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ORIGINAL ARTICLE

Serum inducible kinase is a positive regulator of cortical dendrite development and is required for BDNF-promoted dendritic arborization

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Serum inducible kinase (SNK), also known as polo-like kinase 2 (PLK2), is a known regulator of mitosis, synaptogenesis and synaptic homeostasis. However, its role in early cortical development is unknown. Herein, we show that *snk* is expressed in the cortical plate from embryonic day 14, but not in the ventricular/subventricular zones (VZ/SVZ), and SNK protein localizes to the soma and dendrites of cultured immature cortical neurons. Loss of SNK impaired dendritic but not axonal arborization in a dose-dependent manner and overexpression had opposite effects, both *in vitro* and *in vivo*. Overexpression of SNK also caused abnormal branching of the leading process of migrating cortical neurons in electroporated cortices. The kinase activity was necessary for these effects. Extracellular signal-regulated kinase (ERK) pathway activity downstream of brain-derived neurotrophic factor (BDNF) stimulation led to increases in SNK protein expression via transcriptional regulation, and this upregulation was necessary for the growth-promoting effect of BDNF on dendritic arborization. Taken together, our results indicate that SNK is essential for dendrite morphogenesis in cortical neurons.

***Keywords*:** PLK2; morphogenesis; BDNF; ERK; transcription

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Introduction

Properly arborized dendrites provide structural support for information processing within the nervous system, and dendritic abnormalities are often associated with cognitive diseases, such as mental retardation and autism [1]. Dendritic arborization and complexity is tightly controlled by a combination of extrinsic and intrinsic factors. Different extracellular signals have distinct roles in various aspects of dendrite development, such as orientation, branching, and growth [2]. Extracellular signals are transduced into the cytoplasm via transmembrane receptors, which activate internal effectors. In particular, kinases play crucial roles in signal transduction, due to their ability to modify multiple downstream substrates and integrate upstream signals. Signaling often culminates in cytoskeletal and membrane rearrangements, which lead to morphological changes [3].

Polo-like kinases are key regulators of cell cycle progression [4, 5]. This family of serine/threonine kinases is named after the *Drosophila melanogaster* gene *polo*, the first member discovered [6]. Polo-like kinase 2 (PLK2), also known as serum inducible kinase (SNK) [7], is critical for centriole duplication [8] and is also an important regulator of apoptosis and cancer [9, 10]. The function of polo-like kinase family members has also been studied in the nervous system, mainly in mature neurons [11]. Specifically, SNK has been demonstrated to respond to synaptic activity and play a key role in spine formation, as well as in the regulation of synaptic homeostasis [12, 13]. However, its role in immature neurons has not been reported to date.

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In this study, we examined the function of SNK in early brain development and provide evidences for a critical role of SNK in dendritic arborization during cortex formation. We show that SNK acts downstream of extracellular signal-regulated kinase (ERK) activation in immature cortical neurons undergoing morphogenesis and is necessary for brain-derived neurotrophic factor (BDNF)-promoted dendrite growth and branching.

Results

SNK expression in the developing cortex

snk is expressed in the neocortex, hippocampus, striatum, amygdala and subthalamus of the adult mouse brain [14]; however, the distribution of *snk* in the developing brain has not been reported. Using RT-PCR, we found that *snk* mRNA was expressed in the rat cerebral cortex throughout development, from as early as embryonic day 14 (E14) to adulthood, with peak expression observed around postnatal day 3 (P3; Supplementary information, Figure S1A and S1B). *In situ* hybridization confirmed that *snk* was expressed in the cortical plate (CP) and striatum (STR) at embryonic stages (a1-a2 in Figure 1A), consistent with previous findings [14]. At E18, we found high levels of *snk* in the CP (a3-a4 in Figure 1A), while in neurogenic regions, such as the ventricular/subventricular zones (VZ/SVZ) and the lateral/medial ganglionic eminences (LGE/MGE) [15], staining was not distinguishable from the non-specific staining observed in negative controls (NC, a4 in Figure 1A), indicating that SNK is only expressed in differentiated neurons. After birth, *snk* mRNA was observed in all layers of the CP

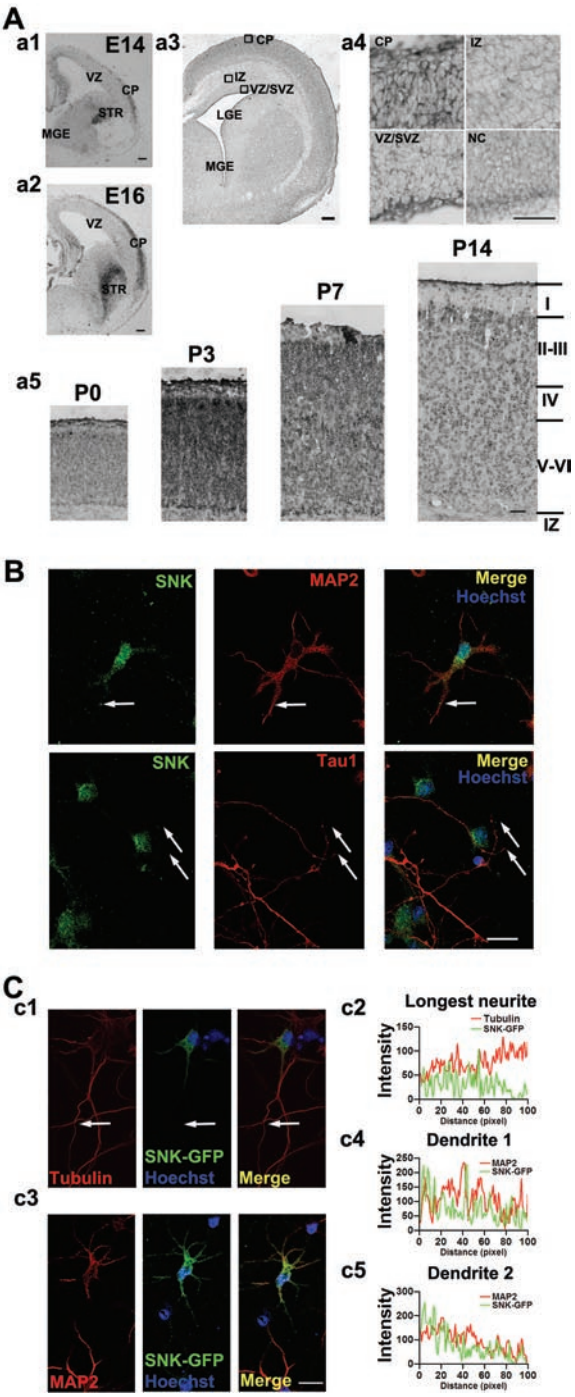

Figure 1 SNK expression in the developing rat cortex and cultured immature neurons. **(A)** *snk in situ* hybridization of rat cortices at E14 (embryonic day 14, a1), E16 (a2), E18 (a3, a4), and at postnatal ages (a5). Scale bar, 200 μm in a1-a3 and 50 μm in a4-a5. STR: striatum; MGE: medial ganglionic eminence; LGE: lateral ganglionic eminence; CP: cortical plate; VZ: ventricular zone; SVZ: subventricular zone; IZ: intermediate zone; NC: negative control incubated with buffer containing no probe during hybridization. Cortical layers boundaries were determined from Hoechst labeling. **(B)** Endogenous SNK (green) staining in DIV3 (3 days *in vitro*) rat cortical neurons after 18 h treatment with 2 μM MG132. Neurons were co-labeled with the microtubule-associated proteins MAP2 (upper panel, red) or Tau1 (lower panel, red) to highlight dendrites and axons, respectively (pointed by arrows), and with Hoechst 33324 (blue) to highlight the nucleus (in **B** and **C**). Scale bar, 20 μm. **(C)** Exogenous SNK-GFP expression in DIV3 neurons. Neurons were immunolabeled with antibodies against GFP (green) and the neurite marker Tubulin (c1, red) or MAP2 (c3, red). Scale bar, 20 μm. Fluorescence intensity was measured from the proximal to the distal end of the neurite, revealing that SNK-GFP was expressed in dendrites, but not in axons (*x*-axis: distance from cell body; *y*-axis: fluorescent emission intensity). The longest neurite in c1 was considered to be the axon (pointed by arrow) and quantified in c2. Dendrite 1 (c4) and 2 (c5) were chosen from c3. Images in **B** and **C** were acquired under 63× oil objective with a numerical aperture (NA) of 1.4.

and was most robustly expressed at P3 (a5 in Figure 1A).

Subcellular localization of SNK in immature cortical neurons

Previous studies have reported that SNK is expressed in the somata and dendrites of mature neurons [12, 14]. We set out to investigate whether its subcellular localization changes during neuronal maturation. Since SNK protein is rapidly degraded, with a half-life as short as 15 min [16], it is almost undetectable by immunohistochemistry [12]. Therefore, we treated cultured cortical neurons with the proteasome inhibitor MG132 (2 μM for 18 h) to allow SNK protein to accumulate. The subcellular localization of both endogenous SNK (Figure 1B) and exogenous GFP-tagged SNK (SNK-GFP; Figure 1C) in neurons cultured for 3 days *in vitro* (DIV3) was similar to that previously reported for mature neurons. We found that SNK co-localized with the soma and dendrite marker microtubule associate protein 2 (MAP2), but not the axon-specific marker Tau1 (Figure 1B). In stage-3 neurons [17], SNK also overlapped with Tubulin at the proximal but not distal end of the longest neurite, which corresponds to the developing axon (arrows in Figure 1C). The localization of the kinase-dead form of SNK (K108M) [12] was similar to that of endogenous SNK and SNK-GFP, indicating that mutating the ATP-binding pocket of the kinase domain does not affect subcellular localization (Supplementary information, Figure S1C).

SNK is necessary for dendrite development

To investigate the role of SNK in developing cortical neurons, we used RNA interference (RNAi) to knock down SNK expression. We generated three independent SNK knockdown constructs: SNK-shRNA1, SNK-shRNA2, and SNK-shRNA3. shRNA1 was found to be the most effective at decreasing SNK expression and shRNA3 the least effective (Figures 2A, 5A-5C and Supplementary information, Figure S3). Control neurons transfected at DIV0 with GFP plus a non-targeting shRNA construct (shRNA-scr) and analyzed at DIV6, exhibited normal dendrite morphogenesis and had similar dendritic complexity compared to neurons transfected with

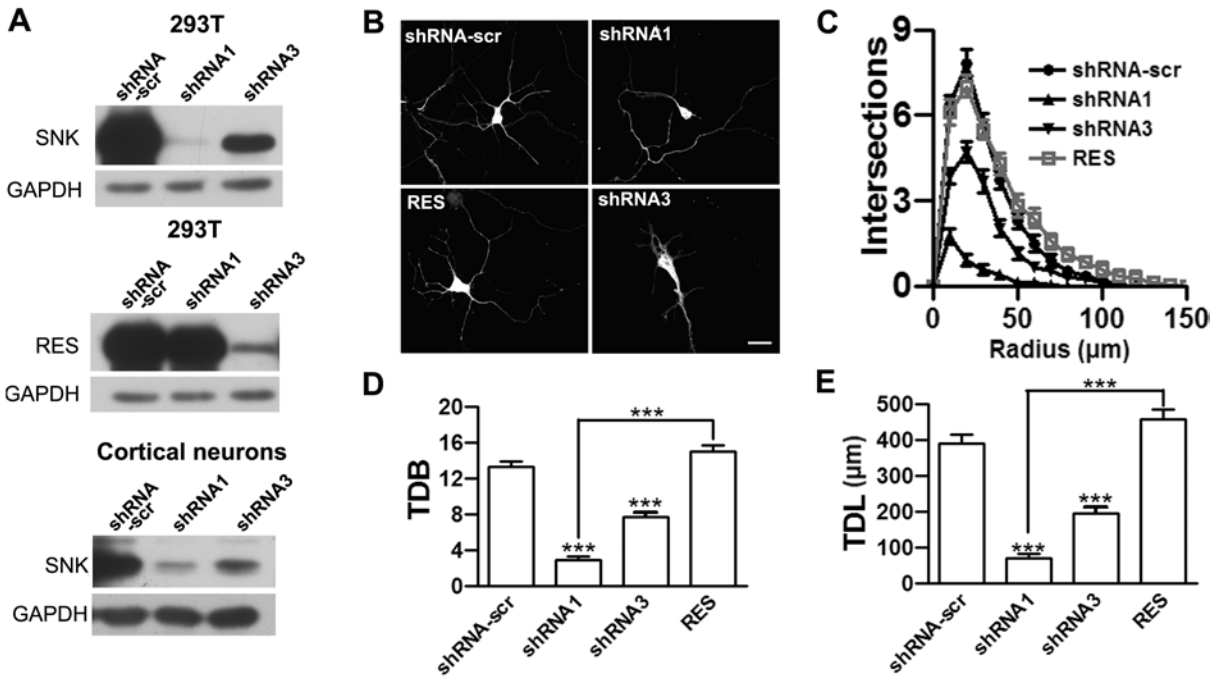

Figure 2 SNK is required for dendrite development *in vitro*. **(A)** The efficiency of short-hairpin RNA constructs, shRNA1, shRNA3, and the control shRNA-scr, in knocking down wild-type SNK and a shRNA1-resistant form of SNK (RES). RNAi efficiency was evaluated by western blotting HEK293T samples after co-transfection of either shRNA together with wild-type SNK (upper panel) or RES (middle panel, here and hereafter, the label RES signifies shRNA1 + RES) by lipo-transfection, or by blotting DIV6 neuron samples (lower panel) after lentivirus-mediated transfection of shRNAs at DIV1. GAPDH was blotted as a loading control. **(B)** Representative DIV6 neurons co-transfected with the indicated constructs and GFP at DIV0. Scale bar, 20 μm. Images were acquired under 63× oil objective, NA = 1.4. **(C-E)** Quantitative analysis of neurons shown in **B**: Sholl analysis **(C)**, analysis of group means for total number of dendrite branches (TDB; **D**), and total dendrite length (TDL; **E**). $n > 40$ per group. ***$P < 0.001$ compared with shRNA-scr using ANOVA with post Newman-Keuls test.

GFP alone (Supplementary information, Figure S2A). In contrast, knocking down SNK impaired dendritic arborization (Figure 2B-2E). The mean number of total dendrite branches (TDB) and mean total dendrite length (TDL) were significantly reduced to 21% and 18%, respectively, in neurons expressing shRNA1 compared to those expressing shRNA-scr (Figure 2D-2E). Knocking down SNK with shRNA3 also inhibited dendritic branching and growth by 42% and 50%, respectively (Figure 2D-E). The effects of shRNA1 were reversed (Figure 2D-2E) by co-transfecting an shRNA1-resistant form of SNK (RES; Figure 2A, middle panel). Sholl analysis further confirmed the remarkable inhibitory effect of SNK RNAi on the dendritic complexity of cortical neurons (Figure 2C).

To analyze the effect of SNK on cortical neuron morphogenesis *in vivo*, we transfected constructs into E16 embryos and then analyzed dendrites after birth. By postnatal day 3 (P3), SNK RNAi had severely impaired apical branching in layer II/III pyramidal neurons (Figure 3A-3D) compared to control. Some shRNA1-expressing neurons were almost devoid of secondary branches, possessing bald, leading process-like apical dendrites (Figure 3B). This effect could be prevented by co-transfecting RES (Figure 3A-3D) and RES even had a growth-promoting effect on dendritic arborization. Knocking down SNK with shRNA3 also caused a significant reduction of dendritic arborization, although to a lesser extent than shRNA1 (Figure 3A-3D) at both P3 (Figure 3A-3D) and P7 (Figure 3E-3H). In the CP of P7 cortices, we could hardly find any GFP-labeled neurons in the shRNA1-transfected group (data not shown), suggesting that long-term suppression of SNK expression leads to cell death, possibly due to morphogenetic defects. These results indicate that SNK is required for dendrite development both *in vitro* and *in vivo*.

We also used calcium phosphate transfection to deliver RNAi constructs at DIV5 and analyzed the morphology of cortical neurons at DIV8, in order to study the function of SNK at a more advanced stage of dendrite development. The results were similar to those obtained at DIV6 (Figure 4). Knocking down SNK with SNK-shRNA1 reduced the mean TDB and TDL to approximately 1/3 of control values, whereas SNK-shRNA3 reduced these values by approximately 25% (Figure 4A and 4B).

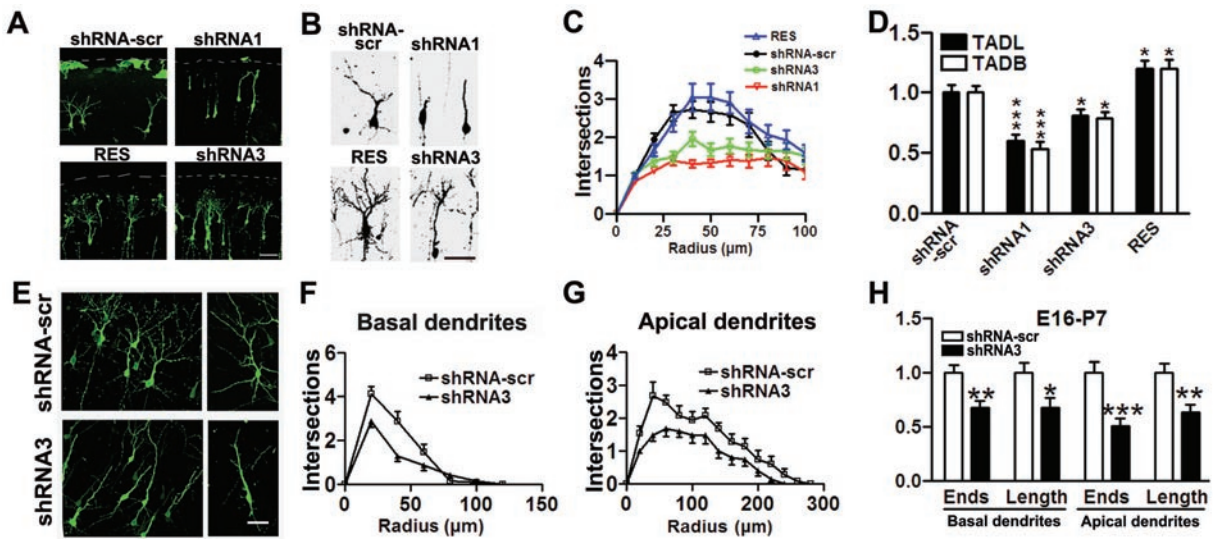

Figure 3 SNK is required for dendrite development *in vivo*. **(A)** Representative P3 cortical sections co-transfected with the indicated constructs and GFP after electroporation at E16. **(B)** Typical appearance of neurons from cortices treated as in **A**. **(C)** Sholl analysis of neurons treated as in **A**. **(D)** Analysis of group means for total number of apical dendrite branch (TADB) and total apical dendrite length (TADL) of neurons shown in **A**. Values represent group means normalized to shRNA-scr. $n >$ 35 neurons from at least five cortices of three rats per group. Values were compared using ANOVA with post Newman-Keuls test. **(E)** Representative P7 cortical sections (left) and typical pyramidal neurons (right) after electroporation with shRNA-scr (upper panel) or shRNA3 (lower panel) at E16. **(F-G)** Sholl analysis of neurons treated as in **E**. **(H)** Analysis of neurons treated as in **E** showing differences in group mean number of basal and apical dendrite branch ends and total length. $n >$ 15 neurons from two cortices of two rats per group. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with shRNA-scr (Student's *t*-test). Scale bar, 50 μm.

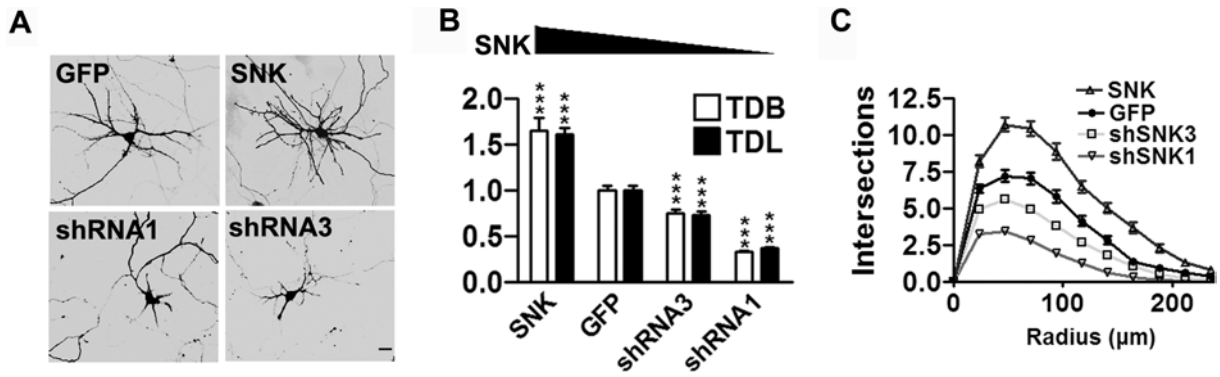

Figure 4 SNK levels correlate with dendritic complexity in DIV8 cortical neurons. **(A)** Typical DIV8 neurons after calcium phosphate-mediated transfection at DIV5 with the indicated constructs and GFP. Scale bar, 20 μm. **(B)** Analysis of neurons treated as in **A**. Values represent group means normalized to the group mean of neurons transfected with GFP alone. $n > 50$ neurons per group. ***$P < 0.001$ compared with the GFP group (ANOVA with post Newman-Keuls test). Relative levels of SNK for each treatment group are shown above the histogram. **(C)** Sholl analysis of neurons treated as in **A**.

SNK is necessary for dendritic but not axonal growth and arborization

The enrichment of SNK in dendrites but not axons prompted us to ask whether SNK regulates dendritic morphogenesis exclusively. We chose to focus our analysis on DIV3 stage-3 neurons transfected on DIV0, when axons are relatively simple to trace. By DIV3, all three shRNA constructs had substantially decreased the level of SNK (Figure 5A-5C and Supplementary information, Figure S3), as well as dendritic growth, arborization and complexity (Figure 5), similar to what we observed at DIV6. Furthermore, Gaussian distribution curves further revealed that the peak mean TDL was shifted from 100-150 μm in control neurons to 50-100 μm in RNAi-expressing neurons (Figure 5F). The shift was positively correlated with the level of SNK, with the most effective RNAi construct, shRNA1, having the greatest inhibitory effect on dendrite outgrowth. Cumulative probability curves of TDL yielded similar results (Figure 5G). Analysis of the mean total number of branch ends and total length revealed that dendritic complexity correlates with the level of SNK (Figure 5H). Importantly, the mean total number of branch ends and total length of axons (TAB and TAL) were not affected by SNK expression (Figure 5I).

SNK kinase activity promotes dendritic arborization

Having established that SNK is required for normal dendrite development, we next investigated whether overexpressing SNK can promote dendritic arborization and whether its kinase activity is involved. We followed the same procedure used for our RNAi experiments to introduce overexpression constructs, wild-type SNK, or the kinase-dead mutant K108M [12, 16], into cultured cortical neurons and layer II/III cortical neurons. At DIV3, SNK overexpression significantly increased dendritic branching (TDB; 1.421 ± 0.013 fold change) and growth (TDL; 1.587 ± 0.018 fold change) compared to neurons transfected with GFP alone (Figure 6C). SNK overexpression starting at DIV5 also increased dendritic branching and growth at DIV8 (1.645 ± 0.009 and 1.608 ± 0.009 fold change, respectively; Figure 4). In P3 cortices *in vivo*, overexpression of SNK significantly promoted the branching and growth of apical dendrites (Figure 6D-6F). Importantly, overexpressing SNK promoted the branching of the leading process of cortical neurons during their migration to the CP (Figure 6G). In contrast, K108M did not promote dendrite development or leading process branching (Figure 6), indicating that kinase activity is necessary for SNK to promote dendritic arborization.

Consistent with our RNAi experiments, the overall length of axons (TAL) was not affected by overexpressing any forms of SNK (Figure 6C), although overexpressing SNK did promote axonal branching (TAB; Figure 6C), possibly due to the ectopic entry of exogenous SNK into axons (data not shown) and the activation of the neurite branching machinery.

BDNF-induced ERK activity promotes SNK transcription

As the level of SNK correlated with dendritic complexity, we asked how SNK levels are regulated during early stages of cortex development. SNK is known to be regulated by numerous extracellular stimuli, including growth factors and neuronal activity, which exert their effects through signaling cascades that vary across different systems [7, 18, 19]. BDNF is widely expressed in the brain and is known to promote neurite branching and

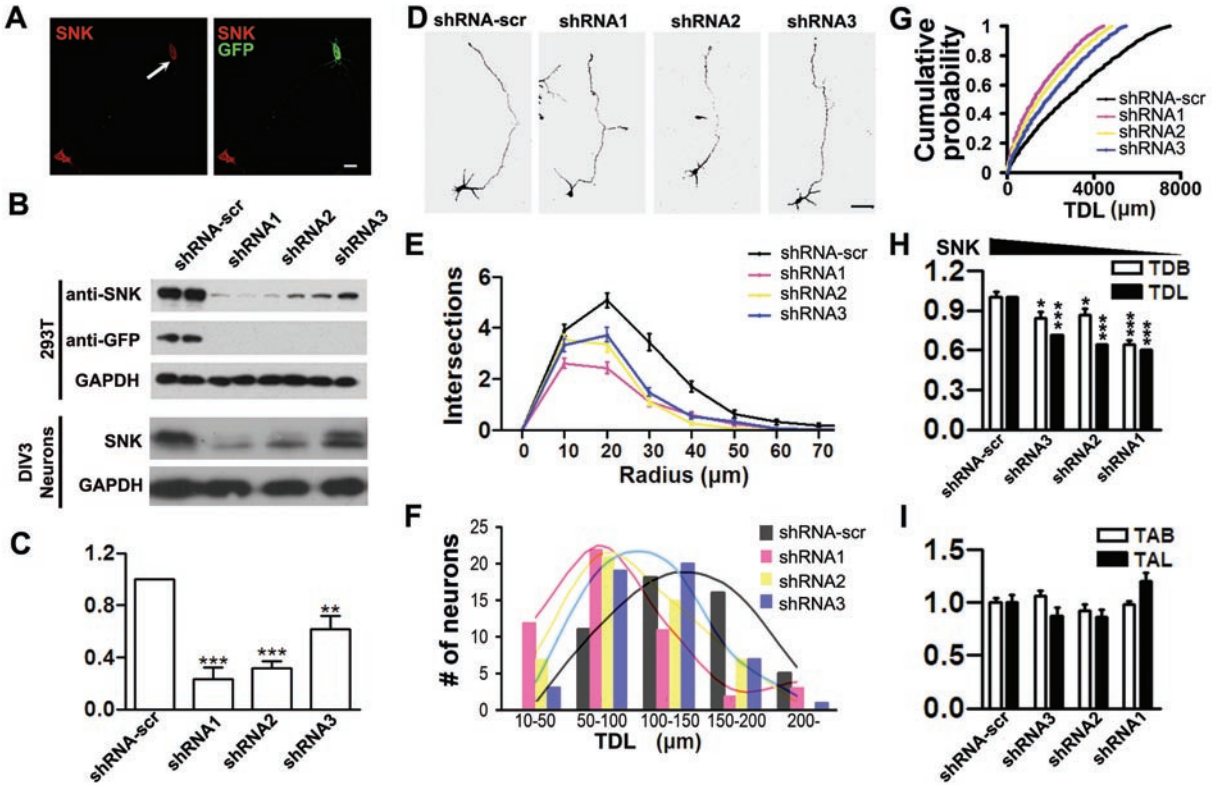

Figure 5 SNK levels correlate with dendritic but not axonal complexity in cultured cortical neurons. **(A)** Representative immunocytochemistry staining of endogenous SNK (red) in DIV3 neurons after 18 h of MG132 treatment. GFP-positive cells (arrow) are neurons transfected with shRNA1 + GFP, while GFP-negative cells indicate untransfected neurons. Images were acquired under a 63× oil objective, NA = 1.4. Scale bar, 20 μm. **(B)** Representative western blot showing the knockdown efficiency of the three SNK-shRNA constructs (shRNA1, shRNA2, shRNA3) and the negative control shRNA-scr in 293T cells co-transfected with SNK-GFP (upper panel), and in electroporated DIV3 cortical neurons (lower panel). Lysates from two independent 293T cultures were blotted for each condition. SNK-GFP levels were detected using anti-SNK and anti-GFP antibodies, and endogenous SNK was detected using anti-SNK. GAPDH was included as a loading control. **(C)** Quantitative analysis of blots shown in lower panel of **B**. Intensity is normalized to shRNA-scr. *n* = 3 blots. **(D)** Typical neurons at DIV3 after electroporation at DIV0 with the indicated constructs and GFP. Scale bar, 50 μm. **(E)** Sholl analysis of neurons treated as in **D**. *n* > 50. **(F)** Gaussian distribution of neurons treated in **E**, binned by total dendrite length. *n* = 50 neurons per group. **(G)** Cumulative probability curve of data presented in **F**. **(H-I)** Quantitative analysis of neurons treated as in **D**. TAB: total number of axon branch ends, TAL: total axon length. *n* > 50 per group. Values represent group means normalized to the shRNA-scr control group. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 compared with shRNA-scr (ANOVA with post Newman-Keuls test). Relative levels of SNK for each treatment group are shown above the histogram in **H**.

growth [20]. We thus asked whether the dendrite morphogenetic activities of SNK and BDNF are interrelated. We found that levels of SNK were rapidly increased after stimulation with 25 ng/ml BDNF at DIV5 (Figure 7A). SNK levels peaked between 2 and 4 h of stimulation, and declined back to the control level after 16 h of stimulation. Since SNK levels are tightly controlled by the ubiquitin-proteasome system (UPS) [12], we tested whether BDNF induced SNK upregulation by inhibiting its degradation. Blocking UPS-dependent degradation with 25 μM MG132 (M) for 2 h increased SNK to a greater degree than 2 h of BDNF stimulation (Figure 7B), and combined treatment with MG132 and BDNF increased SNK levels more than either treatment alone, indicating an additive effect (Figure 7B). Surprisingly, co-administering the transcription blocker actinomycin-D (A) to block transcription during 2 h of BDNF stimulation completely eliminated SNK upregulation (Figure 7B), suggesting that BDNF regulates SNK levels through transcription. This was confirmed by quantitative real-time PCR (qRT-PCR; Figure 7C). Blocking SNK translation with cycloheximide (C) also suppressed SNK upregulation by BDNF, further supporting the conclusion that BDNF regulates SNK synthesis, not degradation.

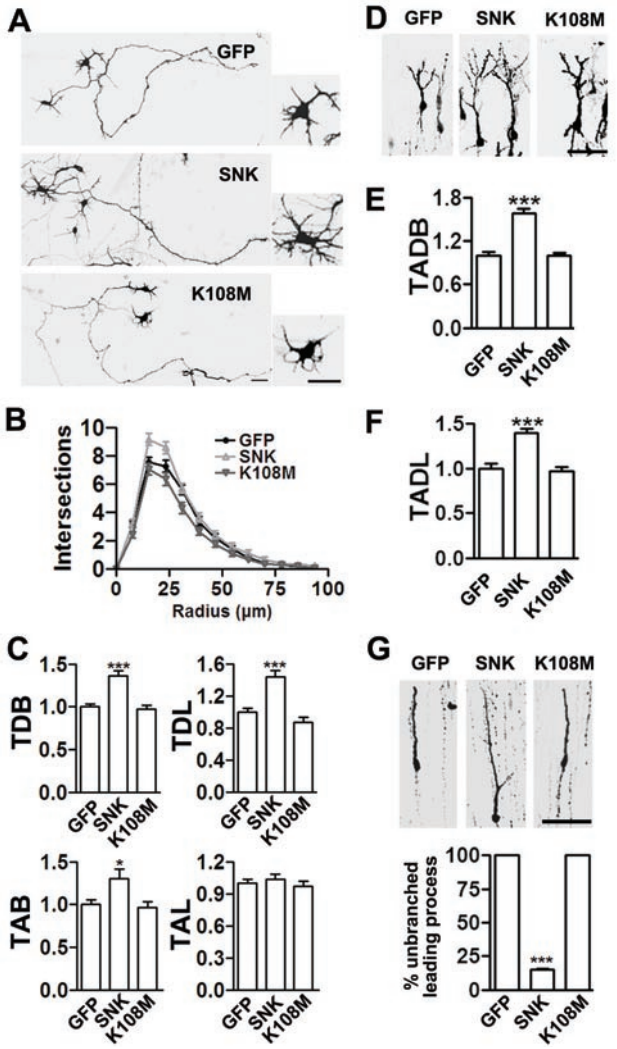

Figure 6 SNK overexpression promotes dendritic arborization in a kinase domain-dependent fashion. **(A)** Representative DIV3 cortical neurons transfected at DIV0 with the indicated SNK constructs and GFP. Scale bar, 50 μm. **(B)** Sholl analysis of neurons treated as in **A**. **(C)** Analysis of group means (normalized to neurons transfected with GFP alone), $n > 40$ neurons per group. **(D)** Representative neurons in P3 cortices electroporated at E16. Scale bar, 50 μm. **(E-F)** Quantitative analysis of neurons treated as in **D**, $n > 45$ from at least five cortices of two rats per group. Means are normalized to GFP control. **(G)** Upper panel, typical appearance of migrating neurons in cortices treated as in **D**. Scale bar, 50 μm. Lower panel, percentage of these neurons with an unbranched leading process. $n = 3$ optical fields per group. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with GFP (Student's *t*-test).

In order to understand the mechanism by which BDNF regulates cellular concentrations of SNK, we selectively blocked the three main downstream effectors of BDNF-TrkB signaling [21] using the following specific inhibitors: K252a (K) to block activation of the BDNF receptor TrkB, U0126 (U0) to block ERK1/2 activation, LY294002 (L) to block AKT phosphorylation and U73122 (U7) to block PLCγ signaling (Figure 7D). We found that BDNF-dependent upregulation of SNK was suppressed after administering K252a and U0126, but not U73122 or LY294002 (Figure 7D), although all reagents were effective (Figure 7D and Supplementary information, Figure S4). This suggests that SNK transcription in developing neurons is positively regulated by BDNF signaling via TrkB-dependent ERK1/2 phosphorylation.

SNK is necessary for BDNF-induced dendritic arborization

We next assessed the physiological significance of BDNF-induced SNK upregulation in cultured cortical neurons. We electroporated cortical neurons at DIV0 with shRNA1, either alone or in combination with RES. BDNF groups were treated with 25 ng/ml BDNF at DIV3 for 24 h before fixation, whereas unstimulated control neurons were fixed at DIV4 without treatment. In control cells, BDNF promoted dendritic branching and growth (Figure 7E-7G). In contrast, SNK-shRNA1 impaired not only normal dendritic arborization, as shown earlier (Figures 2-5), but also BDNF-promoted dendritic arborization (Figure 7E-7G). Co-transfecting RES reversed the inhibitory effect of SNK-shRNA1 on dendritic arborization and led to a net increase in dendritic growth and branching above control levels (Figure 7E-7G). However, RES failed to restore the promoting effect of BDNF on dendritic branching and growth (Figure 7E-7G). This supports our conclusion that BDNF regulates SNK through transcription and that the level of SNK correlates with dendritic complexity, since it is reasonable to believe that the upregulation of endogenous SNK was suppressed by shRNA1 and thus that there was little, if any, increase in the overall level of SNK in stimulated RES + shRNA1-transfected neurons, relative to unstimulated RES + shRNA1-transfected neurons. However, we cannot exclude the possibility that BDNF promotes the activity of SNK through mechanisms distinct from increasing its transcription, since primary dendrite formation was increased after BDNF stimulation of RES + shRNA1-transfected neurons (TPD; Figure 7F). Taken together, these data demonstrate that ERK pathway-mediated upregulation of SNK is crucial for BDNF-promoted dendritic arborization *in vitro*.

Discussion

Novel role of the polo-like kinase SNK in neuronal development

During cerebral cortex formation, neurons develop from progenitor cells, which undergo proliferation,

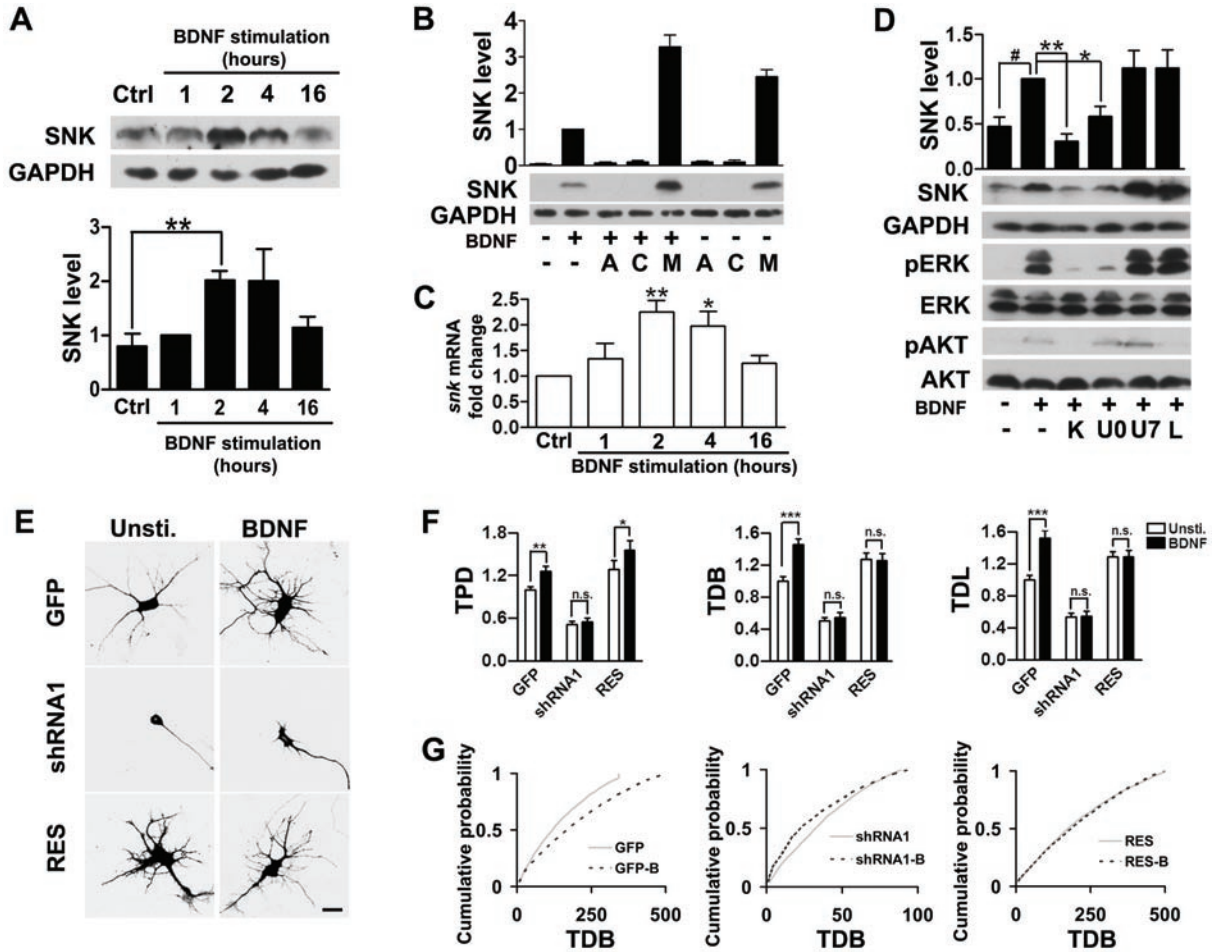

Figure 7 SNK acts downstream of BDNF signaling and is required for BDNF-induced dendritic arborization. **(A)** Representative anti-SNK western blot of unstimulated (unsti.) DIV5 cortical neurons or DIV5 cortical neurons stimulated with 25 ng/ml BDNF for the indicated time period (upper panel). The negative control group (Ctrl) was treated with vehicle solution. Anti-GAPDH was blotted as a loading control. Lower panel, quantification of band intensities normalized to 1 h stimulation (second bar), $n = 4$ blots. **(B)** Anti-SNK western blot of DIV5 cortical neurons treated with the indicated compounds for 2 h (lower panel). BDNF: 25 ng/ml; A: 25 μM actinomycin-D; C: 25 μM cycloheximide; M: 25 μM MG132. Upper panel, quantification of band intensities normalized to BDNF stimulation alone (second bar), $n = 3$ blots. **(C)** Quantitative real-time PCR analysis of SNK mRNA expression in cultured cortical neurons treated as in **A**, normalized to vehicle-treated control. $n = 4$ independent experiments per group. **(D)** Western blot analysis of SNK and BDNF signaling pathway members in DIV5 cortical neurons treated with the indicated compounds for 2 h. BDNF: 25 ng/ml; K: 200 nM K252a; U0: 25 μM U0126; U7: 25 μM U73122; L: 25 μM LY294002. Intensities were normalized to BDNF stimulation alone (second bar). $n = 5$ blots. **(E)** Representative GFP-immunolabeled DIV4 cortical neurons transfected with GFP alone (GFP), GFP + shRNA1 (shRNA1), or GFP + shRNA1 + RES (RES). Cultures were either left unstimulated or stimulated with 25 ng/ml BDNF for 24 h before fixation. Images were acquired under a 40× oil objective, NA = 1.3. Scale bar, 20 μm. **(F)** Quantitative analysis of neurons treated as in **E**. Values represent group means normalized to unstimulated neurons transfected with GFP alone, $n > 35$ neurons per group. TPD: total number of primary dendrites. **(G)** Cumulative probability curve of data presented in **F**. n.s.: not significant; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with GFP alone group using ANOVA with post Newman-Keuls test in **A**-**D**, and Student's *t*-test in **F**.

migration and morphogenesis in a non-overlapping chronological order. Many protein families known to be important in the regulation of cell proliferation, including CDKs, Rho-GTPases, ORCs, and others, have been shown to also participate in the regulation of morphogenesis [22-26]. This pleiotropy provides insight into the mechanisms underlying these cellular events, which share common requirements, such as cytoskeletal remodeling, membrane deformation and cell adhesion dynamics. In this study, SNK expression was observed

in the neocortex and striatum, but not the VZ/SVZ or MGE/LGE regions of the developing rat brain (Figure 1), suggesting that it contributes only to post-mitotic and post-migratory developmental processes. Indeed, overexpressing exogenous SNK in migrating neurons induced arborization of the leading process, indicating that SNK can promote neurite elaboration in post-mitotic neurons and that altering the temporal dynamics of its expression blurs the chronological switch between migratory and dendrite morphogenetic events. Taken together, these findings suggest that SNK is a key regulator of dendritogenesis.

Potential downstream targets of SNK

PLK proteins were originally found to be critical for diverse aspects of mitosis, a cellular event that shares many mechanisms with dendrite morphogenesis, such as the regulated reorganization of microtubules. PLKs, including *Drosophila* Polo [27], mammalian PLK1 [28-30] and PLK3/FNK [31], are associated with tubulins and/or microtubule associated proteins, which are important for both spindle formation during mitosis and microtubule dynamics during morphogenesis. Furthermore, PLK substrates and interacting proteins known to be important for mitosis have also been shown to regulate dendrite development, including β-catenin [32-35] and the GTPase RhoA [36-39]. Although it is thought to associate with microtubules in COS7 cells where its overexpression induces an arborization phenotype [16], SNK has not been reported to interact with these effector proteins. However, given the high level of conservation of the polo-box, it is possible that SNK and other PLK proteins share common subsets of substrates and interacting partners.

SNK has been shown to phosphorylate SPAR [12], a known regulator of spine morphology [40]. SNK may thus affect dendrite growth and arborization in part by regulating the activity of SPAR, which is expressed in immature neurons and has the ability to regulate actin dynamics [40]. However, given that SNK is expressed in the nucleus of immature neurons (Figure 1), we cannot rule out the possibility that SNK affects morphogenesis from the nucleus.

SNK is regulated by BDNF signaling and is critical for the growth-promoting effects of BDNF on dendrites

One key point of this study is that SNK levels were correlated with dendritic complexity and that SNK was rapidly up-regulated in response to BDNF-stimulated ERK activity. This upregulation was necessary for the induction of dendritic arborization by BDNF. BDNF regulates the expression of a variety of proteins and is involved in many important neurological processes [41-43]. Three important molecular pathways (those involving MAPK, PI3K and PLCγ) are known to act downstream of BDNF-TrkB signaling in dendrite development [21]. Among the three, both PI3K- and MAPK-dependent cascades are required downstream of BDNF in the formation of primary dendrites, and Dijkhuizen and Ghosh reported that this activity does not require protein synthesis, at least during the initial 5 h [44]. In contrast, protein synthesis is important for BDNF-promoted dendritic arborization, since BDNF-mediated translational regulation is necessary for this activity [41, 43, 45]. Specifically, mTOR, which plays a key role in translational regulation [45], was found to regulate dendritic growth and branching downstream of PI3K-AKT signaling after BDNF stimulation [41]. However, to our knowledge, BDNF-dependent transcriptional control of a dendrite-regulating protein has not been demonstrated before. In this study, we provide evidence that ERK pathway-dependent transcriptional elevation of SNK is required for the stimulatory effect of BDNF on dendritic branching and growth (Figure 7E and 7F). Therefore, our results provide a new understanding of BDNF signaling in dendritic morphogenesis.

Further implications of SNK function in neural circuit

The results presented herein build on previous work showing that SNK plays a key role in the formation of dendritic spines [12] and in the regulation of circuit plasticity through synaptic scaling [13, 19]. Together, our results reveal that SNK is critical for dendritic morphogenesis throughout neuronal development, which is important for circuit structure and function. BDNF is also essential for many aspects of circuit establishment [46] and functions [47], such as learning and memory (long-term potentiation and long-term depression) [47], synaptic homeostasis [48], and epileptogenesis [49]. In addition, aberrant BDNF signaling is involved in an array of neurological disorders, including mental retardation, autism, epilepsy, and neurodegenerative diseases [47]. Interestingly, SNK is regulated by LTP and epileptic stimuli [14], and is implicated in Parkinson disease and Alzheimer disease via α-synuclein phosphorylation [50-52]. Our work is the first to establish a relationship between BDNF and SNK *in vitro*; however, in this work we only show the effects of exogenous BDNF on SNK. Determining whether or not SNK is essential for physiologically relevant or *in vivo* BDNF-dependent cellular mechanisms will require further study, and it will be interesting to define the extent of the relationship between BDNF and SNK in different contexts, including normal neuronal development and function, and pathological states.

Materials and Methods

Expression vectors and primers

pCAG overexpression vectors (pCAG-GFP-C1, pCAG-mCherry-C1 and pCAG-IRES-GFP) were generated from pCMV-GFP or pCMV-mCherry vectors (Clontech) by replacing the CMV promoter with a CAG promoter. Full-length *Rattus norvegicus* SNK was cloned from cortex-derived cDNA and inserted into pCAG-GFP between the *Eco*RI and *Bam*HI sites. The kinase dead mutant SNK^{K108M} (K108M) construct was generated using the following primer: 5′-ACGCTGCAAtgATTATCCCTCACAGCAGAG-TAGCTAAACC-3′.

Three RNAi sequences targeting rat SNK (SNK-shRNA1: 5′-GACAGATCTGACAAACAAC-3′; SNK-shRNA2: 5′-CAAGGACATGGCTGTGAAT-3′; SNK-shRNA3: 5′-CAGTGAATGCCTTGAGGAT-3′) were synthesized and cloned into pSuper vectors (Oligoengine) following the user instructions. The SNK-shRNA1 resistant form of SNK (RES) contained the following silent point mutations (in lower case) within the target region: 5′-GACAGActTaACtAAtAAt-3′.

Primers (Invitrogen) for RT-PCR and quantitative real-time PCR were SNK: 5′-TGCAGACACAGTGGCAAGAGTC-3′ (forward), 5′-CCATTTGGTGACCCACTGAAAG-3′ (reverse) and GAPDH: 5′-GGTTGTCTCCTGCGACTTCA-3′ (forward), 5′-CCACCACCCTGTTGCTGTAG-3′ (reverse).

Antibodies and reagents

Primary antibodies used were as follows: SNK (Santa Cruz), MAP2 (Chemicon), Tau1 (Chemicon), HRP-GAPDH (Kang Chen), GFP (Invitrogen and Santa Cruz), RFP (Chemicon), ERK (Cell Signaling), pERK (Cell Signaling), AKT (Cell Signaling), pAKT (Cell Signaling), PLCγ (Santa Cruz), and pPLCγ (Santa Cruz).

Secondary antibodies used for western blot were as follows: HRP-anti-rabbit (Kang Chen), HRP-anti-mouse (Kang Chen); and for immunohistochemistry were as follows: Alexa-fluor-488-anti-rabbit (Invitrogen), Alexa-fluor-568-anti-mouse (Invitrogen), Alexa-fluor-488-anti-mouse (Invitrogen), and Alexa-fluor-546-anti-rabbit (Invitrogen).

Reagents used were as follows: BDNF (25 ng/ml; PeproTech), MG132 (25 μM; Sigma), actinomycin-D (25 μM; Beyotime), cycloheximide (25 μM; Beyotime), K252a (200 nM; Sigma), U73122 (25 μM; Calbiochem), U0126 (25 μM; Calbiochem), and LY294002 (25 μM; Calbiochem).

In situ hybridization

In situ hybridization was performed as described previously [53]. Briefly, E14, E16, and E18 rat embryos were incubated overnight in 4% paraformaldehyde (PFA) in 0.1 M RNase-free phosphate buffer (DEPC-PB) at 4 °C. P0, P3, P7, P14, and adult rats were perfused with 4% PFA-PB (DEPC treated), and whole brains were isolated and placed in 4% PFA-PB at 4 °C overnight followed by dehydration in a sucrose gradient (20% and 30% in DEPC-PB). Brains were cut into 30 μm coronal sections with a Cryostat CM1900 (Leica) at −20 °C, and mounted on poly-D-lysine-coated slides (2 mg/ml; BD), and stored at −80 °C. An antisense SNK probe was generated from pGEM-T Easy vector containing the 3′-terminal 681 bases of the SNK coding sequence by *in vitro* transcription using an SP6/T7 transcription kit (Roche). Hybridization was performed using standard protocols; NBT/BCIP (Boehringer) was used for signal detection.

In utero electroporation and morphological analysis

In utero gene transfer was carried out as previously reported [53]. Briefly, pregnant rats were deeply anesthetized on 16 days post-coitum with 12% chloral hydrate and ~1 μg plasmid solution was injected into lateral ventricle of the E16 embryonic CNS with a fine glass micropipette. The brain was then clamped between tweezer-type disc electrodes (CUY-650-5) with the positive end attached to the hemisphere injected with plasmids and given five 50 ms, 50 volt pulses at 1-sec intervals with an electroporator (ECM830). Embryos were then placed back in the womb. All SNK constructs were co-transfected with GFP at a ratio of 2:1, except for RES, which was co-transfected with shRNA1 at a ratio of 1.5:1 and with GFP at a ratio of 3:1.

P3 or P7 pups were perfused and 50 μm sections were prepared with a Cryostat CM1900 at −20 °C followed by anti-GFP immunohistochemistry. Z-stack images were acquired with confocal microscopes (Zeiss LSM510) under a 20× air objective with a numerical aperture (NA) of 0.8. Layer II/III neurons were traced using Neurolucida software and data were exported to Excel, and analyzed and plotted with Prism 4.0 software.

In vitro transfection and morphological analysis

Human embryonic kidney variant HEK293T cells were maintained in Dulbecco modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Cells were transfected with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions [54]. Primary cortical neurons were cultured in accordance with a standard protocol. For *in vitro* electroporation, neurons were suspended in transfection medium (0.2 ml) mixed with plasmids, then transferred into a 2.0 mm electroporation cuvette, and electroporated using an Amaxa Nucleofector apparatus [55]. All SNK constructs were co-transfected with GFP at a ratio of 2:1 (4 μg:2 μg), except for RES (same as *in vivo*). Neurons suspended in plating medium were then placed on coverslips or dishes coated with 1 mg/ml poly-D-lysine. At 2 h after plating, the medium was exchanged for culture medium (neurobasal medium containing glutamax and B27). After the growth period indicated, the neurons were fixed, immunolabeled with anti-GFP, and mounted with Cytomation fluorescent mounting medium (DAKO). For BDNF treatment, neurons were treated with 25 ng/ml BDNF or vehicle control solution on DIV3, fixed, and immunolabeled on DIV4. For calcium phosphate-mediated transfection, neurons at a density of 1×10^5 were transfected on DIV5 as described previously [56], SNK constructs were co-transfected with GFP at a ratio of 2:1, and neurons were fixed on DIV8, then immunolabeled with anti-GFP, and mounted with DAKO mounting medium. Cultured neurons were imaged with confocal microscopes (Zeiss LSM510 or Nikon Eclipse FN1) under either a 20× air objective (default) with numerical aperture (NA) of 0.8 or the objectives specified in the figure legends, and analyzed as described in the previous section.

Quantification and statistical analysis

Western blot and RT-PCR band intensities were quantified using IMAGE QUANT 5.2 software. Morphologies were analyzed with software Neurolucida and Excel. Immunocytochemical inten-

sities were quantified using ImageJ and Image-Pro Plus programs. Data were plotted and significance of differences was analyzed using Prism 4.0 software. Error bars indicate SEM.

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(**Supplementary information** is linked to the online version of the paper on the *Cell Research* website.)