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Netrin-1 and slit-2 regulate and direct neurite growth of ventral midbrain dopaminergic neurons

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We investigated the roles of netrin-1 and slit-2 in regulation and navigation of dopamine (DA) axon growth using an explant culture preparation of embryonic ventral midbrain (embryonic day 14) and a co-culture system. We found that netrin-1 protein significantly enhanced DA axonal outgrowth and promoted DA axonal outgrowth in a co-culture system of netrin-1 expressing cells. Such effects were mediated by the receptor DCC as demonstrated by antibody perturbation of the DCC receptor. In contrast, slit-2 inhibited DA neuron extensions and repelled DA neurite growth. These slit-2 activities required robo receptors since the reduced neurite extension was abolished by addition of excess robo receptors. In this system, netrin-1 stimulated and slit-2 opposed DA neurite growth. Such regulation may be important for DA axonal maintenance, regeneration, and phenotypic target recognition.

Introduction

The cellular and molecular mechanisms underlying trajectory formation and target selection in the development of the nigrostriatal and mesolimbic pathways originating from the ventral midbrain (VM) dopamine (DA) neurons are largely unknown. Two groups of dopamine neurons are located in the ventral midbrain, which are substantia nigra (SN, area 9) and ventral tegmental area (VTA, area 10). Ventral tegmental DA neurons located more medially project to the ventromedial striatum (nucleus accumbens and olfactory tubercle) and cortex, establishing the mesolimbic and mesocortical pathway, respectively (Simon et al., 1976, 1979). In contrast, substantia nigra resides more laterally and selectively sends axons to the dorsolateral striatum (caudate putamen) where axons terminate in an orderly medial–lateral pattern forming the nigrostriatal pathway (Beckstead et al., 1979). The mesolimbic pathway is involved in multiple functions related to emotion, mood, and addiction (Self and Nestler, 1995), whereas loss of the nigrostriatal connectivity is the main neuropathology of Parkinson's disease (PD) characterized by severe motor dysfunction (Bernheimer et al., 1965, 1973). Replacing DA progenitors derived from fetal VM and stem cells into the parkinsonian striatum or substantia nigra has been thought an alternative therapeutic strategy for PD patients (Isacson et al., 2003).

One study in mice showed that genes of EphB1 and EphrinB2 are expressed in a complementary pattern in the ventral midbrain and the striatum, and DA neurite growth derived from SN region appears to be selectively inhibited by ephrinB2 (Yue et al., 1999), suggesting that these paired molecules may confer information for the divergence of axon termination zones of SN and VTA neurons. Examination of DA pathways in the EphB1 or EphrinB2 knockouts may provide strong evidence whether EphB1–EphrinB2 interactions are crucial for target selection of SN and VTA neurons. But loss of EphB1 or EphrinB2 functions might be compensated by other molecules in these mice. If it is the case, it indicates that other known and unknown guidance cues may also play a part in the positional organization of DA axon terminals. Semaphorins are a large family of guidance cues consisting of secreted and membrane-bound proteins that function by binding to their cognate receptors plexins and neuropilins (He and Tessier-Lavigne, 1997; Tamagnone et al., 1999). Semaphorin3 is a well-studied member that is primarily demonstrated as a growth-inhibitory cue for a number of populations of axons in the developing brain (Bagri and Tessier-Lavigne, 2002; Campbell et al., 2001; Raper, 2000). Semaphorin3 is highly expressed in the developing caudate putamen and globus pallidus, but their functions in the development of DA projections have not been evaluated. Growth factors BDNF (Song et al., 1997) and morphogenetic genes sonic hedgehog (Charron et al., 2003) are also involved in axon guiding activities of other pathways. These molecules are also expressed in the ventral midbrain dopamine system (Hynes et al., 1995), but functional characterization of whether these molecules regulate axonal guidance of DA neurons awaits investigations.

Expression of netrin-1/deleted in colorectal cancer (DCC) genes is also observed in the ventral midbrain and the striatum (Livesey

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and Hunt, 1997). Netrin-1 is a well-known chemoattractant for axonal outgrowth by its interaction with the DCC receptor in many brain regions during CNS development (Braisted et al., 2000; Kennedy et al., 1994; Metin et al., 1997), and netrin-1 can also be chemorepellent for other neurons, which requires the UNC5 receptor (Hong et al., 1999). Levels of cAMP in growth cones also regulate responsiveness of the pathfinding axons to netrin-1 (Hopker et al., 1999; Ming et al., 1997). Notably, expression of netrin-1/DCC persists in the adult ventral midbrain and striatum, which may influence maintenance of connections between ventral midbrain DA neurons and striatum. In contrast to the netrin family, the slit family members were initially found to be chemorepellents controlling midline crossing via binding to robo receptors (Brose et al., 1999; Nguyen et al., 1999). Subsequent studies demonstrated that slits could function as attractants in several neural systems (Kramer et al., 2001). Three slit and robo genes have been identified in mammal (Brose et al., 1999). In the developing DA system in the rat brain, the robo1 gene is expressed in both SN and VTA areas, whereas the robo2 gene is preferentially distributed in the SN area. Slit1 is also observed in the lateral ganglionic eminence and the expression becomes much reduced in the caudate putamen. Slit2 is found in the adjacent areas to striatum, such as cerebral cortex and septum, whereas Slit 3 appears at P5 and is also highly expressed in the adult caudate putamen (Marillat et al., 2002). The developmentally regulated expression patterns of slit members suggest that this family may participate in distinct aspects of DA pathway development, for instance, axonal pathfinding, targeting and synaptic plasticity.

We started investigating the roles of netrin-1 and slit-2 in regulation and orientation of DA axonal growth in vitro, providing evidence that these factors influence axonal growth by mechanisms depending on DCC and robo receptors, respectively. Such information is essential for understanding of the molecular mechanisms underlying construction of the anatomical foundation for distinct functions of the midbrain DA system. It is also important for understanding of donor–target recognition and reconnection in neuronal cell therapy studies involving PD patients because these guidance cues continue to be expressed in the mature DA system.

Results

Expression of DCC and robo1-2 in DA neuron subpopulations of the ventral midbrain

In primary culture of the ventral midbrain, we observed that the DCC protein was co-localized with tyrosine hydroxylase (TH, a marker for dopamine neurons) (Fig. 1). DCC was also co-expressed with GIRK2 (Fig. 1D), a potassium channel protein abundantly present in the DA neurons of SN pars compacta, and with much less expression, if any, in most VTA regional DA neurons (Inanobe et al., 1999; Schein et al., 1998). In addition, co-localization of DCC with calbindin $D_{28}K$ was observed in the primary DA cultures (Fig. 1H). Calbindin is an intracellular calcium-binding protein that is mostly expressed in VTA (McRitchie et al., 1996; Neuhoff et al., 2002). The present observations thus revealed that DA cells in both SN and VTA expressed DCC, a receptor required for netrin-1 to enhance axonal outgrowth and to elicit chemoattraction (Geisbrecht et al., 2003).

Using immunohistochemical paradigms, we found that robo-1 was co-localized with calbindin, GIRK2 and TH (Fig. 2),

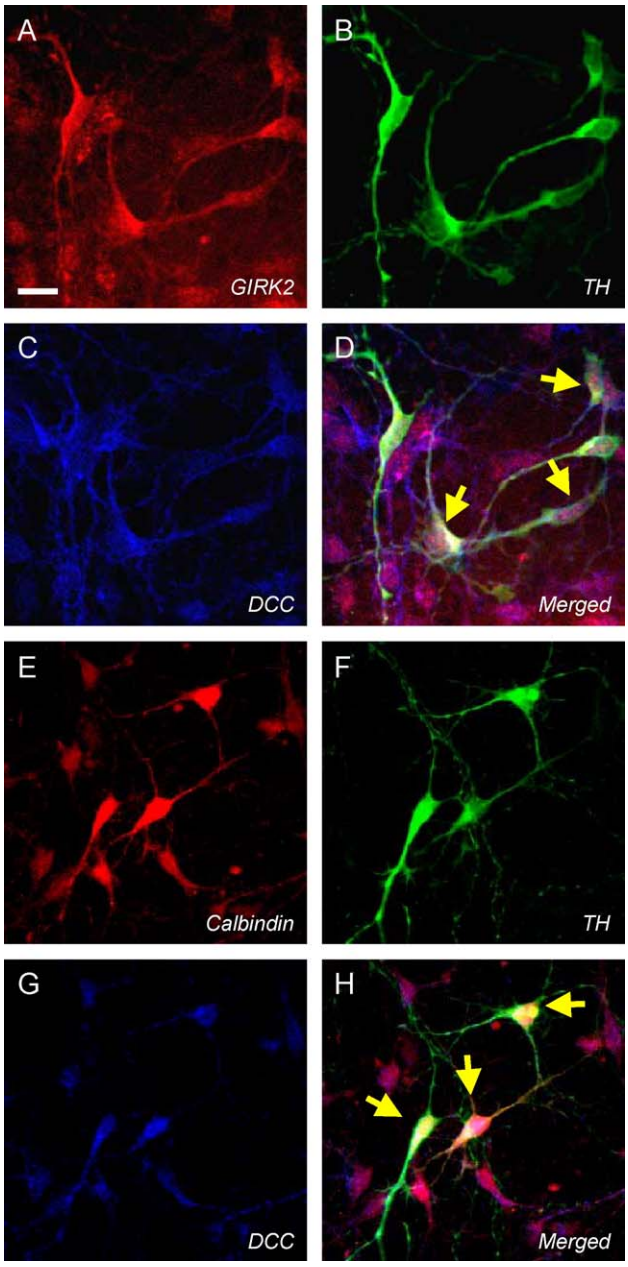

Fig. 1. DCC expression in subpopulations of the TH neurons in the E14 ventral mesencephalic primary culture. (A) GIRK2 (a marker for A9 DA cells). (B) TH. (C) DCC. (D) Merged image shows colocalization of TH, GIRK2 and DCC (indicated by arrows). (E) Calbindin (a marker for A10 neurons). (F) TH. (G) DCC. (H) Merged image shows colocalization of TH, calbindin and DCC (indicated by arrows). Scale bar: A–H, 50 μm.

confirming an expression pattern of robo-1 gene described previously (Marillat et al., 2002). However, we also observed co-expression of robo-2 protein with calbindin and TH, demonstrating a presence of robo-2 protein in VTA area (Fig. 3). Collectively, it appeared that nearly all of the TH neurons expressed DCC and robo receptors.

Promotion and orientation of DA axon outgrowth by netrin-1

To determine effects of netrin-1 on DA axon outgrowth, a recombinant netrin-1 was added to the culture medium at a

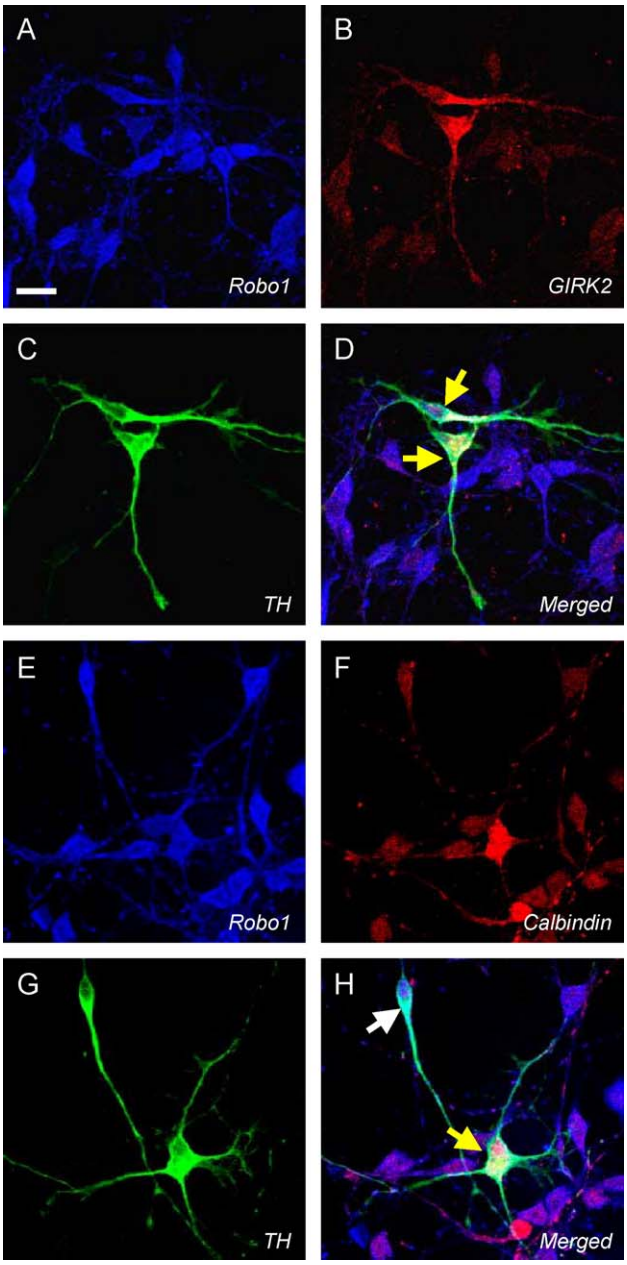

Fig. 2. Expression of robo1 receptor in subpopulations of the TH neurons in the E14 ventral mesencephalic primary culture. (A) GIRK2. (B) TH. (C) Robo-1. (D) Merged image shows that robo1 is co-expressed with GIRK2 and TH (indicated by arrows). (E) Calbindin. (F) TH. (G) Robo1. (H) Merged image shows coexistence of calbindin, TH, and robo1 in this triple staining (indicated by arrows). Scale bar: A–H, 50 μm.

concentration of 200 ng/ml. The concentration used in the present study was based on a previous study that demonstrated an axon growth promoting activity of netrin-1 at a range of 100–400 ng/ml (Metin et al., 1997). We found that explants cultured with netrin-1 (n = 23) apparently exhibited more neurite outgrowth than control explants (n = 23) (Figs. 4A–B). To evaluate whether the DCC receptor mediated such promoting effects, a functional blocking antibody against DCC (clone AF5) was applied at a concentration of 10 μg/ml 1 h before addition of netrin-1 (n = 22). Addition of the species-matched immunoglobulin served as a control (n = 25) (Figs. 5C–D). Quantitative analysis revealed that netrin-1 treatment alone significantly increased neurite covered area when compared to that in explants cultured without netrin-1, with IgG and with the DCC antibody and netrin-1 (Fig. 5E, $*P < 0.03$, one-way ANOVA).

Netrin-1 has been demonstrated to function as an attractant for axon growth through binding to the DCC receptor in many regions in the developing brain (Braisted et al., 2000; Kennedy et al., 1994). To determine its influence on DA axon growth, we performed a guidance assay, in which a VM explant was co-cultured with aggregates prepared from 293-EBNA cells transfected with netrin-1

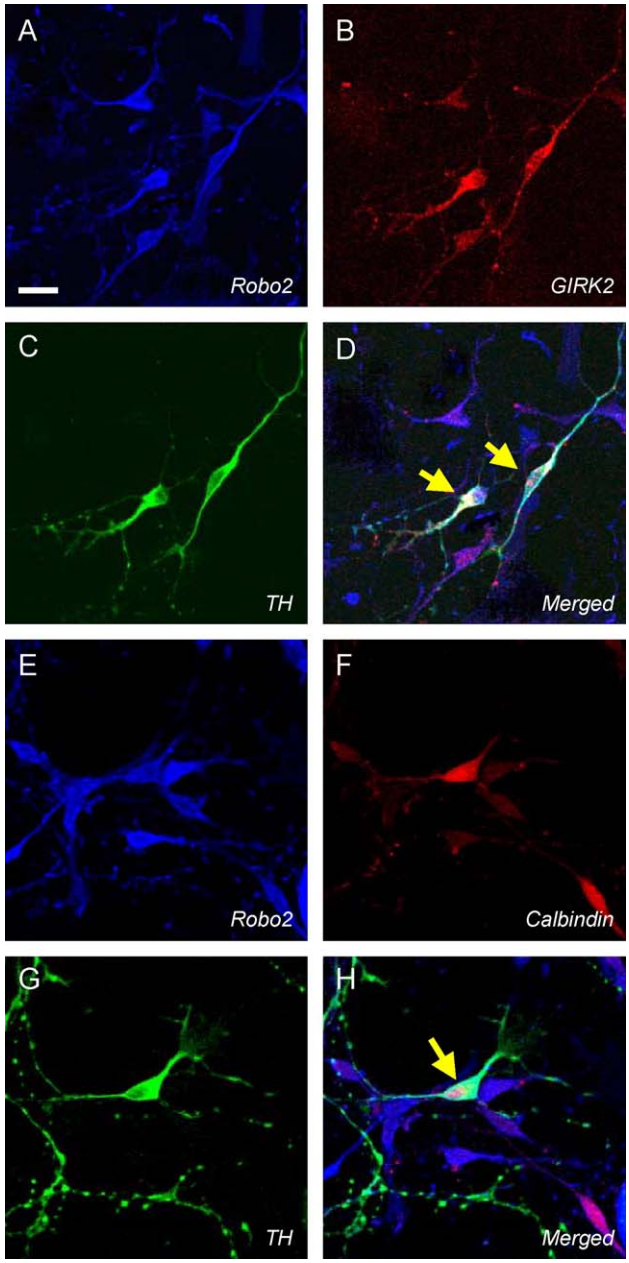

Fig. 3. Expression of robo 2 in subpopulations of the TH neurons in the E14 ventral mesencephalic primary culture. (A–C) Triple immunostaining for GIRK2 (red), TH (green) and robo-1 (blue). (D) Merged image shows coexpression of TH, GIRK2 and robo-2 (indicated by arrows). (E–G) Triple immunostaining for calbindin (red), TH (green) and robo-2 (blue). (H) Merged image shows colocalization of calbindin, TH and robo2 (indicated by arrows). Scale bar: A–H, 50 μm.

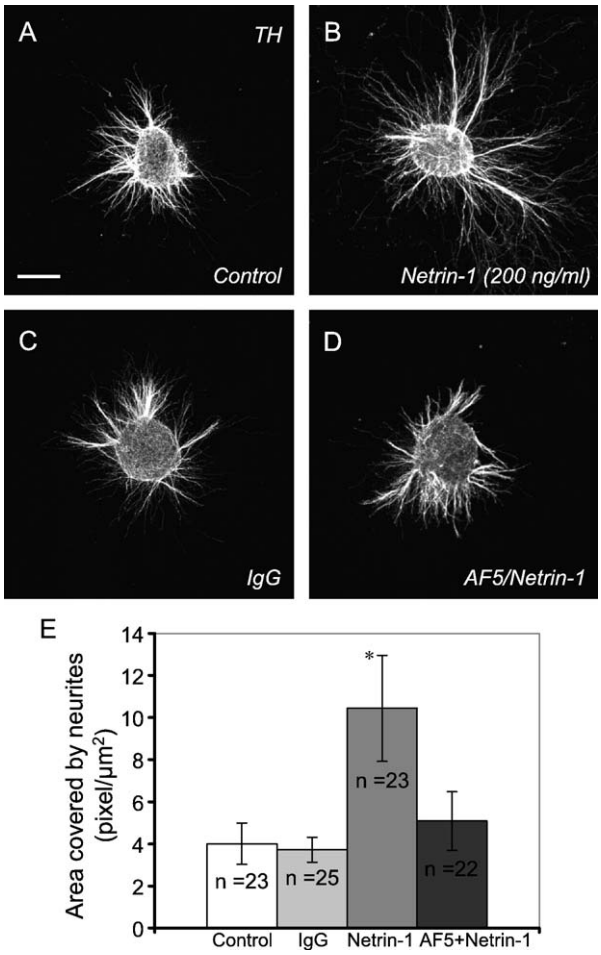

Fig. 4. Netrin-1 promotes neurite outgrowth. (A) Control explants. (B) Explants incubated with Netrin-1 (200 ng/ml). (C) Explants cultured mouse IgG (10 μg/ml). (D) Explants incubated with AF5 (a DCC antibody, 10 μg/ml) and (Netrin-1 200 ng/ml). (E) Graph shows the area covered by neurite measured by using MetaMorph software. Data are presented as pixel per μm^2 explant (mean ± SEM). Statistical analysis shows that the area covered by neurites in netrin-1 treated explants is significantly higher than that in the control explants, $*P < 0.03$ (one-way ANOVA). Scale bar: A–D, 100 μm.

($n = 26$) or control plasmid ($n = 30$) in the collagen gel for 1.5 day. Neurites from explants cultured with control 293-EBNA cells grew radially in all orientations. The control 293-EBNA cells transfected with control plasmid exhibited mild inhibitory effects by themselves on axon growth (Fig. 5A). This phenomenon has also been observed in another study (Metin et al., 1997). In contrast, increased axonal growth toward to netrin-1 expressing cells was observed in explants cultured with netrin-1 aggregates (Fig. 5B). Quantitative analysis was performed by counting axons or fascicles in the proximal and distal quadrants of the explants (Fig. 5C). Ratios of numbers of axons in proximal versus distal area were then calculated (P/D ratio). Statistical analysis showed an approximately 3-fold increase of neurites distributed in the area facing netrin-1 cells compared to that in the control explants ($**P < 0.0001$, Student's t test).

Slit-2 repelled and inhibited DA neurite extension

In co-cultures of VM explant and slit-2 or control cell aggregates, we examined the functions of slit-2 in directing DA axon growth from DA neurons. In the explants cultured with control cell aggregates ($n = 21$), neurites grew well in the collagen gel (see Fig. 6A), and responded to control aggregates in a homogenous way, In contrast, in the VM explants cultured with slit-2 cell aggregates ($n = 41$), most TH positive axons and fascicles were observed in the distal part of the explants, and only few axons were seen in the proximal aspect that faced the aggregates (Fig. 6B). Quantification of the ratio of the number of TH axons and fascicles in the proximal versus the distal aspect showed that only 30 ± 6% (mean ± SEM) neurites were distributed in the proximal aspects of the explants cultured with slit-2 cell aggregates (Fig. 6C), compared to control cell aggregates, indicating that slit-2 was a potent repellent for DA axon growth. In addition, we observed that the neurite length appeared to differ between these two groups. To test whether slit-2 influenced DA neurite growth objectively, dissociated VM cells were seeded on a monolayer of control or slit-2 transfected cells, cultured for 2 days and then stained with a TH

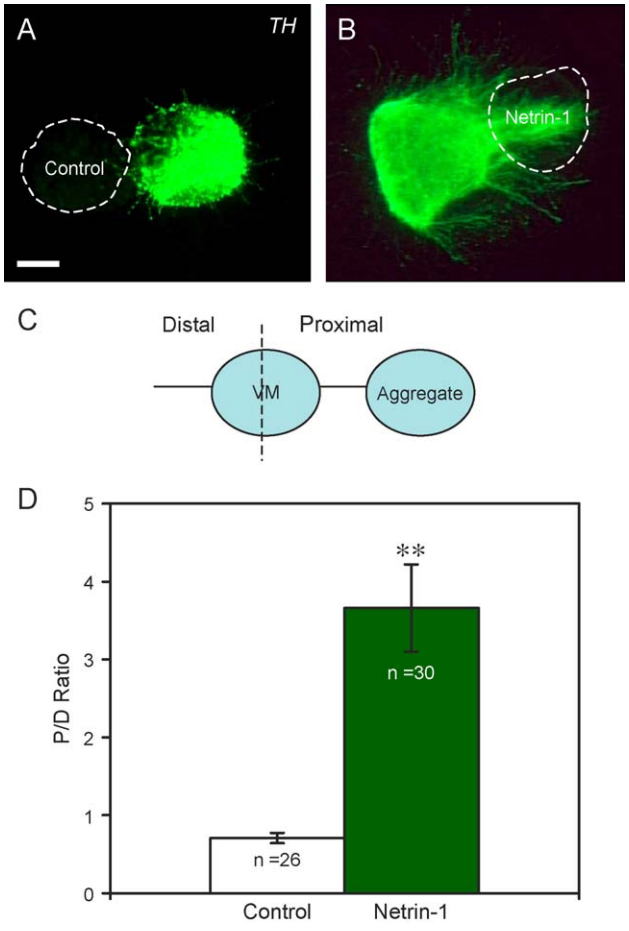

Fig. 5. Netrin-1 promotes DA axonal outgrowth. (A) Coculture of the E14 VM with parental 293-EBNA cells (outlined) exhibits a few axons extending from explant. (B) In contrast, aggregate of 293 cells secreting netrin-1 stimulates TH axonal outgrowth. Majority of axons distributes in front of the aggregates, indicating netrin-1 also navigates DA axon growth. (C) Drawing shows quantitative method. Number of axons and fascicles was counted from the margin area in quadrant proximal or distal to aggregates, respectively. (D) Graph shows ratio of axon and fascicle numbers in proximal versus distal explants (P/D Ratio). Results are presented as mean ± SEM. Statistical analysis reveals that the ratio in co-cultures of VM and netrin-1 aggregates is significantly greater than in the control co-cultures, $**P < 0.000$, Student's t test,). Scale bar: A–B, 150 μm.

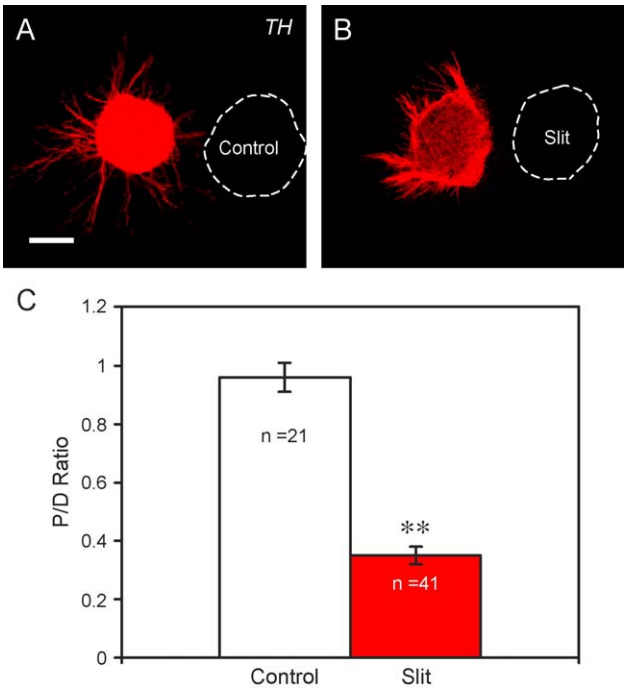

Fig. 6. Slit-2 repels TH axon growth. (A) VM explant cultured with parental HEK 293 cells exhibits radial distribution of axons and fascicles. (B) Most axons and fascicles are observed in the distal part of explants cultured with aggregate of HEK 293 cells secreting slit-2. (C) Ratio of number of neurite bundles in proximal quadrant versus distal quadrant is calculated as described above. Data are presented as mean ± SEM. The P/D ratio is significantly lower in the co-cultures with slit-2 cells than that in the control co-cultures. $^{**}P < 0.0001$, Student's t test). Scale bar: A, B; 100 μm.

antibody. This experiment demonstrated that TH neurite extension was inhibited in co-cultures with slit-2 expressing cells compared to control cells (Figs. 7A–B). We further investigated whether robo receptors were required for such actions of slit-2 on TH neurite extension. Conditioned medium collected from 239-HEK cells expressing N-terminal domain of robo receptor (RoboN, Wu et al., 1999) was added to the co-cultures of VM and slit-2 cells at concentrations of 1:10, 1:5, and 1:2 (Figs. 7C–D). Quantitative analysis was carried out by measuring neurite length using stereology. Statistics (Kruskal–Wallis with post hoc test) showed a significant reduction of DA axonal length in cultures with slit-2 producing cells (48 ± 1.3%, mean ± SEM, $n = 336$) as compared to controls. RoboN reduced the effects of slit-2 producing cells at 1:10 and 1:2, but the values were not significantly different from controls. A concentration of 1:5 neutralized slit-2 elicited inhibitory activity on TH neurite extension (Fig. 7E), indicating that this concentration was optimal for competing with endogenous Robo receptors that bind to slit2. Higher concentrations of RoboN may contain excessive amount of inhibitors that may compete with each other and fail to achieve an optimal blocking effects.

Discussion

In the present study, we investigated molecules that regulate and effectively orient axonal outgrowth of ventral midbrain DA neurons in vitro. We find that netrin-1 is a potent growth-promoter and a stimulant for directed DA axonal outgrowth, and these effects are mediated by one of the netrin-1 receptors, DCC. In contrast, slit-2 is an inhibitory factor for DA neurite extension, and this action is mediated by robo receptors, since addition of roboN reduces or neutralizes slit-2 exerted effects on TH neurite extension in our study. Slit-2 also exhibits a strong repulsive effect on midbrain DA axon outgrowth as examined in the co-culture system. These findings clearly demonstrate that guidance molecules netrin-1 and slit-2 can influence midbrain DA axon outgrowth, suggesting roles of these molecules in the formation of the midbrain DA pathways.

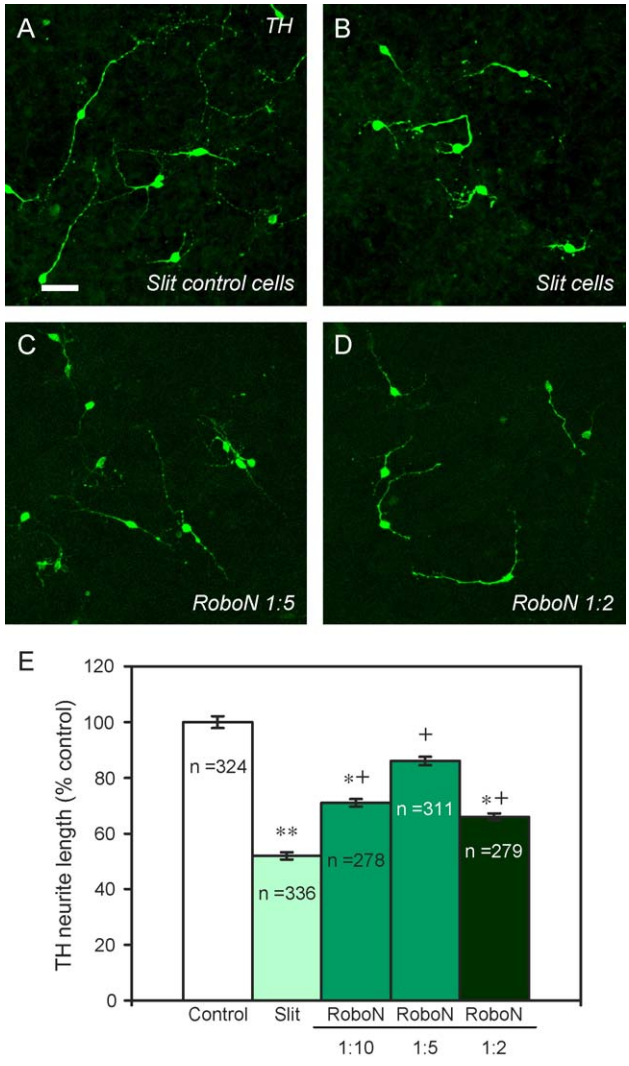

Fig. 7. Slit-2 inhibits DA neurite extension. Dissociated VM cells are cocultured with monolayer of parental or slit-2-transfected cells. (A) TH neurite extension on slit-2 control cells. (B) TH neurite extension on slit-2 transfected cells. (C–D) Conditioned medium of RoboN added at 1:5 and 1:2 partially suppresses inhibitory effects of slit-2 on TH neurite extension. (E) Graph shows DA neurite lengths that are expressed as a percentage of the control, mean ± SEM) in co-cultures ("*" versus control and "+" versus slit-2). TH neurite length cultured with slit-2 cells is significantly lower than that in co-culture with parental control cells $^{*}P < 0.001$. RoboN at concentration of 1:10, 1:5 and 1:2 significantly reduces slit-2 inhibitory effects on DA neurite extension ($^{+}P < 0.001$), but only RoboN at 1:5 can neutralize the inhibitory activity of slit-2, $^{*}P > 0.05$ when compared to co-cultures of control (Kruskal–Wallis ANOVA with post hoc test). Scale bar: A–D, 50 μm.

Roles of netrin-1 in formation of midbrain DA pathways

Using immunohistochemistry, we detected that DCC receptor was co-expressed with TH, confirming a presence of the gene product of DCC mRNA shown by Livesey and Hunt (1997). Complementary expression patterns of DCC and netrin-1 in the developing ventral midbrain and striatum suggest a role of these paired guidance cues in the development of the DA pathway. Furthermore, our functional assays revealed that netrin-1 stimulated DA axon outgrowth toward the experimental targets. These functions required the receptor DCC, which is consistent with a number of studies in other neural systems (Braisted et al., 2000; Deiner et al., 1997; Forcet et al., 2002). Netrin-1 can also repel some axons, which requires the UNC5 receptor (Hong et al., 1999). Loss of netrin-1 functions results in defects in spinal and several forebrain commissural projections (Serafini et al., 1996), and DCC null mice exhibit similar abnormalities in the developing CNS to those observed in netrin-1 deficient mice (Fazeli et al., 1997). In addition to the commissural axon projections, subsequent studies showed that netrin-1/DCC functions are necessary for establishing several other neural pathways, for example, the thalamocortical pathway (Braisted et al., 2000), the hippocampal circuit (Barallobre et al., 2000), and the dorsal column-medial lemniscal system (Kubota et al., 2004). Given the presence of netrin-1 in VM, and the dramatic enhancement of DA neurite outgrowth by netrin-1 in our in vitro study, it suggests that netrin-1 may act on DA axons locally in VM, and distantly as an important factor for DA axonal growth into target regions. Examination of the DA pathways in DCC or netrin-1 null brains would likely provide evidence whether netrin-1/DCC interactions are essential for the development of DA pathways. Since the expressions of DCC and netrin-1 continue in the adult DA pathway (Livesey and Hunt, 1997), they may also play a role in axonal recognition and repair, possibly relevant to PD cellular therapies.

Roles of slit-2 in development of DA pathways

Our immunohistochemical data revealed that robo1 and robo2 receptor proteins were co-localized with the TH and GIRK2 double-labeled DA neurons. Robo2 protein expression was also seen in the TH and Cabindin double-labeled neurons, the VTA group in the ventral midbrain. We could not determine whether expression levels of robo-2 were different in SN and VTA areas using the present paradigm, so one cannot rule out the possibility that a graded expression pattern of robo-2 protein could exist in the VM.

Presence of cognate ligands (slit1–3) for robos has been shown in the developing striatum and in the surrounding areas, and the ligand expression patterns are developmentally regulated in target areas (Marillat et al., 2002), suggesting multiple functions of this family, from the initial formation to maintenance of the ventral DA projections. We found that slit-2 strikingly repelled DA axonal growth in guidance assays and functioned as a potent inhibitor for DA neurite extension, which mediated by robos. These in vitro findings are consistent with morphogenetic phenotypes observed in slit-1 and slit-2, or slit-2 null mice, that exhibit axonal pathfinding errors through diencephalon and telencephalon of the embryonic DA pathway (Bagri et al., 2002). Whether these mutant mice exhibit additional defects in the nigrostriatal and mesolimbic pathways is unknown. Taken together, these findings demonstrate that slit-2 is an important factor in DA axonal pathfinding. Interestingly, slit-3 expression is abundant in the adult caudate putamen (Marillat et al., 2002), indicating that this molecule may influence DA axonal plasticity.

In summary, in the developing VM and striatum, expressions of slits and robos coincide with the onset of the DA pathway formation. The tightly regulated expression patterns of slit members suggest that this family is involved in many aspects of the DA neuron projections, and maybe even regeneration in the adult. Further study is needed to determine functions of slit members at different developmental stages of DA pathway development and in vivo examination of whether loss of function of these genes alters DA neuron projections.

Concluding remarks

It is noteworthy that many guidance molecules are persistently expressed in the adult brain (Encinas et al., 1999; Liebl et al., 2003; Manitt et al., 2001), suggesting that functions of these guidance cues are not limited to control axonal pathfinding and target selection during the initial CNS network formation. These molecules could also play critical roles in structural maintenance, synaptic plasticity, and axonal regeneration in the mature CNS (Moreau-Fauvarque et al., 2003; Pasterkamp et al., 1999). Cell therapeutic studies for PD patients have demonstrated that donor fetal or ES differentiated DA cells grow specifically toward appropriate adjacent and distant host target regions from ectopic or homotopic implantation sites (Bjorklund et al., 2002; Deacon et al., 1999; Isacson and Deacon, 1996), indicating that highly specific guidance mechanisms are preserved by the mature brain that can orientate axonal growth of these new neurons. Our study examined guidance molecules that may also be involved in regenerative processes of new DA neurons derived from fetal VM and stem cells, that are transferred into the striatum of PD animal models as well as PD patients (Isacson, 2003; Isacson et al., 2003). Because netrin-1 and slit-2 are persistently expressed in the adult striatum and adjacent areas (Livesey and Hunt, 1997; Marillat et al., 2002), they may play a part in target-orientated axonal outgrowth of implanted fetal neurons in mature and aged host animals (Isacson and Deacon, 1996, 1997).

Overall, this experiment provides a specific study of guidance molecules involved in axon growth of immature DA neurons. A better understanding of molecular mechanisms controlling DA circuit formation may lead to methods that create favorable environments for axonal and synaptic regeneration and repair in Parkinson's disease.

Experimental methods

Animal

All animal studies were carried out following National Institutes of Health Guidelines and were approved by the Institutional Animal Care and Use Committee at McLean Hospital, Massachusetts General Hospital and Harvard Medical School. Time-pregnant Sprague–Dawley rats at embryonic day E14 (E14) were obtained from Charles River Breeding Laboratories (Wilmington, MA). The day on which a vaginal plug was found was considered as embryonic day 0 (E0).

Primary culture preparation

All culture media were purchased from Invitrogen Corporation (Grand Island, NY), and all other products were obtained from Sigma (St. Louis, MO), unless otherwise specified. E14 pregnant rats were sacrificed by an overdose of pentobarbital (60 mg/kg). Embryos were removed and placed in a cold Hanks' Balanced Salt Solution (HBSS). Ventral midbrains were dissected out and placed in a medium containing 50% DMEM/F12, 50% DMEM, 1.56% glucose, B27, 100 U/ml penicillin and 100 μg/ml streptomycin. The VMs were mechanically dissociated with polished Pasteur pipettes. 2×10^5 cells were seeded on a coverslip precoated with polyethyenimine. The cells were grown for 4 days and then fixed with 4% paraformaldehyde in phosphate buffer (PB, pH 7.4) for 30 min at room temperature.

Preparation of cell aggregates expressing netrin-1 and slit-2

Aggregates were generated as described previously (Kennedy et al., 1994). Briefly, 293-EBNA cells transfected with netrin-1 plasmid were cultured in DMEM containing 10% fetal bovine serum, 2 mM glutamine, 200 μg/ml Hygomycin (Boehringer), 250 μg/ml Geneticin (BRL) and antibiotics. The 293-EBNA cell line transfected with control vector plasmid was used as control. For the slit-2 study, a 293-HEK cell line transfected with human slit-2 or control vector plasmid was cultured in DMEM containing 10% fetal bovine serum and antibiotics as described previously (Sang et al., 2002). The cells were cultured with the growth media for 1.5 day, then harvested, centrifuged and resuspended in 100 μl medium containing 0.5 million cells. Cell aggregates were prepared by pipetting 10 μl cell containing medium on the lid of 35 × 10 mm culture dishes and cultured with DMEM overnight. Expression of slit in the 293-HEK cell line has been demonstrated by western blots previously (Li et al., 1999; Wu et al., 1999). Demonstration of netrin-1 expression in the 293-EBNA transfected cells has been shown previously (Shirasaki et al., 1996).

Explant culture preparation

Explant culture of the ventral midbrain was prepared from E14 embryos. The ventral midbrain was dissected out and cut into small squares in cold DMEM/F12 medium. Squares were transferred into 35 × 10 mm culture dishes. Collagen solution was prepared by mixing (5:1) rat-tail collagen type1 (BD Bioscience, Bedford, MA) with 5× MEM. Thirty-five-microliter collagen solution was pipetted onto the bottom of the dishes and allowed to gel, followed by adding a growth medium DMEM/F12 supplemented with 10% fetal bovine serum and antibiotics. To determine effects of netrin-1 on DA axonal growth, a recombinant netrin-1 (R&D systems, Inc., Minneapolis, MN) was applied to the medium at 200 ng/ml. The concentration of netrin-1 chosen was based on a previous study showing that netrin-1 between 100 and 400 ng/ml promoted axonal growth (Metin et al., 1997). In addition, a blocking antibody against DCC (clone AF5; Oncogene, San Diego, CA) was added to cultures at 10 μg/ml. This antibody solution contains 0.1% sodium azide. The amount of sodium azide contained in the volume of the antibody solution used in the present study did not have an apparent toxic effect on explant growth (data not shown). Species-matched immunoglobulin was used as a control. When preparing the co-culture of VM explants with cell aggregates, cell aggregates were positioned 50–200 μm apart from VM fragments. Cultures were placed in a humidified incubator at 37°C with 5% CO^2 for 1.5 days and were then fixed with 4% paraformaldehyde in 0.1 M PB (pH 7.4) for 30 min at room temperature.

Co-culture of dissociated VM cells with slit-2 expressing cells

HEK-293 cells expressing Slit-2 or control plasmids were plated on coverslips coated with polyornithrine (100 μg/ml) and incubated with the same growing medium described above. VM cell suspension was prepared from E14 embryos and 10^5 cells were seeded per well when slit-2 or control cells became 80% confluent. Cells were cultured for 2 days and fixed with 4% paraformaldehyde in PB (pH 7.4). For function blocking experiment, conditioned medium collected from confluent cultures of the HEK-293 cells expressing extracellular domain of robo (RoboN) was added into culture medium at dilutions of 1:10, 1:5, and 1:2. RoboN carries a hemagglutinin and can bind to slit-2 but is incapable of the stimulating signaling pathway (Wu et al., 1999). RoboN has been used as a competitive blocker for slit-induced activities (Sang et al., 2002; Zhu et al., 1999).

Immunofluorescence

A routine fluorescent immunohistochemistry was performed in VM primary and explant cultures (Sang et al., 2002). In brief, coverslips and explants were pretreated with 10% normal donkey serum (Jackson Laboratories, Maine) and 0.1% Triton X-100 in 0.1 M phosphate-buffered saline (PBS) for 1 h at room temperature, followed by incubation with primary antibodies at 4°C overnight and then with appropriate secondary antibodies conjugated with distinct fluorescence at room temperature for 1 h. Antibodies against calbindin $D_{28}K$ (1:300; Swant, Swiss), DCC (1:50; Pharmingen, San Diego, CA), GIRK2 (1:80; Alomone Labs, Jerusalem, Israel), β III tubulin (1:2000, Covance, Berkeley, CA), TH (1:300, Pel Freez, Rogers, AK) were used. Antibodies against robo-1 and robo-2 (1:50, Santa Cruz Biotechnology, Santa Cruz, CA) used in the study have been shown to be specific (Anselmo et al., 2003). Secondary antibodies conjugated with Alexa 488 and 568 (1:500; Molecular Probes, Eugene, OR) and Cy5 (1:100; Jackson Immunoresearch, West Grove, PA) were used. Omission of primary antibodies or antibodies pre-incubated with excess antigens were served as control. Fluorescent signal was examined by a confocal imaging system (LSM510 META, Carl Zeiss, Thornwood, NY).

Quantitative and statistical analyses

The area occupied by neurites in the VM explants was highlighted by using a function of threshold and measured using MetaMorph software (Universal Imaging Corporation, Downingtown, PA). Data were normalized as pixel/μm^2 explant to exclude size difference of explant culture preparations. Numbers of neurites and fascicles from proximal or distal aspect of the explants cultured with cell aggregates were counted at 20× using stereology software (Microbrightfield, Inc., Williston, VT). Neurite lengths of TH neurons in the co-cultures of dissociated VM cells and slit-2 or control HEK-293 cells were measured using stereology. This measurement was performed on 40× confocal images taken randomly on triplicate coverslips per condition and from duplicate experiments. The neurons with unambiguous extension of neurites

from cell soma were measured. Statistical analyses were carried out using InStat software (GraphPad, Inc, San Diego, CA). P values < 0.05 were considered significantly different.

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