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c-Jun N-terminal Kinase 1 (JNK1) Is Required for Coordination of Netrin Signaling in Axon Guidance*[S]

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Background: The JNK pathway is essential for brain development.
Results: JNK1 plays an important role in Netrin-1-mediated axon guidance in the developing nervous system.
Conclusion: JNK1 is a key signal component in Netrin signaling.
Significance: Unraveling intracellular signal transduction cascades underlying axon guidance will elucidate the molecular mechanisms of neuronal circuit formations in the developing nervous system.

The JNK family of MAPKs is involved in a large variety of physiological and pathological processes in brain development, such as neural survival, migration, and polarity as well as axon regeneration. However, whether JNK activation is involved in axon guidance remains unknown. Here, we provide evidence indicating the JNK pathway is required for Netrin signaling in the developing nervous system. Netrin-1 increased JNK1, not JNK2 or JNK3, activity in the presence of deleted in colorectal cancer (DCC) or Down syndrome cell adhesion molecule (DSCAM), and expression of both of them further enhanced Netrin-1-induced JNK1 activity *in vitro*. Inhibition of JNK signaling either by a JNK inhibitor, SP600125, or expression of a dominant negative form of MKK4, a JNK upstream activator, blocked Netrin-1-induced JNK1 activation in HEK293 cells. Netrin-1 increased endogenous JNK activity in primary neurons. Netrin-1-induced JNK activation was inhibited either by the JNK inhibitor or an anti-DCC function-blocking antibody. Combination of the anti-DCC function-blocking antibody with expression of DSCAM shRNA in primary neurons totally abolished Netrin-1-induced JNK activation, whereas knockdown of DSCAM partially inhibited the Netrin-1 effect. In the developing spinal cord, phospho-JNK was strongly expressed in commissural axons before and as they crossed the floor plate, and Netrin-1 stimulation dramatically increased the level of endogenous phospho-JNK in commissural axon growth cones. Inhibition of JNK signaling either by JNK1 RNA interference (RNAi) or the JNK inhibitor suppressed Netrin-1-induced neurite outgrowth and axon attraction. Knockdown of JNK1 *in ovo* caused defects in spinal cord commissural axon projection and pathfinding. Our study reveals that JNK1 is important in the coordination of DCC and DSCAM in Netrin-mediated attractive signaling.

In the developing nervous system, axon outgrowth and pathfinding are regulated by multiple guidance cues, such as Netrins, Semaphorins, Ephrins, and Slits (1–5). Netrins are a conserved family of canonical guidance cues, functioning as either chemoattractants or chemorepellents in a variety of different species (6–14). Several vertebrate receptors of Netrins have been identified such as deleted in colorectal cancer (DCC)[4] (13, 15), Neogenin (15, 16), Uncoordinated-5 (UNC5) (17, 18), and Down syndrome cell adhesion molecule (DSCAM) (19, 20). Although DCC/UNC40 can mediate attraction and UNC5 mediate repulsion by itself (1, 13, 15, 18, 21–24), assembling of appropriate receptor complexes is essential for the growth cone steering decision in Netrin signaling. For instance, collaboration of DCC with DSCAM mediates Netrin-1-induced axon outgrowth and attraction (19, 20), and interaction of UNC-5 either with DCC or DSCAM mediates repulsion (10, 15, 18, 24–26).

DCC is cleaved by metalloprotease(s) and γ-secretase, generating a membrane-tethered DCC stub and the free intracellular domain, respectively, which are involved in Netrin signaling (27), and the intracellular domain of DCC forms a physical complex with protein synthesis machinery, such as eIFs, ribosomal subunits (40 S, 60 S, and 80 S ribosomes), and monosomes, regulating protein translation and commissural axon projection in response to Netrin-1 (28). Although recent studies have identified a number of key molecules in Netrin signaling (2, 5, 29), including focal adhesion kinase (FAK), Fyn and Src, $p130^{CAS}$, TRIO, and DOCK180, PAK1, the Rho GTPases Rac1, Cdc42, and RhoA, cyclic nucleotides, phospholipase C, PI3K, MAPKs, transient receptor potential channels, calcium, myosin-X, and Enabled/vasodilator-stimulated phosphoprotein, the coordination of signal transduction cascades downstream of different Netrin receptors via these signaling molecules is still unclear.

JNKs, one subfamily of the MAPKs, are involved in a variety of biological processes, including apoptosis, cell proliferation,

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[4] The abbreviations used are: DCC, deleted in colorectal cancer; DSCAM, Down syndrome cell adhesion molecule; UNC5, uncoordinated-5; FAK, focal adhesion kinase; PAK1, p21-activated kinase 1; CAS, Crk-associated substrate; MKK, MAPK kinase; LSD, least significant difference; ANOVA, analysis of variance; PFA, paraformaldehyde.

cell migration, and cytokine production. JNKs are strongly expressed in the nervous system and play important roles in neuronal degeneration, neural development, neuronal migration, polarity, neuronal regeneration, as well as learning and memory (30, 31). JNK activation is triggered by MAPK kinase 4 (MKK4) and MKK7 (32, 33). Mammalian JNKs are composed of JNK1, JNK2, and JNK3 with at least 10 different splice isoforms (34, 35). Although initial studies indicated compensative roles for JNK1 and -2 in neuronal survival and apoptosis during early brain development (36, 37), and *Jnk2* and/or *Jnk3* knockout mice have no obvious brain defects (38), *Jnk1*-deficient mice display an abnormality in anterior commissure formation and axonal microtubule integrity (39), as well as altered dendritic architecture (40), suggesting that the JNK1 pathway may be involved in axon guidance in the developing nervous system.

In this study, we examined the functional importance of JNK1 activation in Netrin signaling through *in vitro* and *in vivo* approaches. Our results indicate that JNK1 is essential for coordination of Netrin/DCC and Netrin/DSCAM signaling and plays an important role in Netrin-1-induced axon outgrowth and pathfinding.

EXPERIMENTAL PROCEDURES

Constructs and Reagents—Plasmids encoding the full-length human DSCAM-FLAG, DSCAMΔN, DSCAMΔC, the full-length human DCC, and DCC truncation mutants (ΔP1, ΔP2, and ΔP3) have been described previously (20, 41). The full-length human JNK1 was PCR-amplified from a human brain cDNA library and subcloned into a pcDNA3 vector. pcDNA3 *FLAG JNK2a2* and pcDNA3 *FLAG JNK3a1* plasmids were provided by Roger Davis (Addgene, Cambridge, MA). The targeted sequences of control shRNA, DCC shRNA, DSCAM shRNA, control JNK1 shRNA, and JNK1 shRNA are as follows: 5′-AATGCATCTCTGCAAGAGGTA-3′ (control DCC shRNA); 5′-CATCCGATGTGCGACTGTA-3′ (DCC shRNA); 5′-AAAGAGTTTAGCTGAAATGCT-3′ (DSCAM shRNA); 5′-CCAGTCAGGCAAGGGATTT-3′ (control JNK1 shRNA), and 5′-CCTTCATTCTGCTGGAATT-3′ (JNK1 shRNA), respectively. The oligonucleotide templates were inserted into the mU6pro vector and verified by sequencing. Mouse JNK2 and JNK3 siRNAs were purchased from Santa Cruz Biotechnology (sc-39102 for JNK2 siRNAs and sc-39104 for JNK3 siRNAs). We used the following antibodies: rabbit anti-FLAG (Abcam, Cambridge, MA); mouse anti-Myc (9E10) and rabbit anti-JNK3 (Upstate Biotechnology, Lake Placid, NY); mouse anti-DCC (BD Biosciences); mouse functional blocking anti-DCC (EMD Millipore Bioscience, Billerica, MA); mouse anti-TAG1 (4D7, the Developmental Studies Hybridoma Bank, Iowa City, IA), and the HRP-conjugated anti-rabbit, anti-mouse, and anti-goat secondary antibodies (Santa Cruz Biotechnology). The rabbit anti-DSCAM was described previously (20, 26, 42) and rabbit polyclonal antibodies (anti-p38, anti-phospho-p38, anti-JNK2, anti-ERK1/2, anti-phospho-ERK1/2, and anti-phospho-JNK) were obtained from Cell Signaling Technology (Danvers, MA). B27, SP600125, DAPI, Alexa Fluor® 555 phalloidin, Alexa Fluor® 488 donkey anti-mouse IgG, Alexa Fluor® 488 donkey anti-rabbit IgG, Alexa Fluor® 647 goat anti-rabbit IgG, and Alexa Fluor® 633 goat anti-mouse IgG were purchased from Invitrogen. Cy3-conjugated anti-mouse IgM was purchased from Jackson ImmunoResearch (West Grove, PA). Purified chick Netrin-1 protein was either obtained from R&D or made from the conditioned media of HEK293 cells as described previously (20, 43, 44). *Clostridium difficile* toxin B and EGF were purchased from Sigma, and KinaseSTAR™ JNK activity assay kit was from BioVision (Milpitas, CA).

JNK Activity Assay and Western Analysis—JNK activity assays were performed following the instructions on the kit (Biovision). Briefly, both transfected HEK293 cells and primary neurons were lysed on ice for 5 min, and the supernatant was immunoprecipitated with anti-JNK antibody. The protein A-Sepharose was mixed with each sample for 1 h at room temperature followed by incubating with c-Jun Protein/ATP mixture at 30 °C for 2 h. The supernatant was collected after brief centrifugation, mixed with protein loading dye, and boiled for 3 min. Protein samples were separated with 7.5% SDS-PAGE, and the Western blot was probed with the rabbit anti-phospho-c-Jun antibody. ERK and JNK activities were also analyzed by incubating the precipitated kinase with substrates (JNK with 2 μg of GST-c-Jun and ERK with 2 μg of GST-Elk, respectively) in the kinase assay buffer in the presence of 10 μCi of [γ-^{32}P]ATP at 30 °C for 20 min. The kinase reactions were analyzed by SDS-PAGE. To examine other protein expression, Western blots were analyzed using specific antibodies, such as anti-DCC, anti-DSCAM, anti-phospho-JNK, anti-phospho-p38, anti-phospho-ERK1/2, anti-p38, anti-ERK1/2, and anti-JNK antibodies. For examining the effect of RNAi knockdown, dissociated primary neurons were cultured on PLL-coated dishes for 2 days after nucleofection, and cell lysates were then analyzed by Western blotting.

Immunostaining—For examining JNK activation in the developing commissural axon, E11 mouse embryos were collected and fixed overnight in ice-cold 4% paraformaldehyde (PFA) in 0.1 M PBS. Samples were cryoprotected in 30% sucrose solution, and 16-μm transverse sections were cut using a cryostat. Slices were post-fixed in 4% PFA solution for 20 min and permeabilized in PBST (0.1% Triton X-100 in 1× PBS) for 15 min. Spinal cord sections were blocked in PBST containing 3% BSA for 1 h and then incubated with primary antibody in PBST overnight at 4 °C (anti-DCC, 1:1000; anti-phospho-JNK, 1:100; anti-TAG1, 1:5). After washing three times in 1× PBS, slices were incubated with the secondary antibody (488 anti-rabbit IgG, 1:200; 647 anti-mouse IgG, 1:200; Cy3 anti-mouse IgM, 1:200) for 2 h at 37 °C. Images were taken using a confocal microscope (Olympus IX71 Fluoview). For immunocytochemistry of phospho-JNK in primary neurons, commissural neurons from E11 mouse spinal cords were cultured overnight, and then the culture medium was replaced with DMEM + B27 + penicillin/streptomycin for 6 h. Primary neurons were treated with either DMSO or different concentrations of SP600125 1 h before stimulation with purified Netrin-1 (250 ng/ml) or PBS for 20 min. After fixation with 4% PFA in DMEM for 10 min, neurons were permeabilized in PBST for 15 min, blocked with 3% BSA in PBST for 1 h, and then incubated with the primary antibody solution for overnight at 4 °C (anti-DCC, 1:1000; anti-phospho-JNK, 1:100). After washing three times, cells were incubated with the secondary antibodies (anti-mouse Alexa

Fluor® 488, 1:800; anti-rabbit Alexa Fluor® 647, 1:250) for 1 h, counterstained with DAPI, and mounted onto slides using Fluorogel (EMS, PA). Images were taken under an epifluorescent microscope (Olympus IX81). Quantifications of phospho-JNK in the growth cone of dissociated commissural neurons were performed using ImageJ software (National Institutes of Health). To measure the total fluorescent intensity of phospho-JNK within the growth cone, a circular region of interest using DCC as the marker was drawn around the growth cone, and then the integrated density for phospho-JNK was measured. Three circular regions of interest near the growth cone were measured and averaged for background subtraction. The following formula was used: integrated density − area of the growth cone region of interest × the mean background (45). At least 80 growth cones per group were measured.

Analysis of Neurite Outgrowth—Primary neuron culture procedures and nucleofection were conducted as described previously (20, 43, 44) with some modifications. E15 cortical neurons were dissociated and co-transfected with Venus YFP and either JNK1 control shRNA, JNK1 shRNA, or shRNA plus wild-type human JNK1 using nucleofection (Amaxa Biosystems). Primary neurons were cultured on PLL-coated coverslips for 18 h after nucleofection in the presence or absence of purified chick Netrin-1 (100 ng/ml). For inhibition of endogenous JNK activity, E15 cortical neurons were cultured with purified Netrin-1 (250 ng/ml) and treated with either DMSO or different concentrations of SP6000125 (1 or 5 μM) for 18 h. These cells were fixed with 4% pre-warmed PFA in 1× stabilization buffer (127 mM NaCl, 5 mM KCl, 1.1 mM NaH_2PO_4, 2 mM KH_2PO_4, 2 mM $MgCl_2$, 5.5 mM glucose, 1 mM EGTA, 10 mM PIPES, pH 6.5) for 20 min and stained with the Alexa Fluor® 555 phalloidin (Molecular Probes) and DAPI. Images were taken under a fluorescent microscope (Olympus IX81). For quantification, the longest neurite length in each clearly polarized neuron was examined using ImageJ software and analyzed using the one-way ANOVA followed by post hoc testing with Fisher's LSD. At least 80 random neurons were measured per group, and experiments were done in triplicate.

For examining the role of JNK activity in Netrin-1-induced commissural axon outgrowth, E13 mouse embryos were removed from anesthetized timed-pregnant mice, and the dorsal half of the spinal cord was collected in cold Hanks' buffered saline solution medium. Explants with similar size (200 μm) were cultured in the collagen gel as described previously (20, 43, 44, 46). After polymerization, explants were cultured overnight in DMEM + B27 + penicillin/streptomycin with or without purified Netrin-1 in the absence or presence of SP600125. Explants were fixed with 4% PFA in 1× PBS, and the number of axon bundles from the spinal cord explants were measured using ImageJ software.

Chick Spinal Cord Commissural Axon Turning Assay—Fertilized White Leghorn chick embryos were staged as described previously (20, 43, 44, 46, 47). Constructs were injected into the neural tube of chick embryos at stage 12–15, and the *in ovo* electroporation was performed with the following program: 25 V, 5 ms, 5 pulses (BTX ECM830) (20, 43, 44, 46). The half spinal cord labeled with YFP fluorescence was isolated from chick embryos at stage 20–21 and trimmed to 200 μm in size, and explants were co-cultured with an aggregate of control or Netrin-1 secreting HEK cells in the mixed gel as described previously (20, 43, 44, 46). Samples were fixed with 4% PFA in 1× stabilization buffer, and images were taken under the confocal microscope (Olympus IX71). The percentage of turning axons was calculated from the numbers of fluorescent axons turning toward the HEK cell aggregate more than 5° divided by the total numbers of fluorescent axons within 300 μm of the HEK cell aggregate. A one-way ANOVA followed by post hoc testing with Fisher's LSD was used for statistical analysis.

Analysis of Chick Spinal Cord Commissural Axon Projection in Vivo—In ovo electroporation was performed as described above. Chick embryos were collected between stages 22 and 23 after electroporation when the commissural axons in the lumbosacral region cross the midline (20, 44, 46, 48, 49). The lumbosacral region of the spinal cord with YFP fluorescence was isolated under an epifluorescence microscope and fixed with 4% PFA in 1× stabilization buffer. Transverse sections of 200 μm were cut and slices mounted in Gel/Mount. Images were taken under the confocal microscope. Statistic analysis was performed on three aspects as follows: percentage of axons reaching the spinal cord midline, average axonal distance from the midline, and percentage of embryos with misguided axons. The percentage of axons reaching the midline was calculated from the numbers of fluorescent axons reaching or crossing over the midline divided by the total numbers of fluorescent axons within 150 μm from the midline (20, 44, 46). The axon distance from the midline was assessed from the average distance of fluorescent axons away from the midline. Statistical significance among different groups was analyzed using the one-way ANOVA followed by post hoc testing with Fisher's least significant difference.

RESULTS

Biochemical Characterization of Cooperation of DCC and DSCAM in Netrin-1-induced JNK1 Activation—Previous studies have shown that activation of extracellular signal-regulated kinase 1/2 (ERK1/2) and p38, two subgroups of MAPKs, is required for Netrin/DCC signaling (50, 51). To test whether JNK is also activated by Netrin-1, plasmids expressing full-length human JNK1 were co-transfected with either full-length human DCC tagged with Myc (DCC-Myc) or DSCAM (DSCAM-FLAG) into HEK293 cells. JNK1 activity was assessed by utilizing a JNK-specific antibody to immunoprecipitate JNK1 from cell lysate, and then the phosphorylation of c-Jun was analyzed by Western blotting using a phospho-c-Jun-specific antibody. Netrin-1 increased JNK1 activity in the presence of DCC (Fig. 1*A*) or DSCAM (Fig. 1*B*). The induction of JNK1 activity by Netrin-1 appeared within 5 min, and the enhancement was sustained up to 20 min in both DCC- (Fig. 1*C*) and DSCAM-transfected cells (Fig. 1*D*). To further determine whether DCC collaborates with DSCAM in this process, we co-transfected JNK1 either with DCC only, DSCAM only, or DCC and DSCAM together into HEK293 cells and JNK activation was assessed. As expected, JNK1 activity was increased in the presence of either DCC or DSCAM after Netrin-1 stimulation (Fig. 1, *E* and *F*). Expression of both DCC and DSCAM further increased Netrin-1-induced JNK1 activity (Fig. 1, *E* and

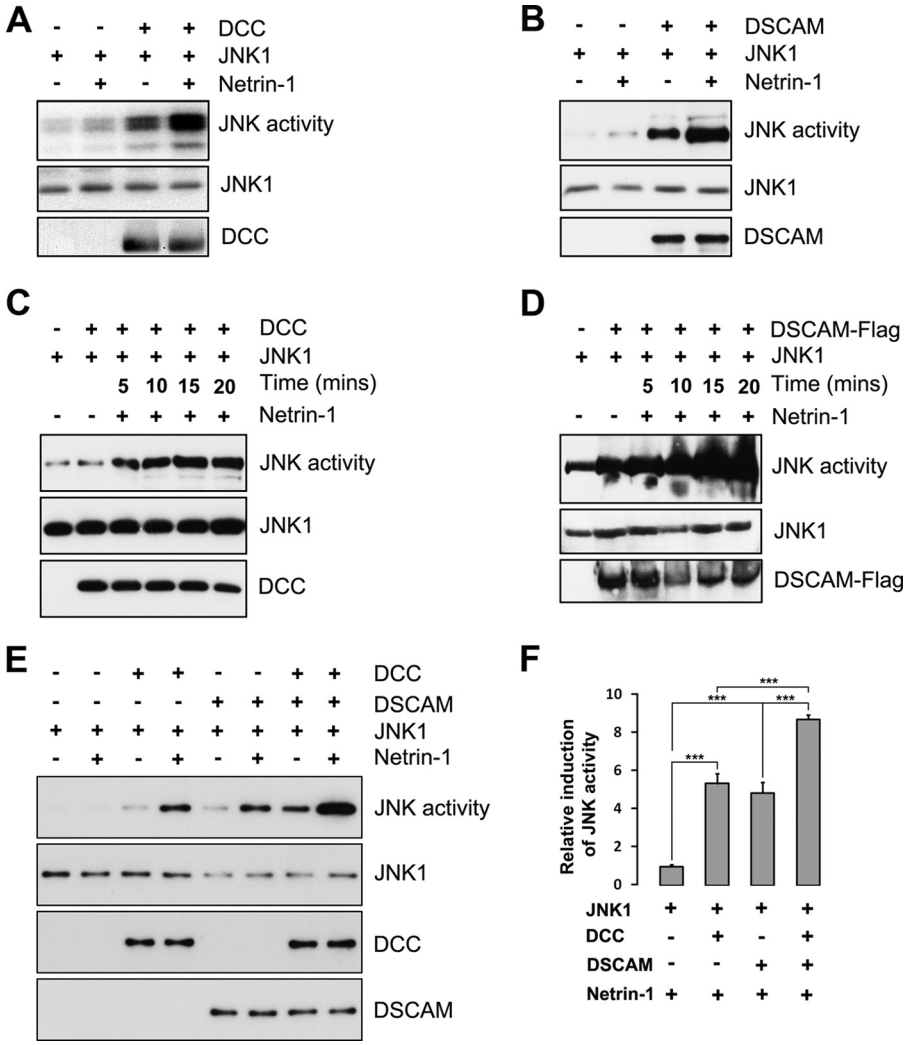

FIGURE 1. **Induction of JNK1 activity by Netrin-1.** *A* and *B,* Netrin-1 increased JNK1 activity in the presence of DCC (*A*) and DSCAM (*B*). Human full-length JNK1 was co-transfected with either DCC-Myc (*A*) or DSCAM-Flag (*B*) in HEK293 cells. JNK1 was immunoprecipitated with anti-JNK and JNK activity performed following the JNK activity assay kit instructions. *C* and *D,* time-dependent induction of JNK1 activity by Netrin-1 in HEK293 cells expressing DCC (*C*) or DSCAM (*D*). Netrin-1 increased JNK1 activity within 5 min, and this induction was persistent over 20 min. *E* and *F,* additive effect of DCC and DSCAM on Netrin-1-induced JNK1 activation. Human full-length JNK1 was co-transfected with either DCC only, DSCAM only, or DCC plus DSCAM in HEK293 cells. Cells were stimulated with purified Netrin-1 (250 ng/ml) for 20 min. *F,* quantification of the relative induction of JNK1 activity of *E*. Netrin-1 increased JNK1 activity in HEK293 cells expressing DCC or DSCAM only, and expression of both DCC and DSCAM together led to an even more pronounced Netrin-1-induced JNK1 activity. The relative induction of JNK activity was examined by the ratio of band intensity of Netrin-1 group *versus* the control. Data are mean ± S.D. from three separate experiments. One-way ANOVA and Fisher's LSD post hoc comparisons were performed. ***, $p < 0.001$.

F), suggesting that DCC may collaborate with DSCAM in this process.

Both DCC and DSCAM are transmembrane proteins belonging to the immunoglobulin (Ig) superfamily. DCC has three conserved intracellular domains (P1, P2, and P3). To identify which domain of DCC is required for Netrin-1-induced JNK1 activity, JNK1 was co-transfected either with the wild-type human DCC-Myc or three distinct DCC truncation mutants, ΔP1 or ΔP2, or ΔP3 into HEK293 cells. Netrin-1 increased JNK1 activity in the presence of the wild-type DCC or truncated DCCs lacking the P1 or P3 domain, whereas deletion of the P2 domain eliminated the Netrin-1 induction (Fig. 2*A*). Biochemical analysis of DSCAM truncation mutants (DSCAMΔC and DSCAMΔN) co-transfected with JNK1 in HEK293 cells also revealed the DSCAM intracellular domain was necessary for the Netrin-1-induced JNK1 activity (Fig. 2*B*). These results suggest that the intracellular domain of DSCAM and the P2 domain of DCC may be required for Netrin-1-mediated JNK1 activation. Expression of a dominant negative form of MKK4, a specific JNK activator, inhibited Netrin-1-induced JNK1 activation in DCC-transfected HEK293 cells (Fig. 2*C*). The JNK inhibitor SP600125 also blocked Netrin-1 effect on JNK1 activation in the presence of DCC (Fig. 2*D*) and DSCAM (Fig. 2*E*), although it did not affect EGF-induced ERK activity (Fig. 2*F*). In addition, Netrin-1 could not increase JNK2 (supplemental Fig. S1, *A* and *C*) and JNK3 activity (supplemental Fig. S1, *B* and *D*), two other JNK family members, in the presence of DCC (supplemental Fig. S1, *A* and *B*) or DSCAM (supplemental Fig. S1, *C* and *D*). These results suggest that DCC and DSCAM are specifically involved in Netrin-1-induced JNK1 activation.

Collaboration of Endogenous DCC and DSCAM in the Induction of JNK Activity by Netrin-1—To determine whether Netrin-1 increases endogenous JNK activity, primary neurons from E15 cortexes were used because these neurons express

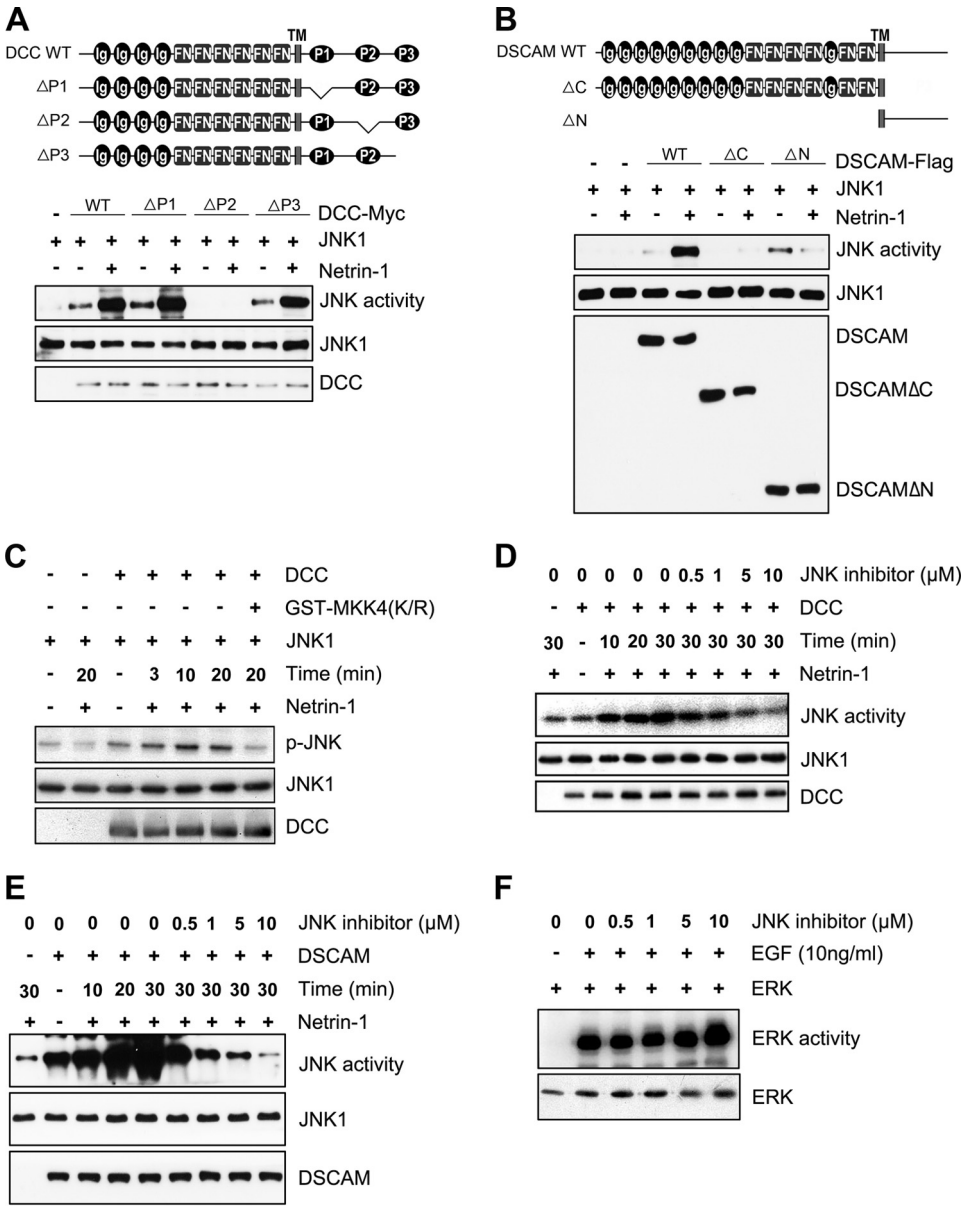

FIGURE 2. **Netrin-1-induced JNK1 activation depended on the intracellular domains of DCC and DSCAM and was inhibited by JNK inhibitor and MKK4 (Lys/Arg).** *A* and *B,* mapping the domains in DCC (*A*) and DSCAM (*B*) responsible for JNK1 activation. DCC-ΔP1, -ΔP2, and -ΔP3 correspond to deletions of residues 1147–1171, 1335–1356, and 1412–1447, respectively. JNK1 was co-transfected with the full-length wild-type DCC (*DCC WT*), different truncated mutants of DCC tagged by Myc or the full-length DSCAM, DSCAM ΔN, or DSCAM ΔC tagged by FLAG into HEK293 cells. The P2 domain in DCC was essential for DCC-dependent JNK1 activation (*A*). Netrin-1-induced JNK1 activation relied on both intracellular and extracellular domains of DSCAM in transfected HEK293 cells. *C,* expression of MKK4 (Lys/Arg), a kinase-inactive form of MKK4, inhibited the induction of JNK1 activation by Netrin-1 in the presence of DCC. *D* and *E*, SP600125, a JNK inhibitor, blocked both DCC-dependent (*D*) or DSCAM-dependent (*E*) induction of JNK1 activity by Netrin-1. *F,* SP600125 did not affect EGF-induced ERK activity in transfected HEK293 cells.

both DCC and DSCAM and respond to Netrin-1 stimulation (20, 43, 44). We examined JNK activation by probing the Western blots with the specific anti-phospho-JNK antibody. JNK phosphorylation was induced by Netrin-1 within 3 min and was sustained up to 20 min (Fig. 3*A*). Although Netrin-1 had no effect on ERK1/2 activation, p38 phosphorylation was increased by Netrin-1 stimulation (Fig. 3*A*). Netrin-1-induced JNK activation was blocked by either addition of the JNK inhibitor SP600125 (Fig. 3*B*) or the anti-DCC function-blocking antibody (Fig. 3*C*). Toxin B, a Rho family small GTPase inhibitor, blocked Netrin-1-induced Rac1 and JNK activities but not p38 phosphorylation (Fig. 3*D*). To determine whether DSCAM is required for endogenous JNK activation, DSCAM shRNA and DSCAM control shRNA constructs were nucleofected into dissociated E15 cortical neurons. Knockdown of DSCAM partially inhibited Netrin-1-induced JNK activation, whereas expression of the control DSCAM shRNA had no effect on Netrin-1 induction (Fig. 3, *E* and *F*). Addition of the anti-DCC function-blocking antibody with expression of DSCAM shRNA in primary neurons together totally abolished the Netrin-1 effect (Fig. 3, *E* and *F*), suggesting that DCC cooperates with DSCAM in this process. Simultaneous knockdown of both DCC and DSCAM also blocked Netrin-1-induced JNK1 activity (Fig. 3*G*), further supporting this hypothesis.

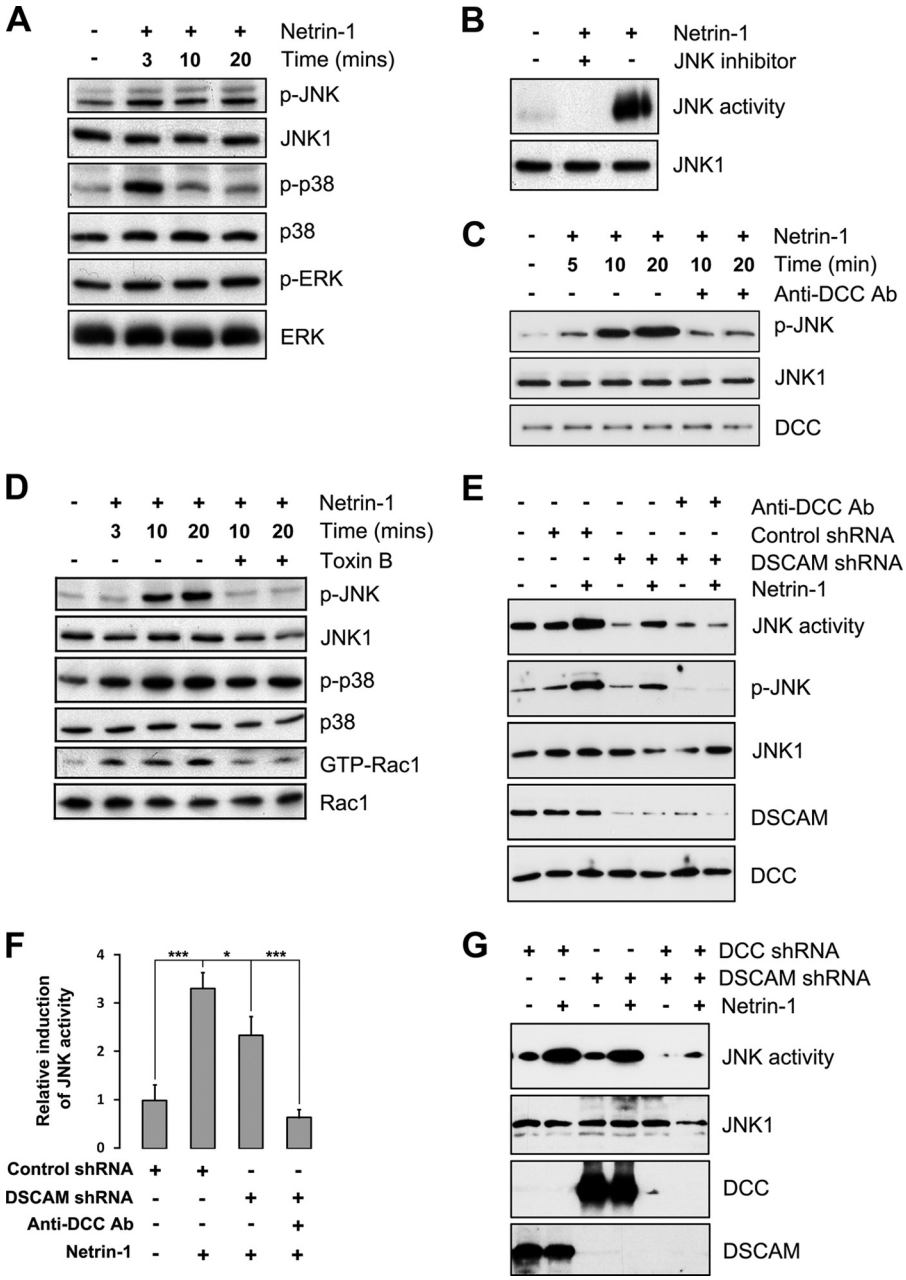

FIGURE 3. **Induction of endogenous JNK1 activity by Netrin-1.** *A,* Netrin-1 increased the levels of phospho-JNK and phospho-p38, but not phospho-p44/42 MAPK (ERK1/2), in primary E13 spinal cord neurons. Neurons were stimulated with purified Netrin-1 (250 ng/ml) for 3–20 min. The cell lysates were blotted with anti-phospho-JNK, anti-JNK, anti-phospho-p38, anti-p38, anti-phospho-ERK1/2, and anti-ERK1/2, respectively. *B,* Netrin-1-induced endogenous JNK activity was blocked by SP600125. E15 cortical neurons were stimulated with purified Netrin-1 (250 ng/ml) for 20 min in the presence or absence of SP600125. *C,* anti-DCC function-blocking antibody inhibited the increase of phospho-JNK levels by Netrin-1 in E15 cortical neurons. *D,* toxin B inhibited Netrin-1-stimulated levels of endogenous phospho-JNK and GTP-Rac1 without affecting phospho-p38 levels in primary E15 cortical neurons. *E,* coordination of endogenous DCC and DSCAM in Netrin-1-induced JNK activation. Primary neurons from E13 dorsal spinal cords were transfected with Venus only, Venus YFP plus control shRNA, and Venus YFP plus DSCAM shRNA. Neurons were stimulated with purified Netrin-1 or the control in the presence or absence of the functional blocking anti-DCC antibody. *F,* quantification of the relative induction of JNK1 activity in *E*. The relative induction of JNK activity was examined by the ratio of band intensity of Netrin-1 group *versus* the control (without Netrin-1), compared with the normalized basal JNK activity change from the control shRNA-transfected *versus* mock transfection group. Data are mean ± S.D. from three separate experiments. One-way ANOVA and Fisher LSD post hoc comparisons were performed. *, $p < 0.05$; ***, $p < 0.001$. *G,* double knockdown of endogenous DCC and DSCAM abolished Netrin-1-induced JNK activity in primary E13 spinal cord neurons.

JNK Activation in Developing Commissural Axons—To examine whether JNK activation specifically occurs in commissural axons, transverse cryosections of E11 mouse spinal cords were immunostained with specific anti-phospho-JNK and DCC or TAG1. Phospho-JNK was strongly expressed in commissural axons before and as they crossed the floor plate (Fig. 4, *A–F*). Interestingly, high levels of phospho-JNK and DCC were also detected in post-crossing commissural axons such as the ventral and lateral funiculi as well as motor columns and the dorsal root entry zone (Fig. 4, *D–F*). To further determine whether JNK activation in developing commissural neurons relies on Netrin-1, primary neurons from E11 mouse dorsal

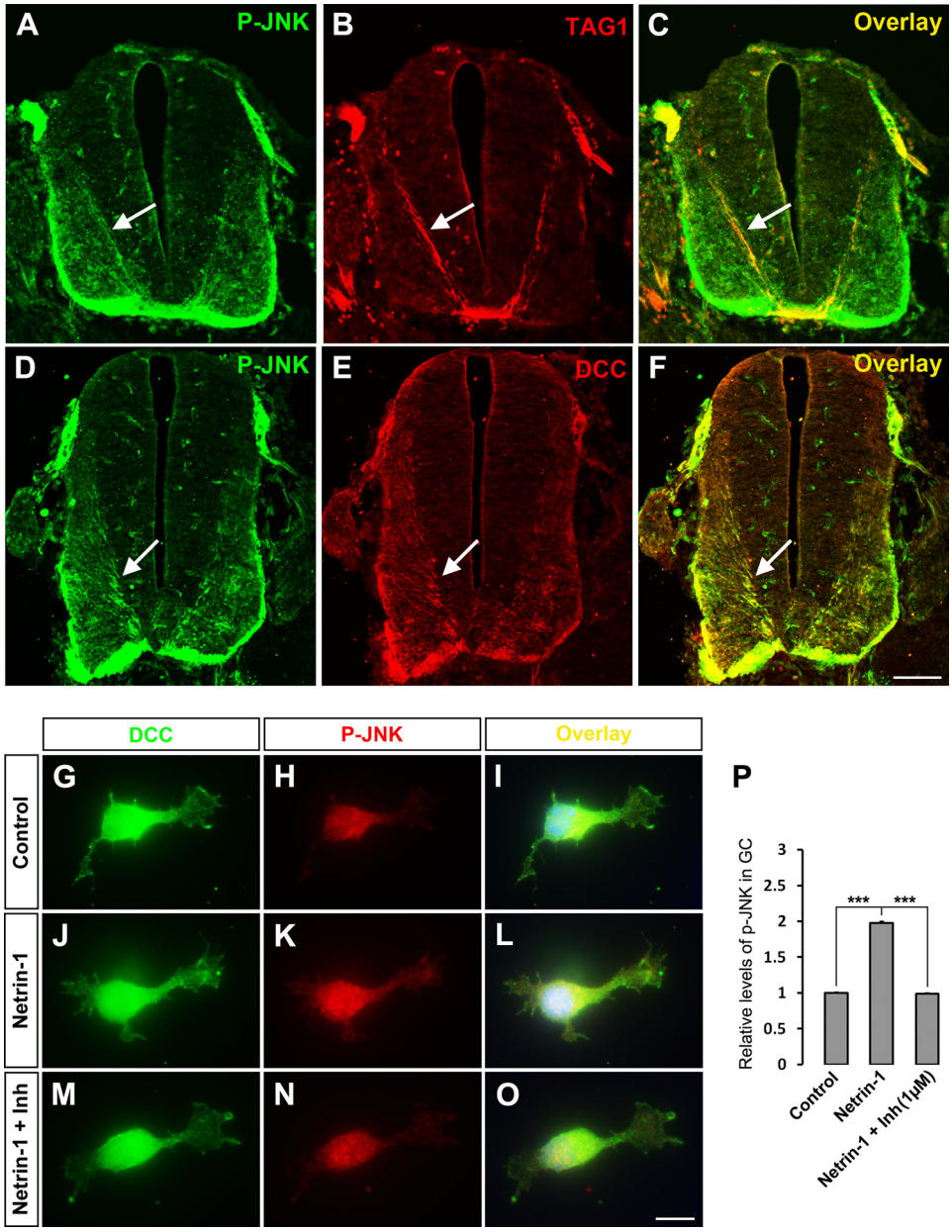

FIGURE 4. **JNK activation in the developing spinal cord.** *A–C,* JNK activation occurs in TAG1-positive precrossing commissural axons of E11 mouse spinal cord. Phospho-JNK and TAG1 were detected by specific anti-phospho-JNK (*A*) and anti-TAG1 (*B*) antibodies. *C,* merged picture of *A* and *B*. *D--F,* co-expression of phospho-JNK and DCC in the developing spinal cord. Transverse sections of E11 mouse spinal cords were double-immunostained with anti-phospho-JNK (*D*) and anti-DCC (*E*). *F,* superimposed images of *D* and *E*. Both phospho-JNK and DCC were strongly expressed in the DREZ, precrossing commissural axons, motor column, and postcrossing commissural axons (ventral funiculus and lateral funiculus). *Scale bar,* 100 μm. *G–P,* Netrin-1 induced phospho-JNK levels in the growth cone of spinal cord commissural axons. Primary neurons from E11 mouse dorsal spinal cords were dissociated and stimulated with purified Netrin-1 (250 ng/ml) (*J–P*) or the control (*G–I* and *P*) in the presence (*M–P*) or absence (*G–L* and *P*) of 1 μM SP600125. Neurons were double-immunostained with anti-phospho-JNK (*H, K,* and *N*) and anti-DCC (*G, J,* and *M*). *I, L,* and *O* are the superimposed images of *G, H, J, K, M,* and *N*, respectively. *P,* quantification of relative induction of JNK activity by Netrin-1 in commissural axon growth cones. Data are mean ± S.D. ***, $p < 0.001$ (one-way ANOVA and Fisher LSD post hoc comparisons). *Scale bar,* 10 μm. *Inh*, inhibitor.

spinal cords were dissociated and cultured. JNK activity in the growth cone of commissural neurons was assessed using immunocytochemistry with the anti-phospho-JNK and anti-DCC antibodies. In the absence of Netrin-1, low levels of phospho-JNK were detected in the growth cone (Fig. 4, *G–I*, quantification in 4P). Netrin-1 stimulation dramatically increased the level of phospho-JNK in the growth cone (Fig. 4, *J–L,* and quantification in *P*). Addition of 1 μM JNK inhibitor abolished Netrin-1 induction (Fig. 4, *M–O,* quantification in *P*). These results suggest that Netrin-1-dependent JNK activation occurs in developing spinal commissural axons.

JNK Activation Is Required for Netrin-1-induced Neurite Outgrowth—To examine whether JNK activation plays a functional role in Netrin-1 signaling, dorsal spinal cord explants from E13 mouse embryos were dissected and cultured in the collagen gel in the presence or absence of Netrin-1. Netrin-1 enhanced commissural axon outgrowth (Fig. 5, *A, B,* and *E*). The JNK inhibitor SP600125 inhibited Netrin-1-induced axon

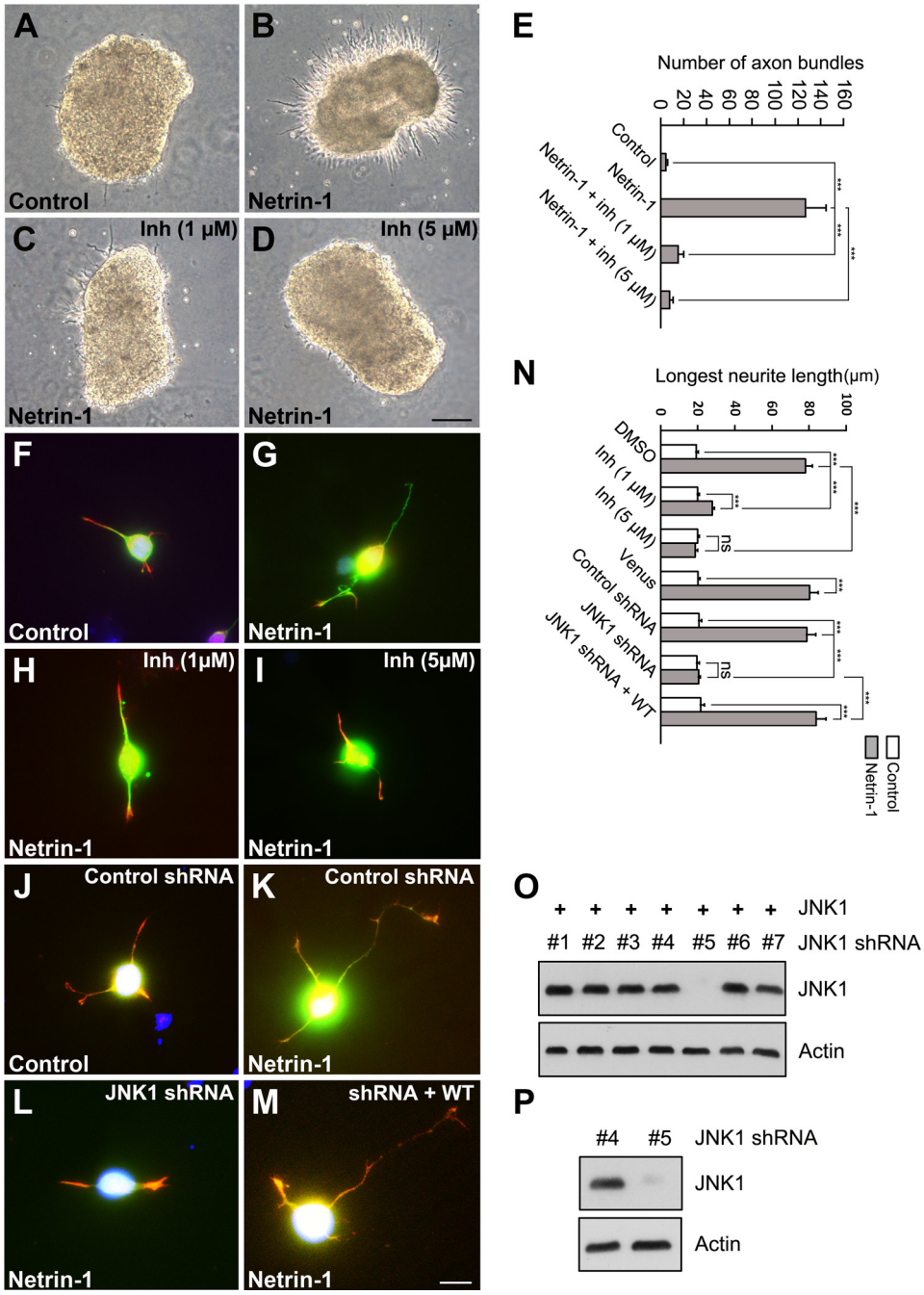

FIGURE 5. **JNK1 inhibition blocks Netrin-1-induced neurite outgrowth.** *A–D,* dorsal spinal cord explants from E13 embryos were cultured in the presence or absence of JNK inhibitor SP600125. *Scale bar,* 100 μm. Explants without Netrin-1 (*A*) showed fewer axon bundles than did explants with Netrin-1 (*B*). Netrin-1-promoted axon outgrowth was inhibited by 1 μM (*C*) and 5 μM (*D*) SP600125. *E,* quantification of axon outgrowth (the number of axon bundles). The numbers of explants tested are as follows: 17 (control group), 20 (Netrin-1 group), 18 (Netrin-1 plus 1 μM SP600125 group), and 16 (Netrin-1 plus 5 μM SP600125 group). Data are mean ± S.E. ***, $p < 0.001$ (Student's *t* test). *F–I,* neurite outgrowth from dissociated neurons. E15 cortical neurons were cultured 18 h in the presence (*G–I*) or absence (*F*) of purified Netrin-1 with (*H* and *I*) or without (*F* and *G*) SP600125. Neurons were stained with TUJ1 (*green*), phalloidin (*red*), and DAPI (*blue*). *J–M,* Venus YFP was co-transfected with control shRNA (*J* and *K*), JNK1 shRNA (*L*), or JNK1 shRNA plus the wild-type human JNK1 (*M*) into E15 cortical neurons. Primary neurons were incubated with (*K–M*) or without (*J*) purified Netrin-1 (100 ng/ml) and stained with Alexa Fluor® 555 phalloidin (*red*) and DAPI (*blue*). *Scale bar,* 10 μm. *N,* quantification of Netrin-1-induced neurite outgrowth of cortical neurons. The *y* axis is the longest neurite length. Data are mean ± S.E. from three separate experiments. ***, $p < 0.001$ (one-way ANOVA and Fisher LSD post hoc comparisons); *ns*, not significant. *O,* JNK1 shRNA (*lane 5*) significantly reduced JNK1 protein levels in HEK293 cells. *P,* endogenous JNK1 protein levels were reduced in dissociated E15 cortical neurons after shRNA transfection. *Inh*, inhibitor.

extension in a dose-dependent manner (Fig. 5, *C–E*). Similar results were observed in primary cortical neurons from E15 mice. As expected (20, 43, 44, 46), Netrin-1 increased cortical neurite extension (Fig. 5, *F* and *G*, quantification in *N*) and SP600125 blocked the Netrin-1 effect (Fig. 5, *H, I,* and *N*). These results indicate that JNK activation is required for Netrin-1-induced axon outgrowth. To further determine the functional role of JNK1 in Netrin signaling, several short hairpin-based RNAi constructs (shRNAs) targeting a sequence common to chick and mouse JNK1 were designed and transfected in both HEK293 cells (Fig. 5*O*) and dissociated E15 cortical neurons (Fig. 5*P*). One shRNA construct (construct 5) could signifi-

cantly knock down the level of JNK1 protein, whereas others could not (Fig. 5, *O* and *P*). We used these RNAi constructs as a JNK1 shRNA and JNK1 control shRNA, respectively. Netrin-1-induced neurite outgrowth was assessed with primary E15 cortical neurons transfected either with Venus yellow fluorescent protein (Venus YFP) only or together with the control shRNA or the JNK1 shRNA. These neurons were stimulated with Netrin-1 and cultured for 20 h. In neurons transfected with Venus YFP or Venus YFP plus the control shRNA, neurite extension was stimulated by Netrin-1 (Fig. 5, *J, K,* and *N*). JNK1 shRNA inhibited Netrin-1-induced neurite outgrowth (Fig. 5, *L* and *N*). The expression of wild-type human JNK1 rescued the defects in Netrin-1-induced neurite outgrowth caused by JNK1 knockdown (Fig. 5, *M* and *N*). In contrast, knockdown of either JNK2 or JNK3 did not affect Netrin-1-induced endogenous JNK activity (supplemental Fig. S2*A*) as well as neurite outgrowth (supplemental Fig. S2, *B–H*). These results indicate that JNK1 is specifically required for Netrin-1-induced neurite outgrowth.

JNK Activation Is Important for Netrin-1-promoted Commissural Axon Attraction—The open book turning assay with commissural axons from the neural tube of chick embryos is a model for studying Netrin-1 attraction (20, 43, 44, 46). To determine whether JNK activation is required for commissural axon turning toward Netrin-1, the Venus YFP construct only or Venus YFP plus the control shRNA or JNK1 shRNA was electroporated into the chick neural tube at stages 12–15. An explant of the neural tube was isolated, and the co-culture with an aggregate of HEK cells was performed as described previously (20, 43, 44, 46). As expected, most commissural axons turned toward HEK cells stably secreting Netrin-1 in explants transfected Venus YFP only (Fig. 6, *B* and *I*) or Venus YFP together with the control shRNA (Fig. 6, *F* and *I*), whereas control HEK293 cells could not attract commissural axons with most axons projecting straight toward the floor plate (Fig. 6, *E* and *I*). SP600125 inhibited Netrin-1-induced commissural axon turning (Fig. 6, *C, D,* and *I*). Co-transfection of Venus YFP with JNK1 shRNA significantly inhibited axon turning towards Netrin-1 (Fig. 6, *G* and *I*). To confirm the role of JNK1, we introduced the wild-type human JNK1 into the neural tube together with JNK1 shRNA and Venus YFP by electroporation. The wild-type JNK1 significantly rescued the defect on axon turning toward Netrin-1 after JNK1 knockdown (Fig. 6, *H* and *I*). Thus, results from both the JNK inhibitor and the JNK1 shRNA demonstrate a requirement of JNK1 activation in axon attraction by Netrin-1.

JNK1 Is Involved in Commissural Axon Projection in Vivo—To determine the functional role of JNK1 *in vivo*, we examined the phenotype of JNK1 knockdown on commissural axon projection in chick embryos. Venus YFP only or Venus YFP plus vector, Venus YFP plus the control shRNA, or Venus YFP plus JNK1 shRNA was introduced into the neural tube of stage 12 chick embryos by *in ovo* electroporation (20, 43, 44, 46). The lumbosacral segments of the spinal cord at stage 23 after electroporation were isolated and transverse sections prepared. Commissural axons projected normally toward the floor plate with most axons reaching the floor plate in the neural tube transfected with Venus YFP alone, Venus YFP plus the vector, or Venus YFP with the control shRNA (Fig. 7, *B–D* and *G–I*). In

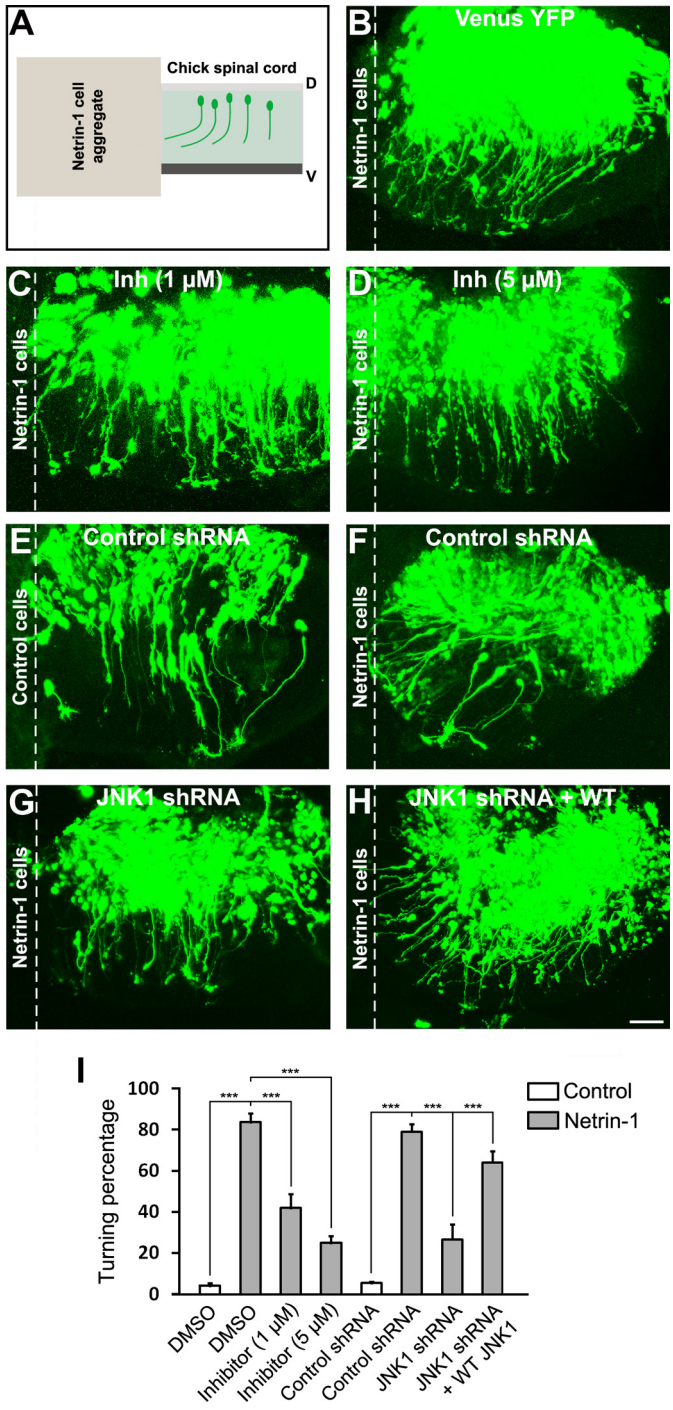

FIGURE 6. **JNK1 is required for Netrin-1-mediated commissural axon attraction.** *A,* schematic diagram of the commissural axon turning assay (20, 43, 44, 46). Venus YFP was electroporated into the neural tube of chick embryos for visualizing commissural axon projection. *B–D,* Netrin-1-induced axon turning was inhibited by SP600125. The neural tube was electroporated with Venus YFP alone and co-cultured with a cell aggregate secreting Netrin-1 in the absence (*B*) or presence of 1 μM (*C*) and 5 μM (*D*) SP600125. *E* and *F,* neural tube explants expressing the control JNK1 shRNA were co-cultured either with control HEK293 cells (*E*) or HEK293 cells secreting Netrin-1 (*F*). *G* and *H,* Venus YFP together with JNK1 shRNA or with JNK1 shRNA plus wild-type JNK1 were electroporated into chick neural tubes and explants cultured with Netrin-1 cells. *Scale bar,* 100 μm. *I,* quantification of axon turning. Data are mean ± S.E. *** indicates $p < 0.001$ (one-way ANOVA and Fisher LSD post hoc comparisons). *Inh,* inhibitor.

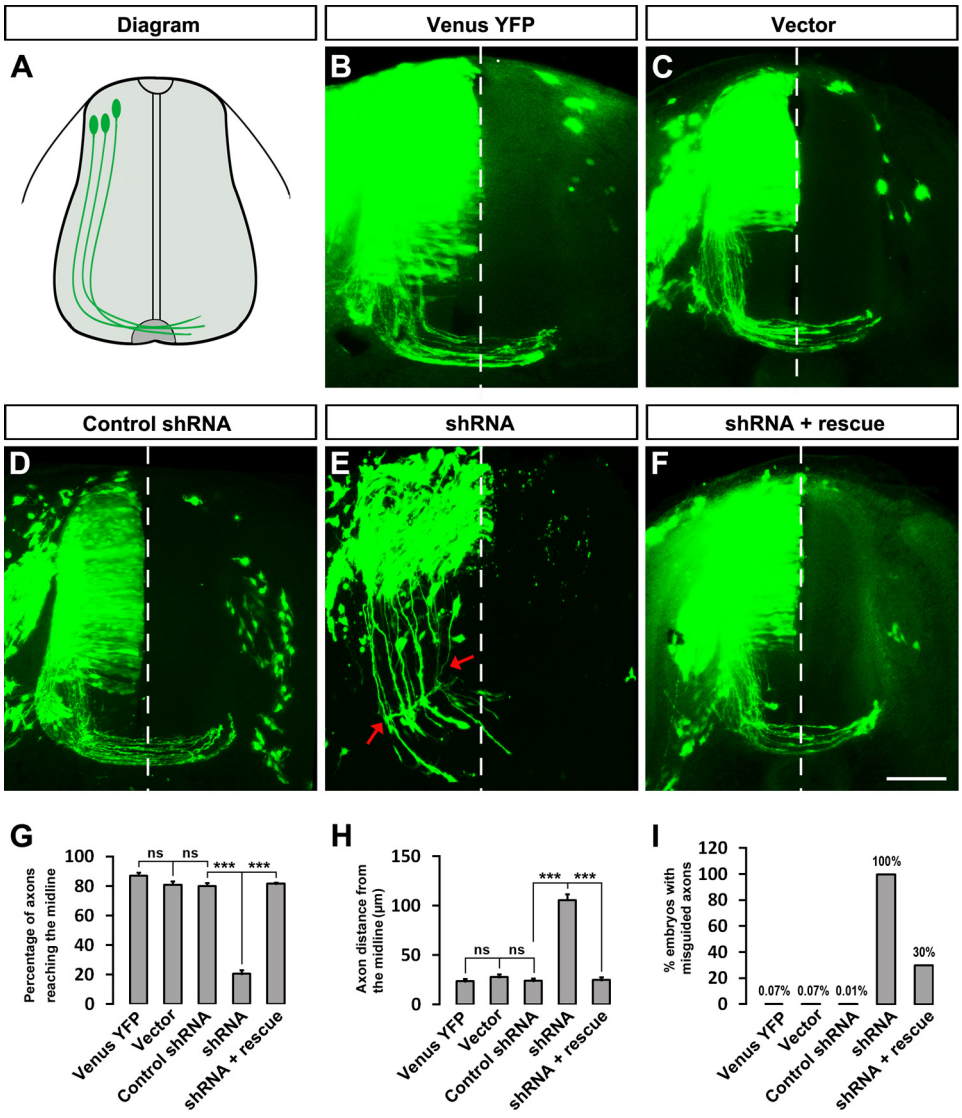

FIGURE 7. **JNK1 is essential for spinal cord commissural axon projection and pathfinding *in vivo*.** *A,* chick spinal cord diagram showing commissural axon projection after electroporation. Venus YFP only or combinations of Venus YFP with other plasmids were electroporated into the chick neural tube *in ovo,* and the lumbosacral region of the spinal cord was collected as described previously (20, 43, 44, 46). *B,* spinal cord electroporated with Venus YFP only. *C,* commissural neurons electroporated with Venus YFP plus the empty vector. *D,* neurons with Venus YFP plus control shRNA. *E,* commissural neurons with Venus YFP plus JNK1 shRNA. JNK1 shRNA not only inhibited the commissural axon extension but also caused aberrant pathfinding. The *red arrowheads* point to misguided axons. *F,* neurons expressing Venus YFP plus JNK1 shRNA and wild-type human JNK1. *Scale bar,* 100 μm. *G,* percentage of axons reaching the midline. *H,* quantification of the average distance of commissural axons away from the midline. Data are presented as the mean ± S.E. in *G* and *H*. ***, $p < 0.001$ (one-way ANOVA with Fisher LSD for post hoc comparisons). *I,* percentage of embryos with misguided axons. $n = 33$ (Venus YFP), 20 (vector group), 15 (control shRNA group), 16 (JNK1 shRNA group), and 18 (JNK1 shRNA + wild-type JNK1).

contrast, expression of JNK1 shRNA not only inhibited commissural axon projection but caused axon misguidance (Fig. 7, *E* and *G–I*). Co-expression of wild-type human JNK1 with JNK1 shRNA rescued the defects in commissural axon projection caused by the JNK1 shRNA (Fig. 7, *F–I*). These findings demonstrate that JNK1 is required for commissural axon projection and pathfinding in the developing spinal cord.

DISCUSSION

Previous studies have shown that JNK activity is essential in axon formation and regeneration (36, 52–54). To determine whether JNK is involved in Netrin signaling, the functional roles of JNK1 were studied by both *in vitro* and *in vivo* experiments. Netrin-1 increased the axon outgrowth from both cortical neurons and mouse dorsal spinal cords. Both the pharmaceutical JNK inhibitor and the JNK1 shRNA inhibited axon outgrowth induced by Netrin-1 (Fig. 5) as well as Netrin-1-induced axon attraction in the chicken "open book" turning assay (Fig. 6), whereas the basal axon outgrowth did not change (Figs. 5 and 6). Expression of the wild-type human JNK1 rescued the defects of JNK1 knockdown on Netrin-1-induced axon outgrowth and attraction (Figs. 5 and 6). In addition, knockdown of JNK2 and JNK3, two other JNK family members, did not affect Netrin-1-induced neurite outgrowth (supplemental Fig. S2). These results indicate that JNK1 is specifically required for Netrin function. In either *Netrin-1*$^{-/-}$ or *Dcc*$^{-/-}$ mice, the projection of commissural axons is defective (13, 55). Similarly, *in vivo* studies with the chick spinal cord show that

knockdown of JNK1 by the RNA interference causes failure of commissural axons to reach the floor plate (Fig. 7), suggesting that JNK1 is required for commissural axon projection and pathfinding in the developing spinal cord. Previous studies have shown that MKK7 deletion decreases the number of TAG1-expressing axons and disrupts contralateral projection of axons by layer 2/3 neurons in the developing cerebrum (56) indicating that JNK activity plays an important role in commissural axon projection. Although *Jnk2* and/or *Jnk3* knock-out mice have no obvious brain defects, *Jnk1*$^{-/-}$ mice exhibit disrupted tract formation of the anterior commissure in the frontal brain (39), further confirming that JNK1 is specifically involved in commissural axon guidance in the developing nervous system. The dual leucine zipper kinase is a MAPK kinase kinase (MAPKKK) for JNK. Recent studies have shown that the dual leucine zipper kinase-JNK1 pathway facilitates axon formation in developing neocortical neurons (52). It is plausible that this pathway may be involved in Netrin signaling. Further investigation is needed to determine the functional role of dual leucine zipper kinase in Netrin signaling.

Many other cues have also been implicated in commissural axon guidance. Sonic hedgehog contributes as a chemoattractant in the floor plate for guiding pre-crossing commissural axon projection (57). Wnt4 guides post-crossing commissural axons along the anterior-posterior axis of the spinal cord (58). Bone morphogenetic proteins function as chemorepellents involved in initial guidance of commissural axons (59, 60). It remains to be determined whether JNK activation is involved in the downstream signaling pathway of these guidance cues.

Both DCC and DSCAM belong to the immunoglobulin subfamily and have similar protein structure. Previous studies have shown that DSCAM functions as a Netrin-1 receptor collaborating with DCC in Netrin signaling (19, 20, 61). Our biochemical data indicate that Netrin-1 increased JNK1 activity in the presence of DCC or DSCAM, and expression of both DCC and DSCAM further increased Netrin-1-induced JNK1 activity (Fig. 1). The JNK inhibitor SP600125 blocked the Netrin-1 effect on JNK1 activation in the presence of DCC and DSCAM, although it did not affect EGF-induced ERK activity (Fig. 2). Endogenous JNK activation was induced by Netrin-1, and this induction was blocked by either addition of the JNK inhibitor SP600125 or the anti-DCC function-blocking antibody (Fig. 3). Knockdown of DSCAM partially inhibited Netrin-1-induced JNK activation, and combination of the anti-DCC function-blocking antibody with expression of DSCAM shRNA in primary neurons together totally abolished the Netrin-1 effect (Fig. 3). In addition, simultaneous knockdown of both DCC and DSCAM also blocked Netrin-1-induced JNK activity (Fig. 3). These results suggest that DCC cooperates with DSCAM through regulating JNK activity in Netrin signaling.

DCC knock-out mice exhibit aberrant commissural axon pathfinding (13). Surprisingly, mice carrying a deletion of exon 1 of DSCAM have no detectable abnormal spinal commissural axon projection (62), whereas RNAi studies in chick and mice as well as evidence in *Drosophila* clearly show that DSCAM is an essential component of Netrin signaling in promoting axon outgrowth and midline crossing (19, 20, 61). This disparity may be caused by compensatory mechanisms of other related guidance receptors and/or signal molecules downstream of DCC and DSCAM after the loss of DSCAM, suggesting that DSCAM may play more complex roles than we expected in Netrin signaling.

Small GTPases of the Rho family are required for regulating actin dynamics and mediating Netrin signaling (63–67). Netrin-1 stimulation recruits multiple signal transduction molecules, such as Fyn, FAK, PAK1, P130^{CAS}, TRIO, and DOCK180, through DCC to form a signaling complex (41, 43, 68–70), which then activates small GTPases Rac1 and Cdc42 (44, 46, 63, 64, 71–73) or inhibits RhoA in Netrin-induced attraction (74). Toxin B, an inhibitor of the small GTPases Rho, Rac, and Cdc42, not only blocked Netrin-1-induced Rac1 activity but inhibited the induction of phospho-JNK1 by Netrin-1 without affecting phospho-p38 (Fig. 3), suggesting that JNK1 is downstream of small GTPases in Netrin signaling. Further investigation is necessary to untangle how small GTPases regulate JNK activity in Netrin-mediated attractive signaling.

The interaction of DCC and UNC5 mediates Netrin-1 repulsion (24). Our recent studies indicate that DSCAM functions as a repulsive receptor cooperating with UNC5C in Netrin signaling (26). These results suggest that combination of DSCAM with DCC or UNC5C plays an important role in Netrin-1-mediated axon attraction or repulsion. The signaling mechanisms downstream of Netrin/DSCAM/UNC-5-mediated repulsion are less understood than those of Netrin/DCC-induced attraction. Several signal molecules, such as FAK and Src family kinases, the tyrosine phosphatase SHP2 (PTPN11), MAX-2, a PAK family member, and MAX-1, an adaptor protein, have been shown to be involved in Netrin/UNC5 repulsion (75–77). PAK1 interacts with DSCAM (78) and Netrin-1 induces phosphorylation of PAK1 and Fyn in the presence of DSCAM (20). Our recent studies have shown that, in the Netrin repulsive signaling, DSCAM cooperates with UNC5C through regulation of endogenous protein kinases activities, such as Src family kinases, FAK, and PAK1 (26). Because expression of DSCAM induces JNK1 kinase activity in mammalian cells (42), it will be interesting to determine whether JNK1 activation is involved in coordinating Netrin/DSCAM and Netrin/UNC5 repulsion.

Previous studies have shown that Netrin-1 increases ERK1/2 activity without affecting p38 and JNK activation (50). Our data indicate that Netrin-1 leads to activation of endogenous JNK1 and p38 but not ERK1/2. In *Xenopus* retinal growth cones, a rapid activation of the p38 MAPK in response to Netrin-1 was also observed (51). The difference in these results may reflect the different cell types used in each study, non-neuronal cells *versus* primary mouse cortical and commissural neurons. Further investigation is needed to determine the functional role of p38 in spinal commissural axon projection.

In conclusion, our data indicate that JNK1 activation plays an important role in coordination of Netrin/DCC and Netrin/DSCAM signaling in the developing nervous system.

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