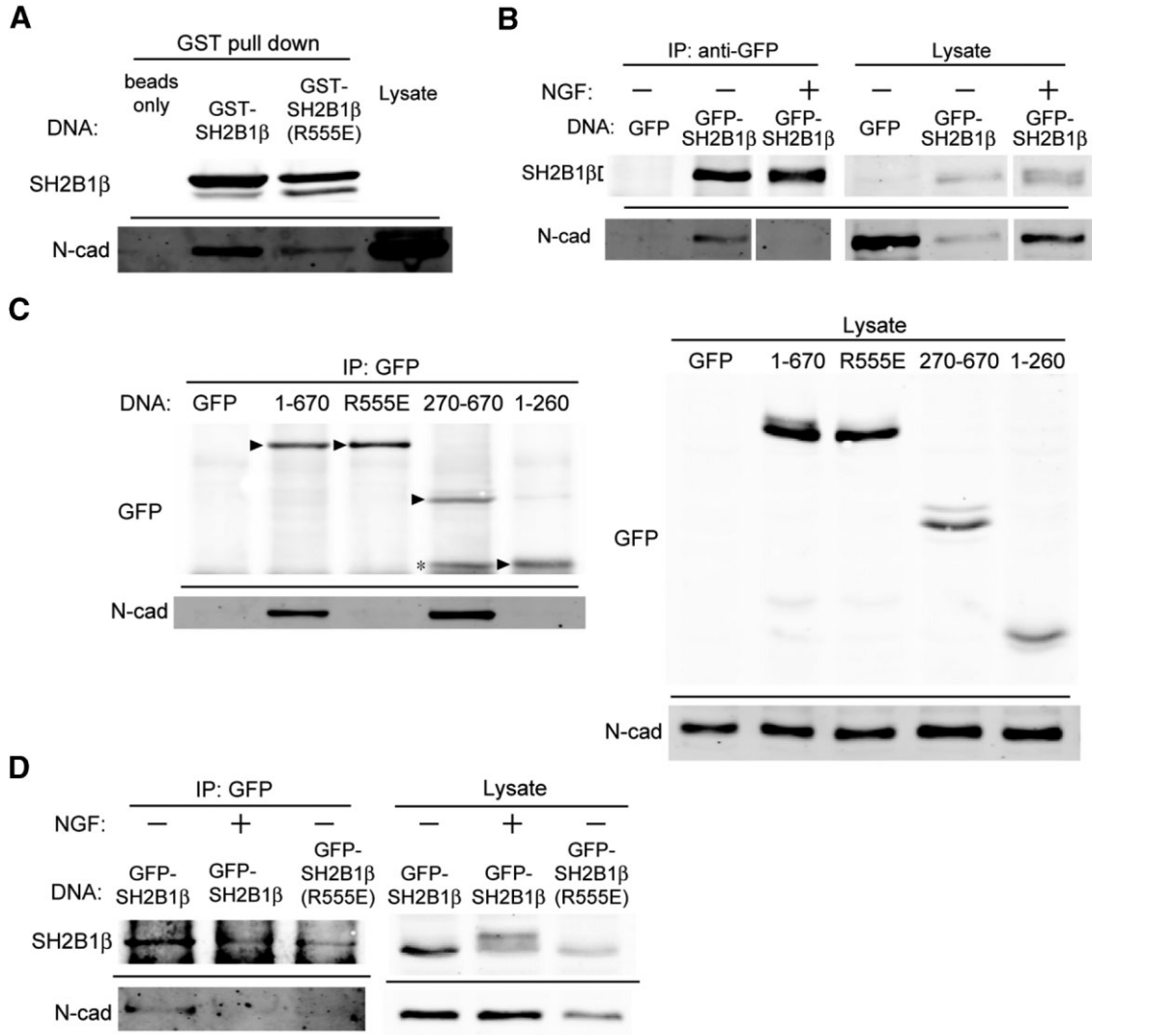

Fig. 8. SH2B1β interacts with N-cadherin. A: GST-SH2B1β or GST-SH2B1β(R555E) was purified via glutathione Sepharose beads. Equal amounts of COS-7 cell lysates were incubated with beads only, GST-SH2B1β, or GST-SH2B1β(R555E), followed by Western blotting using anti-SH2B1 (SH2B1β) or anti-N-cadherin (N-cad) antibody. Representative blots from two independent experiments are shown. COS-7 cell lysate control is shown on the right most lane. B: PC12 cells were transiently transfected with GFP vector or GFP-SH2B1β construct and treated without (−) or with (+) 100 ng/ml NGF for 10 min. Cell lysates were then subjected to immunoprecipitation using anti-GFP antibody and immunoblotted with anti-GFP (SH2B1β) or anti-N-cadherin (N-cad) antibody (left parts). Immunoblots of lysates are shown on the right parts. C: COS-7 cells were transiently transfected with GFP, GFP-SH2B1β, GFP-SH2B1β(R555E), GFP-SH2B1β(270–670) (contains amino acids 270–670), or GFP-SH2B1β(1–260) (contains amino acids 1–260). Cell lysates were immunoprecipitated using anti-GFP antibody and immunoblotted with anti-GFP (GFP) or anti-N-cadherin (N-cad) antibody. Representative blots are shown from three independent experiments. Immunoblots of cell lysates are shown on the right parts. Arrowheads point to GFP-fused SH2B1β constructs. Asterisk represents a non-specific band. D: PC12 cells were transiently transfected with GFP-SH2B1β or GFP-SH2B1β(R555E). Cells overexpressing GFP-SH2B1β were treated without (−) or with (+) 100 ng/ml NGF for 10 min. Cell lysates were immunoprecipitated using anti-GFP antibody and immunoblotted anti-GFP (SH2B1β) or anti-N-cadherin (N-cad) antibody. Immunoblots of cell lysates are shown on the right parts. Lysate control = 5% of input for immunoprecipitation or pull down assays.

SH2B1β interacts with an N-cadherin binding partner instead of N-cadherin directly, no interaction between SH2B1β and β-catenin or Cdk5 was found. Cdk5 has previously been shown to regulate the interaction between various cadherin members and β-catenin through affecting the phosphorylation of β-catenin. In hippocampal neurons, Cdk5 was shown to phosphorylate β-catenin at Y654 to reduce cadherins/β-catenin complex (Schuman and Murase, 2003). It is thus possible that SH2B1β binding, either directly or indirectly, to N-cadherin would prevent a putative phosphatase from de-phosphorylating β-catenin, and thereby reduces N-cadherin/β-catenin complex formation. With reduced binding to N-cadherin, SH2B1β(R555E) cannot efficiently prevent the access of this phosphatase, resulting in reduced pY654 β-catenin levels and increased N-cadherin/β-catenin complex formation. Alternatively, SH2B1β may recruit a kinase that phosphorylates β-catenin at pY654 to reduce the formation of N-cadherin/β-catenin complexes whereas SH2B1β(R555E), with a defective SH2 domain, is not able to do so (Fig. 9). In response to NGF, SH2B1β is phosphorylated and dissociates from N-cadherin. One may predict that this dissociation would contribute to increased stability and thus the

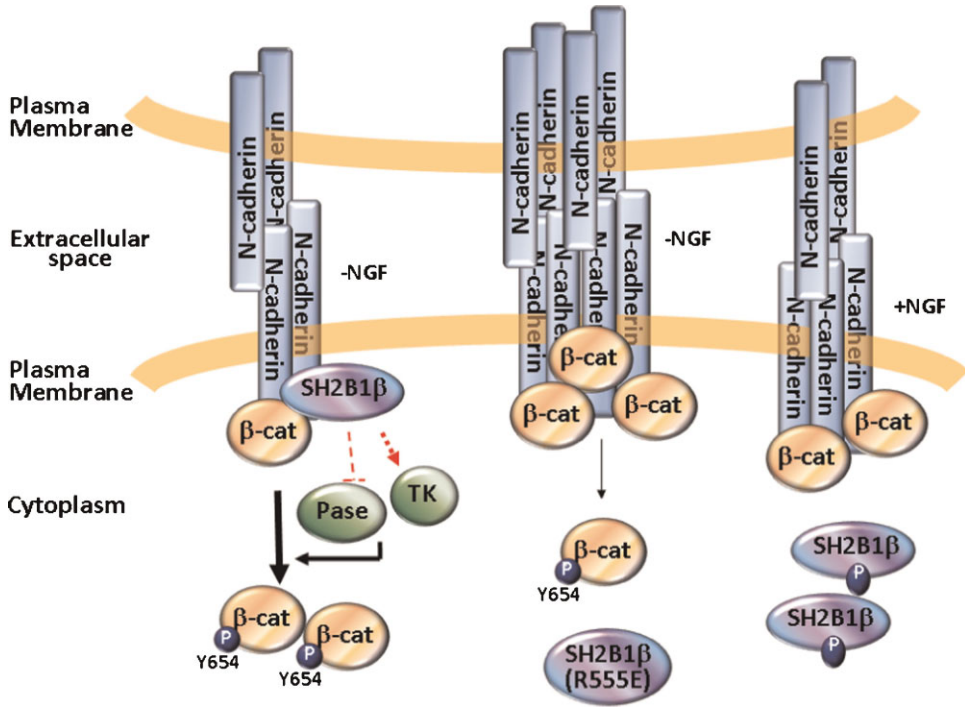

Fig. 9. Working models of how SH2B1β may regulate the formation of N-cadherin/β-catenin complexes. We propose that SH2B1β may inhibit a putative phosphatase (Pase) that de-phosphorylates β-catenin at tyrosine 654, or prevent the access of this Pase to pY654 β-catenin, and thus increases the levels of pY654 β-catenin. Alternatively, SH2B1β may recruit a putative tyrosine kinase (TK) that phosphorylates β-catenin at tyrosine 654, resulting in reduced N-cadherin/β-catenin complex formation and facilitates neurite initiation. In the presence of NGF, SH2B1β is phosphorylated and dissociates from N-cadherin which may in turn increases N-cadherin/β-catenin complex formation.

increased levels of N-cadherin. In fact, N-cadherin levels were found increased during differentiation (data not shown). Therefore, it is plausible that different amount of N-cadherin is required for proper development depending on the stages of differentiation.

The fact that the protein level of N-cadherin in PC12-R555E cells was approximately 11-fold of that in control cells (Fig. 2) suggests that SH2B1β(R555E) contributes to the stability of N-cadherin. Nonetheless, it does not rule out the possibility that SH2B1β and SH2B1β(R555E) would affect the gene expression of N-cadherin. SH2B1β has been shown to shuttle between the cytoplasm and nucleus and regulate a subset of NGF-responsive genes (Chen et al., 2008; Maures et al., 2009). Thus, it would be interesting to determine whether SH2B1β and SH2B1β(R555E) may regulate expression of N-cadherin at the transcriptional level. Alternatively, overexpressing SH2B1β may increase the enzymatic activity of metalloprotease ADAM10, which cleaves N-cadherin (Reiss et al., 2005). However, mRNA and protein levels of ADAM10 were not significantly different between PC12-SH2B1β and PC12-R555E cells (Supplemental Fig. 2).

The current study presents a novel function of N-cadherin in neurite initiation taking the advantage of undifferentiated neuronal model—PC12 cells. It is conceivable that N-cadherin levels may serve to maintain the proper balance of precursor and differentiated cells during neuronal development. High levels of N-cadherin in neural precursor cells may serve to maintain the undifferentiated state whereas reduced level of N-cadherin may favor neuronal differentiation as proper signals present. In line with our findings, a very recent paper elegantly showed that cortical neural precursors regulate their own cell fate through affecting N-cadherin levels (Zhang et al., 2010). The authors suggest that N-cadherin engagement between cells create a self-supportive niche to regulate their own number. N-Cadherin reduction leads to increased neuronal differentiation and increased cell-cycle exit suggesting that N-cadherin interaction between precursor cells serve to maintain the self-renewal ability of neuronal precursors (Zhang et al., 2010).

Acknowledgments

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Literature Cited

Akitaya T, Bronner-Fraser M. 1992. Expression of cell adhesion molecules during initiation and cessation of neural crest cell migration. Dev Dyn 194:12–20.

Chen L, Carter-Su C. 2004. Adapter protein SH2-B beta undergoes nucleocytoplasmic shuttling: Implications for nerve growth factor induction of neuronal differentiation. Mol Cell Biol 24:3633–3647.

Chen L, Maures TJ, Jin H, Huo JS, Rabbani SA, Schwartz J, Carter-Su C. 2008. SH2B1beta (SH2-Bbeta) enhances expression of a subset of nerve growth factor-regulated genes important for neuronal differentiation including genes encoding urokinase plasminogen activator receptor and matrix metalloproteinase 3/10. Mol Endocrinol 22:454–476.

DiFiglia M, Marshall P, Covault J, Yamamoto M. 1989. Ultrastructural localization of molecular subtypes of immunoreactive neural cell adhesion molecule (NCAM) in the adult rodent striatum. J Neurosci 9:4158–4168.
Hatta K, Takagi S, Fujisawa H, Takeichi M. 1987. Spatial and temporal expression pattern of N-cadherin cell adhesion molecules correlated with morphogenetic processes of chicken embryos. Dev Biol 120:215–227.
Kiryushko D, Berezin V, Bock E. 2004. Regulators of neurite outgrowth: Role of cell adhesion molecules. Ann NY Acad Sci 1014:140–154.
Kwiatkowski AV, Weis WI, Nelson WJ. 2007. Catenins: Playing both sides of the synapse. Curr Opin Cell Biol 19:551–556.
Lin WF, Chen CJ, Chang YJ, Chen SL, Chiu IM, Chen L. 2009. SH2B1beta enhances fibroblast growth factor 1 (FGF1)-induced neurite outgrowth through MEK-ERK1/2-STAT3-Egr1 pathway. Cell Signal 21:1060–1072.
Maures TJ, Chen L, Carter-Su C. 2009. Nucleocytoplasmic shuttling of the adapter protein SH2B1beta (SH2-Bbeta) is required for nerve growth factor (NGF)-dependent neurite outgrowth and enhancement of expression of a subset of NGF-responsive genes. Mol Endocrinol 23:1077–1091.
Nakagawa S, Takeichi M. 1995. Neural crest cell–cell adhesion controlled by sequential and subpopulation-specific expression of novel cadherins. Development 121:1321–1332.
Nakagawa S, Takeichi M. 1998. Neural crest emigration from the neural tube depends on regulated cadherin expression. Development 125:2963–2971.
Qian X, Riccio A, Zhang Y, Ginty DD. 1998. Identification and characterization of novel substrates of Trk receptors in developing neurons. Neuron 21:1017–1029.
Radice GL, Rayburn H, Matsunami H, Knudsen KA, Takeichi M, Hynes RO. 1997. Developmental defects in mouse embryos lacking N-cadherin. Dev Biol 181:64–78.
Redies C. 2000. Cadherins in the central nervous system. Prog Neurobiol 61:611–648.
Reiss K, Maretzky T, Ludwig A, Tousseyn T, de Strooper B, Hartmann D, Saftig P. 2005. ADAM10 cleavage of N-cadherin and regulation of cell–cell adhesion and beta-catenin nuclear signalling. EMBO J 24:742–752.
Rhee J, Buchan T, Zukerberg L, Lilien J, Balsamo J. 2007. Cables links Robo-bound Abl kinase to N-cadherin-bound beta-catenin to mediate Slit-induced modulation of adhesion and transcription. Nat Cell Biol 9:883–892.
Rui L, Herrington J, Carter-Su C. 1999. SH2-B is required for nerve growth factor-induced neuronal differentiation. J Biol Chem 274:10590–10594.
Schuman EM, Murase S. 2003. Cadherins and synaptic plasticity: Activity-dependent cyclin-dependent kinase 5 regulation of synaptic beta-catenin-cadherin interactions. Philos Trans R Soc Lond B Biol Sci 358:749–756.
Shoval I, Ludwig A, Kalcheim C. 2007. Antagonistic roles of full-length N-cadherin and its soluble BMP cleavage product in neural crest delamination. Development 134:491–501.
Suzuki SC, Takeichi M. 2008. Cadherins in neuronal morphogenesis and function. Dev Growth Differ 50:S119–S130.
Zhang Y, Zhu W, Wang YG, Liu XJ, Jiao L, Liu X, Zhang ZH, Lu CL, He C. 2006. Interaction of SH2-Bbeta with RET is involved in signaling of GDNF-induced neurite outgrowth. J Cell Sci 119:1666–1676.
Zhang J, Woodhead GJ, Swaminathan SK, Noles SR, McQuinn ER, Pisarek AJ, Stocker AM, Mutch CA, Funatsu N, Chenn A. 2010. Cortical neural precursors inhibit their own differentiation via N-cadherin maintenance of beta-catenin signaling. Dev Cell 18:472–479.