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Expression of the Netrin-1 Receptor, Deleted in Colorectal Cancer (DCC), Is Largely Confined to Projecting Neurons in the Developing Forebrain

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ABSTRACT

Axon guidance mechanisms are crucial to the development of an integrated nervous system. One family of molecules that may be important in establishing axonal connectivity in mammals is the Netrins, and their putative receptors DCC (deleted in colorectal cancer), Neogenin, and Unc-5. Knockout and mutational analyses of some of these genes have shown that they are critically involved in the development of several specific pathways in the developing brain. However, previous expression analyses of these genes have largely been confined to the developing spinal cord. In the present study, we analyzed the expression of DCC in the developing mouse forebrain. We found that DCC protein is expressed in specific axonal populations projecting from the developing olfactory bulb, neocortex, hippocampus, and epithalamus/habenular complex. In the developing olfactory bulb and neocortex, DCC expression is particularly evident during the targeting phase of axon outgrowth and is then rapidly downregulated. As predicted from the knockout and mutational analyses of this gene, DCC is expressed in axonal commissures, in particular the corpus callosum, hippocampal commissure, and the anterior commissure. In addition, we found that DCC is expressed in the habenular commissure, the fasciculus retroflexus, and the stria medularis. Therefore, this analysis implicates a function for DCC in additional axonal guidance systems not predicted from the knockout and mutational analyses. J. Comp. Neurol. 416:201–212, 2000.

Indexing terms: olfactory bulb; cortex; hippocampus; habenula; mouse; axon guidance

Precise development of neuronal connectivity is not rigidly predetermined and "hard-wired" but is profoundly influenced by the environment (Frost and Metin, 1985; Sur et al., 1988; O'Leary and Stanfield, 1989; Schlaggar and O'Leary, 1991). As an axon grows toward its target during development, its growth cone senses molecules within the environment that it uses to determine the correct path of growth (Garrity and Zipursky, 1995). These molecular cues may be soluble and freely diffusing, bound to cellular membranes, or bound to the extracellular environment. Along their way axons use intermediate "guideposts" to find the final target (Tessier-Lavigne et al., 1988). Thus, axons may follow gradients of molecules released by the target itself or the intermediate guideposts. Such molecules may deliver attractive (Placzek et al., 1990), repulsive (Pini, 1993; Luo et al., 1993), or suppressive (Wang et al., 1996) cues to the growth cone.

Some molecules can act as either attractive or repulsive cues for axons (Hedgecock et al., 1990) depending on the expression of separate receptors for attraction or repulsion in the responding cells (Chan et al., 1996). The guidance molecule Netrin-1 can mediate both attraction and repulsion. Netrin-1 was first purified by its ability to attract sensory commissural neurons in the spinal cord (Serafini et al., 1994), but it also repels motor axons (Colamarino

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and Tessier-Lavigne, 1995; Varela-Echavarria et al., 1997). The Netrins belong to a phylogenetically conserved family of long-range guidance molecules with activities in *C. elegans* (UNC-6; Ishii et al., 1992), *Drosophila* (Netrin-A and Netrin-B; Harris et al., 1996; Mitchell et al., 1996), and vertebrates (Kennedy et al., 1994; Serafini et al., 1994; Colamarino and Tessier-Lavigne, 1995; Shirasaki et al., 1996; Richards et al., 1997; Varela-Echavarria et al., 1997).

Netrin-1 is either repulsive or attractive for axons depending on the neuronal population. This bifunctionality correlates with the activity of the *C. elegans* homologue of Netrin-1, Unc-6, which has also been shown to mediate both attractive and repulsive responses (Hedgecock et al., 1990). Unc-6 is required for the dorsal migration of mesodermal cells and for the guidance of circumferential axons (Hedgecock et al., 1990). Two known receptors for Unc-6 are Unc-40 and Unc-5. Genetic experiments have shown that axons are attracted to Unc-6 if they express the Unc-40 receptor but are repelled from Unc-6 if they express the Unc-5 receptor. The mammalian homologues of Unc-40 and Unc-5 have now been cloned. Neogenin (Vielmetter et al., 1994) and deleted in colorectal carcinoma (DCC; Keino-Masu et al., 1996) are homologous to *C. elegans* Unc-40, and Unc-5H1, Unc-5H2 (Leonardo et al., 1997) and rostral cerebellar malformation (rcm; Ackerman et al., 1997) are homologous to *C. elegans* Unc-5 (Lenug-Hagesteijn et al., 1992).

DCC is a transmembrane protein belonging to the immunoglobulin superfamily of cell adhesion molecules (Walsh and Doherty, 1997) and has been shown to bind Netrin-1 in vitro (Keino-Masu et al., 1996). DCC was originally cloned as a tumor supressor gene in colorectal carcinomas (Fearon et al., 1990). In the developing nervous system, DCC has been evolutionarily conserved with homologues cloned in *Drosophila* (Kolodziej et al., 1996), *C. elegans* (Chan et al., 1996), *Xenopus* (Pierceall et al., 1994), rodents (Cooper et al., 1995; Keino-Masu et al., 1996), and humans (Fearon et al., 1990; Nigro et al., 1991).

In the mammalian brain, Netrin-1 mutants and DCC knockout mice do not develop a corpus callosum or a hippocampal commissure and have a greatly reduced or absent anterior commissure (Serafini et al., 1996; Fazeli et al., 1997). However, the mechanism for these pathfinding defects in the brain is not known. In the developing forebrain, Netrin-1 has been shown to attract laterally directed cortical axons in vitro (Metin et al., 1997; Richards et al., 1997), indicating that these molecules are important for additional axonal guidance systems in the forebrain other than commissural axon guidance. Although there has been some expression analysis of DCC and Netrin mRNA in the developing nervous system (Cooper et al., 1995; Serafini et al., 1996; Gad et al., 1997, 1999; Livesey and Hunt, 1997) and some protein expression in the spinal cord (MacLennan et al., 1997), there has been no detailed analysis of the protein expression of either of these genes in the developing forebrain (apart from the developing optic nerve; Deinier et al., 1997).

To investigate the role of DCC in the development of the forebrain, we performed a detailed analysis of DCC protein expression in the telencephalon and diencephalon (and following some of these projections to the mesencephalon). We found that such an analysis implicates DCC in a variety of specific axonal guidance systems in the forebrain in addition to those previously described.

MATERIALS AND METHODS

Animals

Timed-pregnant female CD1 mice were obtained from Charles River Laboratories (Wilmington, MA). Mice were housed at the University of Maryland School of Medicine animal facility. Embryos were staged based on a plug date of embryonic day (E) 0 and were used between E12 and postnatal day (P) 0. Procedures involving animals were approved by the animal care and use committee at the University of Maryland, Baltimore.

Fixation and immunohistochemistry

On the required day of gestation, pregnant dams were anesthetized with sodium pentobarbital (Nembutal, Abbott Laboratories, North Chicago, IL) at 0.07 mg/g body weight and placed on a warming pad during surgery to maintain their body temperature. Once deeply anesthetized, the mother's abdomen was opened to expose the uterus. Pups were removed sequentially and placed on ice until deeply anesthetized before perfusion fixation with either saline, followed by 4% paraformaldehyde/2.5% Acrolein (Polysciences Inc., Warrington, PA) for pups older than E15, or immersed in 4% paraformaldehyde/2.5% Acrolein for younger pups. After perfusion or immersion fixation, the skulls were opened, and the heads were transferred to 4% paraformaldehyde and stored at 4°C until cutting. Deeply anesthetized dams were killed after the pups were removed by a lethal injection of sodium pentobarbital.

All sections were cut at 45 μm on a Vibratome (Leica, Deerfield, IL) and embedded in 3% agarose (Noble Agar, Difco, Detroit, MI). Vibratome sections were placed in phosphate buffered saline (PBS; pH 7.4) and washed before immunostaining. Sections were immunostained free floating but before the staining procedure were incubated in 1% sodium borohydride (Sigma, St. Louis, MO) for 20 minutes to remove the Acrolein and washed 3 × 10 minutes with PBS. The blocking step was done in 0.2% Triton X-100 (Sigma) and 2% normal goat serum (Vector Laboratories, Burlingame, CA) in PBS for 2 hours. The sections were then incubated overnight in anti-DCC antibody 2744 (rabbit polyclonal) at a concentration of 1:30,000 diluted in the blocking solution. Sections were washed 3 × 20 minutes with PBS, incubated for 1 hour with secondary antibody (biotinylated goat anti-rabbit; Vector Laboratories) diluted 1:600 in 0.2% Triton X-100 in PBS, and washed another 3 × 20 minutes. Sections were incubated for 1 hour with AB solution (Vectastain elite kit, Vector Laboratories) made up 1 hour before at 1:500 dilution in PBS with 0.2% Triton X-100 and washed 3 × 10 minutes in PBS. The chromogen solution consisted of 1.25 g of nickel sulfate (Sigma) dissolved in 50 ml of 0.175 M sodium acetate, followed by one 10-mg tablet of 3,3′-diaminobenzidine tetrahydrochloride (DAB; Sigma) dissolved in the same solution. The nickel-DAB solution was filtered, and 5 μl of 30% hydrogen peroxide (Sigma) was added just prior to immersing the sections. The chromogen reaction was terminated by placing the sections in PBS. For double staining with neurofilament and DCC, sections were blocked for 2 hours, and then a polyclonal neurofilament antibody (used at 1:50,000; Chemicon, Temecula, CA) was applied to the sections overnight. The sections were then washed and incubated in AB solution, as described above. The chromogen used was DAB dissolved in Tris buffered

saline (pH 7.2) without nickel sulfate. Procedures for the second day of DCC staining were identical to those described above. Sections were mounted on 2% gelatin-coated glass slides, allowed to air dry, and dehydrated through a series of alcohols before being coverslipped with DPX (Sigma) mounting medium.

Microscopy and production of photomicrographs

Mounted sections were analyzed with an upright light microscope (Leica). Images for figures were scanned with a PowerPhase digital camera (Phase-one, Coppenhagen, Denmark) directly into Adobe Photoshop software (version 4.0) running on a Power Computing Power Center Pro 210 computer. In some cases, alterations of the brightness and/or contrast were then made to the images. Images were then labeled, and collages were made for each figure in Adobe Photoshop. Figures were printed on quality photographic paper with a high-resolution color printer (Fujix Pictrography 3000).

RESULTS

Immunohistochemistry of DCC protein expression was observed only on axonal processes in most cases, in particular those forming large fasciculated axon tracts. Staining of neuronal cell bodies was rarely observed with this antibody.

Expression of DCC protein in the developing olfactory system

Previous studies of DCC mRNA have shown that the DCC message is expressed in the granule cells of the olfactory bulbs at E18 in the mouse (Gad et al., 1997). Staining with the DCC antibody shows that DCC protein is initially expressed on the axons of mitral cells at E14 (Fig. 1A) but is not expressed in the granule cell layer until E17 (Fig. 1B). Staining within the granule cell layer probably represents the dendrites of granule cells that ramify within the granule cell layer (white arrows in Fig. 1C) because these cells do not have axons (Shipley et al., 1995). In addition to the mitral cell layer, DCC is expressed on the processes of cells within an outer ring of the bulb that may represent tufted cell axons (arrow in Fig. 1B). Tufted cell axons project to the anterior olfactory nucleus (AON) and the central olfactory cortex (Shipley et al., 1995). Some diffuse DCC staining is present within the AON and on centrifugal fibers in the central olfactory cortex (data not shown), indicating that tufted cell axons may be labeled with DCC. The mitral cells and the tufted cells together constitute the major efferent projecting cell types of the olfactory bulb (Shipley et al., 1995). DCC is expressed on projecting axon bundles as they fasciculate and leave the olfactory bulb (Fig. 1C) to form the lateral olfactory tract (Fig. 1D,E). At E17, DCC is highly expressed in the lateral olfactory tract on efferently projecting axons from the olfactory bulbs (Fig. 1D) to the piriform cortex (Fig. 1E). However, by E18, the expression in the lateral olfactory tract and the bulb begins to decline (data not shown), indicating that, at least for mitral and tufted cell axons, DCC may only be expressed during the targeting phase of their growth (between E13 and E17 in the rat; Marchand and Belanger, 1991; Lopez-Mascaraque et al., 1996). In addition, we observed some DCC labeling within the olfactory tubercle (arrow in Fig. 2F), which receives inputs from the olfactory bulb via mitral cells but does not send a reciprocal projection back to the olfactory bulb (Shipley et al., 1995). Therefore, this staining probably corresponds to the mitral and tufted cell inputs rather than to an expression within the tubercle itself. DCC was not observed in the olfactory nerve (data not shown), but mRNA expression for DCC has been reported in deep layers of the olfactory epithelium that contain mesodermal and glial cells (Livesey and Hunt, 1997).

DCC is expressed on neocortical axons during the targeting phase of growth

In the developing neocortex, DCC expression appears to be tightly regulated and restricted to axons as they are projecting. DCC mRNA is first expressed at E11.5 in the developing preplate, where its expression is confined to early postmitotic neurons (Gad et al., 1997). From E13, DCC protein is expressed on the axons of neurons projecting within the intermediate zone and on axons within the marginal zone but is not expressed within the cortical plate or ventricular zone (Fig. 2). DCC expression within the marginal zone corresponds to that noted in previous reports showing DCC mRNA in neurons within layer 1, which are likely to be Cajal–Retzius cells (Keino-Masu et al., 1996).

The first axons to project out of the neocortex are the subplate axons that pioneer the internal capsule (McConnell et al., 1989; DeCarlos and O'Leary, 1992). Although we failed to detect DCC protein expression at E12, mRNA for DCC is expressed as early as E11.5 in mouse neocortex (Gad et al., 1997). However, DCC protein is highly expressed by E13 (Fig. 2A) and E14 (Fig. 2B,C), probably representing both the subplate pioneers and some axons of layers 5 and 6. Because DCC is not expressed on dorsal thalamic afferents projecting into the neocortex, DCC expression within the internal capsule appears to be specific for cortical efferent axons within this region. It has been previously shown that the lateral projection from the neocortex extends at E14–E15 in rats, approximately 2 days before the medial projection at E16–E17 (DeCarlos and O'Leary, 1992; Koester and O'Leary, 1994; Richards et al., 1997). Our results show that DCC expression reflects a similar developmental difference in directional targeting in mouse. Initially DCC is highly expressed on the lateral projection as it enters the internal capsule (Fig. 2C); however, by E16, DCC expression is downregulated on these axons, as reflected by a decrease in staining within the internal capsule (Fig. 2E). At the same time, DCC expression is maintained on medially projecting neurons both before and during their targeting phase of growth (compare Fig. 2C with 2E).

Medially projecting neocortical afferents cross the midline at E16.4 in mouse (Ozaki and Whalsten, 1998). At this stage of development, DCC is expressed on these axons as they grow toward and cross the midline (Fig. 2E). At E17, DCC expression at the midline is observed in both a dorsal region of the corpus callosum and a ventral region. The dorsal region (small black arrow in Fig. 2F) probably corresponds to callosally projecting cortical axons because this staining is continuous with the projections from each hemisphere. The ventral staining (white arrow in Fig. 2F) may correspond to a group of axons that defasciculates from the main bundle of axons at the midline, or it may be staining the glial sling, a midline glial "bridge" that directly underlies the corpus callosum at this stage of

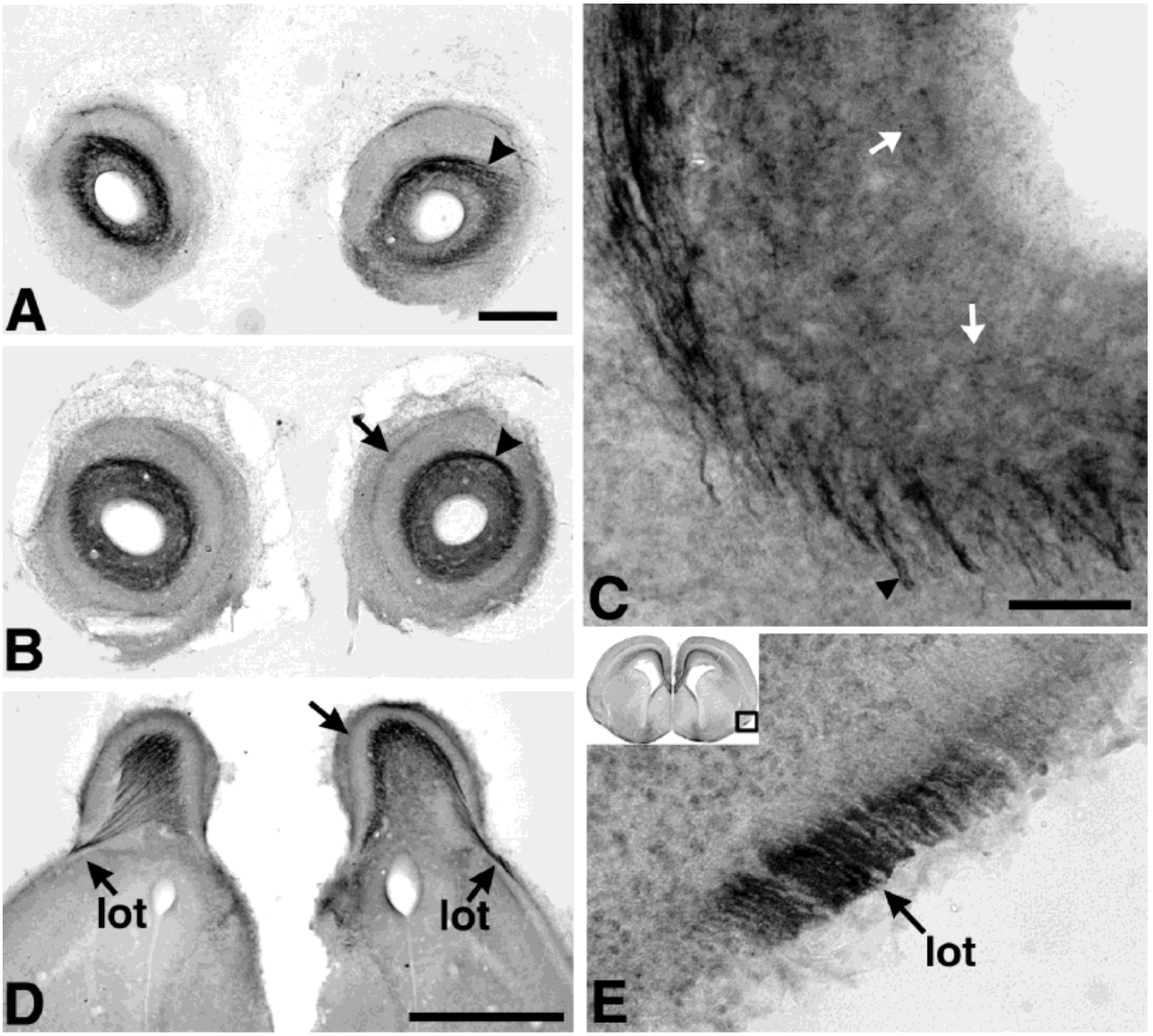

Fig. 1. Deleted in colorectal cancer (DCC) expression in the olfactory system. **A:** In the olfactory bulb, DCC is expressed within the mitral cell axons (arrowhead in A,B) by embryonic day 14 (E14; the earliest age examined). **B:** By E17, DCC is also expressed in the tufted cell axons (arrow) and in the granule cell layer. **C, D:** Mitral and tufted cell axons expressing DCC at E17 form fasciculated bundles (arrowhead in C) as they exit the olfactory bulbs and form the lateral olfactory tracts (lot). **E:** DCC is expressed at E17 within the lot along the entire length of the tract toward the olfactory cortex (E is an enlargement of the boxed area in the inset). A–C and E are coronal sections, and D is a horizontal section. Scale bars = 300 μm in A,B, 30 μm in C,E, 650 μm in D.

Fig. 2. Deleted in colorectal cancer (DCC) expression in the developing cortex. **A:** In the developing cortex, DCC is expressed at embryonic day 13 (E13; the earliest age detected by immunohistochemistry) in the intermediate zone of the neocortex and within the cingulate cortex. **B–D:** As the lateral neocortical projection forms, DCC is highly expressed at E14 (B,C) and E15 (D) on axons within the internal capsule (ic; C is a higher power view of B) and in the dorsal lateral regions of the septum (arrowheads in D). **E:** However, by E16, DCC begins to be downregulated in the internal capsule in more rostral regions initially while still being highly expressed in medial aspects of the neocortex and the cingulate cortex. **F:** At E17, DCC is most highly expressed on the medially projecting cortical axons forming the corpus callosum (small black arrow). At the ventral midline, a second band of expression is seen that may correspond to axonal labeling or labeling of the glial sling (white arrow). At E17, DCC is still highly expressed in the dorsal–lateral regions of the septum (black arrowheads) and in the olfactory tubercle (large black arrow). In more caudal regions such as this, DCC is still expressed in the internal capsule, although this expression is also declining. **G:** By E18, DCC expression has been largely downregulated and only remains on the most dorsal axons of the corpus callosum (cc) and is no longer expressed in the internal capsule. **H:** In the sagittal view at E17, it is evident that DCC expression declines after laterally projecting cortical axons have grown through the internal capsule (ic) and is only visible in the rostral regions of the corticospinal tract (cst). It is not possible to see axons entering the thalamus after passing through the internal capsule. A–G are coronal sections, and H is a sagittal section. Scale bars in A,C,H = 400 μm, in G = 500 μm for B, 400 μm for D,G, 450 μm for F, in E = 650 μm.

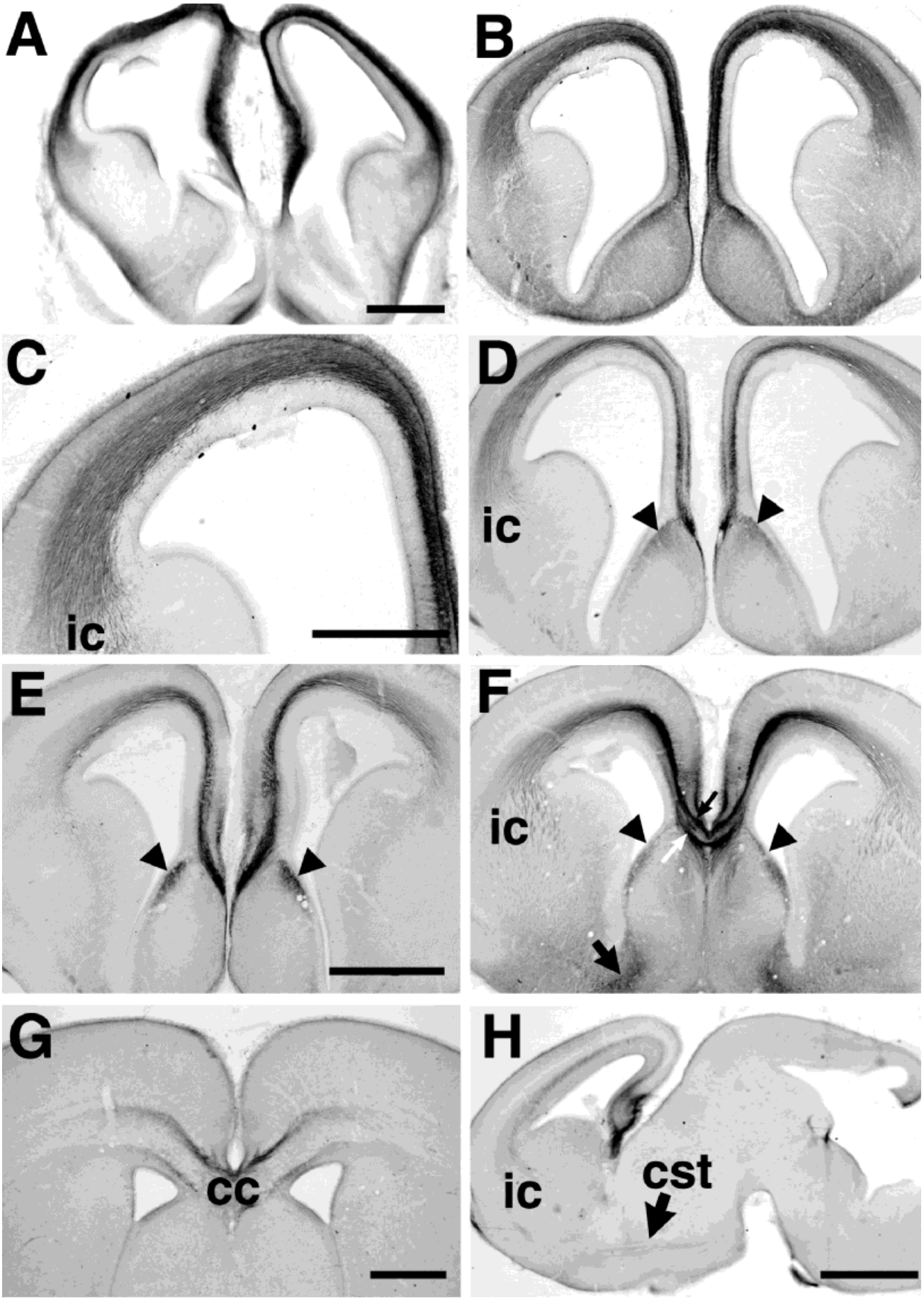

Figure 2

development (Silver et al., 1982). Callosal axons cross the midline in a temporally and regionally specific manner, with lateral axons crossing first in the ventral region of the corpus callosum (CC) and medial axons crossing last in the dorsal region of the CC (Ozaki and Wahlsten, 1992). This is also reflected by the regulation of DCC expression. By E18, DCC expression is downregulated on all other axons and is only present in the dorsal region of the CC (Fig. 2G).

To determine whether DCC expression could be seen in more caudal regions of the corticospinal tract, we cut sections in the sagittal plane of embryos. We found that, after passing through the region of the internal capsule, a small amount of expression was observed in the rostral region of the corticospinal tract (Fig. 2H) but was not found along the entire tract. These same axons were observed in coronal sections in more caudal regions of the brain making up the cerebral peduncles (Fig. 6C). In addition, we did not see DCC expression on cortical axons entering the thalamus (Fig. 2H), even at a range of antibody concentrations (data not shown), indicating that DCC was not even expressed at low levels by these axons.

DCC expression in the hippocampus and the major midline commissures

In horizontal sections of the developing forebrain, DCC expression is most evident in the major midline commissures. DCC is expressed in the hippocampal commissure (Figs. 3E, 4C,D), the CC (Figs. 2F,G, 3E), and the anterior commissure (Fig. 4E,F). Sections alternately labeled with DCC alone (Fig. 3A,C,E) or neurofilament and DCC (Fig. 3B,D,F) show that DCC is expressed before neurofilament in some projecting neurons of the hippocampal and callosal commissures. Figure 3C,D illustrates the reduction in DCC expression within the internal capsule at E16 (Fig. 3C), despite the high expression of neurofilament in the same axons (Fig. 3D).

In the developing hippocampus, DCC is expressed by E13.5 in the hippocampal primordia (Gad et al., 1997) and is clearly evident in the axonal layer by E14 (Fig. 4A) but is not seen in the cell bodies of these cells. DCC is expressed in the fiber layer underlying all regions of the hippocampus from the dentate gyrus and in regions CA1–CA3. Given that these axons project toward and fasciculate to form the fimbria, it appears that these are extrinsically projecting axons (Fig. 4B). DCC continues to be expressed along the entire hippocampal projection toward the midline within the fornix and within both the dorsal and ventral hippocampal commissures (Fig. 4C,D). The hippocampal commissure forms approximately 1 day earlier than the CC but continues to form until birth (Wahlsten, 1981; Valentino and Jones, 1982; Super and Soriano, 1994). DCC is also expressed on these hippocampal axons as they first begin to cross the midline at E14–E15 (data not shown) and during this extended targeting phase of growth.

The anterior commissure is the first major commissure to cross the rostral midline in the mouse at E14 but does not have an extended targeting phase of growth (Silver et al., 1982; Wahlsten, 1981). Coincident with this, DCC is expressed within the axons of the anterior commissure at E14 (Fig. 4E) and E15 (Fig. 4F) but is completely downregulated by E16 (data not shown).

These results in the CC, hippocampal commissure, and the anterior commissure and those described within the neocortical and olfactory bulb projections suggest that DCC is only expressed on these long-range efferent axons while they are growing toward their targets. Upon reaching their targets, DCC is then apparently rapidly downregulated.

DCC expression in the septum

Septal defects have been shown to be associated with agenesis of the CC (Wahlsten and Bulman-Fleming, 1994), a defect seen in both the DCC (Fazeli et al., 1997) and Netrin-1 (Serafini et al., 1996) mutant mice. The ventral septum is a region of high Netrin-1 mRNA expression (K.M. Valentino and L.J. Richards, unpublished observation). Therefore, we wanted to determine where DCC was expressed within this region. The most striking region of DCC expression in the septum is bilaterally in the ventromedial aspects of the lateral ventricles, corresponding to the ventricular zone of the septum (arrowheads in Fig. 2E,F and sep in Fig. 3C). In addition, fibers of the perforant pathway, which run rostrocaudally within the septum, parallel to, and just lateral to the midline, are also labeled with DCC (Fig. 2F).

DCC expression in the diencephalon

The highest DCC expression in the diencephalon is in the epithalamus/habenula complex (Fig. 5). DCC expression was evident from E13 and was highly expressed by E14 (Fig. 5A,B) in this region. DCC is expressed in both medially projecting axons in the habenula commissure (hbc in Fig. 5D) and ventrally projecting axons running in the lateral region of the diencephalon close to the pial surface, called the stria medularis (sm in Fig. 5C), which extend down to the hypothalamus. In addition, DCC is expressed in axons running rostrocaudally through the habenula, seen in cross-section in Figures 5B,D and 6C,E. These axons form two fasciculated bundles of axons called the fasciculus retroflexus (fr; also known as the habenulointerpeduncular tract) that project to the ventral tegmental area (vt), as seen in sagittal sections (Fig. 6A,B,D). DCC-expressing axons are also seen within the proximal region of the mammilotegmental tract (mtg in Fig. 6D).

In the dorsal thalamus, DCC is mostly expressed in medial (or limbic) regions of dorsal thalamus, particularly in the anterior medial thalamus and periventricular thalamic nucleus (Fig. 5E), and in the inferior thalamic radiation (itr; arrowheads in Fig. 6E,F). DCC is also expressed in a band of axons that extend mediolaterally between the dorsal and ventral thalamus in the region of the external medullary lamina (not shown).

In the ventral aspect of the diencephalon, DCC is highly expressed in the optic chiasm (Fig. 5F) and bilaterally in the rostromedial region of the suprachiasmatic nucleus (Fig. 5F). We have also shown DCC protein expression within the developing retina and optic nerve (T. Shu and L.J. Richards, unpublished observations); therefore, the expression of DCC within the optic chiasm is consistent with this finding and suggests that DCC may play a role in axon targeting of the optic nerve in regions distal to the retina.

In more caudal sections, DCC is expressed within the nigrostriatal pathway (nsp; Fig. 6C,E,F), corresponding to previous reports of DCC mRNA expression within the substantia nigra (Livesey and Hunt, 1996; Gad et al., 1997).

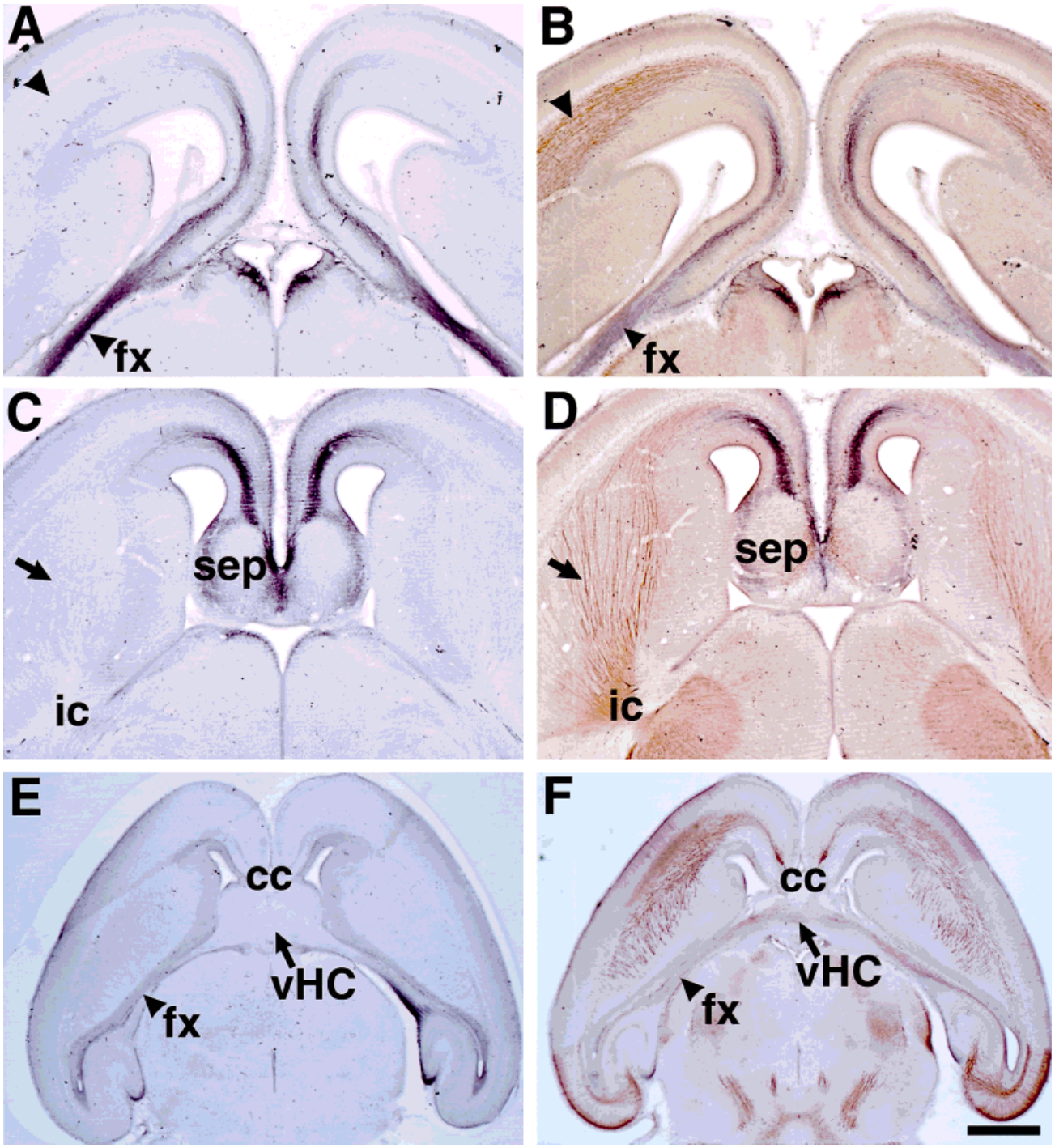

Fig. 3. Deleted in colorectal cancer (DCC) expression in the commissural projections of the forebrain. Comparison of DCC alone (**A,C,E**) and neurofilament and DCC (**B,D,F**) expression in alternate horizontal sections of the developing forebrain at embryonic day 16 (E16; A–D) and E18 (E,F). By E16, DCC expression begins to be downregulated in the laterally projecting axons within the intermediate zone of the neocortex (arrowheads in A,B). In B, neurofilament expression (arrowhead) shows the position of the laterally projecting cortical axons stained with 3,3′-diaminobenzidine tetrahydrochloride (DAB; see Materials and Methods). At the same time, the axons of the fornix (fx) are still highly expressing DCC (stained with Ni-DAB). DCC is expressed on the medially projecting axons prior to neurofilament expression on these axons (compare A,C with B,D). C,D: In a more dorsal section, it is possible to see the downregulation of DCC within the internal capsule (ic; arrows in C,D). Expression of DCC within the lateral regions of the septum (sep) is also evident. E,F: By E18, DCC is no longer expressed in many axon tracts, and despite being expressed in the fornix, DCC is no longer expressed in the ventral hippocampal commissure (vHC) at this stage. cc, corpus callosum. Scale bar = 300 μm in A, 350 μm in B,D, 400 μm in C, 650 μm in E,F.

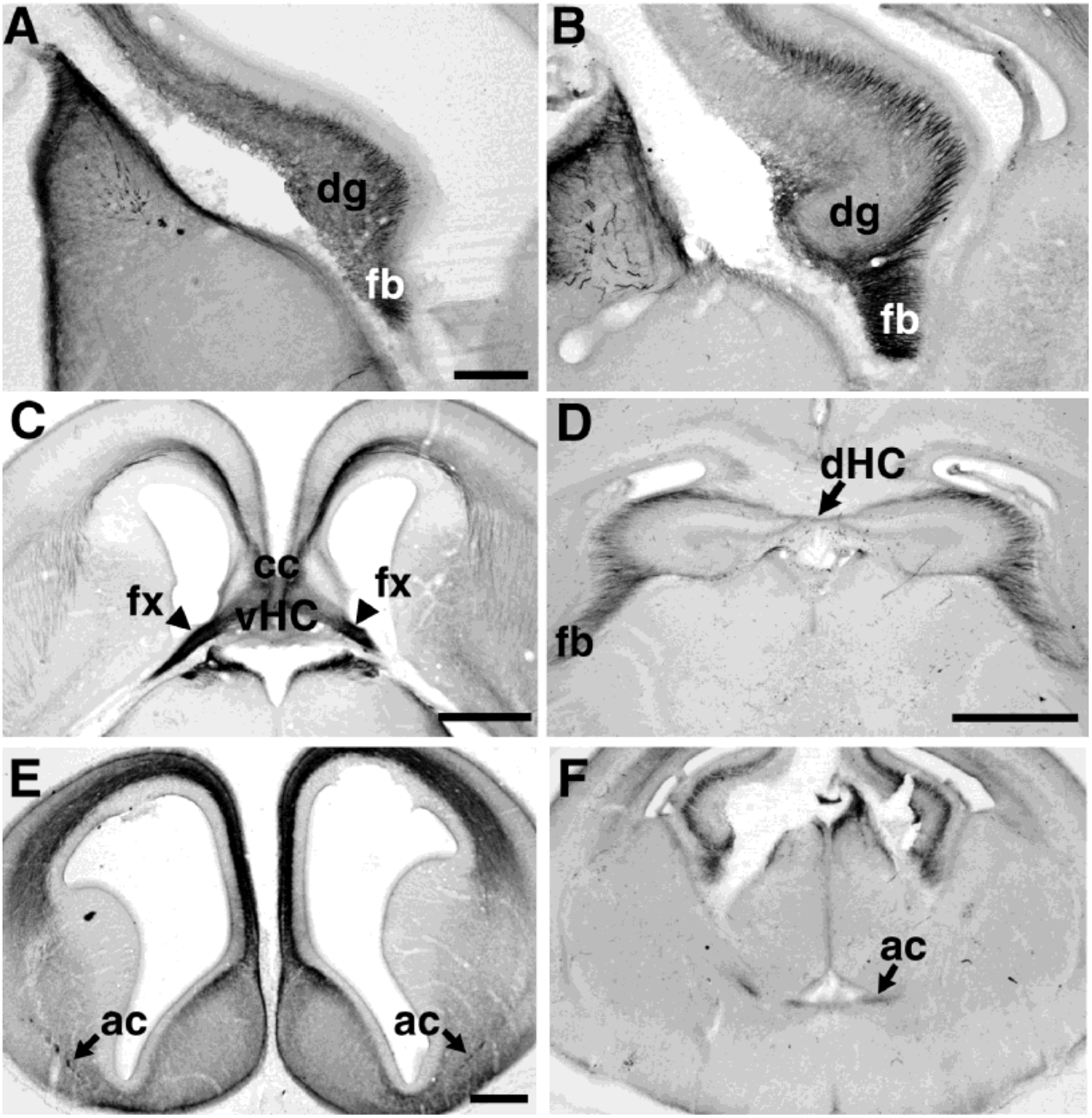

Fig. 4. Deleted in colorectal cancer (DCC) expression in the developing hippocampus and anterior commissure. **A,B:** Coronal sections through the developing hippocampus at embryonic day 14 (E14; A) and E17 (B) show that DCC is expressed exclusively in the axon layer, particularly in those axons leaving the hippocampus via the fimbria (fb). **C,D:** DCC is expressed on both the ventral (vHC; C) and dorsal (dHC; D) regions of the hippocampal commissure and the fornix (fx). (C is a horizontal section at E17, and D is a coronal section at E18.) **E,F:** DCC is expressed in the anterior commissure (ac) as early as E14 (the earliest age examined) and is still expressed at E15 (F; E,F are coronal sections). DCC is no longer expressed in the anterior commissure at E16 (not shown). dg, dentate gyrus; cc, corpus callosum. Scale bars in A = 150 μm, in C = 200 μm for B, 550 μm in C, in D = 600 μm, in E = 300 μm for E,F.

DISCUSSION

The present findings demonstrate that the expression of DCC protein is dynamically regulated by a number of different projecting populations of axons in the developing forebrain. In addition, axons expressing DCC tend to be long-range projecting axons (some exceptions include the granule cells of the olfactory bulb that do not have axons; Shipley et al., 1995). Because DCC immunohistochemistry is largely confined to axonal membranes, DCC protein is located predominantly in large fiber tracts such as the lateral olfactory tract, the internal capsule, the corpus callosum, the anterior commissure, the fimbria/fornix, the fasciculus retroflexus, and the stria medularis. Given the high expression of DCC on midline commissural axons, our

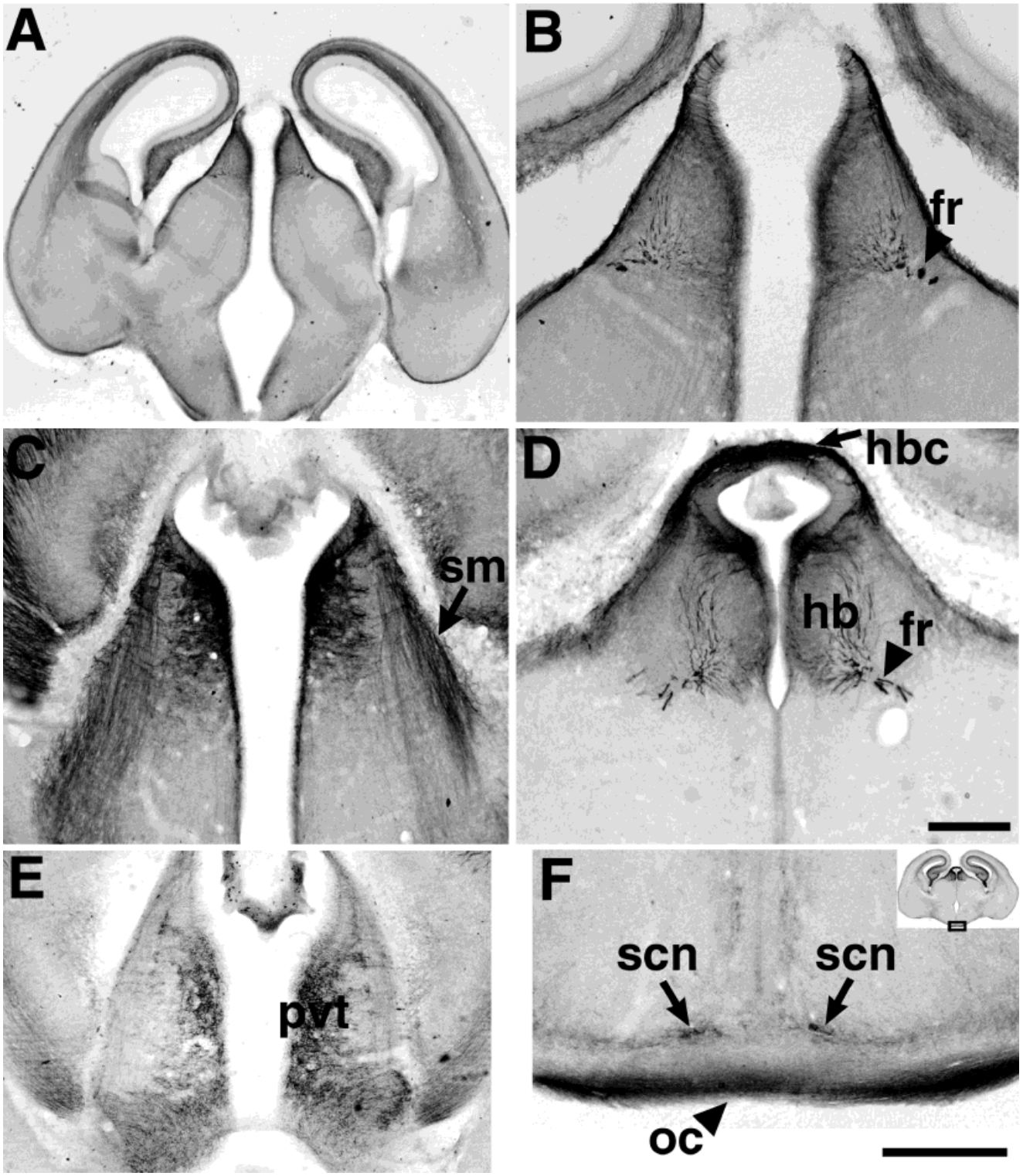

Fig. 5. Deleted in colorectal cancer (DCC) expression in the developing diencephalon. **A–B:** From embryonic day (E14; the earliest age examined), DCC is highly expressed within the epithalamus/habenula complex. B is a high power view of A. Particularly evident are the large fasciculated axon bundles making up the fasciculus retroflexus (fr; B). **C:** In more rostral regions of the diencephalon at E17, DCC-expressing axons are found within the stria medularis (sm). **D:** At E17, DCC continues to be highly expressed within the fasciculus retroflexus and in the habenula commissure (hbc). **E:** In rostral regions of the diencephalon at E16, there is diffuse expression of DCC within the periventricular thalamic nucleus. **F:** High power view of the boxed region of the inset in F. In the ventral diencephalon (shown here at E17), DCC is expressed within the optic chiasm (oc) and within the most rostral, medial, and ventral regions of the suprachiasmatic nucleus (scn). This region may correspond to axons coming directly from the retina making up the retinal hypothalamic pathway. A–F are coronal sections. Scale bars in D = 600 μm for A, 200 μm for B–D, 250 μm for E, in F = 100 μm.

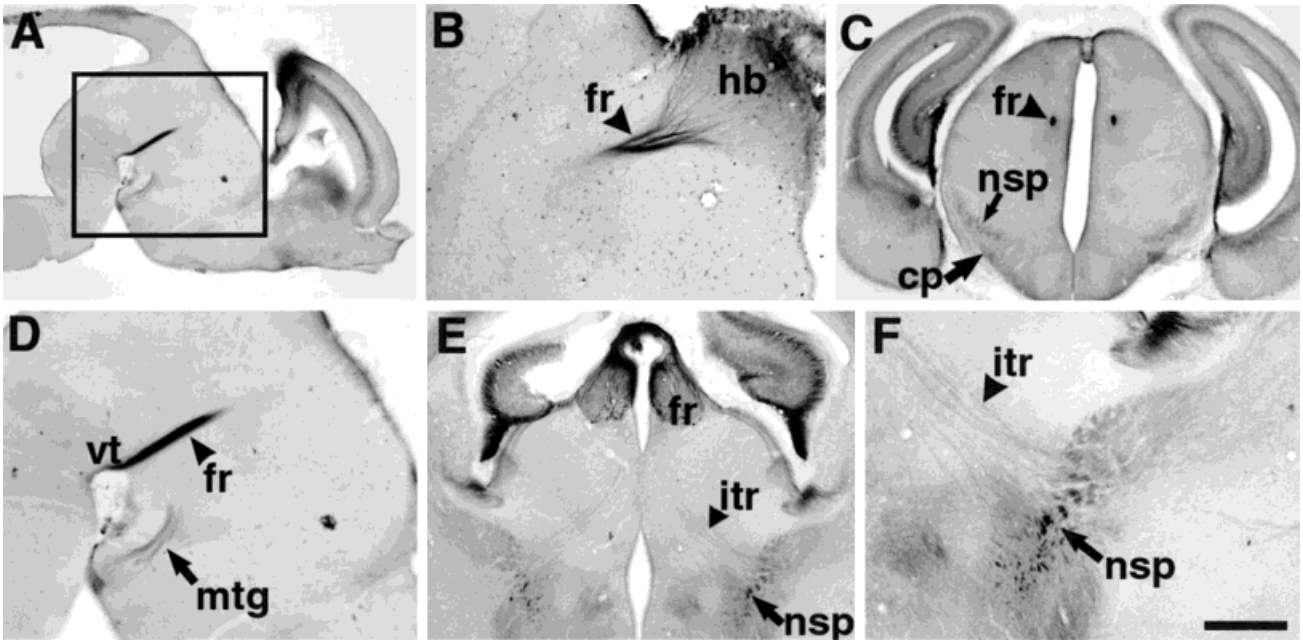

Fig. 6. Projections between the forebrain and midbrain that express Deleted in colorectal cancer (DCC). **A,D:** In the sagittal view, the caudal region of the fasciuclus retroflexus (fr) is clearly evident entering the ventral tegmentum (vt). D is a higher power view of the boxed region shown in A. Also shown in D is some low-level DCC staining within the mammilotegmental tract (mtg), although this staining was seen only within the most proximal regions of the axons. **B:** Rostral view of the fasciculus retroflexus leaving the habenula (hb) in sagittal section. **C:** Coronal section within the caudal diencephalon shows three major tracts: the fasciculus retroflexus (fr), the cerebral peduncles (cp), and the nigrostriatal pathway (nsp). **E–F:** In more rostral regions of the diencephalon, DCC is seen in both the habenula and fasciculus retroflexus, as previously described, and in axons within the inferior thalamic radiation (itr) and the nigrostriatal pathway (nsp). Labeling within the nigrostriatal pathway was seen only within these regions and not within the entire nigrostriatal pathway. A, B, and D are sagittal sections, and C, E, and F are coronal sections. All sections are from embryonic day 17 brains. Scale bar = 350 μm for A, 60 μm for B,D, 600 μm for C, 500 μm for E, 125 μm for F.

results are consistent with previous findings indicating a role for DCC in the development of commissural pathways. However, our results also indicate that the role of DCC in guiding axons may be more generalized to large fiber tracts containing long-range projecting axons (of which these commissural pathways comprise a subset). These results suggest that DCC may be involved in the development of more axon tracts than previously described by DCC gene knockout analysis (Fazeli et al., 1997).

During neocortical development, the lateral axonal projection through the internal capsule is pioneered at E14 in the rat by subplate axons (DeCarlos and O'Leary, 1992). However, the medial axonal projection through the corpus callosum is not pioneered until at least 2 days later in the rat (Koester and O'Leary, 1994). In previous studies, we have shown that this initial laterally directed outgrowth may occur in response to an attractive gradient of Netrin-1 (Richards et al., 1997), which is expressed within the internal capsule (Serafini et al., 1996). In this study, we show two new findings with regard to the development of the efferent neocortical axon projections. First, the Netrin-1-induced attraction of laterally directed neocortical axons may be mediated by DCC expression on these axons during their growth through this region. Second, the expression of DCC on cortical axons reflects this developmental difference between the laterally and medially projecting populations. From at least E13, DCC is expressed throughout the intermediate zone and is highly expressed on laterally directed cortical axons within the internal capsule. However, by E16, DCC expression in the internal capsule begins to decrease, whereas expression on medially projecting neurons remains high as these axons cross the midline and form the corpus callosum. Therefore, DCC is downregulated on the lateral projection first, which is consistent with the idea that DCC is only expressed while axons are growing toward their targets (in this case, an intermediate target such as the internal capsule). Further analysis of this pathway in the DCC knockout mouse is required to determine the role of DCC in the development of the lateral cortical projection through the internal capsule.

DCC is regulated by mammalian homologues of the *seven in absentia* (Siahs) gene (Hu et al., 1997). In this pathway, Siahs regulates DCC expression by degrading the protein via the ubiquitin–proteasome pathway, which targets proteins for rapid degradation. One function of this rapid downregulation in DCC protein may be to inhibit DCC-induced cell-death pathways involving caspases (Mehlen et al., 1998) once the DCC ligand is removed. Our results show that, once cortical axons leave the region of the internal capsule, which expresses Netrin-1, DCC protein is no longer expressed (in axons entering the thalamus) or is greatly downregulated (in the corticospinal tract) on the distal part of the axon that grows beyond the internal capsule. Soon after this time, DCC expression is downregulated on the whole axon. This suggests a localized regulation of DCC expression in the distal part of the axon initially, possibly by Siahs, followed by complete downregulation of DCC in the entire axon. This result also indicates that the DCC/Netrin-1 interaction may only be

involved in guiding cortical axons to this intermediate target and is not required for growth to the final target or the recognition of the final target of the axon.

In further support of a role for DCC in guiding axons to intermediate targets, DCC is highly expressed on many of the major commissural axons within the brain, including the corpus callosum, hippocampal commissure, and the habenula commissure. All these midline structures may be considered to be "intermediate" targets of these axons and implicate DCC in a more general role of commissural formation in the brain and in the spinal cord, as previously described (Keino-Masu et al., 1996). This hypothesis has been supported by the loss of these commissural pathways in the DCC knockout mouse (Fazeli et al., 1997). However, one major difference exists between our findings of DCC protein expression and the phenotype observed in the Netrin-1 and DCC mutant mice. DCC is highly expressed in the habenula commissure, but this structure is present in both mutants. The reason for this difference in findings is unclear. One reason may be that Neogenin may substitute for DCC in the development of the habenula commissure in the DCC knockout, or that DCC may be involved in either habenula axon guidance or other developmental mechanisms apart from guiding axons across the commissure. Because the development of the entire projection of these axons has not been extensively studied in the DCC knockout, we cannot rule out this possibility. Similarly, the lateral olfactory tract has not been studied in the DCC knockout and, therefore, may also show defects not previously described in either the Netrin-1 or DCC knockout.

In the anterior commissure, DCC is expressed only until E15 and is completely downregulated by E16. This result also indicates that DCC expression is only present during the targeting phase of axon outgrowth.

Previous studies have shown the mRNA for DCC to be present by E11.5 in the developing cortex (Gad et al., 1997). We have found that DCC protein expression is not detectable with this antibody until E13 and is expressed within the intermediate zone and not within the cell bodies of these neurons. In addition, we found that DCC protein expression also remains within these axons 1–2 days after the mRNA is no longer detectable. These findings indicate either that DCC mRNA and protein levels may be independently regulated or that a threshold amount of DCC protein must be expressed before we can detect it with this antibody. Further expression analysis with antibodies directed toward different regions of the DCC protein will be required to investigate this possibility.

We have shown that DCC is expressed in particular populations of projecting axons within the developing forebrain. Although some expression analysis of Netrin-1 mRNA has been performed within the developing forebrain (Livesey and Hunt, 1997), there has not been a detailed analysis performed of Netrin-1 protein expression within this region. Given that Netrin-1 may diffuse away from its site of synthesis, a detailed analysis of protein expression may be able to reconcile the expression of Netrin-1 within targeting regions of DCC-expressing axons. At present, there are some inconsistencies with the known mRNA expression patterns of Netrin-1 and DCC protein expression. For example, Netrin-1 is not expressed within the cortical plate, hippocampus, or the developing olfactory bulb, but DCC protein is highly expressed on axons of these regions. Although this may be resolved with better antibodies to Netrin-1, an alternative explanation is that there are additional ligands for DCC that may be expressed along the pathways of these axons.

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