DCC association with lipid rafts is required for netrin-1-mediated axon guidance

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Summary

During development, axons migrate long distances in responses to attractive or repulsive signals that are detected by their growth cones. One of these signals is mediated by netrin-1, a diffusible laminin-related molecule that both attracts and repels growth cones via interaction with its receptor DCC (deleted in colorectal cancer). Here we show that DCC in both commissural neurons and immortalized cells, is partially associated with cholesterol- and sphingolipid-enriched membrane domains named lipid rafts. This localization of DCC in lipid rafts is mediated by the palmitoylation within its transmembrane region. Moreover, this raft localization of DCC is required for netrin-1-induced DCC-dependent ERK activation, and netrin-1-mediated axon outgrowth requires lipid raft integrity. Thus, the presence of axon guidance-related receptors in lipid rafts appears to be a crucial pre-requisite for growth cone response to chemo-attractive or repulsive cues.

Key words: DCC, netrin-1, lipid raft, axon outgrowth, palmitoylation

Introduction

Tessier-Lavigne and colleagues purified netrin-1 as a laminin-related molecule that is expressed by the floor plate of the developing nervous system and that guides/attracts commissural axons (Serafini et al., 1994; Tessier-Lavigne and Goodman, 1996). Netrin-1 has further been shown to be a central axon guidance cue as netrin-1 mutant mice exhibit severe malformations in the brain, believed to be mainly due to aberrant axonal trajectories (Serafini et al., 1996). A variety of functional netrin-1 receptors have been identified including DCC (deleted in colorectal cancer), UNC5H1, UNC5H2, UNC5H3 and the adenosine receptor A2b (Corset et al., 2000; Keino-Masu et al., 1996; Leonardo et al., 1997). DCC, however, appears to be the central receptor for netrin-1-mediated signalling not only because a DCC blocking antibody has been shown to inhibit the biological effects of netrin-1 on axon guidance (Keino-Masu et al., 1996) but also because a DCC mutant phenocopied netrin-1 mutant mice (Fazeli et al., 1997). DCC is a large receptor that is homologous to the NCAM family of proteins and has been proposed to be tumour suppressor because of the loss of DCC expression in a large number of cancers (Mehlen and Fearon, 2004). The proposed function of the netrin-1/DCC interaction in axon guidance is that DCC, expressed on growth cones, is stimulated by an extracellular gradient of netrin-1, leading to the intracellular activation of small GTPase Rac-1 (Li et al., 2002) or the MAPK signalling pathway (Forcet et al., 2002).

Growth cones are highly motile structures known to be submitted to dynamic changes in their actin cytoskeleton and subsequent membrane shape. The importance of the contacts between the extracellular environment, i.e. axonal cues, and the proteins present at the cell plasma membrane, i.e. guidance receptors, led us to investigate if a specific compartimentalized location of DCC to specific plasma membrane domains is required for DCC's efficient signalling. Accumulating evidence suggests that lipid rafts are dynamic, tightly packed and ordered membrane microdomains enriched in sphingolipids and cholesterol (Pike, 2004; Simons and Toomre, 2000; Hueber, 2003). This liquid ordered phase favours the dynamic assembly of different lipid-anchored proteins as well as transmembrane proteins (Simons and Ikonen, 1997; Brown and London, 1998). Recent studies have shown that rafts play an important role in cell signalling, in particular through the organization of surface receptors, signalling enzymes and adaptor molecules into complexes at specific sites in the membrane (Simons and Toomre, 2000). More recently, Guirland and colleagues detected some axonal guidance receptors, including DCC, in lipid rafts (Guirland et al., 2004); a situation required for *Xenopus* axons turning. In this study, we have investigated whether DCC is associated with lipid rafts in mammalian cells and neurons, and propose a mechanism for this lipid raft localization. We have also analysed whether this association in lipid rafts is important for netrin-1-mediated commissural neurons signalling and axon outgrowth.

Materials and Methods

Cells, transfection procedures, immunoblotting, FACs analysis and netrin-1 production

Transient transfections of human embryonic kidney 293 cells were performed as previously described (Forcet et al., 2002). One dimensional immunoblots using different commercially available antibodies raised against DCC, c-myc (netrin-1), Fyn, Fas, Rab5 and TfR were performed as previously described (Forcet et al., 2002; Hueber et al., 2002). Netrin-1 was purified from netrin-1-producing 293-EBNA cells according to the method of Serafini et al. (Serafini et al., 1994). Horseradish peroxidase (HRP)-coupled secondary antibodies was from Jackson Immunoresearch Laboratories. CTB-HRP was from Sigma-Aldrich. FACs analyses were performed as described previously (Mehlen et al., 1998) using a FACsCalibur (BD).

Site-directed mutagenesis and plasmid constructs

Full-length DCC-expressing construct pDCC-CMV-S and netrin-1-expressing construct pGNET1-myc have been described previously (Mehlen et al., 1998); p-HA-DCC-CMV-S encoding full-length DCC with a N-terminal HA motif was derived from pDCC-CMV-S by a Quikchange strategy (Stratagene) using the following primers: 5′-CACAGGCTCAGCCTTTTATCCATATGATGATCCGGATTATGC-3′ and 5′-CATTCAGAAATACATGTTAATGCATAATCCGGTACATC-ATATG-3′. DCC C1121V was obtained similarly by a Quikchange strategy using p-HA-DCC-CMV-S as a template and the following primers 5′-GTG GCT GTG ATT GTC ACC CGA CGC TCT TCA-3′ and 5′-TGA AGA GCG TCG GGT GAC AAT CAC AGC CAC-3′.

Biochemical raft separation

Rafts were isolated as described previously (Hueber et al., 2002). Briefly, PNS (post nuclear supernatant) from (3×10^7) HEK 293 cells or (3×10^6) commissural neurons was solubilized in 1 ml buffer A (25 mM Hepes, 150 mM NaCl, 1 mM EGTA, protease inhibitors cocktail) containing 1% Brij 98 for 5 minutes at 37°C and chilled on ice before being placed at the bottom of a step sucrose gradient (0.9-0.867-0.833-0.8-0.767-0.733-0.7-0.6-1.33 M sucrose) in buffer A. Gradients were centrifuged at 250,000 ***g*** for 16 hours in a SW41 rotor (Beckman Instruments Inc.) at 4°C. One ml fractions were harvested from the top, except for the last one (no. 9) that was 3 ml. The DIM fraction contains pooled fractions 1-4 and the heavy fraction (HF) consists of pooled fractions 8 and 9. For DIM isolation in cold Triton X-100, PNS was solubilized in 1% Triton X-100 at 4°C for 1 hour before centrifugation onto a sucrose density gradient.

In vivo tritium palmitate labelling

[9, 10 (n)-^{3}H]palmitic acid (specific activity 60 Ci/mmol; Amersham Biosciences) was added to the medium of transiently DCC-transfected 293 cells at a concentration of 0.2 mCi/ml in the absence of serum and incubated for 5 hours at 37°C. Cells were harvested, washed, and immunoprecipitation experiments were performed using an anti-HA antibody (16B12, Babco) for DCC pull down or anti-Fyn antibody (Fyn3, Sc16, Santa Cruz) for Fyn pull down.

Commissural neurons culture

The dorsal spinal cord from E13 rat embryos were dissected as described previously (Serafini et al., 1994). The tissues were then dissociated using 5 mg/ml trypsin and 0.1 mg/ml DNaseI (Sigma) in HBSS without calcium and magnesium (Invitrogen), the dissociation was stopped with DMEM and 10% fetal calf serum. Dissociated cells obtained were plated on poly-L-lysine precoated coverslips at 1.2×10^5 cells per well in neurobasal medium supplemented in B27 (Gibco).

Commissural axon outgrowth

Dorsal spinal cord explants from E13 rat embryos were cultured as previously described (Serafini et al., 1994; Corset et al., 2000; Forcet et al., 2002). Axons were stained with an anti-β-tubulin antibody (Babco). When commissural axon outgrowth was induced by floor plate explants, ventral spinal cord were dissected out and included in the collagen matrix near the dorsal explant. Axonal length was quantified as previously described (Corset et al., 2000). Briefly, the total length of axon bundles was measured for each explant and normalized to the values obtained from explants cultured with purified netrin-1.

Cholesterol depletion treatment

HEK293 cells and E13 dissociated commissural neurons were incubated at 37°C in preheated serum-free Hepes buffer (10 mM) containing either 10 mM MβCD (Sigma-Aldrich) for 12 minutes, or 2 U/ml of cholesterol oxidase (CO; Calbiochem) for 2 hours. Following drug treatment, cells were washed once before raft isolation. Spinal cords explants, 1 hour after dissection, were washed once with PBS and incubated in 37°C preheated serum-free explant culture medium in the presence of either 10 mM Hepes and 10 mM MβCD at 37°C for 12 min, or 2 U/ml of CO at 37°C for 1 hour (CO1) or 1 U/ml of CO for 1.5 hours (CO2). Explants were then washed twice and incubated at 37°C with explant culture medium for 16 hours. For the depletion-repletion experiments, explants were treated with cholesterol (1 mM; Sigma-Aldrich) for 30 minutes at 37°C. The unincorporated cholesterol was removed and explants were then washed twice and incubated at 37°C with explant culture medium for 16 hours.

MAPK activity assay

ERK-1/2 phosphorylation was analysed on both HEK293 and E13 commissural neurons using the Face ERK-1/2 kit (Active Motif). ERK-1/2 kinase activity was analysed using a MBP kinase assay as described previously (Forcet et al., 2002). To determine the specificity of the DCC-dependent signal, the blocking Ab-1 anti-DCC antibody was used as described previously (Forcet et al., 2002).

Results and Discussion

We first investigated whether DCC is associated with lipid rafts. These membrane domains can be isolated thanks to their characteristic detergent insolubility upon membrane (DIM) solubilization in either Brij 98 or Triton X-100, and separated from the disordered membrane environments by sucrose density gradient centrifugation.

We found that in HEK293 cells, following solubilization in either Brij 98 (Fig. 1A) or in Triton X-100 (Fig. 1B) a substantial proportion of DCC (52.2%), was found in the lipid raft-containing light fractions that are also enriched in raft markers such as Fyn and GM1 glycosphingolipid (Fig. 1A). The non-raft markers such as Rab5 and TfR (Transferrin receptor) were exclusively detected in heavy non raft fractions (HF; Fig. 1C, and not shown). To further delineate the cholesterol dependence of the buoyant fractions, we tested the effect of methyl-β-cyclodextrin (MβCD). As seen in Fig. 1A, pre-incubation of the cells with MβCD completely abolished the presence of Fyn raft marker and DCC in the buoyant fractions. Moreover, we found that cholesterol oxidase (CO), a cholesterol depleting agent that has been reported to affect more specifically caveolae/raft structure (Okamoto et al., 2000) than does MβCD (Rodal et al., 1999) had a similar effect (Fig. 1A).

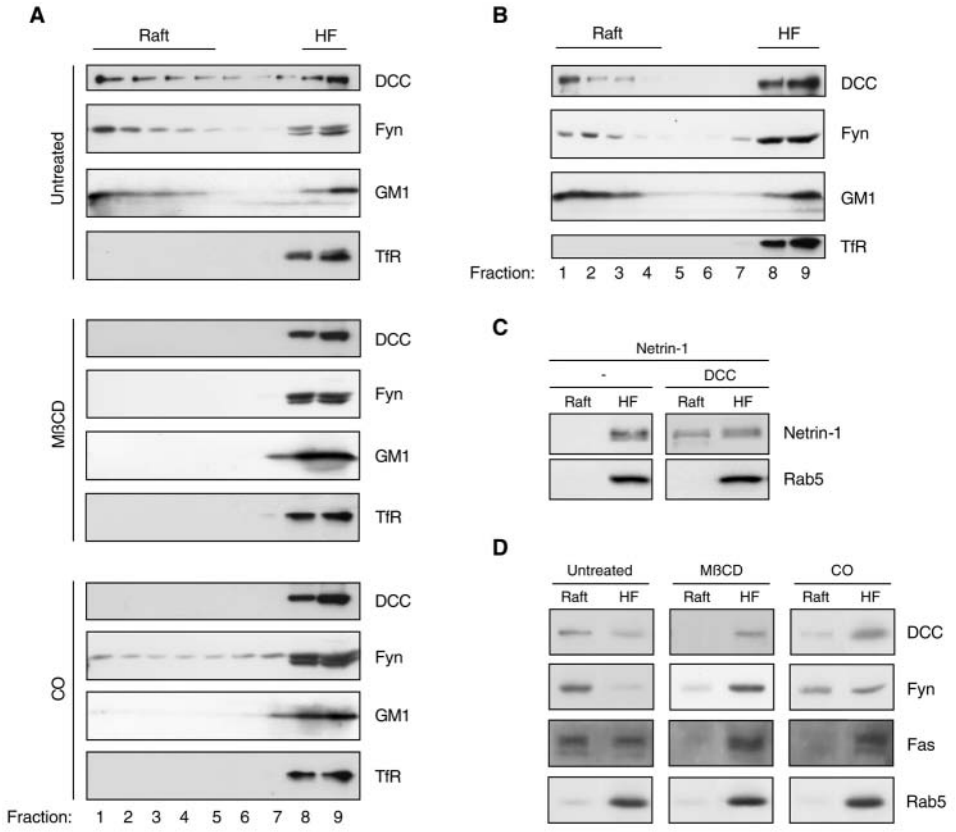

Fig. 1. Ligand-independent partitioning of DCC in lipid membrane rafts. (A) HEK293 cells were transfected with a DCC-expressing construct and 24 hours after transfection, cell were treated or not with either MβCD or CO as described in the Materials and Methods. The cell lysates were solubilized in Brij 98 and subjected to sucrose gradient separation. Immunoblots performed on the different sucrose fractions were revealed with HRP conjugated anti-DCC, anti-Fyn or anti-TfR antibodies, or with cholera toxin B-HRP (GM1). (B) Same as A but Triton X-100 was used instead of Brij 98. (C) HEK293 cells were transfected with netrin-1 and/or DCC-expressing constructs and 24 hours after transfection cell lysates were solubilized in Brij 98 and subjected to sucrose gradient separation. Immunoblots performed on pooled heavy fractions (8 and 9) and light fractions (1-4) were revealed with HRP-conjugated anti-netrin-1 and anti-Rab5 antibodies. (D) DCC is in lipid rafts in commissural neurons. Same as in C but 3×10^6 commissural neurons dissociated from rat E13 embryos were used instead of HEK293 cells. Raft, raft containing fraction; HF, heavy fraction. Raft inhibitors MβCD (10 mM for 12 minutes) or CO (2 U/ml for 1 hour) were included in the incubation at and just before the PNS preparation.

We then investigated whether DCC's ligand netrin-1 is also recruited to DIM. Interestingly, in HEK293 cells forced to express netrin-1, sucrose gradient separation failed to show netrin-1 localization in light fractions unless DCC was co-expressed (Fig. 1C). Thus, netrin-1 moves to lipid rafts when its receptor DCC is present and raft-localized.

The role of the DCC/netrin-1 pair has been mainly studied in developing commissural neurons (Serafini et al., 1994; Serafini et al., 1996; Forcet et al., 2002). Commissural neurons that express DCC are known to extend axons from the dorsal spinal cord to the ventral spinal cord as a result of the chemo-attractivity of netrin-1 synthesized by the floor plate. In order to determine whether DCC, expressed in commissural neurons, is also present in lipid rafts, commissural neurons were dissociated from E13 rat embryos. As shown in Fig. 1D a large fraction of the DCC was detected in DIM fractions together with raft-associated proteins Fyn and Fas. This localization was prevented if cells were pre-incubated with the cholesterol depleting agents MβCD or CO. Taken together, these data demonstrate that part of the DCC is localized in lipid rafts even in the absence of its ligand netrin-1, but netrin-1 is only raft associated in presence of DCC.

We next investigated the mechanism controlling DCC localization in rafts. Many raft-associated molecules have been shown to be palmitoylated (Resh, 1999; Linder and Deschenes, 2003) and DCC has a conserved cysteine residue within its transmembrane domain (Fig. 2A) that could be a potential palmitoylation site. [^{3}H]Palmitate cell labelling, followed by DCC pull-down shows that DCC is indeed palmitoylated (Fig. 2B). Mutation of the transmembrane cysteine C1121 of DCC to valine abrogated DCC palmitoylation (Fig. 2B). Moreover, in sucrose gradient separations, DCC C1121V shows a clear decreased raft localization when compared to wild-type DCC (Fig. 2C). A quantitative analysis performed on pooled raft fractions (Fig. 2D) shows that there was threefold more DCC than the DCC C1121V mutant in lipid rafts, but both DCC and DCC C1121V were present in equal amounts at the HEK293 cell surface as visualized by FACs analysis (Fig. 2D). Thus, DCC palmitoylation is required for lipid raft association.

We then investigated the functional role of DCC raft localization on netrin-1-mediated DCC-dependent axon outgrowth. To determine whether DCC is required in lipid rafts for netrin-1 function, dorsal spinal cord explants from E13 rat embryos were grown in control and raft-disrupted conditions for 16-18 hours in collagen gels with or without purified netrin-1 (Forcet et al., 2002). As previously shown (Serafini et al., 1994; Forcet et al., 2002), the presence of netrin-1 promoted axon outgrowth (Fig. 3). However, addition of CO blocked netrin-1-induced axon extension (P<0.0001; Fig. 3). Similar results were obtained when netrin-1 was provided by floor plate explants (Fig. 3A,B) or when lipid rafts were disrupted by treatment with MβCD (Fig. 3A,B). This effect does not represent a general inhibition of axon outgrowth (e.g. due to the drugs toxicity) but appears to be specific for netrin-1 signalling, since netrin-independent commissural axon outgrowth that can be observed when spinal cord explants are grown for 40 hours (Serafini et al., 1994; Corset et al., 2000), is unaffected by CO [Fig. 3C, total axon length per explant (mean ± s.e.m.), without CO: 427±46 μm

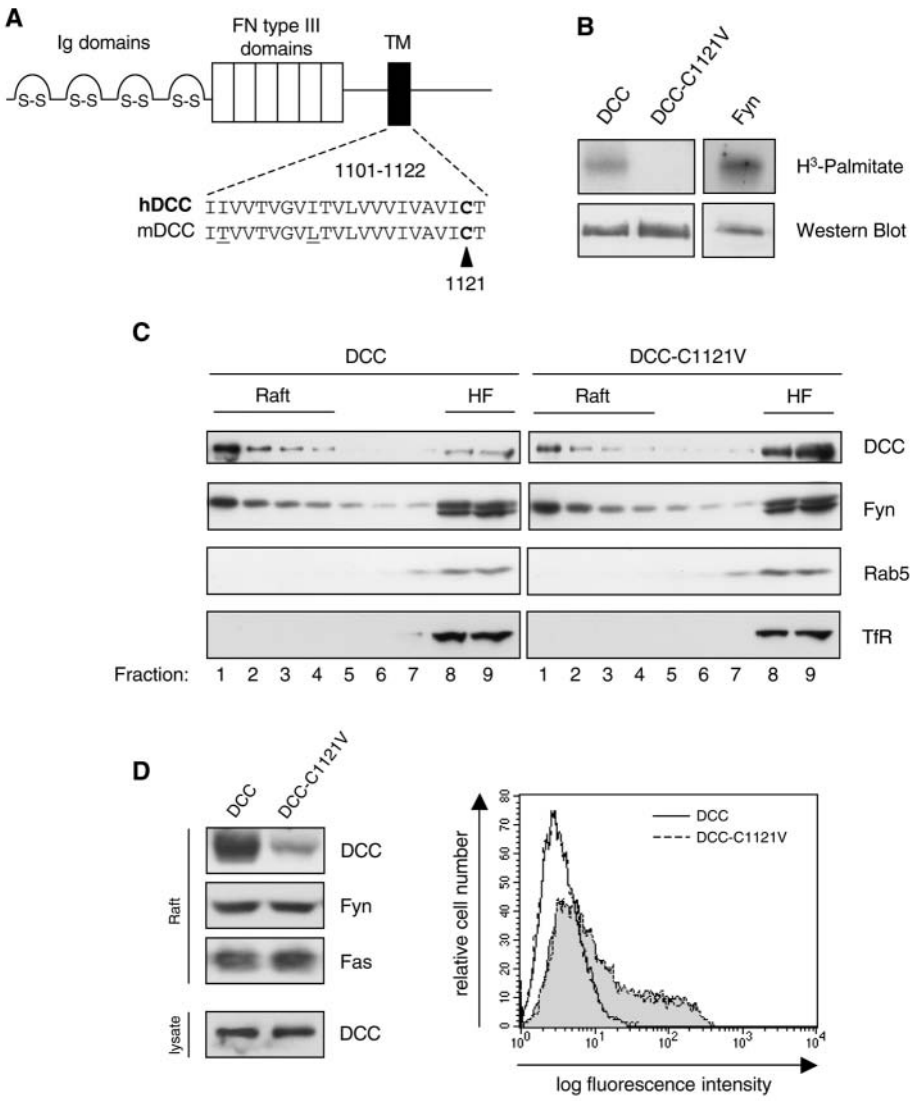

Fig. 2. DCC is palmitoylated. (A) Schematic representation of DCC. (B) Wild-type DCC, mutant C1121V DCC, or Fyn-transfected HEK293 cells were labelled with [^{3}H]palmitate. Cell lysates were immunoprecipitated as described in the Materials and Methods and analysed by both autoradiography and western blotting. (C,D) HEK293 cells were transiently transfected with wild-type DCC or the DCC C1121V mutant. (C) Rafts were then isolated as in Fig. 1A and analysed for DCC, Fyn, Rab5 and TfR expression by western blotting. (D) Raft-containing fractions (Raft) and heavy fractions (HF) were pooled as in Fig. 1C. A quantification of DCC in raft fractions was performed using the GeneTools software. Right: a flow cytometric analysis of DCC expression performed on non-permeabilized wild-type DCC- or DCC C1121V mutant-transfected HEK293 cells (Immunostaining performed using anti-DCC antibody). The experiments shown in C and D are representative of at least three experiments.

(n=7); with CO: 444±86 μm (n=9)]. However, when treated with CO, both the netrin-1-independent grown axons and the few axons grown in response to netrin-1 appear to display a loss of fasciculation, suggesting that lipid raft disruption also affects a general regulator of fasciculation independent of the DCC signalling pathway, e.g. signalling pathways for cell adhesion molecules that were shown to be raft dependent (Niethammer et al., 2002) (Fig. 3C). The inhibition of netrin-1-induced axon outgrowth by cholesterol-depleting drugs is probably directly related to the effect on cholesterol level and not to any other effects of these drugs since the treatment with MβCB followed by repletion of cholesterol only modestly affected netrin-1-mediated axon outgrowth (Fig. 3D). Thus, lipid raft disruption inhibits netrin-1-mediated DCC-dependent commissural axon outgrowth.

We then investigated whether the axonal loss of response to netrin-1 following pretreatment with raft inhibitors was associated with the loss of intracellular signals known to be required for netrin-1-mediated axon guidance. ERK-1/2 MAPK have been shown to be activated upon binding of netrin-1 to DCC and to be required for netrin-1-mediated DCC-dependent axon outgrowth and turning (Forcet et al., 2002; Ming et al., 2002; Campbell and Holt, 2003). Therefore, we analysed ERK-1/2 activity by measuring both ERK-1/2 phosphorylation status and the ability of immunoprecipitated ERK-1/2 to phosphorylate myelin basic protein (MBP) in vitro. In accordance with previous results (Forcet et al., 2002), netrin-1 induced both DCC-dependent ERK-1/2 phosphorylation (Fig. 4A) and ERK-1/2 kinase activity (Fig. 4C). However, ERK-1/2 phosphorylation and kinase activity were completely abrogated when the defective palmitoylation DCC mutant C1121V was expressed instead of wild-type DCC (Fig. 4A). Similar inhibition of ERK-1/2 activation was observed when HEK293 cells were pre-treated with CO (Fig. 4A,C). Netrin-1-mediated DCC-dependent ERK-1/2 phosphorylation observed in E13 commissural neurons was abrogated in CO-treated commissural neurons and was comparable to the level of phosphorylation observed when commissural neurons were pre-incubated with a DCC blocking antibody (Fig. 4B) (Forcet et al., 2002). Moreover, the inhibition of netrin-1-induced ERK-1/2 phosphorylation by CO treatment is probably not just a general toxic effect that affects all intracellular signals because CO treatment failed to inhibit serum-induced ERK-1/2 phosphorylation in commissural neurons (not shown). Thus, DCC localization in lipid rafts is required to mediate guidance effects of netrin-1 most likely because this localization is a pre-requisite for transmission of the correct intracellular signals. Interestingly, it is intriguing to see that both netrin-1-induced ERK-1/2 activation and the requirement for raft integrity occur in a very brief and early/initial window of time, i.e. ERK-1/2 activation is no longer detected 30 minutes after netrin-1 application (Forcet et al., 2002) while the stock of cholesterol is back to normal

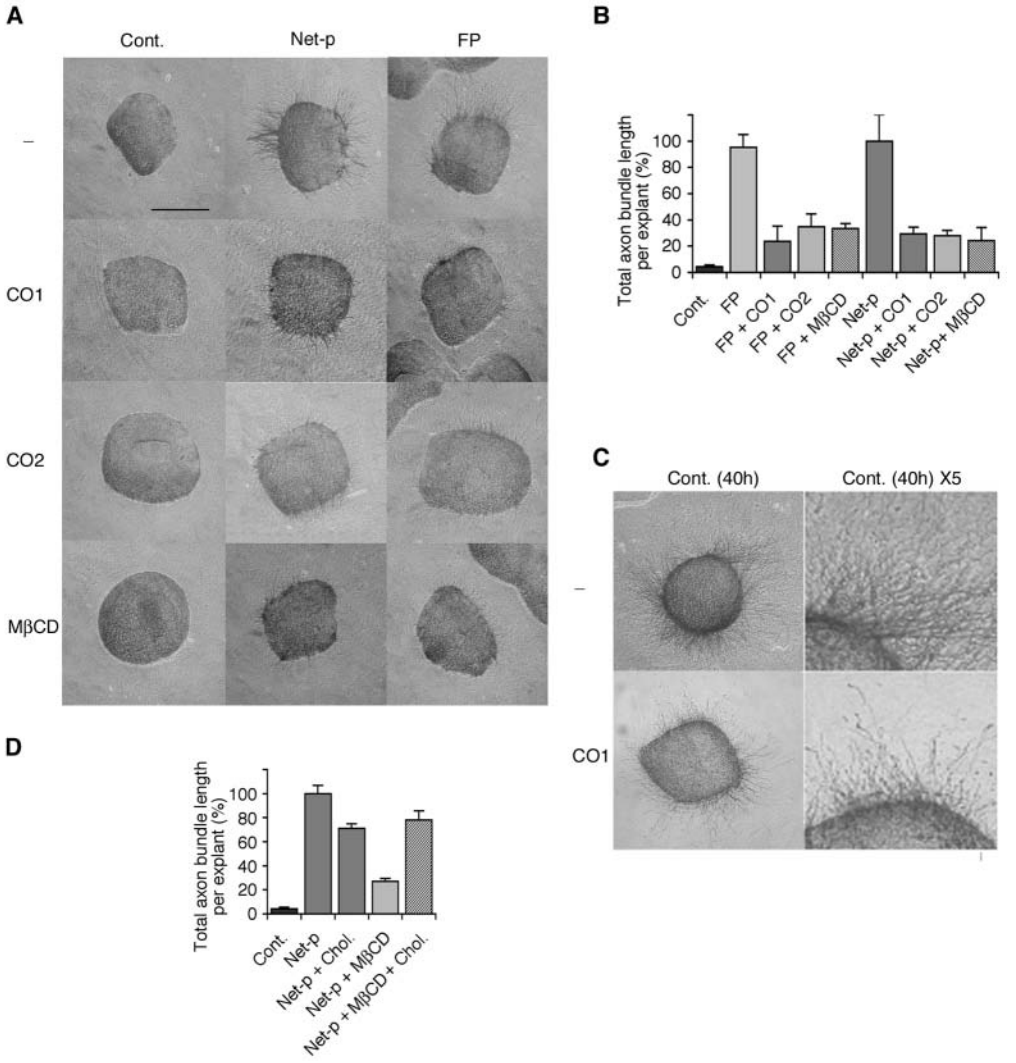

Fig. 3. Netrin-1-mediated DCC-dependent commissural axon outgrowth requires lipid raft integrity. E13 dorsal spinal cord explants were cultured for 18 hours (A,B) or 40 hours (C) in collagen gel either without a netrin-1 source (–), or with purified netrin-1 (Net-p) or next to ventral spinal cord explants (FP). As described in the Materials and Methods, explants were either left untreated (–), treated for 1 hour with 2 U/ml of CO (CO1), 1.5 hours with 1 U/ml of CO (CO2) or 12 minutes with 10 mM MβCD. (B) Quantification of A. The total number of explants that were quantified from four distinct experiments varies from 10 to 15 per tested condition. Values shown are means ± s.e.m. Bars, 170 μm. (D) Quantification (as in B) of the effect of depletion-repletion in cholesterol on E13 dorsal spinal cord explants. Explants were incubated with MβCD as in A and further treated with 1 mM cholesterol and then cultured for 18 hours in collagen gel. Values shown are percentages of netrin-1 treated explants (100%) ± s.e.m.

probably very shortly after the brief treatments for cholesterol depletion. Thus, both DCC localization in lipid rafts and activation of MAPK are probably required for the initiation of neuron outgrowth.

Our results confirm that DCC is a constitutive part of lipid raft microdomains and requires this localization to function as the receptor of the netrin-1 signal. Interestingly, netrin-1 presence does not alter the proportion of DCC in rafts (Fig. 1 and not shown), thus suggesting that growth cones do not reorganize DCC microdomains in response to the source of netrin-1 but rather may adapt an adequate and specific response to netrin-1 because of DCC partitioning in rafts. Thus, by regulating not only receptor expression (Stein and Tessier-Lavigne, 2001) but also lipid raft composition, the growth cone may 'control' the adequate growth response to a cue, i.e. from complete unresponsiveness to chemo-attraction. Interestingly,

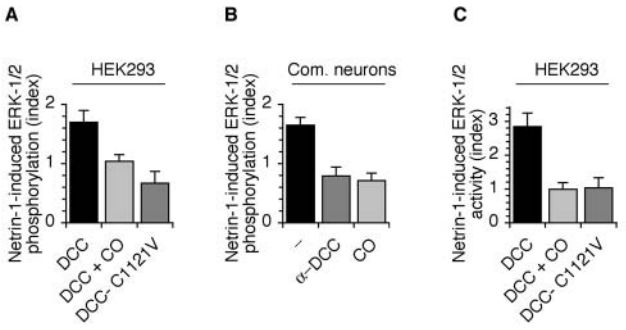

Fig. 4. Netrin-1-induced DCC-mediated ERK-1/2 activation is dependent on DCC association with lipid rafts. (A) Wild-type DCC- or mutant C1121V DCC-expressing HEK293 cells were pre-treated or not for 1 hour with 2 U/ml of CO and then treated for 15 minutes with purified netrin-1. Phosphorylated ERK-1/2 was compared with total ERK-1/2 according to the Face ERK-1/2 kit instructions. An index of ERK-1/2 phosphorylation is presented as the ratio between netrin-1-treated cells and untreated cells. (B) Dissociated E13 commissural neurons were pre-treated or not for 1 hour with 2 U/ml of CO and then treated for 15 minutes with purified netrin-1. To confirm the role of DCC in the ERK-1/2 phosphorylation observed in commissural neurons, a blocking DCC antibody was also added (α-DCC) as previously described (Forcet et al., 2002). Phosphorylated ERK-1/2 was compared with total ERK-1/2 according to Face ERK-1/2 kit instructions. An index of ERK-1/2 phosphorylation is presented as the ratio between netrin-1 treated cells and untreated cells. (C) Same as A except than ERK-1/2 activity was analysed by an in vitro MBP kinase assay following ERK-1/2 immunoprecipitation.

axonal guidance cues such as EphrinA were shown to be raft located through their glycosyl-phosphatidylinositol (GPI) anchors (Gauthier and Robbins, 2003). Similarly, molecules involved in neuritogenesis, such as NCAM, L1 or integrins, have been shown to localized in a ligand-dependent manner in lipid rafts (Kleene et al., 2001; Niethammer et al., 2002). Here we show that the axonal guidance transmembrane receptor DCC is constitutively present in rafts, a localization required for its appropriate growth promoting function. Guirland et al. has recently observed a similar requirement of DCC in lipid rafts for netrin-1-induced axon turning (Guirland et al., 2004). Thus, it is tempting to speculate that other axon guidance pairs such as semaphorin/plexin or Ephrin/EPH may also have functions dictated by receptor localization in rafts. Consequently, local chemical modulation of rafts could be used as an interesting tool for potentiating axon outgrowth or re-growth.

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