8. S. I. Satake, F. Saitow, J. Yamada, S. Konishi, *Nature Neurosci.* **3**, 551 (2000).
9. J. Lerma, *Neuron* **19**, 1155 (1997).
10. M. Frerking, R. A. Nicoll, *Curr. Opin. Neurobiol.* **10**, 342 (2000).
11. M. Casado, S. Dieudonne, P. Ascher, *Proc. Natl. Acad. Sci. U.S.A.* **97**, 11593 (2000).
12. R. Chittajallu *et al.*, *Nature* **379**, 78 (1996).
13. V. R. J. Clarke *et al.*, *Nature* **389**, 599 (1997).
14. H. Kamiya, S. Ozawa, *J. Physiol.* **523**, 653 (2000).
15. D. Schmitz, M. Frerking, R. A. Nicoll, *Neuron* **27**, 327 (2000).
16. A. Contractor *et al.*, *J. Neurosci.* **20**, 8269 (2000).
17. Hippocampal slices were prepared from young adult [postnatal day 20 to 29 (p20-29)] Sprague-Dawley rats. In brief, animals were deeply anesthetized with halothane, decapitated, and the brains removed. Tissue blocks containing the subicular area and hippocampus were mounted on a Vibratome in a chamber filled with cold (~4°C) artificial cerebrospinal fluid (ACSF) containing the following : 119 mM NaCl; 26 mM $NaHCO_3$; 2.5 mM KCl; 1 mM NaH_2PO_4; 2.5 mM $CaCl_2$; 1.3 mM $MgSO_4$; 10 mM glucose, saturated with 95% O_2–5 % CO_2, pH 7.4. Transverse slices were cut at 300-μm thickness and were stored in a submerged chamber for 1 to 7 hours until transferred to the recording chamber, where they were perfused at a high rate of 3 to 4 ml/min. The recording chamber was mounted on an Olympus microscope equipped for infra red–differential interference contrast (IR-DIC) microscopy. At least 1 hour elapsed between slice preparation and recording. For all recordings, $CaCl_2$ and $MgSO_4$ were increased to 4 mM to minimize polysynaptic activation. For whole-cell voltage clamp recordings, the internal solution contained the following: 117 mM Cs-gluconate, 5 mM CsCl, 10 mM TEA-Cl, 8 mM NaCl, 10 mM Hepes, 5 mM EGTA, 4 mM MgATP, 0.3 mM Na_3GTP, 5 mM Hepes, and 5 mM QX-314, pH adjusted to 7.3 with CsOH. Electrode resistances ranged from 1.5 to 5 megohms. Access resistances ranged between 5 and 20 megohms and were continuously checked during the recording. No series resistance compensation was used. Field potential recordings were performed with low-resistance patch pipettes filled with Hepes-buffered external solution placed in stratum lucidum in CA3. Iontophoresis of NMDA (1 M, adjusted to a pH of 9) was accomplished with electrodes pulled from borosilicate glass and having resistances of 70 to 100 megohms. NMDA was ejected using a constant current iontophoresis instrument (WPI, Sarasota, FL) with pulses ranging from 5 to 20 ms. Bipolar tungsten electrodes were placed in the granule cell layer or in the hilus region to stimulate mossy fibers. The NMDARs on the A/C pathway (Fig. 4, D through G) were selectively blocked by using the irreversible, use-dependent antagonist MK-801. Average values are expressed as mean ± SEM. Drugs used were as follows: Kainate (0.002 to 10 μM), GYKI 53655 (20 μM), D-AP5 (20 μM), MCPG (0.5 mM), SCH50911 (20 μM), MK-801 (50 μM), picrotoxin (100 μM), DCG-IV (2 μM), CNQX (10 μM), and NBQX (50 μM).
18. T. Manabe, D. J. A. Wyllie, D. J. Perkel, R. A. Nicoll, *J. Neurophysiol.* **70**, 1451 (1993).
19. D. Schmitz, J. Mellor, R. A. Nicoll, data not shown.
20. P. E. Castillo, R. C. Malenka, R. A. Nicoll, *Nature* **388**, 182 (1997).
21. M. Vignes, G. L. Collingridge, *Nature* **388**, 179 (1997).
22. CNQX (10 μM) and NBQX (50 μM) caused a depression of the NMDA EPSC in approximately 35% of the cells, which were not included in the analysis. There are a number of possible reasons for this effect of CNQX. (i) There is a large underlying KAR-mediated EPSC that is removed. This is unlikely because APV inhibited the EPSC in all cells tested by at least 90%. Furthermore, the EPSC kinetics did not change on addition of CNQX. (ii) CNQX has a direct inhibitory effect on NMDA receptors. This is also unlikely because we see the same depression using NBQX (50 μM), which does not block NMDAR. Moreover, CNQX at 10 μM and NBQX at 50 μM had no effect on NMDAR responses evoked by iontophoresis (n = 4 each). (iii) There is an underlying tonic facilitation of presynaptic release mediated by KAR. Given the high affinity of these KARs (see also Fig. 1D), it is possible that the basal glutamate levels in the slice are sufficient to generate tonic activity in some cases. At present, this is the most likely possibility because we also observe an inhibition of the fiber volley amplitude with addition of CNQX in about 30% of recordings.
23. W. G. Regehr, K. R. Delaney, D. W. Tank, *J. Neurosci.* **14**, 523 (1994).
24. P. A. Salin, M. Scanziani, R. C. Malenka, R. A. Nicoll, *Proc. Natl. Acad. Sci. U.S.A.* **93**, 13304 (1996).
25. J. R. P. Geiger, P. Jonas, *Neuron* **28**, 927 (2000).
26. J. Egeberg S. F. Heinemann, *Proc. Natl. Acad. Sci. U.S.A.* **90**, 755 (1993).
27. N. Burnashev, Z. Zhou, E. Neher, B. Sakmann, *J. Physiol.* **485**, 403 (1995).
28. N. Burnashev, A. Villarroel, B. Sakmann, *J. Physiol.* **496**, 165 (1996).
29. R. S. Zucker, *Annu. Rev. Neurosci.* **12**, 13 (1989).
30. M. Scanziani, R. C. Malenka, R. A. Nicoll, *Nature* **380**, 446 (1996).
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Binding of DCC by Netrin-1 to Mediate Axon Guidance Independent of Adenosine A2B Receptor Activation

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Netrins stimulate and orient axon growth through a mechanism requiring receptors of the DCC family. It has been unclear, however, whether DCC proteins are involved directly in signaling or are mere accessory proteins in a receptor complex. Further, although netrins bind cells expressing DCC, direct binding to DCC has not been demonstrated. Here we show that netrin-1 binds DCC and that the DCC cytoplasmic domain fused to a heterologous receptor ectodomain can mediate guidance through a mechanism involving derepression of cytoplasmic domain multimerization. Activation of the adenosine A2B receptor, proposed to contribute to netrin effects on axons, is not required for rat commissural axon outgrowth or *Xenopus* spinal axon attraction to netrin-1. Thus, DCC plays a central role in netrin signaling of axon growth and guidance independent of A2B receptor activation.

Netrin-1 binds transfected cells expressing DCC, and antibodies to DCC block netrin-1–stimulated axon outgrowth and orientation in vitro (*1–5*), suggesting that DCC is a receptor or a necessary component of a receptor complex required for the actions of netrin-1 on axons. This possibility was supported by the finding that DCC and netrin-1 knockout mice have similar defects in axon guidance and by a comparable coincidence of phenotypes of mutants in DCC family receptors and their respective netrin ligands in invertebrates (*5–9*). Because the results in all species were based on loss of function of DCC family proteins, it was still possible that a coreceptor(s) was required for netrin function. These studies also did not establish whether DCC proteins function in signaling or are merely ligand-binding partners in a receptor complex. Recently, the importance of DCC for netrin signaling has been challenged (*10*, *11*). First, no binding of a soluble DCC ectodomain to netrin-1 was observed in vitro, raising the possibility that DCC does not even bind netrin directly but rather confers binding to transfected cells by complexing with some cellular cofactor (*10*). Second, the report that netrin-1 can activate the adenosine A2B receptor and stimulate cyclic adenosine 3′,5′-monophosphate production by binding its extracellular portion, while a cytoplasmic portion of A2B simultaneously binds the DCC cytoplasmic domain, led to the proposal that A2B is the central mediator of netrin signaling. In support, A2B protein was detected immunohistochemically on netrin-responsive commissural axons in collagen gels, and inhibitors of A2B function blocked commissural axon outgrowth in response to netrin-1 (*11*).

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We revisited these conclusions, first testing for a biochemical interaction of DCC and netrin-1 by generating a soluble form of the DCC ectodomain fused to the Fc portion of a human immunoglobulin molecule. DCC-ecto-Fc precipitated netrin-1 in a solution binding assay (Fig. 1A) (*12*, *13*). The binding appeared specific because DCC-ecto-Fc did not precipitate hepatocyte growth factor (HGF), a ligand for the Met receptor tyrosine kinase, which is of similar size and charge to netrin-1. To eliminate the possibility that a cofactor from the COS cells used to generate the fusion protein contributed to binding, we next used a transmembrane anchored form of the DCC ectodomain [tagged with a hemagglutinin (HA) epitope], translated in vitro in the presence of microsomes (*12*) (Fig. 1B). As predicted, the translated ectodomain was capable of precipitating netrin-1 but not HGF (Fig. 1B). A control, an HA-tagged form of the Met ectodomain, precipitated HGF but not netrin-1 (Fig. 1B). In reverse experiments, we incubated each of the ligands with each of the receptor ectodomains and found that netrin-1 pulled down the DCC but not the Met ectodomain, whereas HGF pulled down the Met but not the DCC ectodomain (Fig. 1C). Thus, netrin-1 can directly and specifically bind the DCC ectodomain.

We next sought to determine whether DCC has a role in netrin signaling. For this, we tested the cytoplasmic domain of DCC for signaling ability by fusing it to the Met ectodomain, taking advantage of the fact that HGF and netrin-1 do not bind each other's receptors. We tested the function of this Met-DCC chimeric receptor by expressing it in stage 22 *Xenopus* spinal neurons (*4*, *14*, *15*). In control cultures from uninjected embryos, stage 22 spinal neurons with monopolar or bipolar morphology never responded to HGF (Fig. 2A). A subset of multipolar neurons did show responses to HGF (*16*), so all subsequent experiments were performed on monopolar and bipolar neurons. The axons of these neurons are netrin-responsive: A gradient of netrin-1 generated by repeated pulsatile release from a glass micropipette attracted these axons and increased their rate of extension (Fig. 2A). Consistent with previous studies (*2*–*4*), these effects require DCC function because they are blocked by addition of an antibody to DCC in the bath or by introduction into these neurons of a truncated form of DCC (comprising its ecto- and transmembrane domains), a presumed dominant negative receptor (Fig. 2A). When the wild-type Met receptor tyrosine kinase was introduced into these neurons, HGF attracted their axons (Fig. 2B) and increased their rate of extension (*17*), consistent with the effects of HGF on Met-expressing rat spinal motor axons (*18*). These effects were not altered by antibodies to DCC (Fig. 2B) (*17*) and required the presence of the cytoplasmic domain of Met, because a truncated receptor comprising the Met ecto- and transmembrane domains could not transduce an attractive response to HGF (and also did not interfere with netrin-mediated attraction in these cells) (Fig. 2C). When the cytoplasmic domain of Met was, however, replaced with that of DCC, the resulting Met-DCC chimeric receptor introduced into the neurons transduced an attractive response to HGF (Fig. 2D). HGF also stimulated the rate of extension of these axons (*17*). Antibodies to DCC blocked the attractive effect of netrin-1 but not of HGF on these axons (Fig. 2D), showing that netrin-1 cannot activate the Met-DCC chimera. These results suggested that the DCC cytoplasmic domain contributes to signaling because it can replace the endogenous Met cytoplasmic domain to elicit an attractive response.

We next tested whether multimerization of the DCC cytoplasmic domain is important for signaling, as it is in other types of receptors (*19*). We first examined whether the wild-type DCC protein undergoes multimerization in response to ligand. For this, we performed cotransfection and coimmunoprecipitation (co-IP) experiments using distinct DCC constructs tagged with myc and HA epitopes, respectively. In transfected COS cells expressing both receptors, netrin-1 induced multimerization of DCC receptors, as assessed by the ability of HA-tagged DCC to coimmunoprecipitate myc-tagged DCC, and vice-versa (Fig. 3A). The multimerization was due in part to the ability of netrin-1 to aggregate DCC ectodomains, because it was also observed with truncated receptors lacking cytoplasmic domains (Fig. 3B). However, netrin-1 also independently stimulated multimerization of DCC cytoplasmic domains: When an HA-tagged full-length DCC construct was coexpressed in COS cells with a myc-tagged, myristoylated form of the DCC cytoplasmic domain targeted to the plasma membrane (Fig. 3C), addition of netrin-1 triggered the association of the myristoylated cytoplasmic domain with the full-length DCC protein (Fig. 3C). Netrin-1 similarly triggered the association of the myristoylated DCC cytoplasmic domain with full-length Met-DCC in cells coexpressing these constructs (*17*). A constitutive association was observed between HA- and myc-tagged myristoylated DCC cytoplasmic domains expressed in COS cells (Fig. 3D), and a similar constitutive association was observed in yeast with the two-hybrid system (Fig. 3E). We conclude that the DCC cytoplasmic domain is constitutively capable of self-association but that this association is normally repressed in the context of the full-length DCC protein. Netrin-1 both stimulates formation of a receptor complex through association of the ectodomains and independently causes a (presumed) conformational change that removes the repression and allows DCC cytoplasmic domains to multimerize. Netrin-1 has similar dual effects in stimulating DCC interactions with repulsive netrin receptors of the UNC5 family (*4*).

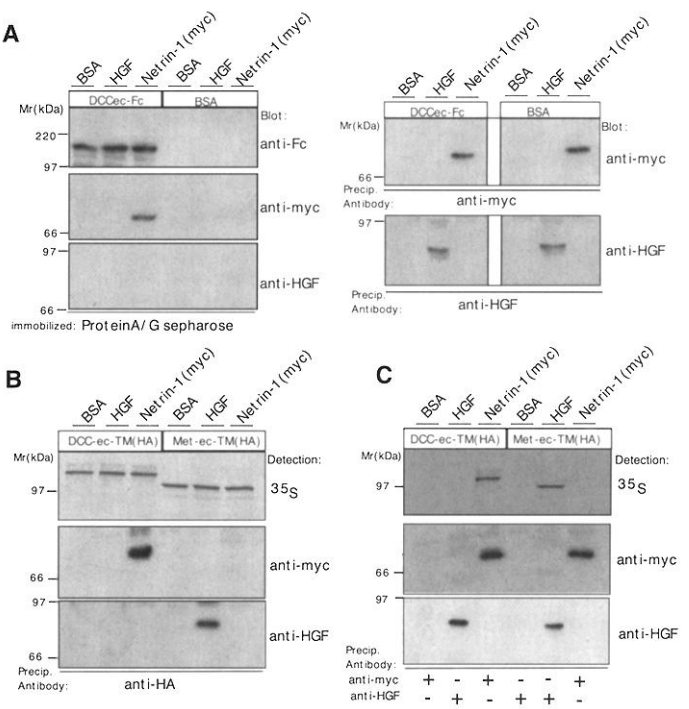

Fig. 1. Netrin-1 specifically binds to the extracellular domain of DCC. (**A**) (Left) Netrin binding to a soluble DCC-ectodomain-Fc fusion protein. Purified DCC-ec-Fc or control [bovine serum albumin (BSA)] proteins were immobilized on protein A/G plus sepharose. Beads were then incubated with purified myc-tagged netrin-1, recombinant HGF (Calbiochem), or BSA for 2 hours at 4°C and washed three times as described (*4*), and the bound proteins were separated by SDS–polyacrylamide gel electrophoresis (PAGE) and analyzed by Western blotting with a mouse antibody to human Fc, a mouse antibody to myc (9E10), or an antibody to HGF. (Right) For ligand IP experiments, ligands were incubated with DCC-ec-Fc or BSA, and one-fifth of the reaction was precipitated with antibodies to the myc tag on netrin-1 or to HGF. Precipitated proteins were analyzed by Western blotting with the indicated antibodies. (**B** and **C**) Transmembrane anchored ectodomain constructs of DCC and Met were transcribed and translated in vitro in the presence of microsomal membranes and ^{35}S-Cys. Translated proteins were incubated with either purified netrin-1, recombinant HGF (Calbiochem), or BSA at 4°C as described (*4*), subjected to IP with the indicated antibodies, and analyzed by SDS-PAGE and autoradiography or Western blot analysis. In vitro translated proteins are of the predicted size.

In deletional analysis, we found that a small region comprising the conserved P3 domain at the COOH-terminus of the cytoplasmic domain, together with a few additional residues, is necessary and sufficient for the self-association of cytoplasmic domains in yeast (Fig. 3E). The domain required for self-association appears to be P3 itself, because deletion of just P3 blocked the ability of HA-tagged DCC and of HA-tagged Met-DCC to recruit the myristoylated myc-tagged DCC cytoplasmic domain in COS cells in response to netrin-1 (Fig. 3F) (*17*). To test whether the cytoplasmic domain association was important for attraction, we introduced the Met-DCC receptor lacking P3 (Met-DCCΔP3) into stage 22 *Xenopus* neurons. In these neurons, HGF did not attract the axons or stimulate their rate of extension (Fig. 3G) (*17*). Deletion from Met-DCC of the P1 domain, which is required for cytoplasmic domain interactions of DCC and UNC5 receptors (*4*) but not of DCC with itself, did not affect the ability of Met-DCC to transduce the turning and outgrowth responses (Fig. 3H) (*17*).

Thus, P3 is required for both self-association and for the function of DCC in chemoattraction. To test whether self-association is the major function of P3, we examined whether another domain capable of self-association, the SAM domain of the EphB1 receptor cytoplasmic domain (*20*, *21*), could substitute for DCC. In the yeast two-hybrid system, the SAM domain could indeed self-associate and mediated self-association of the DCC cytoplasmic domain when it replaced the P3 domain but did not mediate association with the wild-type DCC cytoplasmic domain (Fig. 3I). The SAM domain substitution did not lead to constitutive self-association of the full-length receptor either with itself or with a version of the myristoylated DCC cytoplasmic domain in which P3 was replaced by SAM; rather, the self-association required the presence of the netrin ligand (Fig. 3, J and K). When the SAM domain was added to the Met-DCC chimera lacking P3, the ability of this receptor to transduce an attractive response to HGF was rescued (Fig. 3L), whereas a control receptor comprising the Met ecto- and transmembrane domains, and the SAM domain cytoplasmically, but without DCC sequences, could not transduce a response to HGF (Fig. 3M). Thus, the SAM domain can substitute fully for P3 in mediating ligand-regulated self-association of the DCC cytoplasmic domain and in allowing chemoattractant function.

Taken together, these results suggested that the cytoplasmic domain of DCC can signal attraction after self-association triggered by ligand. It can be activated either by netrin-1 acting on full-length DCC or by a heterologous ligand (HGF) activating a receptor chimera (Met-DCC). Although our results cannot exclude the possibility that a second netrin receptor could be necessary with DCC to mediate the netrin response, they do put two constraints on such a putative coreceptor. First, the fact that HGF attracts

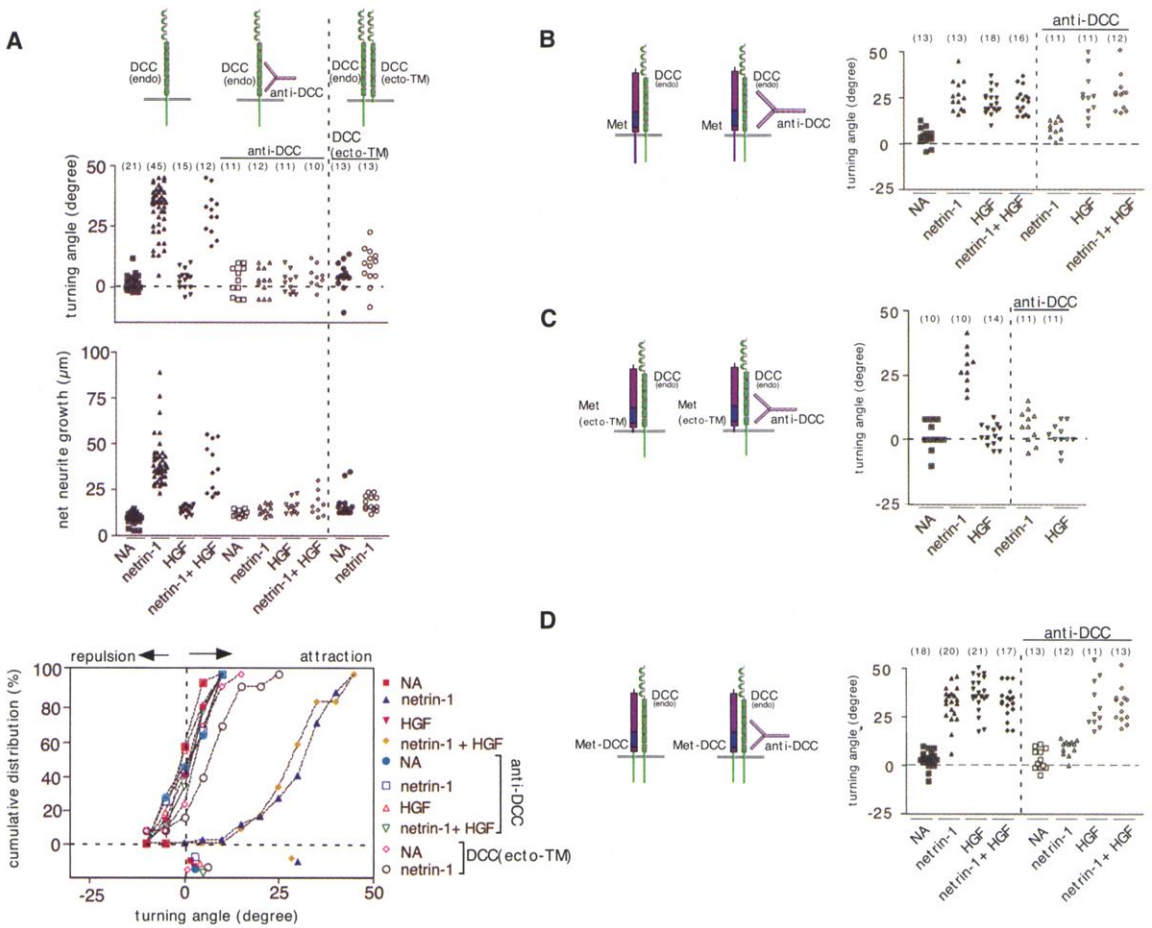

Fig. 2. Chemoattraction induced by activation of a Met-DCC chimera. (**A**) Wild-type monopolar and bipolar neurons do not respond to HGF over a 1-hour period. endo, expressed endogenously. (Top) Distribution of turning angles of all assayed neurons presented as scatter plots (each symbol point represents the response of an individual growth cone) in response to culture medium (NA), netrin-1 (5 μg/ml), HGF (10 μg/ml), and both ligands together, without and with a function-blocking DCC antibody (AF5) (1 μg/ml) in the bath, added 30 min before the experiment. Numbers in parentheses represent the total number of growth cones tested in each condition. (Right of dashed line) Turning angles of stage 22 neurons expressing DCC(ecto-TM), comprising the ecto and transmembrane domains of DCC. (Middle) Net neurite extension during the 1-hour period for the same group of neurons. (Bottom) Cumulative distribution plot of turning angles for wild-type growth cones exposed to the indicated ligands. Percentage value refers to the percentage of growth cones with angular positions less than a given angle. Data points along abscissa are median values for corresponding data shown above. (**B** and **C**). The Met receptor tyrosine kinase can mediate attraction to HGF and does not interfere with netrin-1–mediated attraction. Turning angles of stage 22 growth cones expressing either full-length mouse Met (B) or a presumed dominant negative form of mouse Met [Met(ecto-TM)] (comprising the ecto and transmembrane domains of Met) (C) in the absence or presence of antibody to DCC (AF5). Growth cones were exposed to ligands for 1 hour as in (A). (**D**) HGF can induce turning in Met-DCC–expressing cells. Turning angles of stage 22 growth cones expressing a Met-DCC chimera (ectodomain of Met fused in frame with the transmembrane and cytoplasmic domains of DCC).

axons expressing Met-DCC implies that if there is a necessary netrin coreceptor, then either it can also be activated by HGF or there is an equivalent "HGF coreceptor" that happens to be present in the axons and can substitute for the "netrin coreceptor." Second, the fact that HGF has no effect on wild-type neurons implies that activation of the putative coreceptor by HGF is not sufficient to elicit a response. Similarly, the fact that antibody to DCC can block netrin's actions implies that activation of the putative netrin coreceptor is not sufficient to signal a response. We also discovered a third constraint on the putative coreceptor by performing cross-desensitization experiments (*15*). First, we took advantage of the observation that, for neurons expressing Met-DCC, the attractive effect of a gradient of HGF could be blocked by the presence of netrin-1 uniformly in the bath (and vice-versa) (Fig. 4A), as expected for activation of two receptors that are presumably tapping into the same signaling pathway [note the cross desensitization by brain-derived neurotrophic factor (BDNF) as well, reflecting the previously demonstrated downstream convergence of netrin and BDNF signaling pathways for attraction in these neurons (*15*)]. We found, however, that cross desensitization of the HGF response by netrin-1 could be blocked by antibodies to DCC (Fig. 4A), suggesting that if netrin-1 is activating a necessary coreceptor, this activation is not sufficient for cross desensitization. A similar conclusion was derived from the observation that the attractive effect of netrin-1 on wild-type neurons could not be blocked by a uniform concentration of HGF (Fig. 4A), indicating that activation of a putative coreceptor by HGF also cannot cause cross desensitization. Taken together, all these experiments indicate that, if there is a necessary netrin coreceptor that must be activated by ligand, then (i) there must also be an HGF coreceptor (which could be the same as the netrin coreceptor), but (ii) activation of the necessary netrin and/or HGF coreceptor(s) by ligand is not sufficient by itself either to induce turning (to a ligand gradient) or to cause cross desensitization of the turning response (with ligand present uniformly).

These constraints on a necessary signaling coreceptor for DCC did not appear to be consistent with the hypothesis that activation of the adenosine A2B receptor by netrin is a central and necessary step in the attractive response (*11*). We therefore examined the possible involvement of adenosine receptors more directly using agonists and antagonists (*22*). We first tested the adenosine agonists NECA (which activates all four adenosine receptors, A1, A2A, A2B, and A3) and MECA (more specific for A2A and A2B receptors) but found that when delivered from a pipette (1 mM), they did not induce turning responses or stimulate the rate of extension and, when added to the bath (1 μM), they did not cause cross desensitization of the turning response to netrin-1 or the response of Met-DCC–expressing cells to HGF (Fig. 4B) (*17*). Three adenosine receptor antagonists were also without effect on turning or elongation: alloxazine, an antagonist with specificity toward A2A and A2B receptors, and DPCPX and DPSPX, which together antagonize all four receptors (Fig. 4C) (*17*). A fourth antagonist, enprofylline, reduced the rate of axon extension and blocked turning to netrin-1 (Fig. 4C) (*23*), but we think that this effect is not produced by adenosine receptor antagonism for two reasons. First, the two effects were nonspecific because they were also observed for turning and elongation responses to BDNF, acetylcholine (Ach), and HGF (in Met-DCC–expressing cells) (Fig. 4C) (*23*). Second, similar nonspecific effects on responses to netrin-1 and ACh were observed with a related chemical, IBMX (Fig. 4C) (*23*). Enprofylline and IBMX are both membrane-permeable methylxanthines with multiple pharmacological effects, including phosphodiesterase inhibition and possibly direct effects on some ion channels (*24*). The lack of specificity of their effects, coupled with the lack of effect of the other adenosine receptor agonists and antagonists, strongly implies that (i) the effects of enprofylline and IBMX likely occur through a mechanism other than adenosine receptor antagonism and (ii) adenosine receptor activation is not required for netrin responses in *Xenopus* neurons.

We next turned to mammalian neurons because the report that argued for an involvement of A2B focused on rat spinal commissural neurons (*11*), which require netrin-1 for their growth to the midline between embryonic days 11 and 13 (E11 to E13) (*6*). We could not detect *A2B* mRNA expression by reverse transcriptase–polymerase chain reaction (RT-PCR) in freshly dissected rat dorsal spinal cord at E11 or E13 or in E11 rat spinal cord explants cultured in collagen gels for 40 hours either alone or with netrin-1 (*17*), nor could we detect its expression in commissural neurons by in situ hybridization (*25*) at E12 to E14 (Fig. 5A) (*26*). In the same experiments, *Dcc* mRNA was readily detected by RT-PCR in fresh or cultured dorsal spinal cord tissue and by in situ hybridization in commissural neurons at E12 to E14 (Fig. 5A) (*26*). Expression of *A2B* mRNA was readily detected at high levels in adult mouse lung (*17*). The absence of detectable *A2B* mRNA in commissural neurons contrasts with the report of faint immunoreactivity on commissural axons in collagen gels detected with an antibody to A2B (*11*); we presume that the reported immunoreactivity was nonspecific, as immunostaining of axons in collagen gels is prone to high background.

We next evaluated the requirement for A2B activation in netrin signaling in the mammalian spinal cord. In an assay in which netrin-1 elicits outgrowth of commissural axons from explants of E13 rat spinal cord (*27*, *28*), the four antagonists alloxazine, enprofylline, DPSPX, and DPCPX (at concentrations well in excess of their Kis) did not reduce commissural axon growth in response to netrin-1 (Fig. 5B). In fact, the drugs appeared to cause a significant increase of the response in the cases of enprofylline, DPCPX, and DPSPX (Fig. 5B). Enprofylline was reported to inhibit netrin-stimulated outgrowth (*11*) in an assay with E11 rat spinal cord (*29*), but as with E13 tissue we found that alloxazine (30 μM, 15 times the Ki) and enprofylline (60 and 180 μM, i.e., 10 and 30 times the Ki) instead potentiated the effects of netrin-1 on E11 commissural axons (Fig. 5, C and D). At the highest concentration of enprofylline [600 μM, 100 times the Ki and considerably higher than normally used in adenosine receptor inhibition, but used in (*11*)], we saw a variable response. In two sets of experiments, we saw no change in the response (Fig. 5, C and D). In three sets of experiments, we observed a reduction in the response, but in each case, this was associated with clear necrosis of the explants at that drug concentration (Fig. 5, C and D). In fact, E11 explants are quite sensitive to toxic insults [more so than E13 explants, e.g., see Fig. 1 of (*27*)], probably because at E11 the neurons must first differentiate in the cultures (at E13, they are all differentiated). The variable reduction in axon outgrowth observed with 600 μM enprofylline is therefore likely explained by a toxic effect.

Thus, enprofylline and other adenosine receptor antagonists either potentiate or have no effect on rat spinal commissural axon outgrowth at E13 and E11; the exception is the highest concentration of enprofylline, which can reduce growth at E11 (but not E13) but likely through a toxic effect. Any effect of these antagonists is not likely to be mediated through A2B itself because its mRNA is not expressed in commissural neurons, leaving open how these drugs produce the potentiation. Our results differ from those in (*11*) in which enprofylline was reported to block netrin actions, probably because of two factors. First, in (*11*), only very low levels of netrin were delivered to the explants [compare Fig. 4 of (*11*) with Fig. 5C], so a toxic effect of enprofylline may have been sufficient to abolish the netrin-induced outgrowth. Second, in (*11*) netrin was delivered from aggregates of transfected COS cells rather than as a pure protein. However, we found that enprofylline causes death of COS7 cells (but not human embryonic kidney 293 cells) in a dose-dependent fashion (*30*). Thus, en-

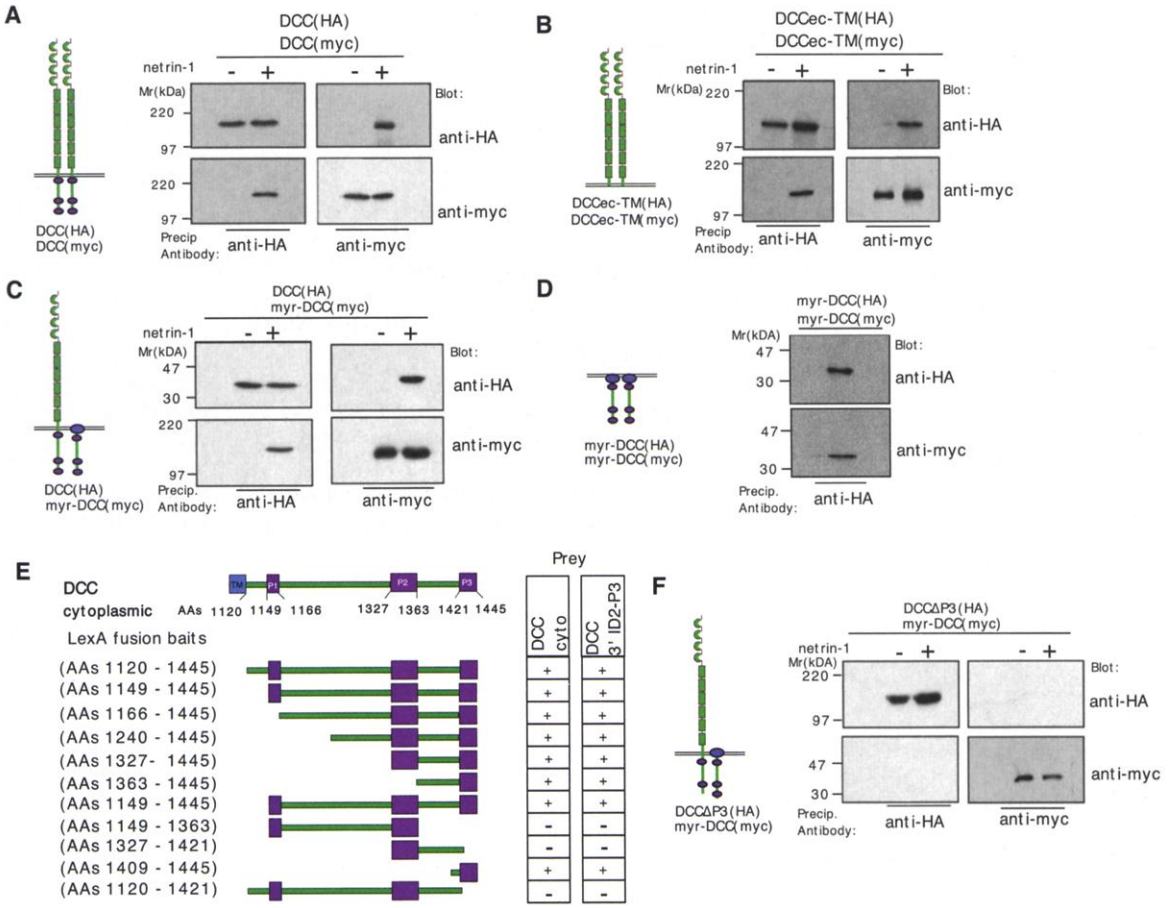

Fig. 3. Netrin-1–mediated attraction requires multimerization of the DCC cytoplasmic domain. (**A**) Ligand-dependent coimmunoprecipitation. HA- and myc-tagged versions of DCC [DCC(HA) and DCC(myc)] were cotransfected into COS cells. Forty hours after transfection, cells were incubated for 20 min at 37°C with either control medium or netrin-1 (0.3 μg/ml), subjected to IP with the indicated antibodies, and analyzed by Western blotting. (**B**) A similar netrin-1–dependent co-IP was observed with DCC constructs comprising just the ecto and transmembrane domain [same conditions as in (A)]. (**C**) Co-IP of a myristoylated form of the cytoplasmic domain of DCC [myr-DCC(myc)] with DCC(HA) in response to netrin-1. (**D**) Constitutive association of myristoylated cytoplasmic domain of DCC with itself [myr-DCC(myc) and myr-DCC(HA)] in COS cells was observed in a ligand-independent fashion. (**E**) Yeast two-hybrid analysis (with a LexA fusion bait and a VP16 fusion prey) to identify the homodimerization domain in the cytoplasmic domain of DCC. Interactions were assessed by the ability to rescue growth on histidine-deficient plates. Schematic representation of DCC cytoplasmic domain deletion constructs and their ability to interact with the entire DCC cytoplasmic domain or a DCC prey fusion encompassing amino acids 1390 to 1445 (3′ID2-P3). Truncation of the P3 domain leads to loss of VP16-DCCcy prey interaction. (+, rescue; −, no rescue). (**F**) Deletion of P3 in full-length DCC [DCCΔP3(HA)] abolishes its netrin-induced association with myr-DCC(myc). (**G** and **H**) The P3 but not the P1 domain in Met-DCC is required for HGF-mediated attraction in *Xenopus* spinal neurons. (**I**) Functional rescue of DCC cytoplasmic domain homodimerization in the yeast two-hybrid system. Interactions were assessed as in (E). Schematic representation of the DCC cytoplasmic domain and various constructs with the EphB1 SAM domain, which were used as LexA bait and VP16 prey fusion constructs. (**J**) A SAM domain in a DCC construct without the P3 domain (Met-DCCΔP3-SAM) does not allow constitutive association of DCC with itself. (**K**) Replacement of P3 with SAM restores the netrin-dependent association of full-length DCC with the myristoylated DCC cytoplasmic domain. (**L**) Met-DCCΔP3-SAM can mediate attraction in response to HGF and does not require DCC function. (**M**) The Met ecto- and transmembrane domain fused to a SAM domain does not mediate attraction in response to HGF.

profylline is likely to have reduced even further the already low level of netrin delivered to explants in the experiments of (*11*). This and the toxic effect on the explants of high enprofylline concentrations likely account for the reduction in axon growth observed in those experiments, which had suggested that enprofylline blocks netrin actions.

Our results reported here provide strong evidence for a direct central role for DCC in netrin signaling. First, we have shown direct binding of netrin-1 to the DCC ectodomain. A previous failure to detect this interaction (*10*) may have reflected some technical limitation. Our data also do not support the idea that activation of the adenosine A2B receptor is essential for netrin signaling in axon growth and guidance. We failed to detect A2B mRNA in rat spinal commissural axons, and our pharmacological results on both rat spinal commissural axons and on *Xenopus* spinal axons do not support a requirement for adenosine receptor activation by netrin to elicit netrin responses. The absence of a requirement for A2B is axon growth and guidance is consistent with the absence of a clear A2B homolog in *Caenorhabditis elegans* (*31*), because any necessary netrin coreceptor might be expected to be evolutionarily conserved. It remains possible, however, that A2B activation by netrin-1 plays a role at any sites of coexpression of receptor and ligand in other neural or nonneural tissues, although it will be important to test whether the formation of a DCC-A2B receptor complex potentiates A2B receptor function or inhibits A2B and/or DCC receptor function.

Although our experiments argue against a requirement for activation of A2B for netrin attraction, they do not eliminate the possibility of some other coreceptor. There could still be a necessary netrin-binding coreceptor, although the results of Met-DCC chimera and cross-desensitization experiments imply that activation of such a coreceptor by ligand would not be sufficient either to elicit any detectable response in the cells or to desensitize the turning response. There could also be a coreceptor that can be recruited by the DCC cytoplasmic domain without requiring netrin binding and activation for its function. Our finding that Met-DCC can mediate outgrowth-promotion and attraction and that multimerization through the P3 domain is required for these functions is consistent with DCC functioning alone but does not preclude the existence of a coreceptor(s) with those properties. Future studies will define the full makeup of the DCC receptor

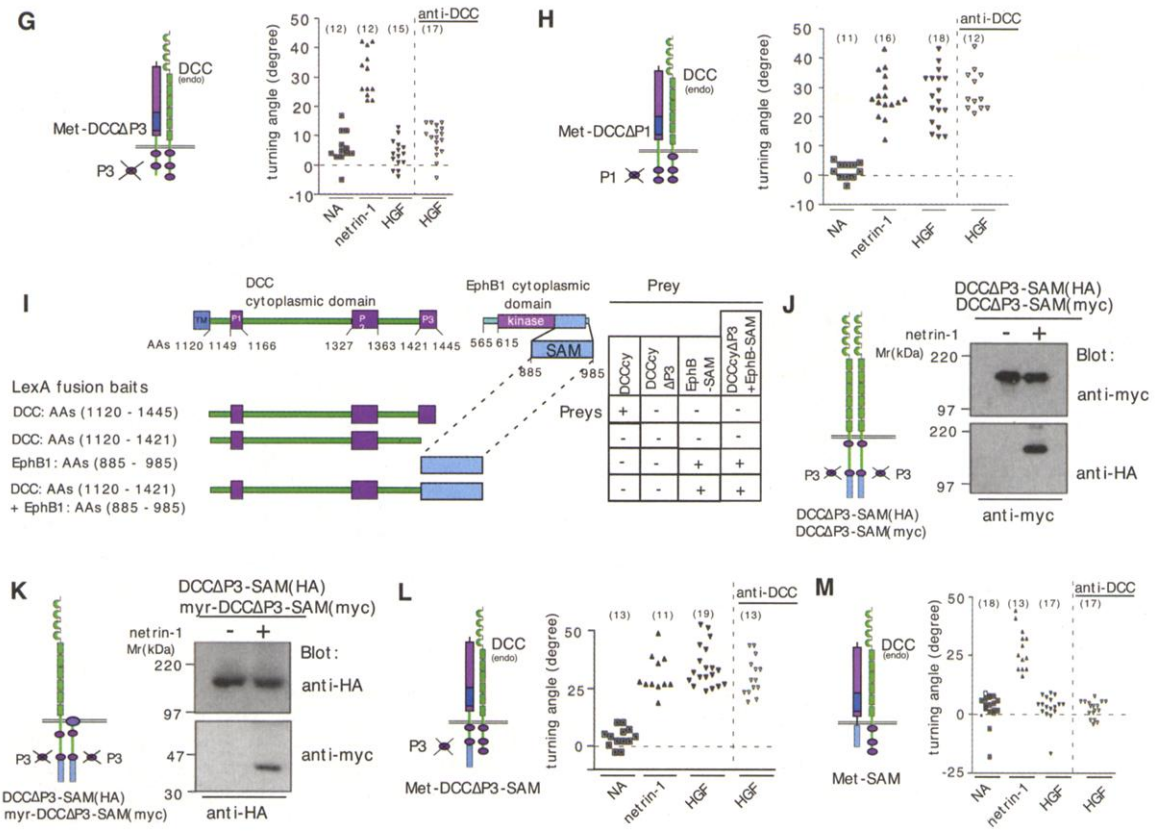

Fig. 3 (*continued*).

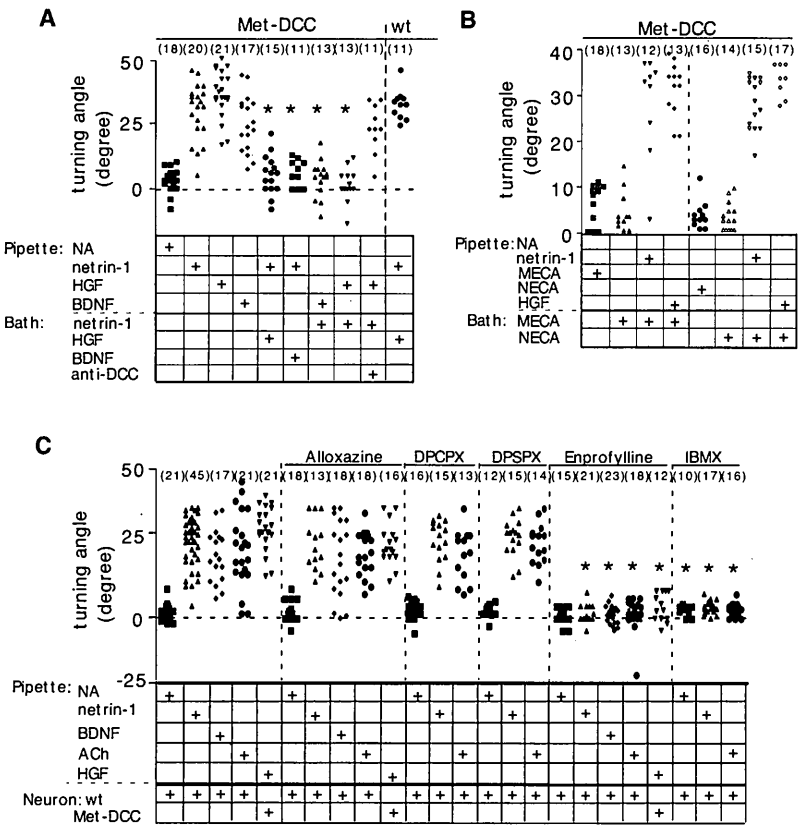

Fig. 4. Test of coreceptor (including adenosine receptor) involvement in netrin responses of *Xenopus* neurons. (**A**) Cross-desensitization experiments with DCC and Met ligands. Turning responses of Met-DCC–expressing growth cones induced by a gradient of the indicated ligand in the pipette, in the presence of uniform concentrations of HGF (50 ng/ml), BDNF (50 ng/ml), netrin-1 (100 ng/ml), and/or antibody to DCC (1 μg/ml) in various combinations in the bath. Cross desensitization of the HGF response by netrin-1 in the bath can be blocked by antibody to DCC. (Right of the dotted line) In wild-type (wt) neurons, bath-applied HGF does not cross desensitize netrin responses. (**B**). Cross-desensitization experiments with adenosine receptor agonists on Met-DCC–expressing neurons. The agonists MECA or NECA were delivered from the pipette (1 mM) or applied in the bath (10 μM). Similar results were observed with other concentrations in the pipette (100 μM and 10 mM of MECA or NECA) or in the bath (100 μM of MECA or NECA) (*17*). (**C**) Test of adenosine receptor antagonists (present uniformly in the bath) on netrin-induced turning. Alloxanine (10 μM), DPCPX (1 μM), and DPSPX (1 μM) have no effect on turning responses induced by netrin-1 or Ach on wild-type neurons. Alloxazine was also without effect on responses to BDNF or HGF (applied to Met-DCC–expressing neurons). DPCPX and DPSPX at 10 μM were also without effect on netrin and Ach-induced turning, and alloxazine at 30 μM was without effect on netrin-induced turning (*17*). Enprofylline (10 μM) and IBMX (10 μM) abolished the turning responses to all the ligands tested, which are indicated in the chart (HGF effects were tested on Met-DCC–expressing neurons). *, statistically significant change compared with no-drug condition ($P < 0.001$, Student's t test).

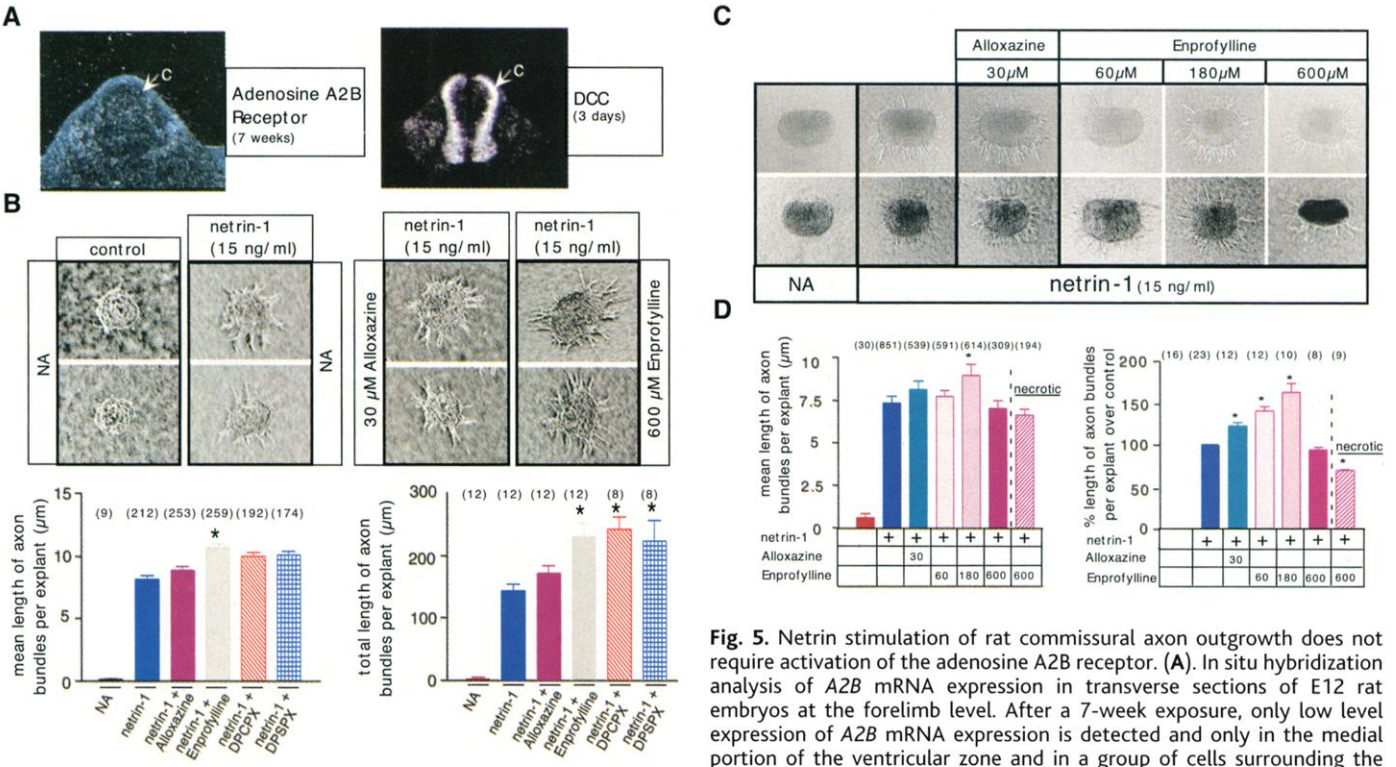

Fig. 5. Netrin stimulation of rat commissural axon outgrowth does not require activation of the adenosine A2B receptor. (**A**). In situ hybridization analysis of *A2B* mRNA expression in transverse sections of E12 rat embryos at the forelimb level. After a 7-week exposure, only low level expression of *A2B* mRNA expression is detected and only in the medial portion of the ventricular zone and in a group of cells surrounding the spinal cord, not in commissural neurons (c, arrow), which instead express high levels of *Dcc* mRNA (detected after a 3-day exposure). (**B**) Axon outgrowth from E13 rat dorsal spinal cord explants elicted by netrin-1 is not decreased by adenosine receptor antagonists. (Top) Micrographs illustrating outgrowth elicted from E13 explants after 16 hours without netrin (control) or with netrin-1 (15 ng/ml) in the absence (NA) or presence of alloxazine (30 μM) or enprofylline (600 μM). (Bottom) Quantification of mean length of axon bundles per explant and total length of axon bundles per explant (from at least four explants in triplicate). Alloxazine, 30 μM; enprofylline, 600 μM; DPCPX, 1 μM; and DPSPX, 1 μM. *, statistically significant change compared with no-drug condition ($P < 0.001$, Student's *t* test). In other experiments in which outgrowth was elicited by 3.5 ng/ml or 7.5 ng/ml netrin-1, DPSPX (1 μM) also potentiated netrin actions (*17*). (**C**) E11 rat dorsal spinal cord explants cultured for 40 hours alone (NA) or in the presence of netrin-1 (15 ng/ml) alone or with the indicated antagonist (all explants oriented dorsal up). Top row shows representative explants from one experiment where 600 μM did not induce necrosis, whereas explants in second row are from a different experiment where that concentration of drug induced clear necrosis (evident as the very dark appearance of the explant on the far right). When enprofylline caused necrosis at 600 μM, some necrosis could also be observed at 180 μM. (**D**) Quantification of the effects in (C) shows mean bundle length (left) and total bundle length per explant (right) (±SEM). Numbers in parentheses indicate the number of axon bundles (left) or the number of explants (right). In control experiments, 0.25% DMSO, the vehicle for the highest concentration of enprofylline, had no effect on netrin-evoked outgrowth (*17*). *, statistically significant change compared with no-drug condition ($P < 0.001$, Student's *t* test).

or receptor complex and its associated signaling components.

References and Notes

1. K. Keino-Masu *et al.*, *Cell* **87**, 175 (1996).
2. G. L. Ming *et al.*, *Neuron* **19**, 1225 (1997).
3. J. R. de la Torre *et al.*, *Neuron* **19**, 1211 (1997).
4. K. Hong *et al.*, *Cell* **97**, 927 (1999).
5. A. Fazeli *et al.*, *Nature* **386**, 796 (1997).
6. T. Serafini *et al.*, *Cell* **87**, 1001 (1996).
7. S. S. Chan *et al.*, *Cell* **87**, 187 (1996).
8. P. A. Kolodziej *et al.*, *Cell* **87**, 197 (1996).
9. K. J. Mitchell *et al.*, *Neuron* **17**, 203 (1996).
10. J. A. Meyerhardt *et al.*, *Cell Growth Differ.* **10**, 35 (1999).
11. V. Corset *et al.*, *Nature* **407**, 747 (2000).
12. See "Solution binding and co-IP" in supplementary material (*32*).
13. See "Construction of recombinant fusion proteins and baits" in supplementary material (*32*).
14. Epitope-tagged heterologous receptors were introduced into stage 22 neurons as in (*15*). Briefly, mRNA encoding the receptor was injected into two blastomeres of embryos at the four-cell stage together with mRNA coding for green fluorescent (GFP). Embryos were allowed to develop to stage 22, and their spinal cords were used to generate cell cultures. Neurons expressing GFP also express the heterologous receptor, as assessed by immunohistochemistry with antibodies to the epitope tag. Detailed methods can be found in the supplementary material (*32*).
15. G. Ming *et al.*, *Neuron* **23**, 139 (1999).
16. A subset of multipolar neurons responded to gradients of HGF with an increase in the rate of elongation of the processes but not, interestingly, in their direction of growth (*17*).
17. E. Stein, Y. Zou, M. Poo, M. Tessier-Lavigne, data not shown.
18. A. Ebens *et al.*, *Neuron* **17**, 1157 (1996).
19. A. Weiss, J. Schlessinger, *Cell* **94**, 277 (1998).
20. E. Stein *et al.*, *Genes Dev.* **12**, 667 (1998).
21. J. Schultz, C. P. Ponting, K. Hofman, P. Bork, *Protein Sci.* **6**, 249 (1997).
22. NECA, alloxazine, and DPCPX act at adenosine receptors in *Xenopus* [P. Brown, N. Dale, *J. Physiol.* **525**, 665 (2000)]. It is likely that MECA and DPSPX do too, although this has not been reported. All have been well characterized for their effects on mammalian adenosine receptors [V. Ralevic, G. Burnstock, *Pharmacol Rev.* **50**, 413 (1998)].
23. See Web fig. 1 in supplementary material (*32*).
24. I. M. Fearon *et al.*, *Eur. J. Pharmacol.* **26**, 342 (1998).
25. In situ hybridization was performed as described (*6*) with either a DCC fragment encoding amino acids 1145 to 1445 or the entire murine A2B cDNA, which was amplified from NIH3T3 cells and confirmed by sequence analysis.
26. See Web fig. 2 of supplementary material (*32*).
27. T. Serafini *et al.*, *Cell* **78**, 409 (1994).
28. E11 and E13 rat dorsal spinal cord explants were dissected and cultured as described (*27*, *29*). Enprofylline was either dissolved in dimethyl sulfoxide (DMSO) as a 100 mM stock or in 0.1 M NaOH. Alloxazine was resuspended in either DMSO or 0.1 M NaOH before addition to the culture medium.
29. M. Tessier-Lavigne, M. Placzek, A. G. Lumsden, J. Dodd, T. M. Jessell, *Nature* **336**, 775 (1988).
30. See Web fig. 3 in supplementary material (*32*).
31. C. Bargmann, personal communication.
32. Supplementary material is available on *Science* Online at www.sciencemag.org/cgi/content/full/291/5510/1976/DC1.

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