

Lesion-Induced Regulation of Netrin Receptors and Modification of Netrin-1 Expression in the Retina of Fish and Grafted Rats

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To determine whether netrin receptors (DCC, UNC5H1, UNC5H2) and netrin-1 are present in the adult rat retina and may affect regeneration of retinal ganglion cell (RGC) axons into peripheral nerve (PN) grafts, *in situ* hybridization (ISH) and immunostaining experiments were performed in normal and operated rats. Netrin-1 expression was not found in the optic nerve head of adult rats, normal and postlesion, but appeared transiently at 7 and 14 days after PN grafting. ISH signals of netrin receptors, however, disappeared from RGCs within 2 days after lesion and remained absent after PN grafting except for UNC5H2, which transiently occurred in a few RGCs. Netrin-1 expression was observed in the optic nerve head of adult fish, normal and postlesion, and the netrin-1 Fc fusion protein bound to young growing and all regenerating axons. Thus, the netrin-1-dependent guidance system continues to function in fish but apparently no longer operates in adult rats.

INTRODUCTION

In contrast to their counterparts in fish, retinal ganglion cells (RGCs) in mammals fail to spontaneously regenerate their axons after optic nerve lesion. This has been attributed to several factors: the nonpermissive glial cell environment, the lack of growth-encouraging elements, and the inability of the affected neurons to express the full set of growth-related molecules required for growth and guidance. Replacement of the nonpermissive optic nerve by a growth-supportive peripheral nerve allows roughly 5% of the RGCs origi-

nally present to regenerate long axons into the graft (Aguayo *et al.*, 1991). These axon-regenerating RGCs express the growth-related proteins GAP-43, L1, and reggie-1 and -2 but lack others, i.e., TAG-1 and SC-1, which accompany axon growth in the embryo (Jung *et al.*, 1997; Lang *et al.*, 1998).

The grafted retinæ contain—aside from the 5% of axon-regenerating RGCs—cells whose axons do not gain access to the graft, apparently because of difficulties in traversing the optic nerve head. This is particularly evident in rats receiving injections of neurotrophins (NT). This therapy rescues RGCs from lesion-induced death, which otherwise abolishes 90% of the RGCs by 28 days (Cohen *et al.*, 1994; Mansour-Robey *et al.*, 1994). Repeated injection of BDNF or NT-4/5 increases the incidence of profuse axonal sprouting around the optic disk/optic nerve head region and induces long intraretinal axon branches which often turn at the optic disk/optic nerve head and grow toward the periphery (Sawai *et al.*, 1996). This suggests lack of a growth-encouraging signal or presence of a repelling signal at the optic disk/nerve head region.

In the rodent embryo, passage of axons from intraretinal territories into the optic nerve requires the presence of netrin-1 in the optic nerve head region and the presence of the netrin-1 receptor DCC (Deleted in colon-rectal cancer) on the RGC axons (Keino-Masu *et al.*, 1996; Deiner *et al.*, 1997). It is conceivable that the failure of the lesioned axons to grow across the nerve head into the graft results from defects in this growth/guidance system. This system includes, aside from DCC, UNC5H family receptors (Leonardo *et al.*, 1997; Przyborski *et al.*, 1998). DCC cooperates with UNC5H receptors in translating the signal derived from the

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binding of netrins to the extracellular portion of the receptor proteins into the axon. The association of the cytoplasmic domain of UNC5H with the cytoplasmic domain of DCC converts the attractive response into a repellent one (Hong *et al.*, 1999; Bashaw and Goodman, 1999), resulting in the deflection of the growth cone away from the netrin-1 source. UNC5H1 and UNC5H2 were reported in neurons in which DCC is expressed (Leonardo *et al.*, 1997) and this includes the embryonic eye cup. So it is possible that both receptor types, DCC and UNC5H, participate in netrin-1-dependent RGC axon guidance and are—together with netrin-1—subject to lesion-induced expressional changes so that axotomized RGCs have difficulties in growing across the optic nerve head.

To explore the possibility that the netrin-1/netrin receptor-dependent guidance system is defective or has undergone substantial changes in the visual system of adult rodents, we analyzed the expression of DCC, UNC5H1, and UNC5H2, the netrin receptors present in the mammalian visual system (Livesay and Hunt, 1997; Leonardo *et al.*, 1997), and of netrin-1 by *in situ* hybridizations and/or immunocytochemistry in normal adult rats, after optic nerve lesion, and following PN grafting. Moreover, if netrin-1 does play a role in axon regeneration one might expect its presence in the fish visual system, well known for its ability to regenerate RGC axons after lesion. To assess the situation in adult fish, we examined the goldfish retina using anti-netrin-1 antibodies and a netrin-1 Fc fusion protein as a reliable indicator of the presence or absence of netrin receptors on the surface of RGC axons (Keino-Masu *et al.*, 1996).

RESULTS

Expression of Netrin-1 and of Netrin Receptors during Development

In confirmation of earlier work (Deiner *et al.*, 1997; Strähle *et al.*, 1997), the optic nerve head region in the E13/14 embryo was labeled by anti-netrin-1 antibodies (Fig. 1a) and exhibited netrin-1 *in situ* hybridization signals (not shown). Accordingly, DCC mRNA was expressed in the embryonic RGC layer (Fig. 2a) (Deiner *et al.*, 1997) and the RGC axons, known to be L1 positive, were brightly labeled with anti-DCC antibodies (Figs. 1c and 1e). In addition, developing RGCs apparently coexpress mRNAs of the UNC5H receptor family as demonstrated by the *in situ* hybridization signals in the RGC layer with the UNC5H1 and H2 receptor probes (Figs. 2d and 2g). This indicates that RGC axons

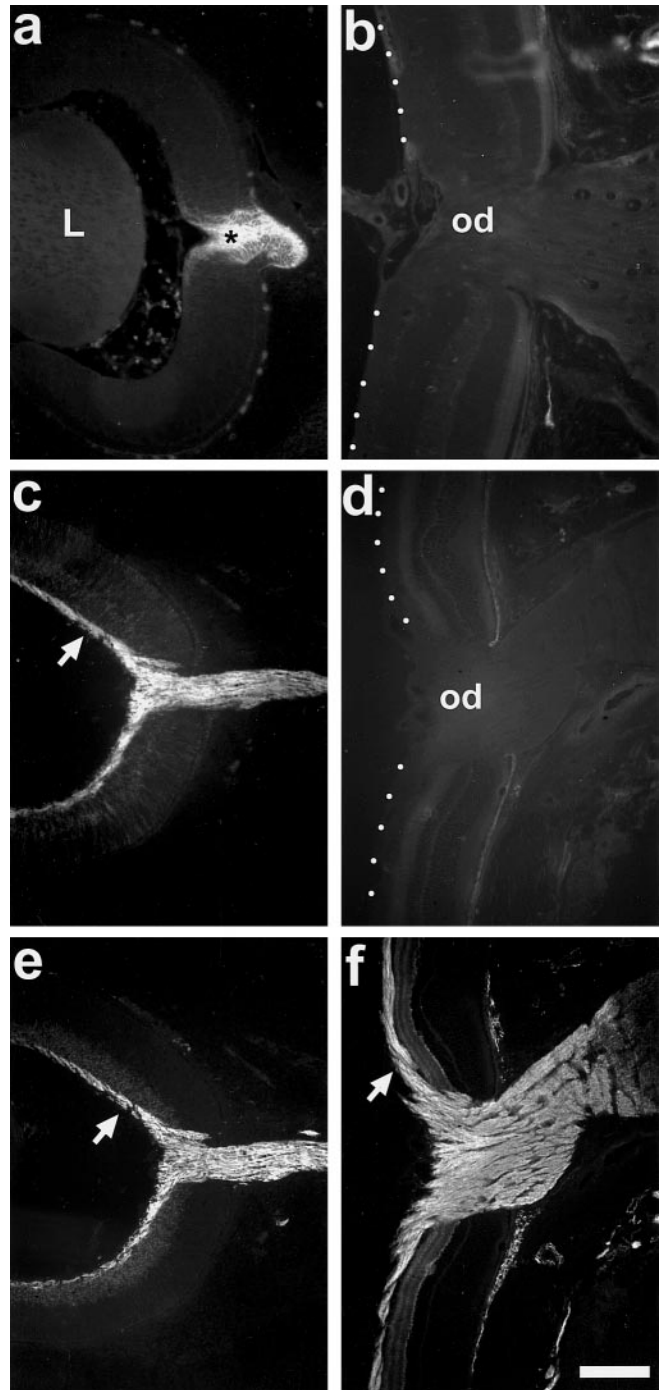


FIG. 1. Expression of netrin-1 and DCC during development. (a) Anti-netrin-1 stains the optic disk/nerve head region (od) in the embryonic (E14) rat eye (asterisk), but no longer in the adult (b). The dotted line indicates the inner limiting membrane above the RGC axon layer. L, lens; scale bar, 100 μm . (c) Anti-DCC labels RGC axons (arrow) in the embryo (E16) but not in the adult retina (d). Scale bar, 200 μm (c), 125 μm (d). (e and f) Anti-L1 labels RGC axons in the embryo (e) and in the adult retina (f). Scale bar, 200 μm (e), 125 μm (f).

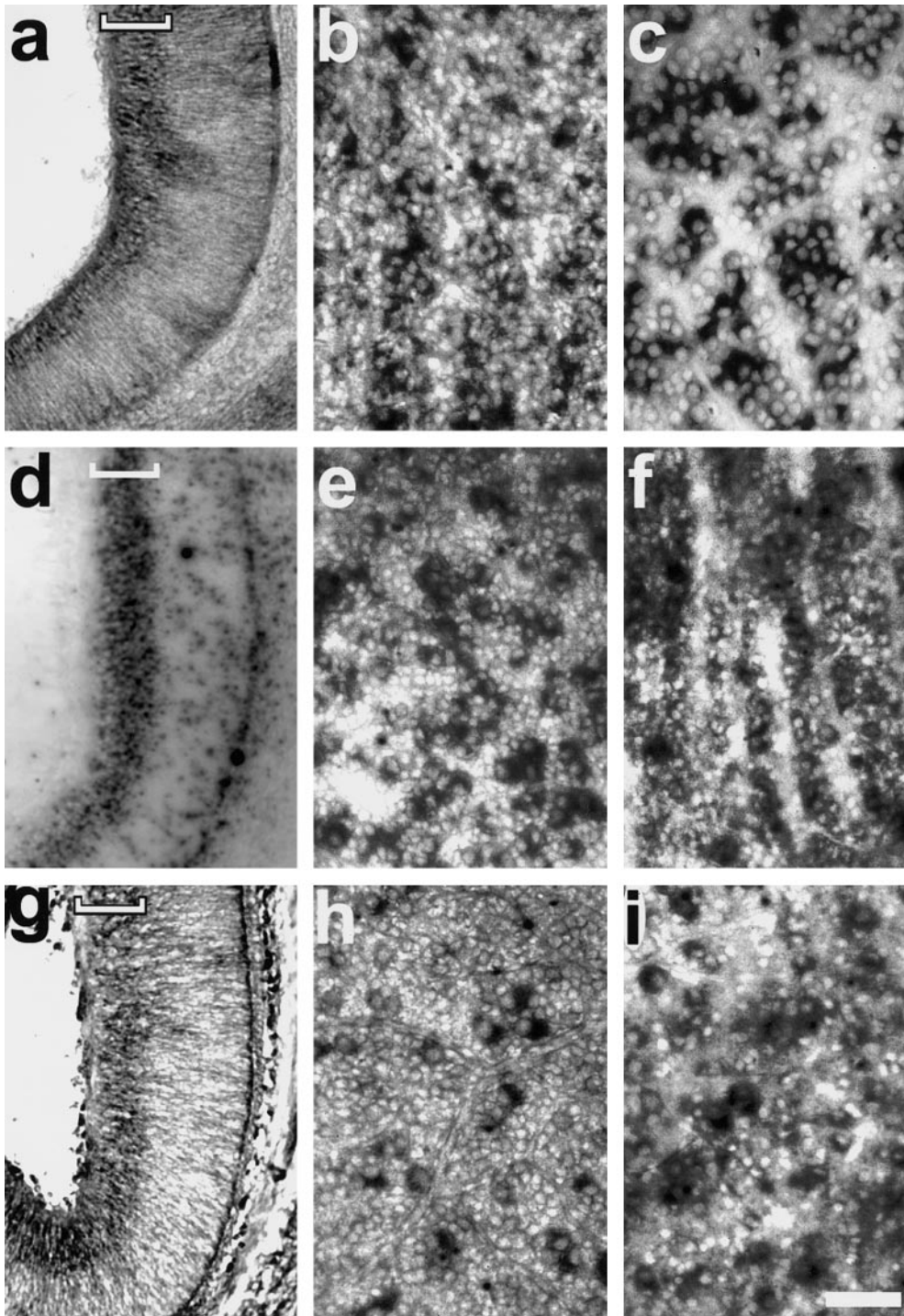


FIG. 2. Expression of netrin receptors in RGCs during development. *In situ* hybridizations with cRNA probes of DCC (a, b, c), of UNC5H1 (d, e, f), and of UNC5H2 (g, h, i) give labeling of the developing RGC layer (brackets) in the E16 embryonic eye (a, d, g). Staining is also observed in many RGCs in the P15 (b, e, h) and adult (e, f, i) retina. Scale bar, 40 μm for a, d, g; 60 μm for b, c, e, f, h, and i.

can interpret netrin-1 as a growth-encouraging or -discouraging signal depending on the context.

The three netrin receptor mRNAs, DCC, UNC5H1, and UNC5H2, continued to be expressed by many (but apparently not all) RGCs over postnatal stages and into adulthood (Figs. 2b, 2c, 2e, 2f; 2h, and 2i). In the adult retina, anti-DCC staining of axons was not apparent (Fig. 1d), whereas that of anti-L1 persisted (Fig. 1f).

Despite the continuous expression of netrin receptor mRNAs by RGCs, no netrin-1 *in situ* hybridization signals were found in cells of the optic nerve head in the eye of adult rats and anti-netrin-1 immunoreactivity was undetectable (Fig. 1b). Therefore, netrin-1, which is crucial for guidance of embryonic axons (Deiner *et al.*, 1997) seems to be developmentally regulated and is no longer synthesized by glial cells of the optic nerve head in normal adult rats. As axons can regulate the level of receptor protein expression depending on the concentration of the relevant ligand (Kidd *et al.*, 1998a,b), it is conceivable that loss of netrin-1 accounts for the downregulation of DCC protein on RGC axons. As antibodies against UNC5H1 and UNC5H2 are not available, the expression of these receptor proteins could not be examined.

Downregulation of Netrin Receptor Expression and Absence of Netrin-1 from the Optic Nerve Head Region after Optic Nerve Lesion

To determine if netrin-1 expression is reinduced by optic nerve crush, the optic nerve head region was analyzed in adults at 2, 7, 21, and 28 days postlesion. *In situ* hybridization signals and anti-netrin-1 immunoreactivity remained absent from the optic nerve head (see Fig. 7a). This correlates with the disappearance of *in situ* hybridization signals for netrin receptor mRNAs from RGCs within 2 days after nerve crush, and no *in situ* hybridization signals for DCC, UNC5H1, or UNC5H2 were detected in RGCs at later stages after optic nerve lesion (Figs. 3a–3c). At 7 days after crush, retinae exposed to a Gap-43 cRNA probe, however, show clear and distinct *in situ* hybridization signals in RGCs (Fig. 3d) in correspondence with earlier data (Doster *et al.*, 1991; Jung *et al.*, 1997). This indicates that expression of DCC, UNC5H1, and UNC5H2 is downregulated by RGCs in response to lesion. Moreover, downregulation of netrin receptor mRNAs occurs rapidly as in the case of TAG-1 mRNA (Jung *et al.*, 1997).

When segments of adult rat retina are explanted 7 days after optic nerve lesion and kept in culture, RGCs are capable of regenerating axons (Bähr *et al.*, 1988). Consistent with the *in vivo* observations, these *in vitro*

regenerating RGC axons were anti-DCC negative (Fig. 4d), but brightly labeled by anti-Gap-43 and anti-L1 (Fig. 4c). Axons extending from explants of embryonic eyes (E16), however, were labeled by anti-L1 and anti-DCC antibodies (Figs. 4a and 4b).

These findings give reason to suspect that the guidance system which depends on optic nerve head-associated netrin-1 and netrin receptor expression in RGCs fails to function after lesion. The crucial question addressed next concerns netrin-1 and netrin receptor expression in RGCs of grafted rats in which only a small population of RGCs is capable of regenerating axons into the sciatic nerve graft.

Netrin-1 and Netrin Receptor Expression in Grafted Rats

RGC axon regeneration into PN grafts takes time. Therefore, most earlier studies (Aguayo *et al.*, 1991), including our own (Jung *et al.*, 1997), analyzed RGC axon regeneration at around 28 days after surgery when axons are found in the transplant. As demonstrated previously, axon-regenerating RGCs express Gap-43 and L1 mRNAs (Jung *et al.*, 1997). We therefore performed double *in situ* hybridization experiments pairwise with a Gap-43 cRNA probe (to identify RGCs with regenerating axons) and cRNA probes of either of the netrin receptors. In addition, *in situ* hybridization experiments with cRNA probes for DCC, UNC5H1, or UNC5H2 alone were also carried out.

No *in situ* hybridization signals were obtained in 28-day grafted retinae with either of the netrin receptor probes whether applied alone or pairwise with the GAP-43 cRNA probe in double *in situ* hybridization experiments (Figs. 5a–5e). The Gap-43 mRNA was detected in RGCs (Figs. 5a–5c) at the same intensity and in a number of cells comparable to that found in earlier studies (Jung *et al.*, 1997, 1998) and most or all RGCs expressing Gap-43 mRNA showed signals for the presence of L1 mRNA in the double *in situ* hybridizations (Fig. 5f), confirming that the method can successfully be applied in this tissue. These findings therefore suggest that axon-regenerating RGCs fail to upregulate netrin receptor expression.

The absence of *in situ* hybridization signals with the DCC receptor probe in RGCs of grafted rats correlated with the absence of anti-DCC immunostaining on regenerating RGC axons. The anterograde labeling of regenerating RGC axons (by intraocular injections of biotin) allowed identification of these axons in sections through the sciatic nerve graft (Jung *et al.*, 1997). The RGC axons so labeled were stained by anti-Gap-43 and

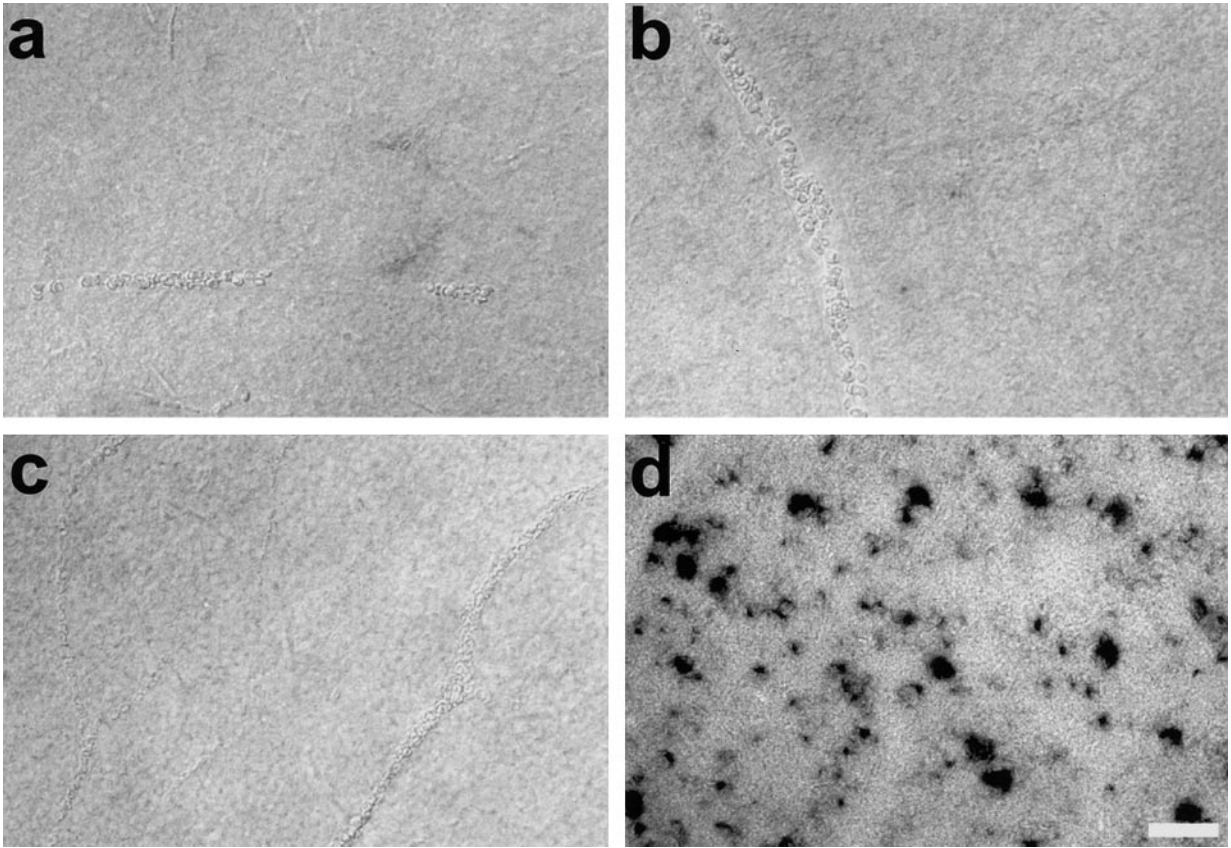


FIG. 3. Lesion-induced downregulation of netrin receptor expression. *In situ* hybridizations with cRNA probes of DCC (a), UNC5H1 (b), UNC5H2 (c), and Gap-43 as control (d) in retinas 7 days after optic nerve lesion. No staining is detectable with any of the netrin receptor probes (a–c) but does appear in RGCs with the Gap-43 cRNA probe (d). Scale bar, 30 μ m.

L1 antibodies, but they were anti-DCC negative (Figs. 6a–6d).

Transient Reexpression of Netrin-1 in Grafted Rats

To explore the possibility that a transient upregulation of netrin-1 and netrin receptors at earlier stages after PN transplantation may influence regrowth of severed axons into the graft, the optic nerve head region and retinae were probed for the presence of netrin-1 and netrin receptors, respectively. In contrast to the situation after lesion alone (Fig. 7a), sections through the optic nerve head exhibited netrin-1 immunoreactivity in this region at 7 days after PN grafting (Fig. 7b), which was nearly as intense as in the embryo in three (of four) rats, but was weak in the remaining animal. Within the optic nerve head region staining intensity decreased with distance from the retina. At 14 days after grafting, anti-netrin-1 immu-

noreactivity was weak in two of the operated rats and undetectable in the other two, suggesting that netrin-1 expression decreases with time. Attempts to analyze the optic nerve head region in the 7- and 14-day grafted rats by *in situ* hybridization failed because the crucial region and much of the surrounding area exhibited nonspecific background staining, probably as a result of the mechanical stress exerted on the tissue during the grafting procedure. This was no longer a problem in the rats 28 days after surgery. In these rats, however, much as in normal adults, no *in situ* hybridization signals were detected at the optic nerve head.

Thus, netrin-1 appears to be transiently upregulated in optic nerve head glial cells (most likely through the influence of the PN graft) and could potentially encourage or discourage growth of lesioned axons into the graft, if the RGC axons express the relevant receptors. RGC axons in these grafted rats, however, were anti-

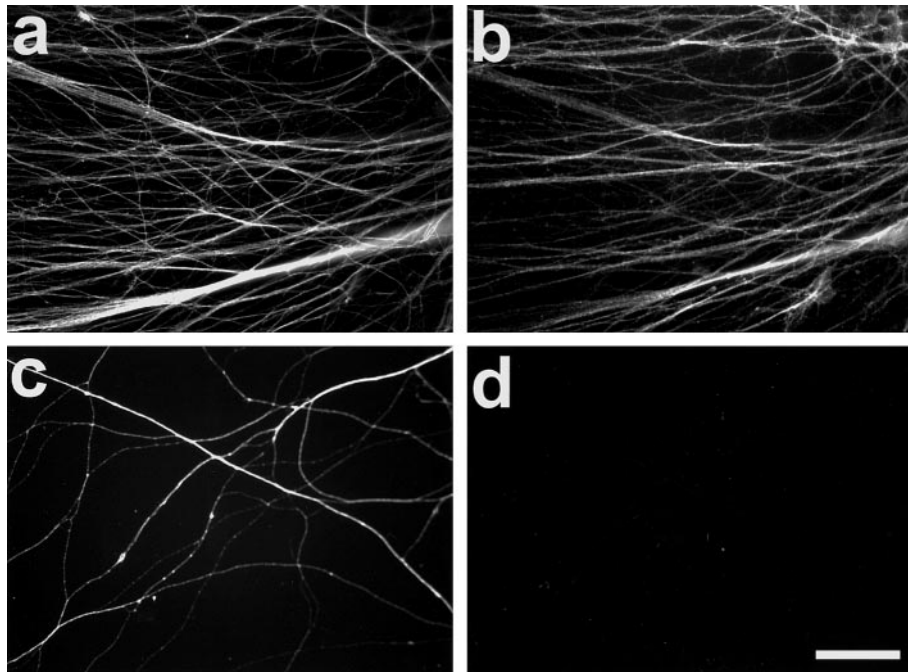


FIG. 4. RGC axons regenerating *in vitro* are anti-DCC negative. (a and b) RGC axons extending from E16 retina explants after 3 days in culture are anti-L1 (a) and anti-DCC positive (b). (c and d) Explants of adult retinae, 7 days postlesion, extend regenerating RGC axons. These axons are labeled by anti-L1 (c) but not by anti-DCC antibodies (d). Scale bar, 50 μm .

DCC negative (Fig. 7c) but brightly labeled by anti-L1 (Fig. 7d).

RGCs in retinae 7 days after transplantation did not exhibit *in situ* hybridization signals with either the DCC or the UNC5H1 receptor probe (Figs. 8a and 8b). The UNC5H2 receptor probe, which always produced staining of increased intensity compared to the other probes, gave a fine granular staining in the cytoplasm of a fraction of the RGCs expressing GAP-43 mRNA (Figs. 8c and 8d). This may indicate the presence of UNC5H2 receptor mRNA in a subpopulation of RGCs but is unlikely to be of functional relevance (see Discussion).

Together, these results show that the netrin-1/netrin receptor guidance system undergoes substantial changes in the adult and lesioned rat visual system. Still, if netrin-1 is important for developing and regenerating RGC axons, one might expect it to be expressed in the fish visual system, known for its capacity for functional repair.

Netrin-1 and Presence of Netrin Receptors in Fish

The adult fish retina, in contrast to the mammalian retina, continues to grow throughout life by adding

new RGCs at its peripheral margin (Johns, 1977). Axons extending from these new RGCs elongate toward the optic disk (Bastmeyer *et al.*, 1995; Ott *et al.*, 1998) and require guidance cues for transit through the optic nerve head.

The fish optic disk/nerve head was examined for the presence of netrin-1 by applying anti-netrin-1 antibodies to the retina of normal adults and after optic nerve transection. Anti-netrin-1 stained elongate cells (Fig. 9a), which, when viewed in whole mounts, occur in a ring around the optic disk (Fig. 9b) through which the axons pass (Fig. 9c). These young growing axons selectively express cell surface proteins required for axon growth and guidance and are visualized here by staining retina whole mounts with anti-neuroilin antibodies (Ott *et al.*, 1998). To examine whether growing RGC axons express netrin receptors, the netrin-1 Fc fusion protein was applied to retinae of normal and nerve-transected fish. Figure 10 shows retina segments halfway between the retinal margin and the optic disk. The path of the young growing axons, representing a small subpopulation of all axons in radially oriented fascicles, is seen by anti-neuroilin staining (Fig. 10a). In the unlesioned retina, netrin-Fc binds selectively to the young RGC

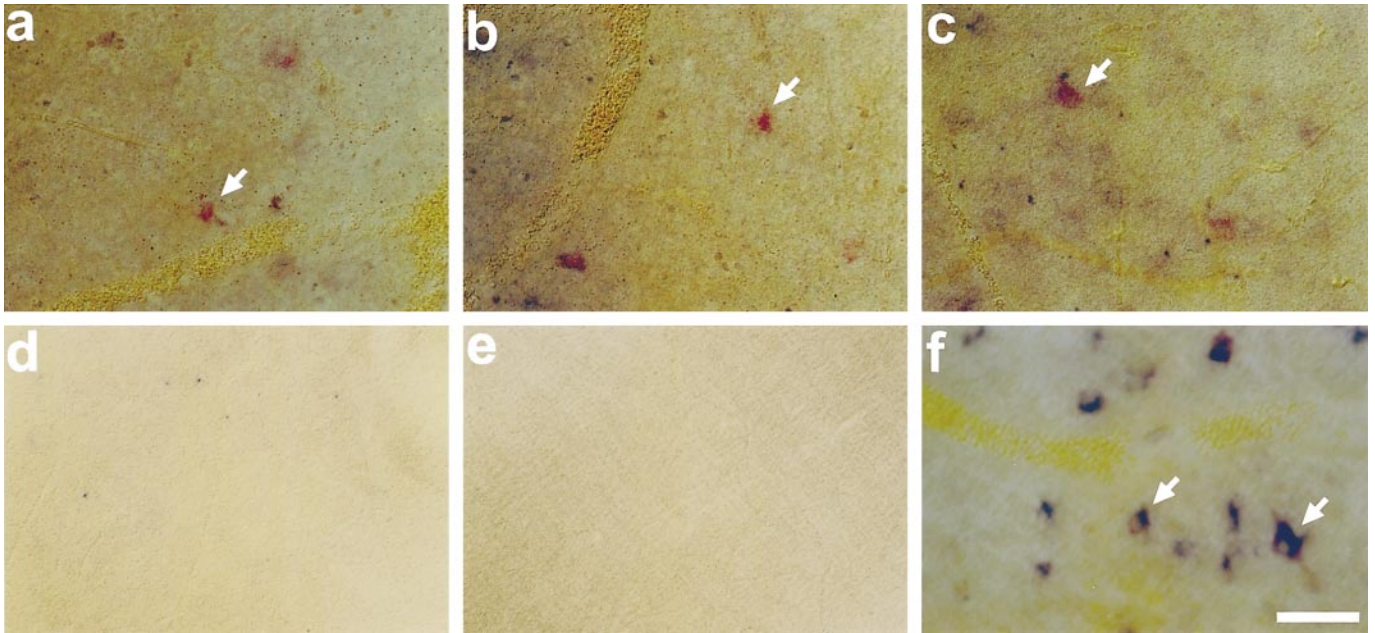


FIG. 5. Absence of netrin receptor mRNAs from RGCs in grafted rats. Retinae of grafted rats (28 days after surgery) were subjected pairwise to a double *in situ* hybridization protocol with either of the netrin receptor cRNA probes and with the Gap-43 cRNA probe (a, b, and c). RGCs exhibit Gap-43 mRNA (red, arrows) but no indications of DCC, UNC5H1, or UNC5H2 mRNAs. Also, no signs of DCC and UNC5H1 mRNAs are detected in RGCs after *in situ* hybridization with the corresponding cRNA probes alone (d and e). Double *in situ* hybridization with L1 and Gap-43 cRNA probes, however, results in double-labeled RGCs (arrows) (f; Gap-43 mRNA, red; L1 mRNA, blue). Scale bar, 60 μm .

axons derived from the retinal margin (Fig. 10b). In the retina 14 days after nerve transection, however, diffuse staining appeared on all RGC axons (Fig. 10c). The axons from young RGCs are labeled more brightly than the older axons, which apparently re-express netrin-1 receptors. Expression by young axons only and reexpression by all axons after optic nerve lesion is typical for several growth-associated cell surface proteins in the goldfish visual pathway (Stuermer *et al.*, 1992). Staining of the optic disk by anti-netrin-1 remained unchanged during RGC axon regeneration.

It thus appears that netrin-1 is available in adult fish at the optic disk for guidance of new axons into the nerve and continues to be present during axon regeneration following optic nerve transection. Moreover, in correlation with the addition of new RGC axons to the preexisting ones there is a regulated expression of netrin-1 receptors which is confined to young growing axons. Lesion, however, induces up-regulation of netrin-1 receptor expression in all RGC axons, which correlates with the well-known ability of the fish RGCs to regenerate lesioned axons.

DISCUSSION

The present results demonstrate that the timely and coordinated expression of netrin-1 and of netrin receptors which is observed during development is disrupted in the lesioned visual pathway of the adult rat. This implies that lesioned RGC axons do not receive the growth-encouraging signal required for passage into the optic nerve and for regeneration into the PN transplant. In fish, in contrast, severed axons reexpress netrin receptors. This and the uninterrupted expression of netrin-1 correlate with the success of axonal regeneration in the fish visual pathway.

Netrin-1 expression is apparently developmentally regulated and is not detectable in the optic nerve head in the normal adult rat retina or after optic nerve lesion, but is transiently upregulated after PN transplantation. Thus, netrin-1 is not available to RGC axon sprouts emerging in response to lesion alone but may influence such sprouts after PN transplantation. Netrin-1 receptor mRNA expression by RGCs continues into adulthood, but *in situ* hybridization experiments show that DCC, UNC5H1, and UNC5H2 are rapidly downregulated af-

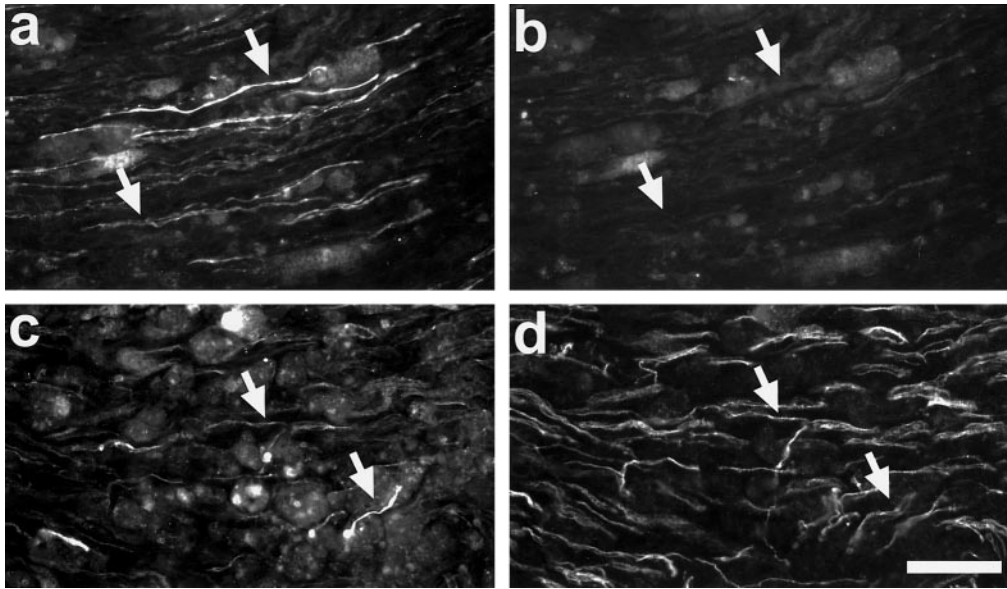


FIG. 6. Regenerating RGC axons in the graft are anti-DCC negative. (a and b) 28 days after PN transplantation, the regenerating RGC axons (arrows) in the graft are identified by biotin labeling (a) but the same axons are anti-DCC negative (b). (c and d) The biotin-labeled regenerating RGC axons (c, arrows) are labeled by anti-L1 (d). Scale bar, 50 μ m.

ter lesion. Moreover, DCC and UNC5H1 do not appear in RGCs of grafted rats. Only a fraction of the RGCs transiently possess low level UNC5H2 receptor mRNA 7 days after PN transplantation but no longer at 28 days. This makes it unlikely that netrin-1 is recognized as a growth-encouraging signal by axons in grafted rats. Instead, if UNC5H2 mRNA is of functional relevance, the interaction of receptor-expressing RGC axons with netrin-1 should result in the deflection of the axons (Hong *et al.*, 1999). Thus, downregulation of netrin receptors, and in particular of DCC, in the axotomized rat retina correlates with the very limited ability of RGCs in rats to grow across the optic nerve head and to regenerate long axons into PN grafts.

Loss of netrin-1 and netrin receptor downregulation in the adult lesioned rat retina implies that the netrin-dependent guidance system is apparently no longer operating. In this respect, the intraretinal sprouts and axons of lesioned RGCs resemble developing RGC axons in netrin-1-deficient and DCC knockout mice (Deiner *et al.*, 1997).

In grafted rats, however, netrin-1 transiently reappears in the optic nerve head region so that a potentially growth-encouraging signal, i.e., netrin-1, is available for a limited period. Yet, we find no sign of upregulation of DCC, and this receptor is required to translate netrin binding into a positive response of the axon (Keino-Masu *et al.*, 1996; Deiner *et al.*, 1997). UNC5H receptors

can elicit an avoidance reaction by the axons in response to netrin-1 (Leonardo *et al.*, 1997; Hong *et al.*, 1999). That netrin-1/UNC5H receptor interactions sufficiently account for the failure of many axons to grow across the optic nerve head into the graft seems unlikely for several reasons: First, only one of the two UNC5H receptors (UNC5H2) is detectable in RGCs of grafted rats. Second, its level of expression is low, judging from the intensity of the color reaction from the nonradioactive *in situ* hybridization method. Third, the granular reaction product is detectable in only a few RGCs, which are a small fraction of RGCs exhibiting the GAP-43 *in situ* hybridization signal. Finally, the turning of intraretinal axons away from the optic nerve head (Sawai *et al.*, 1996) also occurs in lesioned rats without PN graft in which DCC and both UNC5H receptors are downregulated and in which netrin-1 is absent from the optic nerve head. Overall, it appears that downregulation of netrin-1 and of the netrin receptors partially accounts for the inability of lesioned axons to grow across the optic nerve head. This response may also be attributed to other growth and guidance systems, such as that involving Robo and Slit family members (Kidd *et al.*, 1988a,b, 1999), which were identified in the rodent visual system (Yuan *et al.*, 1999). Expression or upregulation of repulsive/inhibitory molecules such as semaphorins and members of the ephrin/Eph receptor fam-

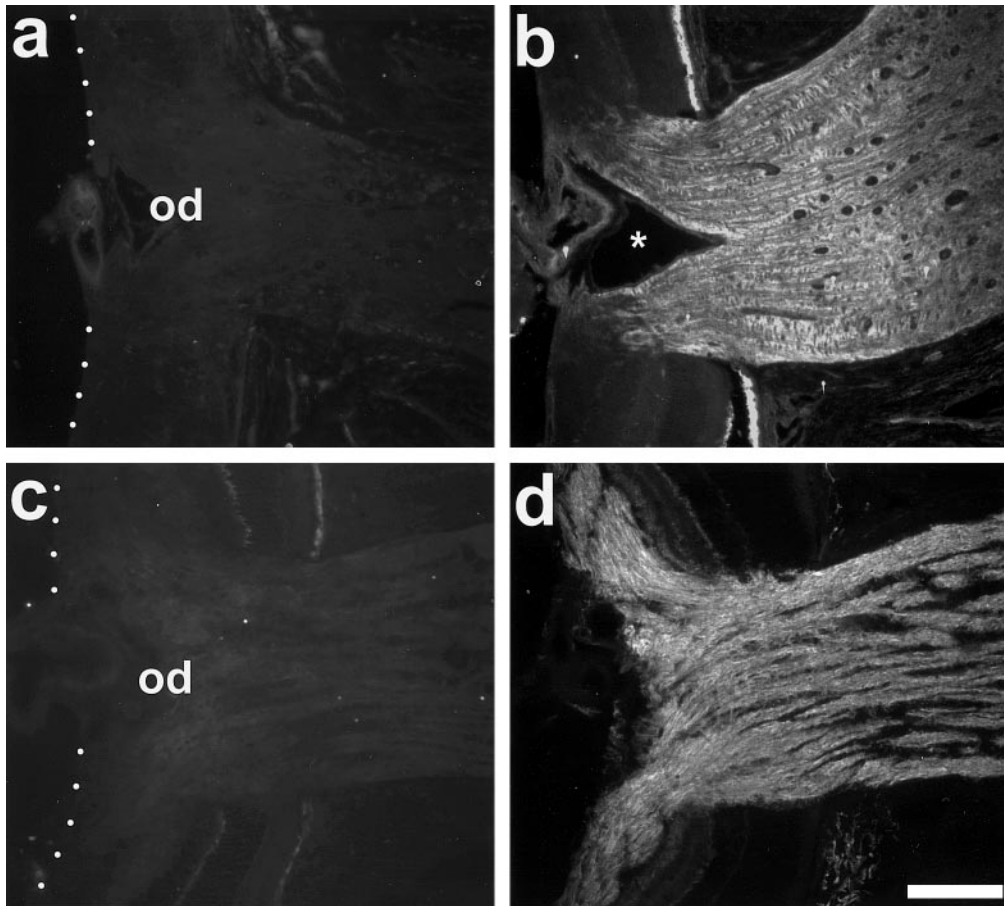


FIG. 7. Transient upregulation of netrin-1 and loss of DCC in grafted rats. (a and b) Netrin immunoreactivity is absent from the optic disk/nerve head (od) 7 days after optic nerve crush (a) but reappears in this region 7 days after PN transplantation (b). Asterisk, retinal artery. The dotted line indicates the inner limiting membrane above the RGC axon layer. (c) Anti-DCC fails to label RGC axons 7 days after PN transplantation, but (d) the axons are stained by anti-L1. Scale bar, 100 μm .

ily (for review see Mueller, 1999) could also be involved.

That netrin-1 and netrin receptor expression may be advantageous for RGC axon regeneration is suggested by the uninterrupted presence of netrin-1 at the optic nerve head/disk in adult fish. That optic nerve head cells continue to express netrin-1 may be explained by the fact that newly added axons during the continuous growth of the goldfish retina require the appropriate guidance cues (Vielmetter *et al.*, 1989; Ott *et al.*, 1998). The cDNAs of DCC and UNC5H receptors have not been cloned in goldfish, and the DCC antibody does not recognize the corresponding protein in fish. The netrin-Fc fusion protein, however, binds to RGC axons in the fish retina in a characteristic manner: only young growing axons from the retinal margin are labeled in the normal unlesioned retina, but all RGC axons in the

retina are labeled after optic nerve lesion. Although we do not know which netrin receptor types are expressed and reexpressed by fish RGC axons, the overall pattern revealed by the netrin-1 antibodies and netrin-Fc fusion protein is consistent with the general rule that lesioned axons reexpress the proteins associated with developing axons in the embryo and in the adult and receive the relevant guidance cues (Stuermer *et al.*, 1992).

RGCs in grafted rats which successfully regenerate an axon into the graft were previously found to express only some of the growth-related proteins present on RGC axons in the embryo. At 28 days after grafting, the RGCs with axons in the graft synthesize β -tubulin (McKerracher *et al.*, 1993), Gap-43, L1 (Jung *et al.*, 1997), and reggie-1 and -2 (Lang *et al.*, 1998), indicating that these growth-associated proteins are required for regeneration of long axons into the graft. They do not

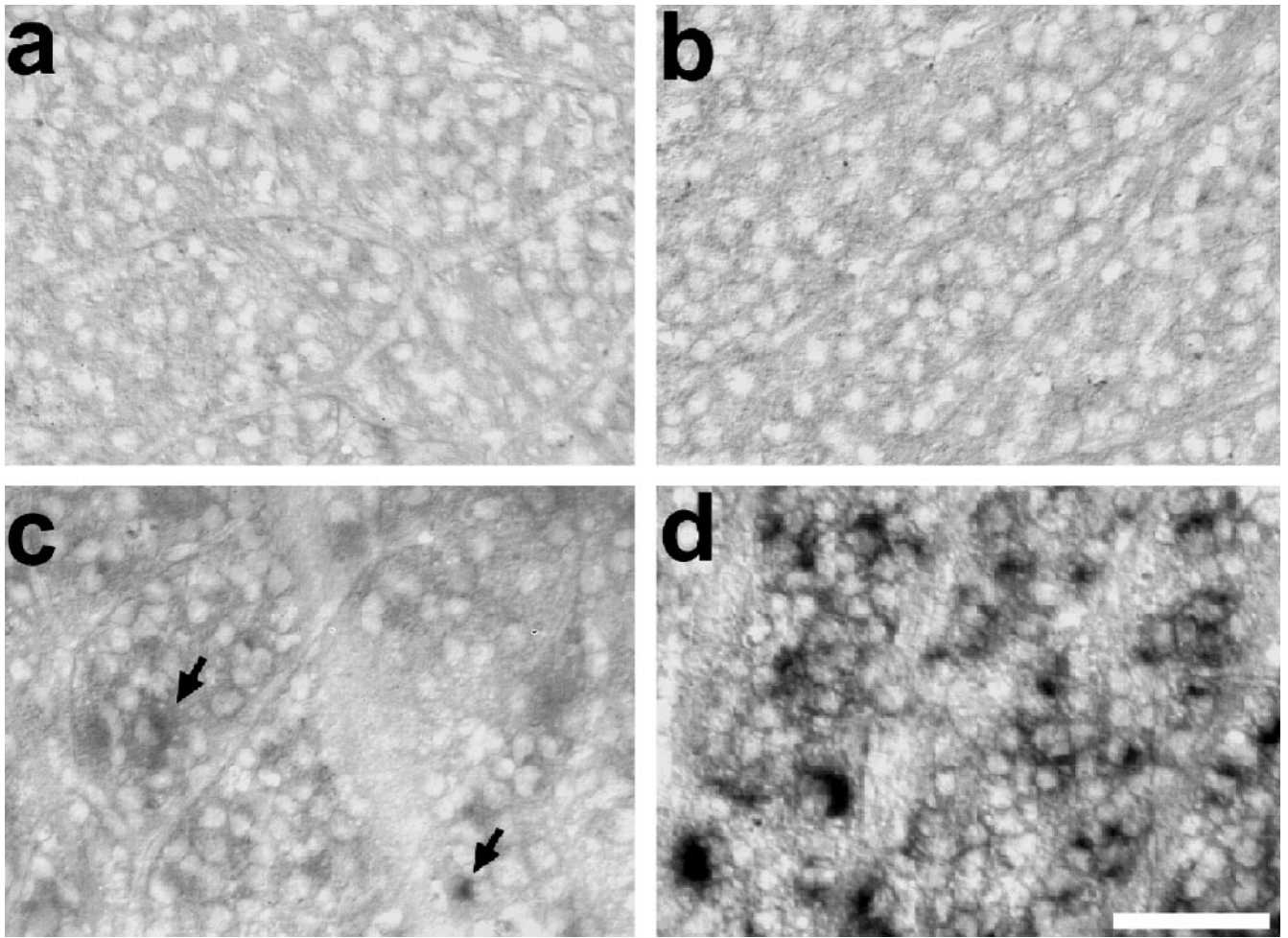


FIG. 8. No upregulation of netrin receptor mRNAs in 7-day grafted rats. *In situ* hybridization with DCC (a) and UNC5H1 (b) cRNA probes produce no staining in RGCs of grafted rats. (c) A small fraction of RGCs show granular staining after exposure to an UNC5H2 receptor probe (arrow). These represent a subpopulation of the RGCs present which all show intense staining with the GAP-43 cRNA probe (d). Scale bar, 50 μ m.

express TAG-1 mRNA (Jung *et al.*, 1997), which was in fact downregulated within 2 days after lesion, much as are the netrin receptor mRNAs, so that netrin receptors do not appear to play an essential role in the elongation of these axons. In light of the finding that axons downregulate the relevant receptors the role of netrin-1, which is transiently expressed in the optic nerve head region of grafted rats, is less apparent. It has recently been shown that Schwann cells in sciatic nerves are capable of upregulating netrin-1 following lesion (Madison *et al.*, 2000). This suggests that netrin-1 may also be present in PN transplants but how this expression affects lesioned axons which lack the relevant receptors is unclear. It has, however, been demonstrated that the graft exerts a positive influence on the axotomized RGCs (Dezawa and Adachi-Usami, 2000), promotes

RGC survival (Bähr *et al.*, 1992), encourages the growth of RGC axons, and, as shown here, leads to a transient upregulation of netrin-1 in the optic nerve head. All these effects are probably caused by factors derived from the graft. It is less likely that Schwann cells pass from the PN graft into the optic nerve head region and beyond this structure since peripheral glial cells seem to become inhibited of migrating into CNS fiber tracts by contacts with astrocytes (Franklin and Blakemore, 1993).

The question as to why a small fraction of RGCs (5%) regenerate axons into a graft remains unanswered (Aguayo *et al.*, 1991). It is possible that they represent a fraction of those that were able to form sprouts directly at the lesion site and in the vicinity of the PN graft (Richardson *et al.*, 1982) instead of being retracted back

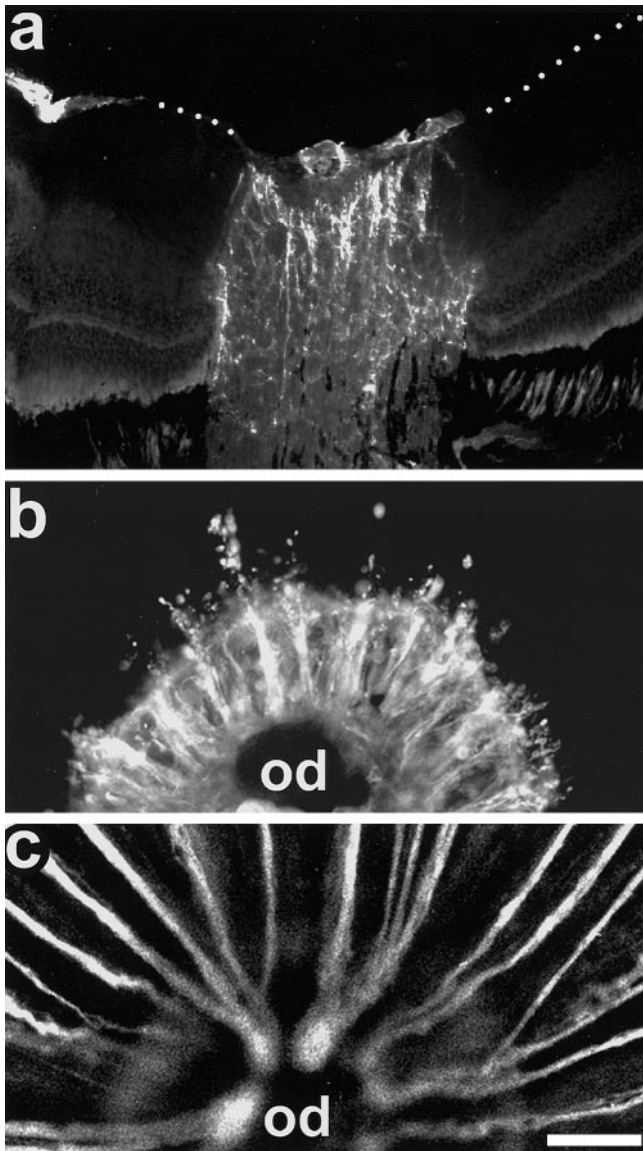


FIG. 9. Netrin-1 in the goldfish retina. (a) Sections through the center of the goldfish retina show anti-netrin-1 staining of elongate cells in the optic nerve head. The white dots mark the inner limiting membrane of the retina. (b) Retina whole mount. The anti-netrin-1 labels glial cells which form a ring around the optic disk (od). (c) The young growing RGC axons, visualized by anti-neuroilin in the same retina shown in (b), are assembled into fascicles and pass over the netrin-1-positive glial cells (shown in b) to enter the optic nerve head. Scale bar, 100 μm for a; 50 μm for b and c.

into the retina. Or, there is a subpopulation of RGCs capable of traversing the nerve head, independent of the netrin guidance system, as is the case in netrin-1- and DCC-deficient mice (Deiner *et al.*, 1997).

Where mRNA expression (detected by nonradioac-

tive *in situ* hybridization method employed here) was compared with expression of the respective proteins, congruent results were obtained (Laessing and

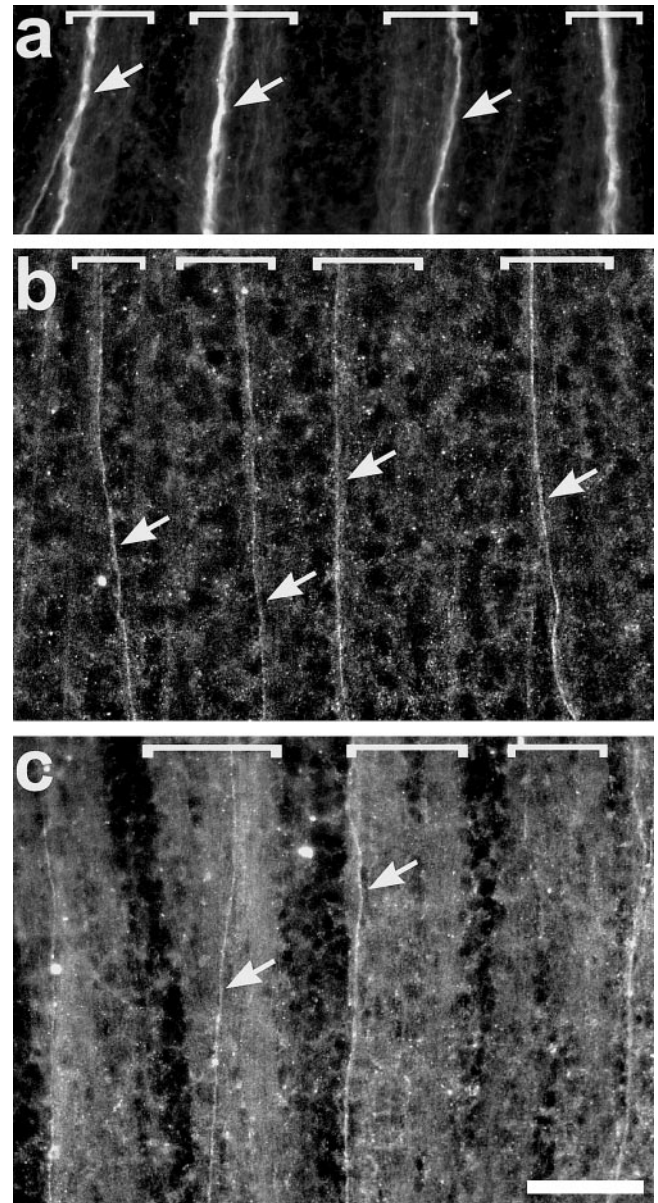


FIG. 10. Expression of netrin receptors on growing RGC axons in fish. Segments of retinae at midretinal levels. (a) Anti-neuroilin antibodies label young growing RGC axons (arrows), which represent a subpopulation of all RGC axons present. The width of RGC axon fascicles is marked by brackets (a-c). (b) In the normal adult goldfish retina, netrin-Fc binds selectively to young growing RGC axons (arrows) derived from the retinal margin. (c) After optic nerve lesion, netrin-Fc stains all RGC axons, but the young ones are stained more intensely than regenerating axons. Scale bar, 100 μm .

Stuermer, 1996; Jung *et al.*, 1997; Ankerhold *et al.*, 1998) generally supporting the conclusion that netrin receptors are downregulated in response to lesion. An exception found in the present study is the presence of *in situ* hybridization signals with the DCC receptor probe in normal adult rat RGCs while the RGC axons in the retina and the proximal portion of the optic nerve showed no anti-DCC labeling above background. In the embryo, however, anti-DCC did stain axons (here and Deiner *et al.*, 1997) of RGCs exhibiting DCC mRNA. The purchased anti-DCC antibody, which was raised against a peptide of human protein, may have insufficient affinity for the rat proteins and thus fail to detect lower levels of proteins, or, the RGCs may for some reason (see Results) cease DCC protein translation.

The presence of DCC and UNC5H receptors in embryonic rat RGCs suggests that netrin-1 can attract or repulse RGC growth cones (de la Torre *et al.*, 1997). One may imagine that netrin-1 first encourages axon growth along the optic nerve head cells and then, through UNC5H receptor interaction with the DCC receptor, repels the growth cones. Avoidance of this region may then result in axon growth out of the eye and into the optic nerve. Such a sequential effect of netrin-1 on RGC growth cones has been suggested to occur in *Xenopus* (Höpker *et al.*, 1999). Here, the avoidance response seems to result from changes in the intracellular cAMP concentration elicited by laminin/ β -integrin signaling. Our present finding that UNC5H receptors are expressed during RGC axon elongation in embryonic rats suggests that UNC5H receptors also participate in guiding growth cones through the nerve head and possibly at other points along their path.

EXPERIMENTAL METHODS

Surgery. Adult female Wistar rats weighing 250–300 g were anesthetized by intraperitoneal injection of Kemint/Rompun (Alvetra/Bayer; 0.25 ml Kemint/0.05 ml Rompun per 300 g body weight) for all surgical procedures including intravitreal injections of biotin. All surgery was in compliance with animal welfare legislation. In one group of rats, the left optic nerve was crushed as described by Doster *et al.* (1991) to analyze the changes of mRNA and protein production in RGCs at 2, 5, 7, 14, 21, and 28 days after optic nerve lesion. In a second group of rats (grafted rats) an autologous sciatic nerve segment was attached to the orbital stump of the transected optic nerve (Jung *et al.*, 1997) according to procedures by Vidal-Sanz *et al.* (1987). In brief, the posterior pole of the left eye and optic nerve were

exposed and the dural sheath longitudinally excised. The optic nerve was transected approximately 0.5 mm behind the eye, taking care to avoid damage to the ophthalmic artery. A segment of the common peroneal nerve, 2 cm long, was removed and sutured to the sclera of the optic nerve stump. The distal end of the PN nerve, unconnected to the brain, was left subcutaneously over the posterior part of the skull. Grafted rats were sacrificed 7, 14, and 28 days after surgery. The retinae of all operated rats were examined ophthalmoscopically and those with signs of vascular damage were discarded.

For immunohistochemistry, grafted rats 28 days after surgery received an intraocular injection of 5 μ l *n*-hydroxysuccinimidobiotin ester (5 mg in 10 μ l ethanol plus 10 μ l DMSO) (Halfter *et al.*, 1987) 2 days before sacrifice to anterogradely label regenerating RGC axons in the graft (Jung *et al.*, 1997).

Preparation of retinal whole mounts. Eyes of postnatal and adult rats (normal and operated) were removed, briefly rinsed in 70% methanol, and transferred to HBSS. The retina was isolated and attached by suction to a nylon filter (Hybond N+; Amersham) with the photoreceptor layer down. The basal lamina overlying the RGC layer was removed. The retina was fixed in 4% PFA in PBS at 4°C (overnight), rinsed in PBS, and stored in methanol at –20°C (at least 2 h).

Production of cRNA as hybridization probes and *in situ* hybridization. The following plasmids were used: pCRII vector from Invitrogen containing a 0.8-kb insert of rat DCC; Bluescript pBS vectors from Stratagene containing a 0.8-kb insert of *unc5h1* cDNA, a 1.5-kb insert of *unc5h2* cDNA, and a 2.7-kb insert of a part of the 3'UTR of mouse netrin-1 cDNA (these cDNAs were kindly provided by M. Tessier-Lavigne); pGEM-3 containing a 0.75-kb insert of GAP-43 (kindly provided by J. H. P. Skene); and pBKSII M13 containing a 5.2-kb insert of L1/NILE (kindly provided by W. Stallcup). From the 5'-terminal region of L1/NILE, 1.3 kb was subcloned in pBSKII(+) using the unique sites of *Bam*HI/*Xho*I. The plasmids were linearized with the appropriate restriction enzymes, treated with proteinase K (Boehringer Mannheim), extracted with phenol/chloroform, precipitated, and labeled by *in vitro* transcription with a digoxigenin (DIG) RNA labeling kit (Boehringer Mannheim) using SP6, T3, or T7 RNA polymerases.

In situ hybridization was carried out on cryostat sections through the heads of embryos (E14–E19), on retina whole mounts of postnatal P0–P15 and adult rats, and on cryostat sections of adult rat retinae, as described previously (Jung *et al.*, 1997). The heads of em-

bryos were fixed in 4% PFA in PBS at 4°C for 6 h, transferred to 30% sucrose in PBS overnight, embedded in Tissue Tek, frozen in liquid nitrogen, cut in 20- μ m transverse sections in a cryostat, air-dried for at least 2 h, stored at -20°C, and then subjected to *in situ* hybridization procedures. The sections and whole mounts were brought to room temperature (RT) and then fixed with 4% PFA (in PBS), treated with proteinase K (10 μ g/ml, RT, 10 min), fixed again in 4% PFA (in PBS), and prehybridized at 55°C for 2 h prior to overnight hybridization (at 55°C) in 500 μ l hybridization buffer (50% formamide, 2 \times SSCT, 10% dextran sulfate, and 1 mg/ml t-RNA) containing the probe at concentrations ranging from 50 to 150 ng/ml hybridization buffer. The next day the tissue was washed at 55°C in 50% formamide/2 \times SSCT, 2 \times SSCT, and subsequently in 0.2 \times SSCT. After a blocking step (PBST, 2% sheep serum (Sigma), 2% goat serum (Vector Laboratories), 2 mg/ml bovine serum albumin (Sigma)), the tissue was incubated with anti-DIG antibodies (1:2000 in PBST) for 2 h at RT and thoroughly washed in PBST prior to the signal detection with BM purple AP substrate (Boehringer Mannheim). Sections and whole mounts were mounted in Mowiol (Hoechst).

For double *in situ* hybridizations, the hybridization solution contained both a DIG- and a fluorescein-labeled riboprobe (150 ng/ml each). First the DIG-labeled probe was detected using the anti-DIG-AP developed with BM purple as described above. After washing steps in PBST the AP was inactivated by heat (80°C, 20 min). The fluorescein-labeled riboprobe was then detected by incubation with an AP-coupled antibody against fluorescein (1:2000), which was developed after washing in 0.1 M Tris-HCl/0.1% Tween with fast red (Boehringer Mannheim) according the manufacturer's instructions.

All *in situ* hybridizations were made as doublets with the antisense and sense probes as control. Signals were found with the antisense probe and the sense probe controls were void. Data were collected from at least four adult rats, four embryos, and four postnatal rats for each time point after ONL and after PN grafting, with each of the cRNA probes described above.

Immunohistochemistry. The following antibodies were used: IgG fraction of polyclonal antibodies against mouse L1 (kindly provided by F. Rathjen) at a concentration of 1.6 μ g/ml, Mab No. AF5 (IgG1) against human DCC (Calbiochem) at 1 μ g/ml, polyclonal antibody 11760 raised against a peptide corresponding to a conserved sequence of chick netrin-1 and netrin-2 and mouse netrin-1 (Deiner *et al.*, 1997, kindly provided by M. Tessier-Lavigne and T. Kennedy) at 1:100, and Mab

N518 against goldfish neurolin (Leppert *et al.*, 1999) at 30 μ g/ml. Secondary antibodies were Cy3-conjugated donkey anti-mouse IgG (Jackson Immunoresearch) at 1:1000, Cy3-conjugated donkey anti-rabbit IgG (Jackson Immunoresearch) at 1:1000, Alexa-488-conjugated goat anti-rabbit IgG (Molecular Probes) 1:1000, and dichlorotriazinyl amino fluorescein-coupled goat anti-rabbit IgG (Jackson Immunoresearch), tetramethylrhodamine isothiocyanate (TRITC)-coupled goat anti-mouse IgG (Jackson Immunoresearch), and TRITC-coupled goat anti-human IgG Fc (Jackson Immunoresearch), all at 1:200.

Immunostaining with anti-DCC and anti-L1 antibodies was carried out on 10- μ m cryostat sections of unfixed tissue embedded in Tissue Tek (Miles) and frozen in liquid nitrogen. Immunostaining with anti-netrin-1 was performed on paraffin sections after fixation in Carnoy's fixative and immersion in Paraplast. Horizontal sections were made through the heads of E14 and E16 rat embryos, including both eyes, and cross sections through the eyes of adult rats after removal of the lens and vitreous. The sciatic nerve graft with the eye attached was sectioned longitudinally. Cryostat sections were collected on SuperFrost Plus slides (Menzel), air-dried at room temperature, fixed in methanol (5 min, -20°C), rinsed in PBS, and incubated with the primary antibodies either 1.5 h at 37°C or overnight at 4°C. After rinses in PBS, sections were treated with the appropriate secondary antibodies (1.5 h at 37°C), rinsed again, and coverslipped with Mowiol containing *n*-propyl gallate (Sigma) as an anti-fading reagent. Anti-netrin-1 immunostaining was carried out on 10- μ m paraffin sections as described in Deiner *et al.* (1997). In brief, sections were boiled for 10 min in 0.01 M citrate buffer at pH 6.0, cooled down to room temperature, dipped in 0.1% Triton X-100/PBS, and then exposed to the anti-netrin-1 antibodies for 6 h at RT. After rinses in PBS, sections were incubated with a fluorescent secondary antibody (1.5 h at 37°C), rinsed again, and coverslipped with Mowiol.

All immunostainings were performed with controls in which secondary antibodies were applied alone. The sections and cultures were examined in a Zeiss Axio-phot equipped with the appropriate set of filters and either photographed directly with a conventional camera or monitored by a digital camera and transferred to the computer equipped with Metamorph software. Images were processed with Adobe PhotoShop software.

Preparation of retinal explants. The eyes of adult rats were removed 7 days after optic nerve crush, briefly rinsed in 70% ethanol, and transferred to HBSS. The retina was isolated and attached to a nylon filter (Hybond N+;

Amersham Pharmacia Biotech) with the photoreceptor layer down. The basal lamina overlying the RGC layer was removed and retina and filter were cut in 400- μ m strips. The strips were placed on poly-l-lysine/laminin-coated coverslips with the RGC layer down and cultured for 7 days in serum-free medium (Jung *et al.*, 1997) at 37°C in the presence of 5% CO₂. Retinae from E16 embryos were isolated and explanted in the same way, on the same substrates, and under the same culture conditions as retina explants of adults but were stained after 3 days in culture. Retinal explant cultures were rinsed in 10% FCS/DMEM (Gibco), fixed in methanol (5 min, -20°C), rinsed in PBS, incubated with the primary antibody (overnight at 4°C), rinsed again in PBS, incubated with a fluorescent secondary antibody (1.5 h at 37°C), rinsed again in PBS, and coverslipped with Mowiol.

Preparation and immunostaining of goldfish retina. Immunostainings of goldfish retinae, normal and 14 days after optic nerve transection, were performed on whole mounts and cryostat sections as previously described (Ott *et al.*, 1998; Leppert *et al.*, 1999). In brief, to obtain whole mounts the isolated retina (photoreceptor layer down) was attached to a Hybond nylon filter by suction, the vitreous and the inner limiting membrane together with retinal blood vessels were removed, and the retina was fixed in methanol (-20°C, 10 min). To obtain cryostat sections, the isolated retina was embedded in Tissue Tek (Miles) and frozen in liquid nitrogen. Sections (12 μ m) through the optic disk/optic nerve head region were collected on uncoated coverslips, air-dried for 1 h, and fixed in methanol (-20°C, 5 min). Whole mounts and sections were exposed to Mab N518 against neuroilin and to the polyclonal netrin-1 antibodies overnight at 4°C (whole mounts) or for 2 h at RT (sections) and subsequently treated with the appropriate secondary antibodies (as described above).

To determine if goldfish RGC axons express netrin-1 receptors a netrin-1 Fc fusion protein (kindly provided by M. Tessier-Lavigne) was used since neither cDNAs encoding netrin-1 receptors in fish nor the relevant antibodies are available. This fusion protein consists of chick netrin domain V and VI fused to the constant (Fc) region of a human immunoglobulin molecule (Keino-Masu *et al.*, 1996). Retinal whole mounts from normal goldfish and 14 days after optic nerve lesion were obtained as described above. Unfixed retinae were incubated for 1 h at RT in 5% FCS (fetal calf medium) in Leibovitz (L15) medium containing 10 μ g/ml netrin-1 Fc. After two rinses in L15, retinae were fixed in methanol (-20°C, 5 min), rinsed in PBS, and then exposed to a TRITC-conjugated goat anti-human IgG Fc antibody for 2 h at RT, rinsed again in PBS, and coverslipped with Mowiol.

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