Supplementary Information

KIF21A-mediated axonal transport and selective endocytosis underlie the polarized targeting of NCKX2

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Supplementary Materials and Methods

Cell culture

Hippocampal neurons were cultured on coverslips suspended above an astrocyte feeder layer. The protocol for low-density neuron-glia co-culture is described in ref. (Kaech & Banker, 2006). All work with animals was conducted in accordance with the animal welfare guidelines of Seoul National University. In brief, 2 weeks before neuronal culture, astrocytes were obtained by passing a cortical cell suspension from postnatal day (P) 1 Sprague-Dawley (SD) rat through a cell strainer (40 μm mesh, BD Falcon), and then cultured in glial medium [minimum essential medium (MEM; Invitrogen) supplemented with 0.6% glucose, 1 mM pyruvate, 2 mM GlutaMAX-I (Invitrogen), 10% horse serum (HS; Invitrogen) and penicillin-streptomycin (PS; Invitrogen)]. Hippocampi from embryonic day (E) 18 SD fetal rats were dissected in Hank's balanced salt solution (Invitrogen) and dissociated with papain (Worthington) and then triturated with a polished half-bore Pasteur pipette. The neurons in plating medium (MEM supplemented with 0.6% glucose, 1 mM pyruvate, 2 mM GlutaMAX-I, 10% fetal bovine serum (FBS; Invitrogen) and PS) were plated on poly-D-lysine (Sigma)-coated glass coverslips (Marienfeld) in a 60-mm culture dish at a density of 0.7-1.4x10^4 cells per cm^2. Paraffin dots ('feet' to suspend the coverslips above the glial feeder layer) were previously applied to the coverslips. Next day coverslips were transferred above the glial culture preincubated in Neurobasal A medium (Invitrogen) supplemented with 0.5 mM GlutaMAX-I and 2% B-27 supplement (invitrogen) for 1 day. To prevent proliferation of glial cells, 5 μM of 1-β-D-cytosine-arabinofuranoside (Ara-C; Sigma) was added at DIV4. HEK293 cells (ATCC) were plated at a density of 5x10^4 cells per 100-mm culture dishes and maintained in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 10% FBS.

Transfection

Primary hippocampal neurons (DIV3-6) were transfected using calcium phosphate (Ryan et al, 2005). Before transfection, the conditioned culture medium was saved and neurons were incubated with 2 ml of Neurobasal A containing 25 mM HEPES (pH 7.3). The DNA/calcium phosphate precipitate was prepared by mixing one volume of DNA (up to 15 μg) in 250 mM $CaCl_2$ with an equal volume of 2x HBS (280 mM NaCl, 50 mM HEPES, 1.5 mM Na_2HPO_4, pH 7.1) using a vortex mixer. Then 200 μl DNA/calcium phosphate mixture was added drop-wise to the cultured neurons, and neurons were incubated at 37°C for 15 min. After the incubation, DNA/calcium phosphate precipitates were washed out three times with fresh Neurobasal A for 5 min and the cells were returned to the saved original medium. For neurons, NCKX2-GFP and DsRed with or without either of KIF21A, dnKIF21A or dnKIF21B were transfected. NCKX2-GFP and either of the three kinesin constructs were co-transfected in a ratio of 1:4. NCKX2-FLAG and wild-type or mutant dynamin-1 constructs were co-transfected in a ratio of 1:1. HEK293 cells were also transfected using calcium phosphate. The procedures were essentially the same except the medium was not changed before and after adding of DNA/calcium phosphate mixture to the culture. For HEK293 cells, myc-NCKX2-loop with either of KIF21A, KIF21B, dnKIF21A or dnKIF21B were transfected.

Co-immunoprecipitation

Hippocampal neurons from E18 SD rats were plated at a density of 4.5x10^4 cells per cm^2 on 100-mm culture plates coated with 100 μg/ml poly-D-lysine and cultured for 2 weeks. The neurons at DIV14 were washed once with Dulbecco's

phosphate-buffered saline (DPBS; Invitrogen) and lysed for 30 min on ice in lysis buffer containing 25 mM Tris (pH 7.4), 10 mM NaCl, 1% sodium cholate hydrate, 1% Triton X-100 (v/v), 1 mM phenylmethyl sulfonyl fluoride (PMSF; Sigma) and 0.2% protease inhibitor cocktail (Sigma) (Hong et al, 2009; Oh et al, 2006). Cell lysates were then centrifuged at 8,200 x *g* for 15 min at 4°C and the protein concentrations were determined by Bradford protein assay (Bio-Rad). The supernatants containing 500 μg total proteins were incubated with rabbit anti-KIF21A IgG or rabbit anti-$NCKX2_{loop}$ IgG immobilized on the amine-reactive gel according to the manufacturer's instructions (ProFound co-immunoprecipitation kit; Thermo/Pierce). Non-immune rabbit IgG (Calbiochem) was used as control. For co-immunoprecipitation, we used rabbit polyclonal anti-$NCKX2_{loop}$ or anti-KIF21A. The immunoprecipitated protein complexes were subjected to SDS-PAGE and western blot analysis with anti-$NCKX2_{ext}$ and anti-KIF21A antibodies.

HEK293 cells were seeded in 100-mm culture dishes at about 70% confluence and transfected with myc-NCKX2-loop and FLAG-tagged KIF variants, either KIF21A, KIF21B, dnKIF21A or dnKIF21B, using calcium phosphate. After culture for 20-48 h, the cells were washed twice with DPBS, solubilized in ice-cold lysis buffer containing 20 mM Tris (pH 7.4), 140 mM NaCl, 10% sucrose, 1 mM EDTA, 1 mM Na_3VO_4, 1% NP-40 (v/v) and 0.2% protease inhibitor cocktail. Cell lysates were clarified by centrifugation at 8,200 x *g* for 15 min at 4°C. The supernatants were incubated with anti-c-myc antibody-conjugated agarose beads (Sigma) for 1 h. The beads were then washed three times with lysis buffer for 5 min, and the immunoprecipitated protein complexes were denatured in 2X SDS sample buffer and subjected to SDS-PAGE and western blot analysis with anti-FLAG.

Western blotting

Cell lysates or immunoprecipitated proteins were separated by SDS−PAGE and transferred onto a polyvinylidene difluoride membrane (Millipore). The resulting blots were blocked for 1 h in phosphate-buffered saline (PBS) plus 0.1% Triton X-100 (0.1% PBST) containing 5% skim milk (Difco). The blots were incubated overnight at 4℃ with specific primary antibodies: rabbit polyclonal anti-$NCKX2_{ext}$ (1:100, Thermo/Affinity BioReagents), rabbit polyclonal anti-KIF21A (1:500), mouse monoclonal anti-FLAG M2 (1:5000, Sigma), mouse monoclonal anti-c-Myc (1:500, Santa Cruz Biotechnology) or mouse monoclonal anti-β-actin (1:2500, Abcam) as loading controls. After washing three times with 0.1% PBST, the blots were incubated at room temperature (RT) for 1 h with the corresponding horseradish peroxidase (HRP)-conjugated secondary antibodies: goat anti-rabbit IgG (1:2000, Abcam) or goat anti-mouse IgG (1:5000, Jackson ImmunoResearch). After washing three times with 0.1% PBST, detection was performed using enhanced chemiluminescence (ECL) reagent (Amersham Bioscience). The membranes were then exposed to BioMax X-ray films (Kodak). Films were digitally scanned and signals were quantified using densitometric analysis software, Multi Gauge (Fujifilm).

Immunocytochemistry

For detecting total (both cytosolic and surface) protein, the cells were fixed with ice-cold 4% paraformaldehyde (PFA) or 3.8% formaldehyde in PBS for 20 min, washed with PBS, and then permeabilized with 0.1% PBST for 5 min at RT. The cells were further washed in PBS and blocked in blocking solution (5% donkey or goat serum in 0.1% PBST) for 1 h at RT. Primary antibodies were diluted in blocking solution, and incubated with samples for 1 h at RT or overnight at 4℃. Primary antibody dilutions were as follows: rabbit polyclonal anti-$NCKX2_{ext}$ (1:100), mouse monoclonal anti-FLAG M2 (1:2500),

mouse monoclonal anti-synaptophysin (1:100, Sigma), mouse monoclonal anti-Tau-1 (1:500, Sigma), mouse monoclonal anti-microtuble-associated protein 2 (MAP2; 1:500, Millipore/Chemicon) and rat monoclonal anti-neurofilament H (NF-H; 1:500, Millipore). After three washes in PBS, cells were incubated with secondary antibodies diluted in blocking solution for 1 h at RT in the dark. Secondary antibodies were used as follows: Alexa Fluor 488-conjugated goat anti-rabbit IgG (1:400 dilution, Invitrogen/Molecular Probes) for immunostaining of endogenous NCKX2; Cy5-conjugated donkey anti-rabbit IgG (1:400, Millipore/Chemicon) for NCKX2-GFP; rhodamine-conjugated donkey anti-mouse IgG (1:200, Jackson ImmunoResearch) for FLAG-KIF21A (Figure 2), NCKX2-FLAG (Figure S1) and MAP2 (Figures 6Bb and 7A); Cy5-conjugated donkey anti-mouse IgG (1:200, Jackson ImmunoResearch) for NCKX2-FLAG (Figure 7D), MAP2 (Figures 3, 4, 6A and 7C), Tau-1 (Figures 3 and 6A) and synaptophysin (Figure 6A); Alexa Fluor 568-conjugated goat anti-rat IgG (1:400, Invitrogen/Molecular Probes) for NF-H. Finally, the cells were washed three times in PBS and mounted with fluorescent mounting medium (DakoCytomation). In some experiments of Supplementary Figure S1, nuclear staining by DAPI was performed to visualize untransfected cells. Before mounting, cells were incubated with DAPI in PBS (1:5000, Sigma) for 5 min and washed with PBS then mounted.

For surface immunostaining of NCKX2, live cells were incubated with rabbit anti-$NCKX2_{ext}$ (1:100) or rabbit anti-GFP (1:100, Millipore/Chemicon) in serum-free culture medium for 15 min at 36°C or at 4°C, rinsed with culture medium, fixed with ice-cold 4% PFA or 3.8% formaldehyde in PBS for 20 min and washed with PBS. For double-immunostaining with MAP2, Tau-1, synaptophysin or NF-H, cells labeled with anti-NCKX2 antibody and then fixed were subsequently permeabilized with 0.1% PBST for 5 min at RT. The fixed cells were incubated in blocking solution for 1 h at RT, and then with anti-MAP2, Tau-1, synaptophysin or NF-H antibodies diluted in blocking solution (Bel et al, 2009). Subsequent steps were the same as those for total protein immunostaining. The immunostained cells were imaged with FV300 (Olympus) or TCS-SP2 (Leica) confocal laser scanning microscopes with 60x or 63x water-immersion objectives, and then processed using Fluoview or Leica Lite.

Electrophysiological recording and calcium imaging

Cultured hippocampal slices were placed in a submerged recording chamber and perfused with artificial cerebrospinal fluid (ACSF) composed of (in mM): 124 NaCl, 26 $NaHCO_3$, 3.2 KCl, 2.5 $CaCl_2$, 1.3 $MgCl_2$, 1.25 NaH_2PO_4 and 10 glucose with pH adjusted at 7.4 by saturating with carbogen (95% O_2, 5% CO_2). For calcium imaging, whole-cell patch-clamp mode was attained on the mRFP-expressing dentate granule cell soma under visual control using differential interference illumination in an upright microscope (BX51WI; Olympus, Tokyo, Japan). The expression of mRFP was detected using a HcRed filter set (part# 41043, Chroma, VT). The whole-cell recordings were made using an EPC-10 amplifier (HEKA Elektronik, Lambrecht, Germany) with a pipette solution containing (in mM) 140 K-gluconate, 5 di-Tris-phosphocreatin, 5 NaCl, 4 MgATP, 0.4 Na_2GTP, 15 HEPES, 2.5 Na-pyruvate (pH 7.3, adjusted with KOH) together with 200 μM Oregon Green 488 BAPTA-5N (OGB-5N, K_D ~50 μM) or 50 μM Oregon Green 488 BAPTA-1 (OGB1, K_D ~200 nM) (Invitrogen/Molecular probes). Both Ca^{2+}-indicators were excited at 488 nm. Calcium imaging was performed using a confocal laser-scanning system (FV300; Olympus) and a 60x water-immersion objective (numerical aperture, 0.9; LUMPlanFl/IR; Olympus). Ca^{2+}-transients (CaTs) at hippocampal mossy fiber *en passant* boutons, somata, dendritic spines and shafts of dentate granule cells were evoked by applying high frequency stimulation, a train of short depolarizing pulses (2 ms in duration) from -80 mV to 0 mV at 33 Hz or

100 Hz for 5 s, via whole-cell patch pipette on the soma, and recorded in XY scan mode at 5 Hz for 20 s. A single action potential-induced CaT was recorded in line-scan mode at 540 Hz for 2.8 s. Suprathreshold current was typically 600~900 pA for 12 ms.

Supplementary Table

: Mean [Ca^{2+}] increments induced by high-frequency stimulation (HFS) of hippocampal dentate granule cells

Cell group	location	n	number of cells	$\Delta F/F_0$
NT control	Somata	18 (6)	18 (6)	0.299 ± 0.015 (0.741 ± 0.021)
	Dendritic spines	34 (11)	14 (4)	0.357 ± 0.022 (0.603 ± 0.046)
	Dendritic shafts	28 (9)	14 (5)	0.368 ± 0.026 (0603 ± 0.046)
	MFBs	64	12	1.302 ± 0.050
shKIF21A	Somata	19 (5)	19 (5)	0.300 ± 0.018 (0.724 ± 0.043)
	Dendritic spines	45 (11)	13 (5)	0.364 ± 0.029 (0.672 ± 0.047)
	Dendritic shafts	35 (11)	13 (5)	0.306 ± 0.027 (0.673 ± 0.062)
	MFBs	46	15	2.161 ± 0.079**
shNCKX2	Somata	7	7	0.268 ± 0.022
	Dendritic spines	15	4	0.329 ± 0.033
	Dendritic shafts	10	4	0.358 ± 0.037
	MFBs	26	5	2.171 ± 0.148**

[Ca^{2+}] increments presented as $\Delta F/F_0$ of OGB-5N. Ca^{2+} transients were induced by HFS at 33 Hz or 100 Hz (parenthesized numbers). $\Delta F/F_0$ values of a Ca^{2+} transient were read at the end of HFS. NT, non-targeting; MFB, mossy fiber bouton; The statistical values are presented as mean ± SEM. ** Statistical significance compared with control value ($p < 0.01$).

Supplementary Figures

Fig. S1

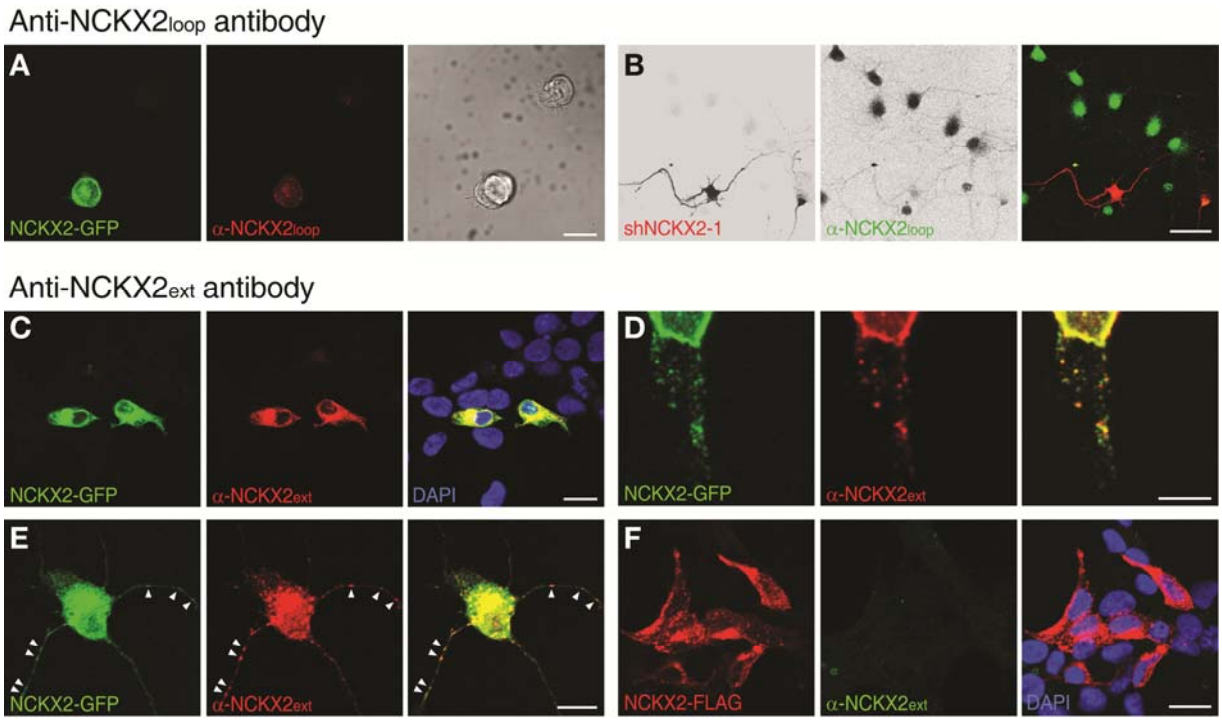

Figure S1. Reliability test of NCKX2 antibodies. (A) Anti-NCKX2$_{loop}$ antibody (red) specifically immunolabeled the HEK293 cell expressing NCKX2-GFP (green). The untransfected cell is shown in the transmitted image. Scale bar: 20 μm. (B) When endogenous NCKX2 was immunolabeled with anti-NCKX2$_{loop}$ (green), The NCKX2-depleted hippocampal neurons using shNCKX2 (See Supplementary Figure S5) were not immunostained with anti-NCKX2$_{loop}$. The shNCKX2-transfected cells were identified by co-expressing mRFP fluorescence (red). Scale bar: 50 μm. (C) The HEK293 cells expressing NCKX2-GFP (green) were specifically labeled with anti-NCKX2$_{ext}$ antibody (red). Untransfected cells were visualized by DAPI (blue). Scale bar: 20 μm. (D) Higher magnification images of HEK293 expressing NCKX2-GFP. The green fluorescence of NCKX2-GFP is completely colocalized with red immunofluorescent labeling. Scale bar: 5 μm. (E) Puncta labeled with anti-NCKX2$_{ext}$ (red) were well matched with NCKX2-GFP (green) in neurites (arrowheads) of the cultured hippocampal neuron. Scale bar: 10 μm. (F) Because the anti-NCKX2$_{ext}$ antibody was raised against a synthetic peptide within the N-terminal extracellular region (residues 90 to 102), HEK293 cells expressing NCKX2-FLAG, in which the FLAG tag (^{90}DYKDDDDK^{97}) replaces the epitope's N-terminal 8 amino acids, were not immunoreactive to the anti-NCKX2$_{ext}$ (green). Expression of NCKX2-FLAG was detected by anti-FLAG (red), and untransfected cells were visualized by DAPI (blue). Scale bar: 20 μm.

Fig. S2

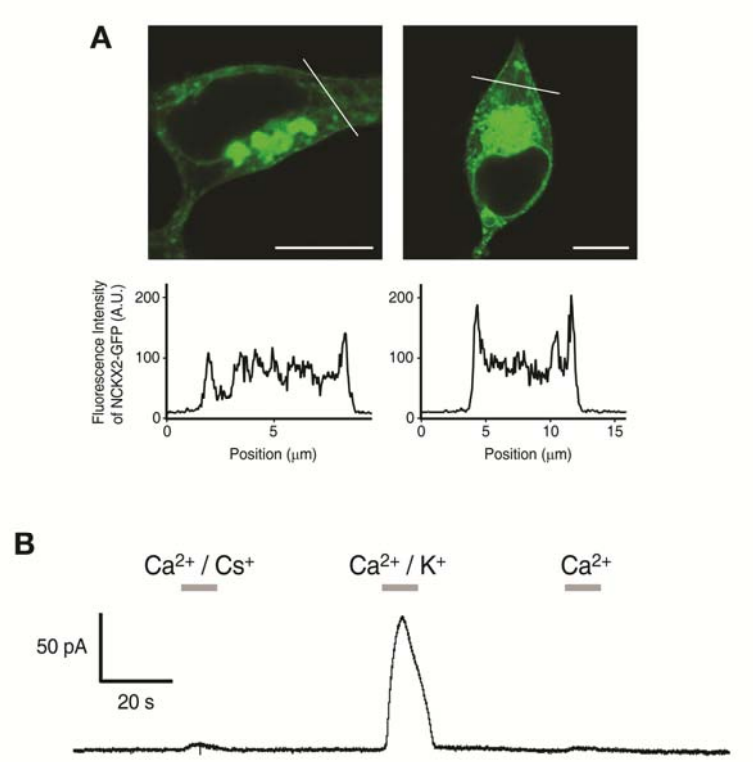

Figure S2. Test for functional competence of NCKX2-GFP. (A) Overexpressed NCKX2-GFP was localized both in the cytoplasm and the plasma membrane in the HEK293 cells. Line profiles along the white line in upper figure were shown below each image. Scale bar: 10 μm. (B) NCKX activity of NCKX2-GFP was tested by recording Ca^{2+} and K^+-dependent reverse mode exchange current in the HEK293 cell perfused with high Na^+ internal pipette solution using whole-cell patch-clamp technique. Similar to the activity of wild-type NCKX2 (Lee et al., 2006), reverse mode NCKX current could be induced by bath application of Ca^{2+} plus K^+, but not by Ca^{2+} plus Cs^+ or Ca^{2+} only. The procedure for recording reverse-mode (outward) NCKX currents was previously described in detail in (Lee et al., 2006). In brief, whole-cell patch clamp recordings were attained on the HEK293 cells expressing NCKX2-GFP with a pipette solution containing the following (in mM): 120 Na-gluconate, 10 KCl, 20 HEPES, 1 EGTA, 4 MgATP, 0.3 Na_2GTP at pH 7.2 (adjusted with NaOH). Current recordings in baseline conditions were obtained while the cells were superfused with Ca^{2+}- and K^+-free bath solution containing the following (in mM) : 140 LiCl, 0.5 EGTA, 1 $MgCl_2$, 10 HEPES, 10 glucose at pH 7.4 (adjusted with LiOH). Reverse-mode of NCKX currents were induced by bath application of test solution. Ca^{2+}/K^+ (or Cs^+) test solution contained 140 mM KCl (or 140 mM CsCl) and 1 mM $CaCl_2$ instead of 140 mM LiCl and 0.5 mM EGTA. Ca^{2+} test solution contained 1 mM $CaCl_2$ instead of 0.5 mM EGTA.

Fig. S3

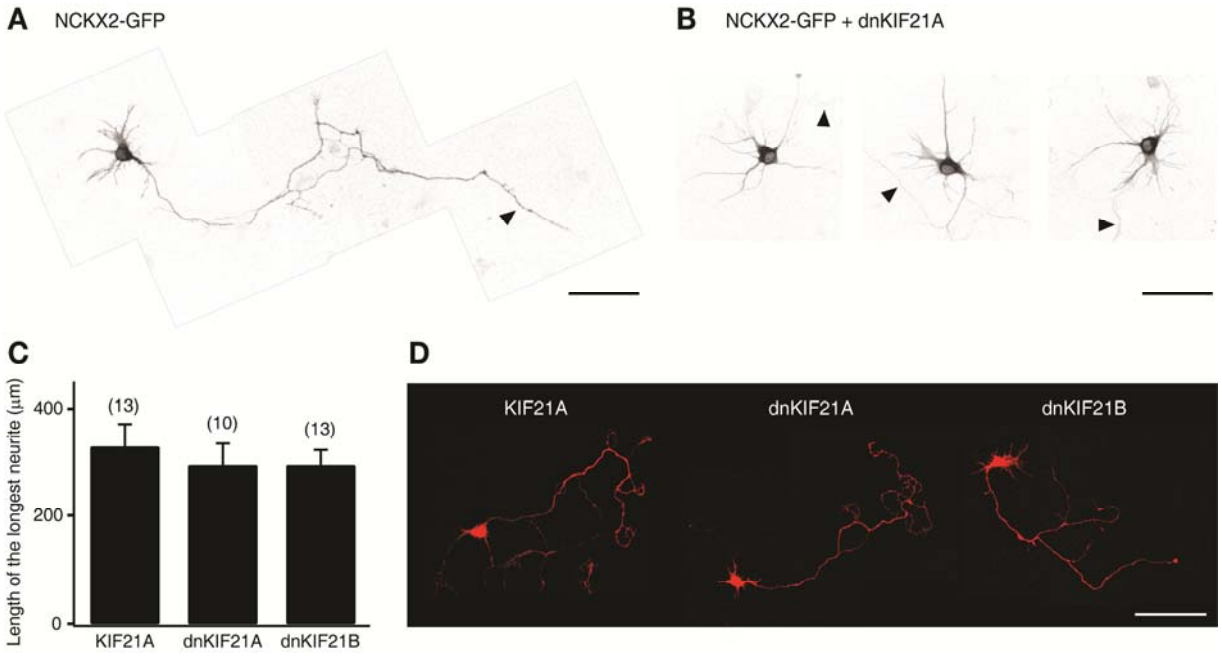

Figure S3. Overexpression of dnKIF21A suppresses the axonal transport of NCKX2-GFP without affecting the length of the longest neurites. (A-B) Overexpression of NCKX2-GFP alone (A) or co-expression of NCKX2-GFP and dnKIF21A (B) in the hippocampal neurons. GFP fluorescence was inverted into gray color to improve contrast. Scale bar: 100 μm. (C-D) Length of the longest neurites from KIF21A, dnKIF21A and dnKIF21B overexpressed hippocampal neurons. (C) Length of the longest neurites was not significantly different between KIF21A, dnKIF21A and dnKIF21B overexpressed groups ($p > 0.5$). (D) Representative images each of KIF21A, dnKIF21A and dnKIF21B co-expressed with DsRed for visualizing the whole morphology of a neuron. Scale bar: 100 μm.

Fig. S4

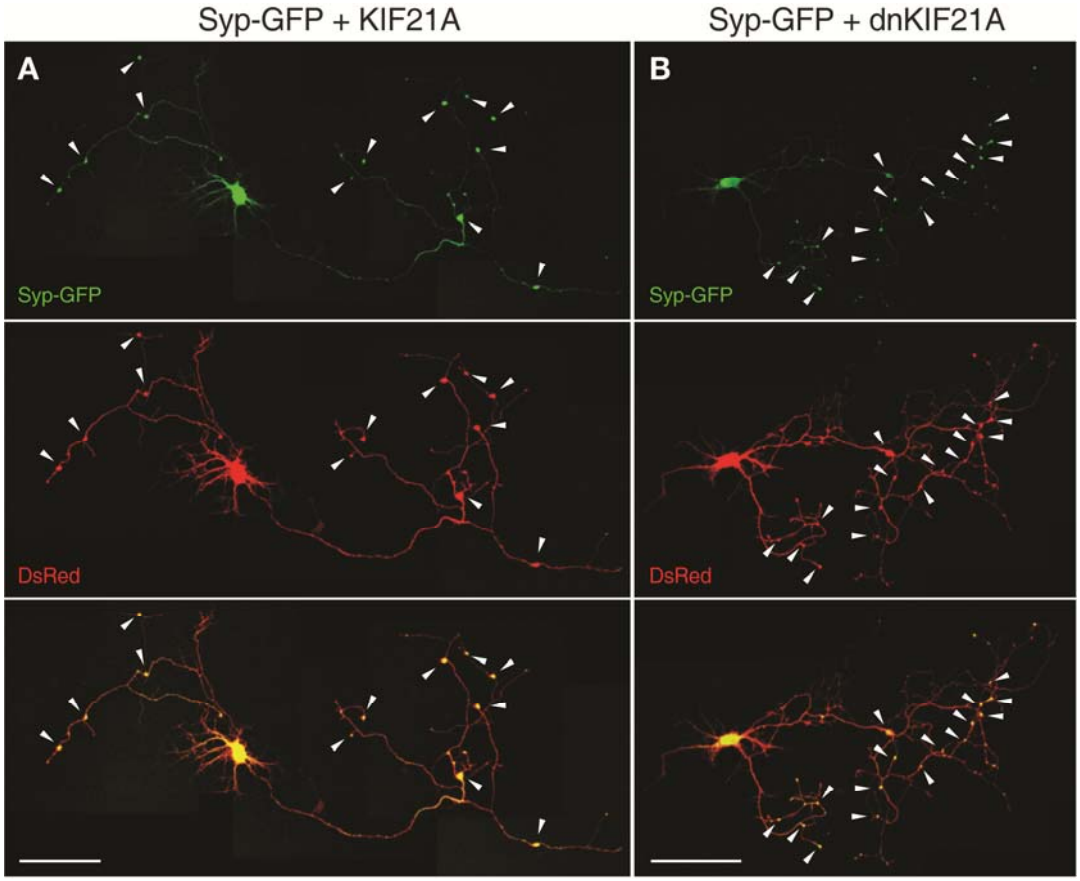

Figure S4. Axonal transport of synaptophysin-GFP was not inhibited by dnKIF21A. To visualize the distribution of synaptophysin, EGFP was fused to the C-terminus of synaptophysin (Syp-GFP, green). Cell morphology was visualized using DsRed fluorescence (red) in the same way as Figure 3A-B. Syp-GFP was well distributed at putative axon terminals (arrowheads), regardless of co-transfection with wild-type KIF21A (A) or dnKIF21A (B). Merged images are shown in the bottom panels. Scale bar: 100 μm.

Fig. S5

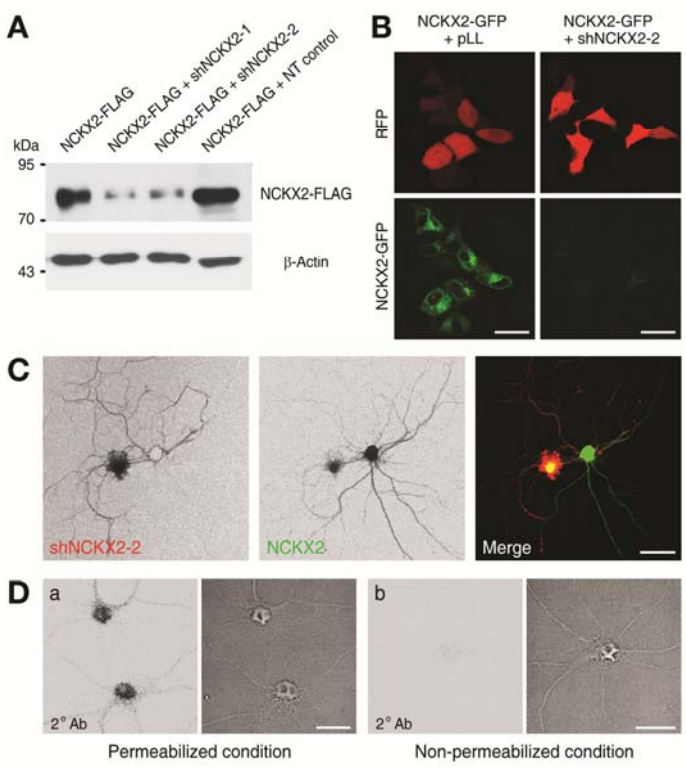

Figure S5. shRNA-mediated depletion of NCKX2. (A) FLAG-tagged NCKX2 (NCKX2-FLAG) was transfected alone or co-transfected with one of two different types of shRNA targeting NCKX2 mRNA (shNCKX2-1 or shNCKX2-2) or non-targeting (NT) control to HEK293 cells. Both shNCKX2-1 and shNCKX2-2 efficiently depleted NCKX2-FLAG. (B) pLentiLox3.7 plasmids (pLL) encoding RFP alone (red, left) or RFP + shNCKX2-2 (red, right) were co-transfected to HEK293 cells with NCKX2-GFP (green). Fluorescence imaging of the cells confirmed that shNCKX2-2 effectively knocked-down NCKX2-GFP. Scale bar: 30 μm. (C) Endogenous NCKX2 was distinctly depleted by shNCKX2-2 on the 15th day after transfection. The hippocampal neuron transfected with shNCKX2-2 was identified by co-expressing mRFP fluorescence (red). Endogenous NCKX2 was immunolabeled with anti-$NCKX2_{ext}$ (green) under the permeabilized condition. The persistent green fluorescence confined in the soma of shNCKX2-2-transfected cell is caused by non-specific staining of secondary antibody (2° Ab). Scale bar: 50 μm. (D) DIV8 hippocampal neurons were incubated with Alexa Fluor 488-conjugated goat anti-rabbit IgG (Invitrogen) without prior incubation with primary antibody. In the permeabilized condition somata (especially nuclei) were non-specifically stained (Da), but not in the non-permeabilized condition (Db), indicating that non-specific signal of the secondary antibody is responsible for the somatic green fluorescence of the neurons shown in C. Scale bar: 20 μm.

Supplementary References

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