Amyloid Precursor Protein Regulates Netrin-1-mediated Commissural Axon Outgrowth

Nicolas Rama1, David Goldschneider1, Véronique Corset, Jérémy Lambert, Laurent Pays, and Patrick Mehlen2

From the Apoptosis, Cancer and Development Laboratory, Equipe labellisée ‘La Ligue,’ Centre de Cancérologie de Lyon, INSERM U1052-CNRS UMR5286, Université de Lyon, Centre Léon Bérard, 69008 Lyon, France

Received for publication, November 17, 2011, and in revised form, July 2, 2012. Published, JBC Papers in Press, July 10, 2012, DOI 10.1074/jbc.M111.324780

Background: The biological role of amyloid precursor protein (APP) during development is unknown. Results: We show that APP modulates netrin-1-mediated DCC-induced signaling. We also show the requirement for APP in netrin-1-mediated commissural axon outgrowth. Conclusion: APP is important for the netrin-1 role during neuronal navigation. Significance: In addition to its known role in Alzheimer disease, APP appears to be a co-receptor for neuronal wiring during development.

The multifunctional protein netrin-1 was initially discovered as the main attractive cue for commissural axon guidance by acting through its receptor DCC. Recently, we have shown that netrin-1 also interacts with the orphan transmembrane receptor amyloid precursor protein (APP). APP is cleaved by proteases, generating amyloid-β peptide, the main component of the amyloid plaques that are associated with Alzheimer disease. Our previous work demonstrated that via its interaction with APP, netrin-1 is a negative regulator of amyloid-β production in adult brain, but the biological relevance of APP/netrin-1 interaction under non-pathological conditions was unknown. We show here that during commissural axon navigation, APP, expressed at the growth cone, is part of the DCC receptor complex mediating netrin-1-dependent axon guidance. APP interacts with DCC in the presence of netrin-1 and enhances netrin-1-mediated DCC intracellular signaling, such as MAPK activation. Inactivation of APP in mice is associated with reduced commissural axon outgrowth. Thus, APP functionally acts as a co-receptor for DCC to mediate axon guidance.

The diffusible laminin-related molecule netrin-1 is now recognized as a multifunctional protein with roles as diverse as tissue morphogenesis, developmental angiogenesis, and tumor progression (1–3). However, netrin-1 remains the first identified guidance cue implicated in neuronal navigation (4, 5). Specifically, netrin-1 is known to be expressed by the ventral structure of the neural tube, the floor plate, to guide/attract commissural axons during nervous system development (4, 5). Definitive evidence supporting netrin-1 as a central axon guidance cue was provided by the description of netrin-1 mutant mice (17). Similarly, it has recently been shown that the sonic hedgehog receptor Boc participates in the guidance of commissural axons, DCC, and Boc co-localizing at the growth cone (18, 19). In a recent search for proteins interacting with DCC, we identified, through a proteomic approach, the amyloid precursor protein (APP) receptor, a protein known mostly as the precursor for amyloid-β (Aβ) peptide in Alzheimer disease, and we described APP as a novel receptor for netrin-1 (20).

Alzheimer disease, the most common form of dementia, is a progressive neurodegenerative disorder characterized by extracellular deposits of Aβ peptide in senile plaques, intraneuronal

* This work was supported by an institutional grant from CNRS, University of Lyon, Centre Léon Bérard, and by the Ligue contre le Cancer, L’Institut National du Cancer (INCa), and L’Agence Nationale de la Recherche (ANR).
1 This article contains supplemental Figs. 1–3.
2 To whom correspondence should be addressed. Fax: 33-47-878-2887; E-mail: patrick.mehlen@lyon.unicancer.fr.

© 2012 by The American Society for Biochemistry and Molecular Biology, Inc. Published in the U.S.A.
neurofibrillary tangles, synapse loss, and cognitive decline (21). It is widely believed that the accumulation of Aβ, a small peptide with a high propensity to form oligomers and aggregates, is central to the pathogenesis of Alzheimer disease. Aβ derives from the proteolytic cleavage of APP (22). We thus demonstrated that netrin-1 functions as a ligand for APP and that it negatively regulates Aβ peptide production in Alzheimer model transgenic mice. We also provided the proof of concept that netrin-1 brain administration may represent an appealing strategy to improve the Alzheimer phenotype (20).

However, although much is known about interacting proteins and processing events for APP, the physiological role(s) of APP and its related family members, APLP1 and APLP2 (amyloid precursor-like proteins 1 and 2), is still poorly understood (22, 23). APP has been proposed to function in cell adhesion and motility, as well as synaptic transmission and plasticity (for review, see Ref. 23). Of interest, analysis of APP-null mice revealed defects similar to those observed in both netrin-1 and DCC mutant mice. These defects, the severity of which depends on the genetic background, include abnormalities in the developing corpus callosum and other commissures (24). Here, we hypothesized that these similar defects argue for a role of APP in mediating netrin-1-dependent axon guidance, and we thus analyzed whether APP participates in the guidance of commissural neurons.

**EXPERIMENTAL PROCEDURES**

**APP Mutant Mice**—Pregnant OF1 mice were purchased from Charles River Laboratories. APP mutant mice were genotyped as described (20). Genotyping of APP embryos was performed by PCR using primers 5′-GAGACGAGACAGCT-CAGTCCCTAGGG-3′, 5′-ATACACTGGTTCTTAATCAGAG-GCCC-3′, and 5′-CGAGATCGAGACCTCTGTTCACACA-3′.

**Immunohistochemistry and in Situ Hybridization**—Mouse embryos were fixed overnight in 4% paraformaldehyde at 4 °C and equilibrated in PBS containing 10% sucrose. Thereafter, mouse embryos were included in 7.5% gelatin and 10% sucrose. Thereafter, embryos were fixed overnight in 4% paraformaldehyde at 4 °C and equilibrated in PBS containing 10% sucrose. Thereafter, mouse embryos were included in 7.5% gelatin and 10% sucrose. Transversal sectioning was performed with a cryostat. DCC and APP immunolocalization was performed on 20-μm sections for the mouse spinal cord. The sections or commissural neurons were permeabilized of 30 min in PBS containing 0.1% Triton X-100 and blocked for 4 h in PBS containing 3% normal donkey serum, 2% BSA, and 0.1% Triton X-100. Sections were then incubated overnight at 4 °C with anti-APP antibody (0.5 μg/ml, clone A20) or anti-APP antibody (0.15 μg/ml, A8717, Sigma). For immunoprecipitation, B103 cells were transfected with calcium phosphate. The γ-secretase inhibitor N-[N-(3,5-difluorophenylacetyl)-l-alanyl]-[(S)-phenylglycine t-buty] ester (DAPT) and the proteasome inhibitor benzyloxycarbonyl-Leu-Leu-Leu-al (MG132) were purchased from Sigma and used at 2 and 1 μM, respectively.

**Immunoprecipitation and Western Blotting**—Co-immunoprecipitations were processed as described (14) with anti-DCC antibody (0.5 μg/ml, clone A20) or anti-APP antibody (0.15 μg/ml, A8717, Sigma). For immunoprecipitation, B103 cells were transfected with calcium phosphate. The γ-secretase inhibitor N-[N-(3,5-difluorophenylacetyl)-l-alanyl]-[(S)-phenylglycine t-buty] ester (DAPT) and the proteasome inhibitor benzyloxycarbonyl-Leu-Leu-Leu-al (MG132) were purchased from Sigma and used at 2 and 1 μM, respectively.

**Image Analysis**—For the determination of commissural axon bundle size, images were acquired with Zeiss Axiowert 200M microscope and analyzed with Zeiss AxioVision software. The width of commissural axon bundles was quantified at d/2 (see Fig. 1C). The widths of the neural tube side (a) and commissural axon bundles (b) were measured. The b/a ratio was calculated. For co-localization analysis, acquisition was performed on a Zeiss Axioplan 2 LSM 510 confocal microscope, and images were analyzed using ImageJ software with the plugin JACoP.

**Plasmids**—For cell culture experiments, the constructs used were as follows: pCMV-DCC, pCR-DCC-IC, and pCMV-DCCΔEC (26); pcDNA3-APP, pcDNA3-APP-C100, pcDNA3-APP-C83, pGLUC, pCMV-Renilla, pMST, pMST-APP-Gal4, pCMV-Fe65, pcDNA3-APLP1, and pcDNA3-APLP2 (20); and pCMVneo-DCC-GIC, pGNT1-Myc, pcDNA3.1-UNC5H2, and pCS2-Notch (4, 27, 28).

**Cells, Transfection, and Reagents**—Transfections of B103 rat neuroblastoma cells were performed using Lipofectamine reagent (Invitrogen) according to the manufacturer’s instructions. For co-immunoprecipitation, B103 cells were transfected with pCMV-DCC, pCR-DCC-IC, and pCMV-DCCΔEC. Immunoblotting was performed using different commercially available antibodies: anti-phospho-ERK1/2 (Cell Signaling Technology); anti-ERK1/2, anti-c-Myc, and anti-HA (Sigma); anti-DCC (clone G97-449); anti-APP; and anti-APLP1 and anti-APLP2 (Abcam). Quantification of band intensity was carried out with ImageJ software.

**Primary Culture of Spinal Cord Commissural Neurons**—Commissural neurons were obtained from E11.5 mouse embryos by dissecting out the dorsal spinal cords. The tissues were then dissociated as described previously (29). The dissociated cells were plated on poly-l-lysine-precoated coverslips at 3 × 10^5 cells/well on a 24-well plate. Commissural neurons were cultured for 18 h in Neurobasal medium containing 2% horse serum. Commissural neurons were then cultured for 30 min in Neurobasal medium containing 300 μg/ml netrin-1 (AG-40B-0075, Adipogen) and fixed in 4% paraformaldehyde. Immunocytochemistry against APP and DCC was performed, followed by a co-localization assay.

**siRNA and Electroporation**—APP was silenced using commercialized Silencer® select siRNA (s62516, Life Technologies). A nonspecific scrambled siRNA, Silencer® select negative control No. 1 siRNA (4390843, Life Technologies), was the negative control. Transfection was performed by electroporation on E11.5 commissural neurons using a Neon™ system (MPK10096, Invitrogen) with 50 pmol of siRNA/1 × 10^6 cells.

**Transactivation Assays**—Transactivation assays were performed as described (27, 30).
Axon Outgrowth Assays—Commissural axon outgrowth assays were performed as described previously (9, 14). The outgrowth from E11.5 dorsal explants of mouse embryos was quantified by measuring the total length of all axon fascicles leaving the explant (mean length of axon bundles per explant).

Turning Assays—E10.5 whole mouse spinal cord explants were dissected, embedded in three-dimensional collagen matrices, and cultured for 18–20 h as described (18) together with an E10.5 mouse floor plate on its side. After TAG-1 immunostaining, whole mouse spinal cord explants were acquired with a Zeiss Axiovert 200 microscope, and the turning of commissural axons toward the ectopic floor plate was analyzed using ImageJ software.

APP/DCC/Netrin-1 Interaction Detection by ELISA—96-well plates were coated for 1 h at room temperature with α-APPs (the APP ectodomain up to the α-secretase cleavage, 6.25 nm, Sigma) in PBS. After 2 h of blocking at room temperature with 5% FCS in PBS, wells were washed with 0.05% Tween 20 in PBS and then incubated with the DCC ectodomain (R&D Systems) at 3.125–25 nm. This was done at room temperature for 1 h with or without netrin-1 at 5 nm. The washed wells were incubated with 0.1 μg/ml anti-DCC antibody (Ab1, Calbiochem) in the blocking buffer for 1 h at room temperature. Horseradish peroxidase-conjugated anti-mouse antibody (Jackson Immunoresearch Laboratories) was added at 0.8 μg/ml for 1 h at room temperature. The washed wells were then incubated with a chemiluminescent substrate (Pierce ECL Western blotting substrate). Luminescence was read on a Tecan Infinite P500 luminometer.

RESULTS

We first analyzed whether APP is expressed in developing commissural neurons. In situ hybridization was performed on E10.5 and E11.5 mouse spinal cord sections and revealed APP expression in commissural neurons (Fig. 1A). Immunohistochemistry was performed on tissue sections from E11.5 mouse embryos. As shown in Fig. 1B, APP was detected and enriched in commissural axon tracts. Confocal analysis further confirmed the expression of APP in these axons, as APP co-localized with DCC (with DCC being expressed only in commissural neurons at this stage) (Fig. 1B). This observation fits with the pan-genome expression analysis performed on commissural neurons at this stage (Fig. 1B). This observation fits with the pan-genome expression analysis performed on commissural neurons at this stage (Fig. 1B). This observation fits with the pan-genome expression analysis performed on commissural neurons at this stage (Fig. 1B).

We next assessed whether APP is somehow implicated in commissural axon guidance by analyzing commissural neuron bundles in APP mutant mouse embryos. Although we failed to notice any major defect in the number of bundles or in the overall pathway of commissural extension, we did observe a change in the distribution of axon bundles, with significantly thicker bundles in APP−/− embryos than in wild-type animals (Fig. 1, C–E). Such a phenotype resembles that observed in Boc mutant mouse embryos (19), thus supporting the view that APP is required for adequate commissural axon guidance.

We thus undertook to determine the precise role of APP in commissural axon guidance. We initially investigated APP following a search for DCC interactors by a proteomic approach (20). We thus analyzed whether APP interacts with DCC. B103 cells, which do not constitutively express APP and DCC, were forced to express APP and DCC in the presence or absence of netrin-1. As shown in Fig. 2 (A and B), DCC immunoprecipitation pulled down APP in the absence of netrin-1. However, the presence of netrin-1 strongly enhanced the interaction of APP and DCC. A similar interaction was observed with reverse co-immunoprecipitation using an APP pulldown (supplemental Fig. 1A). We further analyzed APP/DCC interaction by confocal analysis in a primary culture of commissural neurons from E11.5 mice. Whereas DCC was detected mainly in neuronal extensions, APP was detected in the growth cone and soma. Netrin-1 addition was associated with increased APP/DCC colocalization at the level of the growth cones (Fig. 2C and supplemental Fig. 1, B and C). The interaction between DCC and APP was also detected in vivo, as pulldown with anti-APP antibody allowed the detection of DCC in brain lysate from E11.5 mouse embryos (Fig. 2D). Interestingly, we investigated APP/DCC interaction in ventral spinal cords from E11.5 embryos, and as shown in Fig. 2E, DCC was detected in the APP pulldown. Thus, APP and DCC are expressed in developing commissural neurons, and the presence of netrin-1 strengthens APP interaction with DCC.

APP has two family members, APLP1 and APLP2, which have been proposed to play a partially redundant function in vivo (31). We thus investigated whether DCC could also interact with APLP1 and APLP2. As shown in Fig. 2 (F and G), in B103 cells forced to express DCC and APLP1 or APLP2, netrin-1 treatment induced a robust interaction between DCC and APLP1/APLP2.

We thus mapped the domains in DCC and APP implicated in APP/DCC interaction. Immunoprecipitations were performed using B103 cells forced to express different deletion mutants of either DCC or APP (Fig. 3A). The intracellular domain of DCC failed to interact with APP (Fig. 3B). The deletion of the DCC intracellular domain had no impact on APP/DCC interaction (Fig. 3B and data not shown). Thus, the domain of interaction of DCC with APP lies in the ectodomain of DCC. Of interest, a DCC mutant (i.e. DCCΔEC) that contains the complete intracellular domain of DCC plus the transmembrane domain (approximately up to the α-secretase cleavage of DCC) (30, 32) still interacted with APP, thus suggesting that the transmembrane region of DCC is important for APP/DCC interaction. However, netrin-1 treatment of cells expressing DCCΔEC and APP failed to strengthen APP/DCC interaction (Fig. 3B), further confirming that the complete ectodomain of DCC is required for the netrin-1 effect on the APP-DCC receptor complex.

While searching for the APP domain implicated in APP/DCC interaction, we noticed that the C-terminal fragment C83 derived from the γ-secretase cleavage of APP failed to interact with DCC (Fig. 3C), hence showing that DCC interacts with the APP ectodomain. Interestingly, the C100 protein, derived from the β-secretase cleavage of APP (Fig. 3A), was pulled down with DCC when netrin-1 was added (Fig. 3C and supplemental Fig. 1D), supporting the view that a major binding region of APP is localized in the Aβ domain of APP. We thus investigated

4 M. Tessier-Lavigne, personal communication.
whether the APP ectodomain directly interacts with DCC. The interaction of recombinant αAPPs with increasing concentrations of recombinant DCC ectodomain was thus assessed by ELISA in the presence or absence of netrin-1. As shown in Fig. 3D, whereas in the absence of netrin-1, DCC failed to interact with APP, in the presence of netrin-1, there was a strong DCC/αAPPs interaction. Together, these data support the view that netrin-1 enhances the interaction of APP and DCC that occurs via their respective ectodomains and includes, for APP, the 17 N-terminal residues of Aβ from the β-cleavage site to the α-cleavage site.

Because APP interacts with DCC and because DCC is known to transduce netrin-1 signaling in commissural neurons (11, 14, 15, 33), we investigated the role of APP in DCC signaling. Two distinct general types of mechanisms for transducing downstream signals have been reported for DCC: 1) nuclear signaling upon DCC cleavage by γ-secretase, allowing DCC intracellular domain-mediated gene transcription (30, 34), and 2) cytoplasmic signaling involving classic signaling pathways, such as activation of the ERK1/2 MAPK pathway, the latter being key for commissural axon guidance (14, 15).

We first assessed the relative importance of APP in DCC nuclear signaling. B103 cells were transfected with DCC and/or APP and treated or not with netrin-1. DCC immunoblotting was performed to monitor the α- and γ-cleavage of DCC. DAPT, a potent inhibitor of γ-secretase, was used as a control to block γ-cleavage and to reveal α-cleavage, whereas MG132, a

---

**FIGURE 1. APP is expressed in commissural neurons and is important during commissural axon navigation.** A, in situ hybridization of APP in spinal cords at E10.5 and E11.5 in wild-type mice and at E11.5 in APP−/− mice as a negative control. C, commissural neurons; M, motoneurons; P, neuronal progenitors; V, ventricular zone; DRG, dorsal root ganglion. B, immunostaining of DCC and APP protein performed on sections of embryonic spinal cords from wild-type or APP mutant mice. APP was detected on commissural axons (arrows), which are DCC-positive axons (arrow). An enlargement of the ventral spinal cord is also shown. Merge indicates the co-localization of the two proteins. C, general diagram used for the measurement of commissural axon bundle width. D, TAG-1 immunostaining of an E11.5 spinal cord shows an increase in commissural axon width in APP mutant compared with wild-type embryos. E, quantification of D according to the method shown in C (Mann-Whitney statistical test).
potent proteasome inhibitor, was used to enhance detection of the unstable /H9253-fragment of DCC. Of interest, in DCC-expressing B103 cells, APP expression efficiently blocked the /H9253-secretase cleavage of DCC (Fig. 4A and supplemental Fig. 2, A and B). Such an inhibitory effect of APP on DCC cleavage is specific, as UNC5H2, known to interact with DCC (35), had no effect on DCC cleavage whereas Notch had no effect on DCC cleavage (Fig. 4A). To further study the APP inhibition of DCC nuclear signaling, B103 cells were transiently transfected with DCC fused at its C terminus to Gal4 in the presence or absence of APP. As shown in Fig. 4B, the transcriptional activity of the DCC-Gal4 fusion protein in a Gal4-luciferase reporter assay was efficiently blocked upon treatment with DAPT, supporting the general view that the /H9253-cleavage of DCC leads to the formation of an intracellular fragment with transcriptional activity. Of interest, the presence of APP mimicked DAPT treatment,
leading to a strong decrease in DCC transcriptional activity (Fig. 4B). This effect is a specific effect of APP on DCC and not vice versa, as DCC expression had no effect on the transcriptional activity of the APP-Gal4 fusion protein (Fig. 4C).

In an attempt to further correlate the inhibition of DCC nuclear signaling during commissural axon guidance, we monitored the relative presence of α- and γ-DCC fragments in E11.5 spinal cords (when commissural axons grow toward the floor plate) and E12.5 spinal cords (when commissural neurons have reached the floor plate). As shown in Fig. 4 (D and E), the presence of the γ-fragment was decreased in E12.5 embryos, supporting the view that while reaching the floor plate where netrin-1 is abundant and where DCC interacts with APP, commissural axons shut down DCC nuclear signaling.

We thus analyzed whether APP could modulate DCC cytoplasmic signaling. ERK1/2 has been shown to be activated upon netrin-1 addition in DCC-expressing cells (14). We first analyzed ERK1/2 phosphorylation in DCC-expressing B103 cells in response to netrin-1 in the presence or absence of APP. As shown in Fig. 5A, DCC expressed in B103 cells triggered ERK1/2 phosphorylation in response to netrin-1 in a very transient manner, i.e. maximum at 5–10 min and no longer detected at 15 min. In the presence of APP, DCC-dependent ERK1/2 phosphorylation was enhanced (Fig. 5, A and B, and supplemental Fig. 3, A and B). We thus investigated whether APP could be implicated in DCC-mediated ERK1/2 activation through a loss-of-function experiment in commissural neurons. Primary commissural neurons from E11.5 embryos were cultured, and APP was silenced or not via a siRNA strategy and then treated or not with netrin-1. Transfecting APP siRNA reduced the level of APP in primary commissural neurons (Fig. 5C). As shown in Fig. 5 (D and E) and supplemental Fig. 3 (C and D), whereas netrin-1 triggered a robust ERK1/2 phosphorylation in scrambled siRNA-transfected primary neurons, this effect was inhibited in APP siRNA-transfected neurons. Thus, APP is implicated in netrin-1-induced DCC-mediated MAPK activation.

Because netrin-1-induced DCC signaling appears to involve DCC multimerization (36, 37), we analyzed whether APP could modulate netrin-1-induced DCC multimerization. Two tagged versions of DCC were transfected in B103 cells, and HA immunoprecipitation was performed to reveal Myc-DCC. Although netrin-1 strongly enhanced Myc-DCC pulldown with HA-DCC as already reported, the presence of APP did not and had no effect on the basal DCC multimerization status nor the netrin-1-induced DCC multimerization (Fig. 5F), thus suggesting that APP affects DCC downstream of receptor multimerization. Taken together, these data support the view that APP partici-
pates in DCC signaling by blocking DCC nuclear signaling while enhancing DCC cytoplasmic signaling as exemplified by ERK1/2 activation.

We thus analyzed whether APP could be implicated in commissural axon guidance triggered by netrin-1. Netrin-1 is known to guide commissural axons and, as such, triggers both commissural axon outgrowth and orientation. Axon outgrowth was monitored using dorsal spinal cord explants from E11.5 mouse embryos grown for 16–18 h in collagen gels in the presence or absence of netrin-1. As shown in Fig. 6, spinal cord explants from E11.5 wild-type mouse embryos showed robust axon outgrowth induced by netrin-1. When a similar experiment was performed using APP mutant embryos, axon outgrowth was significantly reduced (p < 0.0001) (Fig. 6). Thus, together with the in vitro data and with the detection of thicker bundles of commissural neurons in APP mutant mice, these data support the involvement of APP in netrin-1-mediated commissural axon navigation.

**DISCUSSION**

Our data support the view that APP (and possibly APLP1 and APLP2) is part of the DCC complex that was shown to mediate netrin-1 signaling during commissural axon navigation. We have shown that APP interacts with DCC both in vitro and in vivo in commissural neurons. We demonstrated by both gain-of-function and loss-of-function experiments that APP is...
required for adequate netrin-1-induced DCC-mediated MAPK activation. We finally demonstrated that inactivation of APP is associated with abnormal distribution of commissural neurons in the developing neural tube and is associated with reduced commissural axon outgrowth. Using E10.5 whole mouse spinal cord explants dissected and embedded in three-dimensional

![Image](https://example.com/image.png)

**FIGURE 5.** APP enhances netrin-1-induced DCC-mediated ERK1/2 signaling. A, APP collaborates with DCC to strengthen ERK1/2 phosphorylation in response to netrin-1. B103 cells transfected with a mock vector, DCC, APP, or both APP and DCC were treated with netrin-1 for 5, 10, and 15 min, and immunoblots against phospho-ERK1/2 and ERK1/2 are shown. B, quantification of A. A.U., arbitrary units. C, APP siRNA (siAPP) decreases the expression of APP in commissural neurons. APP and β-actin immunoblots are shown. Scr, scrambled siRNA. D, APP silencing in commissural neurons decreases netrin-1-induced ERK phosphorylation. Phospho-ERK1/2 and ERK1/2 immunoblots are shown. as, aspecific band. E, quantification of D. F, APP does not modulate netrin-1-induced DCC multimerization. HA-based immunoprecipitation (IP) was performed on B103 cells cotransfected with APP and HA- and Myc-tagged DCC and treated or not with netrin-1. The c-Myc immunoblot shows the ability of Myc-DCC to interact with HA-DCC in the presence of netrin-1. *, p < 0.05 (Mann-Whitney statistical test). Tot., total.

**FIGURE 6.** APP is required for commissural axon outgrowth. Dorsal spinal cord explants were dissected out from E11.5 wild-type or APP mutant embryos and cultured in collagen in the presence or absence of 375 ng/ml netrin-1. A, representative images of axon outgrowth under the different conditions tested are shown. KO, knock-out; w/o, without. B, the total number of explants that were quantified from five distinct experiments varied from 8 to 12 per tested condition. The values shown are means ± S.E. A Kruskal-Wallis test was used to compare the overall condition (p = 0.001). A Mann-Whitney test was also used to compare +/+ versus +/− (p = 0.036) and +/+ versus −/− (p < 0.001).
APP Potentiates Netrin-1 Signaling

collagen matrices and cultured for 20 h closed to a mouse floor plate as described by Charron et al. (18), we failed to detect any effect of APP inactivation on commissural axon turning toward the grafted floor plate (data not shown). Even though this has to be explored further, it suggests that APP plays a regulatory role in axon outgrowth rather than turning.

The detection of significantly thicker bundles of commissural neurons in APP−/− embryos compared with wild-type animals supports the view that APP is not the main player in netrin-1 navigation compared with DCC, i.e., commissural neurons are completely disorganized in DCC−/− embryos (11), but is instead a co-receptor that regulates netrin-1 navigation. Along this line, APP can be added to other players in netrin-1-mediated navigation of commissural neurons such as DSCAM (10), A2b (9, 38), and sonic hedgehog/Boc (18, 19). The involvement of APP in netrin-1 signaling is, however, of large interest, as not much is known regarding the biological role of APP, beside its implication in Alzheimer disease. It is noteworthy that netrin-1 mutant mice display profound defects in the developing nervous system and that a major portion of this phenotype has been attributed to the role of netrin as an axonal cue for its receptor, DCC, because DCC mutant mice exhibit similar defects in the developing nervous system. However, APP-null mice also display similar neural developmental defects, the severity of which depends on genetic background: these defects include abnormalities in the developing corpus callosum and other commissures (24). Together with the fact that APLP1 and APLP2 may compensate for APP biological function (31), this strongly suggests that one APP function during development is to specify netrin-1-induced DCC signaling. Of interest, it was reported that TAG-1, a protein known to be expressed in commissural neurons, may modulate APP intracellular signaling (39). Thus, by regulating netrin-1-induced DCC signaling, APP could represent a DCC co-receptor that integrates signals emanating from other factors.

Along this line, we have shown here that while amplifying DCC-mediated MAPK activation, APP shuts down DCC nuclear signaling, which has been speculated to be important for midline crossing (34). Interestingly, the APP effect on DCC-mediated ERK1/2 activation appears to be stronger both in intensity and in duration. Thus, the regulation of APP expression may be a determining factor that dictates or orients the adequate response to the presence of netrin-1. Further work will have to establish the precise interplay of APP and DCC in the netrin-1 response during development.

This interplay between netrin-1, DCC, and APP may thus have at least two distinct functions. First, during embryonic development, this trio is important for neuronal wiring, with APP being in this system mainly as a modulator of the netrin-1 response. Second, in adult brain, DCC may be the regulator and APP the main receptor. Indeed, we have shown that in adult brain, netrin-1 is a functional ligand of APP and negatively regulates the formation of the amyloid peptide (20). This interaction may be important in the regulation of Alzheimer disease, and it may then be important for a putative therapeutic strategy to understand the relative importance of DCC in netrin-1/APP interaction in normal and pathological adult brains.

Acknowledgments—We thank Marc Tessier-Lavigne and Alain Checodile for critical review of this work.

REFERENCES
2. Castets, M., and Mehlen, P. (2010) Netrin-1 role in angiogenesis: to be or not to be a pro-angiogenic factor? Cell Cycle 9, 1466–1471
SUPPLEMENTARY MATERIAL

THE AMYLOID PRECURSOR PROTEIN REGULATES NETRIN-1 MEDIATED COMMISSURAL AXON OUTGROWTH

Nicolas Rama, et al.

Apoptosis, Cancer and Development Laboratory - Equipe labellisée ‘La Ligue’, Centre de Cancérologie de Lyon, INSERM U1052-CNRS UMR5286, Université de Lyon, Centre Léon Bérard, 69008 Lyon

Supplementary Figure 1: APP and DCC co-immunoprecipitate and co-localize.
A. Netrin-1 promotes APP-DCC complex formation in B103 cells. Reverse immunoprecipitation as Fig.2A. APP immunoprecipitation (IP) performed in B103 cells transiently co-transfected with an APP-expressing vector with or without a DCC-expressing construct in presence or not of netrin-1. Immunoblots using different DCC or APP antibodies are shown. B. The colocalization between APP and DCC shown in the merge panels in Fig.2C was quantified with JACoP plugin of ImageJ. Netrin-1 increases the colocalization. C. Control staining of Fig.2C showing immunostaining without DCC or APP antibody. D. APP-C100 and APP-C83 failed to interact with DCC without netrin-1. Same as Fig.3C but without netrin-1 treatment.

Supplementary Figure 2: APP prevents the γ-cleavage of DCC.
Densitometric analyses of DCC α (A) and γ (B)-fragments are shown. Statistical test: Mann Whitney, *: p<0.05.

Supplementary Figure 3: APP does not modulate the level of ERK expression.
A. and B. respectively show the densitometric analysis of ERK and P-ERK upon overexpression of APP and/or DCC in B103 cells. C. and D. respectively show the densitometric analysis of ERK and P-ERK silencing of APP. In ABCD, analysis has been done on at least 3 independent experiments. Statistical test: Kruskall-Wallis, *: p < 0.01.
Fig1 sup.
Fig 2 sup.
Fig3 sup.
Amyloid Precursor Protein Regulates Netrin-1-mediated Commissural Axon Outgrowth
Nicolas Rama, David Goldschnieder, Véronique Corset, Jérémy Lambert, Laurent Pays and Patrick Mehlen

doi: 10.1074/jbc.M111.324780 originally published online July 10, 2012

Access the most updated version of this article at doi: 10.1074/jbc.M111.324780

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

Supplemental material:
http://www.jbc.org/content/suppl/2012/07/10/M111.324780.DC1.html

This article cites 39 references, 13 of which can be accessed free at
http://www.jbc.org/content/287/35/30014.full.html#ref-list-1