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Netrin-1 Mediates Neuronal Survival Through PIKE-L Interaction With the Dependence Receptor UNC5B

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Abstract

Netrins, a family of secreted molecules, play critical roles in axon guidance and cell migration during neuronal development 1,2. In addition to its role as a chemotropic molecule, netrin-1 also acts as a survival factor 3–7. Both UNC5 (i.e. UNC5A, B, C or D) and DCC are transmembrane receptors for netrin-1^{8,9}. In the absence of netrin-1, DCC and UNC5 act as dependence receptors and trigger apoptosis 3,6,¹⁰. However, how netrin-1 suppresses the apoptotic activity of the receptors remains elusive. Here, we show that netrin-1 induces interaction of UNC5B with the brain specific GTPase PIKE-L. This interaction triggers activation of PI 3-kinase signaling, prevents UNC5B's pro-apoptotic activity and enhances neuronal survival. Moreover, this process tightly relies on Fyn as PIKE-L is tyrosine phosphorylated in response to netrin-1 and the netrin-1-mediated interaction of UNC5B with PIKE-L is inhibited in Fyn null mice. Thus, PIKE-L acts as a downstream survival effector for netrin-1 through UNC5B in the nervous system.

PIKE-L is a brain specific GTPase, which binds and stimulates PI 3-kinase in a GTP-dependent manner 11,¹². PIKE-L binds Homer, an adaptor protein for metabotropic glutamate receptor (mGluR). Activation of mGluRs enhances formation of an mGluRI-Homer-PIKE-L complex, leading to activation of PI 3-kinase and prevention of neuronal apoptosis ¹³. PIKE is also a substrate for caspases. PIKE can be phosphorylated on tyrosine residues by Fyn, leading to its resistance to caspase cleavage 14. To search for PIKE-L-binding proteins, we conducted yeast two-hybrid screening using GTPase domain as bait. Four out of twelve clones are both His and β -gal positive, one of which encodes the C-terminus of UNC5B (Figure 1A). In HEK293 cells, transfected GFP-PIKE-L selectively binds to 569–946 fragment of UNC5B but not other fragments. Compared to the binding by the C-terminal motif (a.a. 569–946), truncation of death domain (a.a. 854–946) decreases UNC5B affinity to PIKE-L. Reciprocal immunoprecipitation reveals that the interaction occurs no matter PIKE-L or UNC5B is precipitated by its antibody (Figure 1B, middle panels). Full-length UNC5B and its C-terminal fragment released after caspase cleavage ^{5,7} specifically interact with GTPase domain but not with other regions of PIKE-L, consistent with our yeast two-hybrid findings (Figure 1B, right panels). We also

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observed the robust interaction between endogenous PIKE-L and UNC5B in both the cortex and hippocampus of rat brain (Figure 1C). Immunostaining of hippocampal and cortical primary neurons reveals that PIKE-L and UNC5B colocalize in the cell body and throughout all neuronal processes (Figure 1D, left panel). The staining is specific as GST-PIKE-L (a.a. 268–384) antigen but not control GST abolishes PIKE-L staining in neurons (Figure 1D, right panels).

To examine whether netrin-1 modulates PIKE-L interaction with UNC5B, we cotransfected UNC5B into HEK293 cells with wild-type or dominant-negative PIKE-L-KS (K413AS414N). This mutant cripples PIKE-L's GTPase activity. It binds PI 3-kinase but fails to activate it^{11, 12}. Compared to control, netrin-1 elicits PIKE-L interaction with UNC5B; by contrast, PIKE-L-KS fails to bind UNC5B regardless of netrin-1 stimulation (Figure 2A), indicating that GTPase activity is essential for the association. Coimmunoprecipitation demonstrates that PIKE-L does not bind to death domain containing p75NTR or UNC5A, but it faintly associates with UNC5C irrespective of netrin-1 treatment (data not shown), suggesting that PIKE-L specifically interacts with UNC5B in response to netrin-1.

PIKE can be phosphorylated on tyrosine residues by Fyn¹⁴. Netrin-1 has also been shown to trigger its receptor DCC tyrosine phosphorylation^{15–18}. To test whether phosphorylation on tyrosine modulates the interaction between PIKE-L and UNC5B, we pretreated the cotransfected HEK293 cells with several tyrosine kinase inhibitors, followed by netrin-1. Netrin-1-mediated PIKE-L/UNC5B interaction is selectively disrupted by Genistein, PP2 and compound 5. PP2 is a Fyn selective inhibitor, whereas Genistein and compound 5 are tyrosine kinase inhibitors. By contrast, Daidzein and PP3, inactive controls for Genistein and PP2, respectively, fail to dissociate the complex (Figure 2B, top panel). Accordingly, netrin-1 triggers tyrosine phosphorylation on both PIKE-L and UNC5B, which are selectively blocked by Genistein, PP2 and Compound 5 (Figure 2B, 2nd panel). In primary cortical cultures, netrin-1 elicits endogenous PIKE-L to bind UNC5B, which is blocked by PP2 pretreatment, and Genistein also decreases the interaction (Figure 2C, top panel). PIKE-L tyrosine phosphorylation is substantially abolished by PP2 or Genistein pretreatment. UNC5B tyrosine phosphorylation is evidently blocked by PP2 and partially prevented by Genistein (Figure 2C, 2nd and 5th panels).

To explore whether Fyn contributes to phosphorylate UNC5B or PIKE-L, we cotransfected UNC5B into HEK293 cells with various tyrosine kinases. Coimmunoprecipitation shows that constitutively active FynA but not kinase-dead FynD or other tyrosine kinases including Src and Pyk2 interacts with UNC5B (Supplemental Figure 1A, left panels). Moreover, FynA also elicits robust tyrosine phosphorylation on UNC5B. The 100 kDa band below UNC5B might be an unknown tyrosine phosphorylated protein associated with UNC5B (Supplemental Figure 1A, top middle panel). We conducted the similar experiments with PIKE-L, and found that FynA but not FynD or other tyrosine kinases selectively bound and phosphorylated PIKE-L (Supplemental Figure 1B). Coimmunoprecipitation demonstrates that PIKE-L tightly binds UNC5B in FynA but not FynD cotransfected cells even in the absence of netrin-1, no matter PIKE-L or UNC5B is immunoprecipitated (Supplemental Figure 1C, top left panels). Both UNC5B and PIKE-L are potently phosphorylated in FynA but not FynD transfected cells (Supplemental Figure 1C, middle panels). Noticeably, compared to FynD cells, PIKE-L apoptotic cleavage is partially decreased in FynA cells (Supplemental Figure 1C, right lower panel), an effect similar to netrin-1-treated cells. PIKE-A is mainly phosphorylated by Fyn on Y681 and Y774 residues¹⁴, which correspond to Y1032 and Y1124 in PIKE-L. Immunoprecipitation assay reveals that wild-type PIKE-L strongly interacts with UNC5B, whereas Y1032F, Y1124F and Y1032, 1124F mutants fail to interact with UNC5B. As expected, PIKE-L wild-type is evidently tyrosine phosphorylated, which is abolished in PIKE-L mutants (Figure 3A, top and 2nd panels). Reciprocal immunoprecipitation reveals similar

results (Figure 3A, 4th and 5th panels), suggesting that PIKE-L tyrosine phosphorylation is necessary for its association with UNC5B. To explore further whether Fyn regulates the association between PIKE-L and UNC5B, we conducted immunoprecipitation with wild-type and Fyn $-/-$ mice. UNC5B robustly interacts with PIKE-L in $+/+$ but not $-/-$ mouse brain (Figure 3B), indicating that Fyn is required for the association. PY99 antibody but not control IgG selectively pulls down UNC5B and PIKE-L from $+/+$ but not $-/-$ brain lysates, suggesting that UNC5B and PIKE-L are strongly tyrosine phosphorylated in wild-type but not Fyn-null mice (Figure 3B, left panels). To explore whether netrin-1 triggers Fyn activation through UNC5B, we tested this notion in HEK293 cells, which were transfected with PIKE-L or UNC5B alone or in a combination. Netrin-1 elicits a UNC5B-dependent Fyn activation. Cotransfection of both PIKE-L and UNC5B enhances Fyn activation upon netrin-1 treatment (Figure 3C, top left panel). Depletion of UNC5B in cortical neurons abolishes netrin-1-provoked Fyn activation (Figure 3C, bottom right panel), underscoring that UNC5B is critical for Fyn activation. Collectively, Fyn plays a critical role in regulating UNC5B/PIKE-L complex formation in mouse brain.

Netrin-1 activates PI 3-kinase, which is essential for netrin-1's role in axon guidance^{19–21}. To assess whether UNC5B/PIKE-L complex plays any role in mediating netrin-1-provoked PI 3-kinase activation, we transfected HEK293 cells with PIKE-L and UNC5B. In the absence of UNC5B, netrin-1 fails to provoke PI 3-kinase activity in PIKE-L transfected cells while netrin-1 elicits robust PI 3-kinase activity in wild-type PIKE-L and UNC5B cotransfected cells. This netrin-1-dependent PI 3-kinase activation is decreased in Wortmannin or LY294002 pretreated cells or when a dominant-negative PIKE-L mutant is transfected (Figure 4A, bottom panel). These data suggest that netrin-1-triggered PI 3-kinase activation is mediated by PIKE-L. To explore whether PIKE-L is necessary for this action, we infected cortical neurons with various adenovirus expressing sh-RNA of PIKE, wild-type PIKE-L or PIKE-L-KS and monitored Akt phosphorylation. Netrin-1-provoked PI 3-kinase activity is substantially reduced when PIKE-L is depleted. Infection of PIKE-L increases netrin-1's stimulatory effect on PI 3-kinase, which is massively attenuated in PIKE-L-KS infected cells (Figure 4B, 2nd panel).

To evaluate whether Fyn mediates netrin-induced PI 3-kinase activity, we pretreated primary neurons with PP2, and stimulated cells with netrin-1 for 30 min. Netrin-1 induces potent Akt activation, which is completely blocked by PP2 (Figure 4C, top panel), suggesting that Fyn is essential for netrin-1 to elevate PI 3-kinase activity. Compared to wild-type neurons, netrin-1-triggered Akt activation is only slightly decreased in DCC $-/-$ neurons, indicating that DCC is not the major receptor for this effect (Figure 4C, 3rd panel). Moreover, activation of Src family tyrosine kinases is partially diminished in DCC-null neurons (Figure 4C, bottom panel), suggesting that DCC contribute to Src family tyrosine kinases activation by netrin-1. To explore the physiological role of Fyn in regulating PI 3-kinase activation by netrin-1, we monitored Akt activation in wild-type and Fyn knockout neurons. Netrin-1 strongly elicits PI 3-kinase activation in wild-type neurons and PIKE-L further enhances its effect. Strikingly, PIKE-L (Y1032, 1124F) fails to increase further Akt activation by netrin-1 (Figure 4D, top panel), indicating that PIKE-L binding to UNC5B is essential for PI 3-kinase activation by netrin-1. As expected, netrin-1 fails to activate Akt in Fyn $-/-$ neurons, regardless of PIKE-L expression (Figure 4D, 3rd panel). PI 3-kinase activation status tightly couples to Akt phosphorylation (data not shown). Depletion of UNC5B in cortical neurons evidently attenuates Akt activation. Nonetheless, PIKE-L expression level remains constant irrespective of UNC5B alteration (Figure 4E). Taken together, this finding supports that UNC5B but not DCC plays an essential role for mediating netrin-1-provoked PIKE-L dependent PI 3-kinase/Akt signaling activation.

To determine whether PIKE-L modulates netrin-1's anti-apoptotic action, we infected primary neuronal cultures with various adenoviruses and provoked apoptosis with glutamate, a broadly

used excitatory amino acid (EAA) neurotransmitter for neuronal cell death. In control neurons, netrin-1 evidently blocks glutamate-triggered apoptosis. Infection of wild-type PIKE-L strongly suppresses apoptosis, and netrin-1 further enhances the protective effect. By contrast, the anti-apoptotic effect by netrin-1 is reduced in PIKE-L-KS-infected neurons. Moreover, knocking down of PIKE-L almost completely abolishes netrin-1's survival activity. The shRNA resistant PIKE-L mutant infection restores the survival effect, suggestive of the specificity of the siRNA effect. Fyn phosphorylation deficient mutant PIKE-L(Y1032, 1124F) also attenuates apoptosis, but it is irresponsive to netrin-1 (Figure 5A). Thus, PIKE-L binding to UNC5B is necessary for netrin-1's anti-apoptotic effect. The anti-apoptotic activity by PIKE-L couples to its stimulatory effects on PI 3-kinase in netrin-1-treated cells. In Fyn $-/-$ neurons, netrin-1's anti-apoptotic effect is substantially impaired. PIKE-L(Y1032, 1124F) modestly represses apoptosis in a netrin-independent manner. Wild-type PIKE-L decreases apoptosis, but netrin-1 fails to enhance its survival activity (Figure 5B, upper panel), supporting that Fyn is required for PIKE-L to mediate netrin-1's anti-apoptotic effect. Interestingly, depletion of PIKE-L slightly decreases UNC5B but not DCC expression level. Overexpression of PIKE-L enhances UNC5B and DCC expression, indicating that PIKE-L stabilizes netrin-1 receptors in neurons (Figure 5B, lower panels). In the absence of netrin-1, depletion of PIKE-L enhances UNC5B cleavage and PARP fragmentation in wild-type neurons elicited by glutamate, which are attenuated by PIKE-L overexpression. Netrin-1 further elevates PIKE-L's anti-apoptotic effect. In the absence of PIKE-L, netrin-1 fails to block PARP and UNC5B apoptotic cleavage (Figure 5C, left panel). In contrast, in Fyn $-/-$ neurons, netrin-1 loses its anti-apoptotic effect as PARP and UNC5B are robustly cleaved no matter netrin-1 is present or not. Nevertheless, overexpression of PIKE-L slightly decreases UNC5B cleavage (Figure 5C, right panels).

Excitotoxic neuronal cell death in brain is often induced experimentally by the administration of kainic acid (KA), a potent agonist of the α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid/kainate class of glutamate receptors²². Depletion of PIKE-L in hippocampus by injecting adenovirus expressing its shRNA into mouse brain enhances UNC5B cleavage triggered by KA, whereas overexpression of PIKE-L completely suppresses UNC5B degradation. Accordingly, PARP cleavage, a marker for apoptosis, tightly correlates with PIKE-L expression levels (Figure 5D). Since UNC5B knockout mice are embryonic lethal²³, we can not use the UNC5B deficient mice to explore its role in neuronal apoptosis. Alternatively, we deplete UNC5B in mouse brain utilizing adenovirus expressing its shRNA. Compared to control, elimination of UNC5B in hippocampus evidently blocks KA-provoked apoptosis in mouse brain (Figure 5E), underscoring that UNC5B implicates in neuronal cell death *in vivo*. Collectively, our data show that netrin-1 triggers PIKE-L/UNC5B complex formation, which is regulated by Fyn tyrosine kinase, and represses neuronal apoptosis.

Our finding that PI 3-kinase is coupled to UNC5B through PIKE-L in response to netrin-1, suppressing neuronal apoptosis, provides a molecular mechanism that explains netrin-1's stimulatory effect on PI 3-kinase. Conceivably, such active effect by netrin-1 on PI 3-kinase is associated with the activation by PIKE-L of PI 3-kinase. PIKE activates PI 3-kinase in a GTP-dependent manner¹¹. PLC- γ 1 acts as a physiological guanine nucleotide exchange factor (GEF) for PIKE¹². Recently, it has been shown that netrin-1 induces activation of PLC- γ 1, which is mediated by DCC but not UNC5B or neogenin²⁴. Presumably, PLC- γ 1 is activated by netrin-1 and functions as a GEF for PIKE-L. The *in vitro* binding study reveals that the cytoplasmic domain of DCC associates with PIKE-L and coimmunoprecipitation also indicates that DCC interacts with PIKE-L in brain lysates, for which Fyn might not be essential (Supplemental Figure 2A–C). These data provide a molecular mechanism explaining PIKE-L activation in response to extracellular stimuli such as netrin-1. Netrin-1 provokes the association between DCC and UNC5B²⁵. Strikingly, we found that PIKE-L or Fyn triggers DCC/UNC5B complex formation even in the absence of netrin-1. Netrin-1 further elevates the

binding between DCC and UNC5B in the presence of PIKE-L or Fyn (Supplemental Figure 2D). Conceivably, Fyn and PIKE-L play critical roles in mediating netrin-1-triggered DCC/UNC5B heterodimer formation in neurons. UNC5B mediates apoptosis through DAP-kinase (DAPK) ⁷. However, neither PIKE-L nor Fyn affects the association between UNC5B and DAPK (data not shown). In addition, PIKE-L does not regulate UNC5B-dependent DAPK activation either (Supplemental Figure 2E).

At E12 in mutant mice deficient for netrin-1 expression, apoptosis is strongly enhanced in the whole brainstem and the ventricular zone ⁵. However, Hinck et al., reported *Ntn1* is not a necessary survival factor for UNC5A-expressing neurons *in vivo*, as the hypomorphic netrin-1 mutant mice failed to show increased apoptosis in neural tube ventral neurons ²⁶. To investigate netrin-1's role in neuronal survival *in vivo*, we conducted TUNEL staining on E18 brain sections. Evident TUNEL-positive cells in *Ntn1* ^{-/-} mice are found in ventrolateral periaqueductal area, where both UNC5B and DCC are expressed (Supplemental Figure 3). Thus, our findings suggest that netrin-1 contributes to suppressing neuronal cell death in region close to the Aqueduct in mouse brain.

Previous studies reveal that PKC/PICK1 complex mediates UNC5A membrane surface removal upon PKC activation, which inhibits netrin-1 dependent collapse of hippocampal growth cones ²⁷. Since PIKE-L does not bind UNC5A, we conducted immunofluorescent staining on hippocampal neurons to explore whether PIKE-L plays any role in UNC5B surface expression. UNC5B surface expression is not altered by PMA treatment; nevertheless, depletion of PIKE-L enhances its surface distribution, and PMA further escalates it. By contrast, overexpression of PIKE-L decreases UNC5B surface expression, PMA and netrin-1 combined treatment further attenuates its surface residency (Supplemental Figure 4A), supporting that PIKE-L might promote UNC5B internalization. Netrin-1 provokes UNC5B internalization, but PI 3-kinase inhibitors display negligible effect on UNC5B surface expression, indicating that PI 3-kinase signaling is not implicated in this event (Supplemental Figure 4B). Interestingly, knockout of Fyn elicits UNC5B surface insertion, suggesting that netrin-1-triggered Fyn but not PI 3-kinase signaling mediates UNC5B trafficking. On the other hand, depletion of PIKE-L abolishes netrin-1-triggered actin filaments in cortical neurons (Supplemental Figure 5). Together, these data support that PIKE-L might not only be involved in the survival activity of netrin-1 but also in its putative role in neuronal cell adhesion and migration. Collectively, in this report, we show that Fyn is sufficient and necessary for triggering PIKE-L and UNC5B tyrosine phosphorylation and association upon netrin-1 treatment. Our finding that PIKE-L selectively associates with UNC5B but not other family member of UNC5 or p75NTR provides strong evidence indicating that PIKE-L/UNC5B complex is responsible to activate PI 3-kinase cascade and suppress apoptosis upon netrin-1 stimulation.

Methods

Cells and Reagents

HEK293T cells were maintained in DMEM, supplemented with 10% fetal bovine serum (FBS), 2 mg/ml glutamine and 100 units penicillin-streptomycin at 37°C with 5% CO₂ atmosphere in a humidified incubator. Stable HEK293 cells expressing myc tagged chicken netrin-1 were provided by Dr. Yi Rao (Northwestern University). The purified anti-PIKE-N antibody is against the N-terminus from residues 1 to 384 in PIKE-L. Anti-HA-HRP, anti-DAP-Kinase antibody was from Sigma. Anti-PY99, anti-p110, anti-DCC, anti-GFP, anti-Akt antibodies were from Santa Cruz Biotech. Anti-p-Akt473 antibody was from Cell Signaling. Recombinant chicken netrin-1 protein and anti-UNC5B antibody were from R&D system. Anti-UNC5B (clone 1A9, antigen 27 aa-127 a.a.) was from Novus Bio. Glutathione Sepharose 4B was supplied by Pharmacia Biotech. All the tyrosine kinase inhibitors and Anti-myc antibody were

from Calbiochem. The primer sequence for preparing siRNA resistant PIKE-L: GCA CTT AT (C)T TG(C)T ATC GAA TG. The C nucleotides in the parenthesis were changed into T. The siRNA sequence for UNC5B (sense) is: 5' AGA CTG GAT TCC AGC TCA A 3'. Protein A/G-conjugated agarose beads were from Sigma.

Yeast Two-hybrid Screen

The yeast two-hybrid screen was performed using the MATCH-MAKER Two-hybrid System 2 (Clontech) according to the manufacturer's protocol. The GTPase domain of PIKE was subcloned downstream of the Gal4 DNA-binding domain in pAS2-1 and was used as bait to screen a human Fetal Brain cDNA Library in pACT2. Clones that grew on plates lacking leucine, tryptophan and histidine with 50 mM 3-aminotriazole were selected and assayed for β -galactosidase activity ²⁸.

Protein-protein interaction assays

Ten-cm dishes of HEK293T cells were transfected with 10 μ g DNA by the calcium phosphate precipitation method. In 48 h, the transfected cells were treated as indicated, collected and washed once in PBS, lysed in 1 ml lysis buffer (50 mM Tris, pH 7.4, 40 mM NaCl, 1 mM EDTA, 0.5% Triton X-100, 1.5 mM Na₃VO₄, 50 mM NaF, 10 mM sodium pyrophosphate, 10 mM sodium β -glycerophosphate, 1 mM phenylmethylsulfonyl fluoride (PMSF), 5 mg/ml aprotinin, 1 mg/ml leupeptin, 1 mg/ml pepstatin A), and was centrifuged for 10 min at 14,000 $\times$ g at 4°C. Experimental procedures for co-immunoprecipitation were carried out as described ²⁹. Immune complexes were resolved by SDS-PAGE and were subjected to immunoblotting. GST pull down assay was carried out as described previously ¹⁴.

PI 3-kinase assay

The cell lysate was prepared as described above. PI3-kinase was immunoprecipitated with rabbit anti-p110 from the cell extract, and washed with the following buffers: 3 times with buffer A (PBS, 1% NP-40, 1 mM dithiothreitol (DTT)); 2 times with buffer B (PBS, 0.5 M LiCl, 1 mM DTT); and 2 times with buffer C (10 mM Tris-HCl, pH 7.4, 0.1 M NaCl, 1 mM DTT). Subsequent steps were performed as described ¹².

Primary rat cortical neuron culture and apoptotic assay

Primary cultured mouse cortical neurons were prepared as follows. P1 mouse pups were decapitated and cortex was extirpated, cross chopped and suspended by pipetting for separation in 5% fetal calf serum (FCS), 5% horse serum (HS) Dulbecco's modified Eagle's medium (DMEM) gently. The cell suspension was then centrifuged at 250 $\times$ g for 5 min. This operation was repeated again. Cells were seeded into polyethyleneimine-coated 10 cm dishes and 12-well plates including coated-coverslips and incubated at 37 °C in 5% CO₂/95% air. After 3 h, culture medium was changed to Neurobasal containing B-27 supplement (Invitrogen) and incubated for 4 days. For maintenance, a half medium is change to fresh Neurobasal/B27 in every 4 days. After 1 week, the dish cultured neurons are employed in various experiments. After infection with various adenovirus expressing PIKE-L, PIKE-L-KS, PIKE-L (Y1032, 1124F) adenovirus and control virus, wild-type cortical neurons were treated with 200 ng/ml netrin for 30 min before 80 μ M glutamate was introduced. In 16–18 h, propidium iodide and Hoechst 33342 were added. The stained neurons were fixed and studied under fluorescent microscope. The neuronal apoptosis was also verified by an independent approach: caspase-3 activated fluorescent dye MR(DEVD)2. Due to the sensitivity, 80 μ M glutamate was added into Fyn $-/-$ cortical neurons to trigger apoptosis for 12–14 h.

Adenovirus injection into mouse hippocampus

We determined the viral titer by measuring the number of infected HEK293 cells expressing GFP, and adjusted all viral stocks to 1×10^8 plaque-forming units/ml before use. For injection of adenoviral vectors into the hypothalamus, we anesthetized male C57BL/6J mice aged 8–12 weeks ($n = 6$ per group) with 2.5% avertin. We performed standard surgical procedures to inject adenoviral vectors expressing either PIKE-L or UNC5B-specific siRNA with GFP or GFP alone using a stereotaxic table (David Kopf Instruments). The experiment was conducted as previously described³⁰. In 4 days, we intraperitoneally administrated 25 mg/kg kainic acid. In 5 days, the hippocampal regions were dissected and homogenated and analyzed by immunoblotting.

Statistical analysis

The results were expressed as means $\pm$ S.D. calculated from the specified numbers of determination. A Student's t-test was used to compare individual data with control value.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. PIKE-L interacts with UNC5B

(A). PIKE-GTPase interacts with UNC5B (569–946 a.a.) in yeast two-hybrid system. (B). Interaction of PIKE-GTPase and UNC5B in HEK293T cells. Diagram of PIKE-L and UNC5B was shown in the left panel. In one set of experiments, fragments of UNC5B were cotransfected with GFP-PIKE-L to HEK293T cells. In the other set, fragments of PIKE-L were cotransfected with UNC5B-HA. Immunoprecipitation assay showed that UNC5B 569–946 residues interacted with PIKE-L and truncation of death domain (a.a. 855–946) decreased the binding affinity (middle top panels); Reciprocal immunoprecipitation of UNC5B confirmed the finding (middle 3rd panels). PIKE-GTPase domain bound to UNC5B (right top and 3rd panels). (C) Interaction of PIKE-L and UNC5B in rat brain tissues. Brain homogenates from different

sections were subjected to western blot using UNC5B antibody and PIKE-N antibody respectively (5th and bottom panels). UNC5B antibody was incubated with brain homogenates and the immunocomplexes were probed with anti-PIKE-N antibody. PIKE-L strongly bound to UNC5B in cortex and hippocampus (top panel). Reciprocal immunoprecipitation with anti-PIKE-N showed the similar results (3rd panel) (see Supplementary Fig. S6 for a bigger image). Verification of PIKE-L and UNC5B in the immunocomplex (2nd and 4th panels). (D) PIKE-L and UNC5B colocalize in the cell body and neuronal processes of rat hippocampus and cortical neurons (left panels). Bar Scale: 10 μ m. GST-PIKE-L (a.a. 268–384) antigen but not control GST preincubation abolished PIKE-L neuronal staining (right panels).

Figure 2. Netrin-1 mediates the interaction between PIKE-L and UNC5B through tyrosine phosphorylation

(A) netrin-1 enhances the binding by PIKE-L but not dominant-negative PIKE-L-KS to UNC5B. Both full-length UNC5B and its fragments strongly bound to PIKE-L (top and 3rd panels). Equal amount of HA-UNC5B and GFP-PIKE-L was immunoprecipitated (2nd and bottom panels). (B) PP2 and tyrosine kinase inhibitors disrupt the interaction between PIKE-L and UNC5B in HEK293T cells. GFP-PIKE-L and UNC5B-HA were co-transfected to HEK293T cells. 24 h later, cells were treated with different tyrosine inhibitors (10 nM PP2; 5.4 μ M PP3, an inactive analog of PP2; 1 μ M Genistein; 300 μ M Daizein, an inactive analog of Genistein ; 0.4 μ M compound 5) for 30 min, followed by 200 ng/ml netrin-1 treatment for

24 h. UNC5B and PIKE-L were immunoprecipitated by anti-HA and anti-Myc antibodies, respectively. PIKE-L weakly bound to UNC5B under control condition, netrin-1 enhanced the association. Genistein, PP2 and Compound 5, which substantially abolished PIKE-L and UNC5B phosphorylation, blocked the interaction between PIKE-L and UNC5B; in contrast, the inactive compounds failed (top, 2nd, 4th and 5th panels) (see Supplementary Fig. S6 for a bigger image). Verification of immunoprecipitated PIKE-L and UNC5B (3rd and bottom panels). (C). Tyrosine kinase inhibitor PP2 disrupts the interaction between endogenous PIKE-L and UNC5B in neurons. Rat cortical neurons were treated with various tyrosine kinase inhibitors. Endogenous UNC5B and PIKE-L were immunoprecipitated, respectively. PP2 completely disrupted the interaction between PIKE-L and UNC5B in rat cortical neurons, and Genistein partially decreased the association (top and 4th panels). Netrin-triggered UNC5B and PIKE-L tyrosine phosphorylation was inhibited by PP2 and decreased by Genistein (2nd and 5th panels) (see Supplementary Fig. S6 for a bigger image). Verification of immunoprecipitated UNC5B and PIKE-L (3rd and bottom panels).

Figure 3. Interaction of PIKE-L and UNC5B is regulated by Fyn tyrosine kinase

(A) Tyrosine phosphorylation of PIKE-L is required for the interaction between PIKE-L and UNC5B. HEK293T cells were co-transfected with UNC5B and PIKE-L wild-type, Y1032F, Y1124F, Y1032FY1124F, respectively, followed by treatment with netrin-1. Only PIKE-L but not tyrosine phosphorylation deficient mutants selectively associated with UNC5B (top and 4th panels). Netrin-1 provoked tyrosine phosphorylation of PIKE-L (2nd panel). Verification of immunoprecipitated and transfected constructs (3rd, 5th and 6th panels). Netrin treatment decreased wild-type PIKE-L but not mutant cleavage (bottom panel). (B) Tyrosine phosphorylation of PIKE-L and UNC5B is required for interaction in mice brain. Brain homogenates from wild-type and *fyn*^{-/-} mice were immunoprecipitated with control IgG and

anti-PY99, respectively, followed by immunoblotting with anti-UNC5B or anti-PIKE-N. Tyrosine phosphorylated UNC5B can be detected only in wild-type but not fyn-null brain lysates (left upper panel). PIKE-L tyrosine phosphorylation was diminished in fyn-null sample (left lower panel). The interaction between PIKE-L and UNC5B can be detected only in wild-type but not fyn $-/-$ brain (middle panels). PIKE-L, DCC and UNC5B expression levels remained similar in both wild-type and Fyn $-/-$ brain (right panels). (C) Netrin-1-triggered Fyn activation is UNC5B dependent. HEK293 cells were transfected with Myc-PIKE-L and HA-UNC5B alone or in a combination. The transfected cells were treated with netrin-1 for 24 h. Endogenous Fyn was immunoprecipitated and its activation was verified by immunoblotting with anti-PY99. Netrin-1 provoked fyn activation only in cells expressed UNC5B. Cotransfection of PIKE-L and UNC5B elicited Fyn activation in the absence of netrin (left top panel, lane 5). Netrin-1 treatment further enhanced fyn phosphorylation. Equal amount of fyn was pulled down (2nd panel). Verification of transfected UNC5B and PIKE-L (left 3rd and bottom panel). Depletion of UNC5B diminished netrin-1's stimulatory effect on fyn. Cortical neurons were electroporated with miRNA of UNC5B, followed by netrin-1 stimulation. In control neurons, netrin-1 triggered Fyn activation, which was diminished in UNC5B-depleted neurons (right bottom panel) (see Supplementary Fig. S6 for a bigger image). Verification of UNC5B expression and immunoprecipitated Fyn (right top and 2nd panels).

Figure 4. PIKE-L and Fyn are necessary for netrin-1-triggered PI 3-kinase activation

(A) Netrin-1 enhances PIKE-L-induced PI 3-Kinase activity in HEK293T cells. HEK293T cells were transfected with myc-PIKE-L in the presence or absence of UNC5B and treated as indicated. P110 α was immunoprecipitated and PI 3-kinase assay was conducted with the immunocomplex. In the absence of UNC5B, no PI 3-kinase activity was detected regardless of netrin-1 treatment. Netrin-1 increased PI 3-kinase activity when PIKE-L and UNC5B were cotransfected. PI 3-kinase activity was decreased by wortmannin (10 nM) or dominant-negative PIKE-L-KS (left bottom panel). Verification of transfected constructs (left top and 2nd panels). Wortmannin (50 nM) and LY294002 (10 μ M) completely blocked netrin-1 triggered PI 3-kinase activity (right bottom panel). (B). PIKE-L is necessary for netrin-1-

induced PI 3-kinase activation in neurons. Cortical neurons were infected with control adenovirus, Si-PIKE, PIKE-L-wt or PIKE-L-KS adenovirus followed by netrin-1 treatment as indicated. Compared to control, depletion of PIKE-L abolished netrin-triggered Akt phosphorylation, which was enhanced in PIKE-L but not PIKE-L-KS infected neurons (2nd panel). The expression of PIKE-L was verified (top panel). AKT and loading control tubulin were verified (3rd and bottom panels). (C) PP2 blocks Akt activation. Cortical neurons were pretreated with PP2, followed with netrin stimulation. Netrin-provoked Akt phosphorylation was abolished by PP2 (top panel). Netrin-provoked Akt phosphorylation was slightly decreased in DCC^{-/-} neurons (3rd panel). Activation of Src family tyrosine kinases were attenuated in DCC knockout mice, as manifested by anti-PY416, an antibody specific for active src tyrosine kinases (5th panel). (D) Netrin-triggered PI 3-kinase/Akt activation is crippled in fyn ^{-/-} neurons. Wild-type and fyn ^{-/-} cortical neurons were infected with adenovirus expressing wild-type PIKE-L or PIKE-L (Y1032, 1124F) mutant, and treated with netrin-1 for 30 min. (E) UNC5B is critical for netrin-1-triggered Akt activation. Depletion of UNC5B diminished Akt activation by netrin-1 in cortical neurons (2nd panel). PIKE-L remained intact regardless of UNC5B expression levels (top and bottom panels).

Figure 5. PIKE-L and Fyn are essential for the neuronal survival effect by netrin-1

(A). Netrin-1 facilitates the anti-apoptotic effect of PIKE-L. Cortical neurons were infected with adenovirus at DIV7 as indicated and treated with 80 μ M glutamate for 16–18 h in the presence or absence of netrin-1. Quantification of apoptotic assay (upper panel). Verification of siRNA resistant PIKE-L clones (lower panels). (Error bars represent s. d. * $P < 0.005$; # $P < 0.01$; P values were calculated using student t-tests). (B) Apoptotic assay with Fyn ^{-/-} neurons. Wild-type and Fyn ^{-/-} neurons were treated with or without netrin-1, followed by 80 μ M glutamate for 12–14 h. The anti-apoptotic effect of netrin-1 was impaired in fyn ^{-/-} neuron (upper panels). (Error bars represent s. d. * $P < 0.01$; # $P < 0.005$; P values were calculated using student t-tests). Verification of DCC and UNC5B expression levels when PIKE-L was

overexpressed or depleted in cortical neurons (lower panels). (C) PIKE-L and Fyn are required for the anti-apoptotic activity of netrin-1. The cortical neurons were infected and treated as described above. (D) PIKE-L prevents UNC5B proteolytic cleavage, suppressing apoptosis. Mouse brain was injected with adenovirus expressing PIKE-L or its shRNA. In 5 days, the mice were intraperitoneally injected with 25 mg/kg KA. In 4 days, the samples were analyzed by immunoblotting. PIKE-L overexpression inhibited kainic acid-triggered UNC5B cleavage, whereas depletion of PIKE-L escalated UNC5B degradation and PARP cleavage (see Supplementary Fig. S6 for a bigger image). (E) Elimination of UNC5B attenuates apoptosis. Mouse brain was injected with control adenovirus or adenovirus expressing shRNA of UNC5B. In 5 days, KA was injected to trigger neuronal cell death in the mice. Five mice were employed in each group for both control and shRNA of UNC5B. Adenoviral vector independently expressed green fluorescent protein (GFP). Brain sections were examined under fluorescent microscope. Cells labeled with GFP in brain also expressed UNC5B or PIKE-L-specific siRNA.