

Rapid, Widespread, and Longlasting Induction of Nestin Contributes to the Generation of Glial Scar Tissue after CNS Injury

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Abstract. Neuronal regeneration does generally not occur in the central nervous system (CNS) after injury, which has been attributed to the generation of glial scar tissue. In this report we show that the composition of the glial scar after traumatic CNS injury in rat and mouse is more complex than previously assumed: expression of the intermediate filament nestin is induced in reactive astrocytes. Nestin induction occurs within 48 hours in the spinal cord both at the site of lesion and in degenerating tracts and lasts for at least 13 months. Nestin expression is induced with similar kinetics in the crushed optic nerve. In addition to the expression in reactive astrocytes, we also observed nestin induction

within 48 hours after injury in cells close to the central canal in the spinal cord, while nestin expressing cells at later timepoints were found progressively further out from the central canal. This dynamic pattern of nestin induction after injury was mimicked by *lacZ* expressing cells in nestin promoter/*lacZ* transgenic mice, suggesting that defined nestin regulatory regions mediate the injury response. We discuss the possibility that the spatiotemporal pattern of nestin expression reflects a population of nestin positive cells, which proliferates and migrates from a region close to the central canal to the site of lesion in response to injury.

INJURIES to the nervous system lead to a number of changes in cellular function, both in neurons and in surrounding cells. The proximal part of the severed axon elongates in an attempt to regrow to the target. In contrast, the distal part of the injured axon, which has lost contact with the neuronal cell body, disintegrates and the phenotype of the surrounding glial cells are altered in the degenerating tract, i. e., Wallerian degeneration. The central (CNS)¹ and peripheral (PNS) nervous systems respond differently to injury, for reasons that are only partially understood. A distinct difference is that neuronal regeneration occurs almost exclusively in the PNS. The fact that axons from CNS neurons can grow for long distance in peripheral nerve grafts demonstrates that the failing regeneration in the CNS is not primarily a result of an inherent weak regenerative capacity by these neurons

(Aguayo et al., 1991). Rather, the glial environment in the CNS fails to support, or may even inhibit, axon growth. Although astrocytes support neurite growth in vitro and locally at injuries, astrocytes as well as oligodendrocytes seem to inhibit axonal growth in vivo (Schwab et al., 1993).

In the CNS, scar tissue, referred to as the glial scar, builds up at the site of the injury. Glial scar is largely formed by astrocytes which produce high levels of the intermediate filament (IF) protein glial fibrillary acidic protein (GFAP). The GFAP network at the scar area is very compact and it has been proposed that glial scar may act as a physical barrier to neurite outgrowth (for review see Eng et al., 1992; Hatten et al., 1991; Reier et al., 1989). GFAP, like other IFs, builds a cytoskeletal network by an initial dimerization of two protein monomers followed by higher order assembly leading to the formation of 10 nm fibers in the cytoplasm (Heins and Aebi, 1994).

In addition to GFAP, a number of other IFs are expressed during nervous system development. Differentiated neurons express the three forms of neurofilaments, newly differentiated neurons express α -internexin and neurons in the peripheral nervous system express peripherin (Liem, 1993). Progenitor cells in most parts of the CNS and PNS express vimentin (Liem, 1993) and the recently identified IF nestin (Lendahl et al., 1990). Nestin was originally defined by the monoclonal antibody Rat.401,

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This work is dedicated to the memory of our beloved friend and colleague Jonas Dahlstrand.

1. *Abbreviations used in this paper:* bFGF, basic fibroblast growth factor; CNS, central nervous system; DRG, dorsal root ganglion; GFAP, glial-fibrillary acidic protein; IF, intermediate filament; IR, immunoreactivity; NGF, nerve growth factor; PNS, peripheral nervous system.

which identifies an epitope expressed by neuroepithelial cells and cells in the myotome (Hockfield and McKay, 1985). Cloning of the rat (Lendahl et al., 1990) and human (Dahlstrand et al., 1992b) nestin genes revealed that nestin encodes an IF most closely related to neurofilaments and α -internexin. Nestin is predominantly expressed by CNS progenitor cells (Dahlstrand et al., 1995) and cells early in muscle development (Sejersen and Lendahl, 1993). Nestin expression is downregulated in the adult nervous system (Dahlstrand et al., 1992a, 1995) and very low expression levels are observed except in choroid plexus (Dahlstrand et al., 1995), in certain Schwann cells (Stemple and Anderson, 1993) and in endothelial cells (Dahlstrand et al., 1992a). In addition, nestin is expressed in adult CNS stem cells, both in vitro and in vivo in the subventricular zone (Morshead et al., 1994). Regulatory regions in the nestin gene controlling expression in CNS progenitor cells and early muscle cells have been identified in transgenic mice (Zimmerman et al., 1994).

The nestin gene is, in addition to its normal expression during CNS development, reactivated in different situations of cellular stress or induced proliferation. Nestin expression is thus induced in CNS tumors, in particular in more malignant tumors (Dahlstrand et al., 1992a; Tohyama et al., 1992). Immortalization of CNS progenitor cells results in cell lines expressing nestin (Frederiksen et al., 1988; Redies et al., 1991; Renfranz et al., 1991). Furthermore, cells that are removed from adult striatum and grown in primary culture express nestin and can differentiate to neurons and glial cells (Reynolds and Weis, 1992). These data suggest that cells originally derived from a nestin expressing population can resume nestin expression when subjected to various stimuli.

To learn whether the nestin gene is induced upon injury to the nervous system we analyzed nestin expression at different timepoints after traumatic injury in CNS and PNS, both at the site of injury and in the area of Wallerian degeneration. To define regulatory regions mediating an injury response we analyzed by the same injury paradigm transgenic mice carrying a reporter gene linked to the nestin promoter. Our data show that nestin is induced only in CNS and that induction in CNS suggests a role for nestin in the formation of glial scar. Finally, the temporal and spatial distribution of nestin expression after injury indicates that a nestin expressing cell population migrates from potentially proliferative areas in the subventricular zone to the site of injury.

Materials and Methods

Animals and Surgery

Adult male Wistar rats were anaesthetized with chloral hydrate (300–350 mg/kg) and adult male C57BL/6 mice (20–30 g) were anaesthetized with 2.5% Avertin. In the mice and a group of rats, a laminectomy was made at the mid thoracic level and the dorsal funiculus was cut transversely with microsurgical scissors and the lesion was extended rostrally by a superficial longitudinal incision in the dorsal funiculus, as previously described (Frisén et al., 1992). In another group of rats the left optic nerve was crushed 5 mm behind the eye by compression with a forceps for 10 s. In a third group of rats the left sciatic nerve was cut and the proximal and distal stumps were ligated.

Immunohistochemistry

Rats which underwent spinal cord, optic nerve, or sciatic nerve surgery

were allowed to survive for 2 or 4 days, 1, 2, or 4 wk after the injury (two animals at each survival time and operative procedure). In addition, two spinal cord injured rats were sacrificed 13 mo after the injury. Four spinal cord injured mice (survival time 1 or 10 d), two uninjured mice, and two uninjured rats were also used for immunohistochemistry. The animals were anaesthetized as above and perfused with Tyrode's solution, followed by 4% formaldehyde and 0.4% picric acid in 300 mOsm phosphate buffer. Cryostat sections (14 μ m) were incubated in a humid atmosphere at +4°C overnight with rabbit antiserum to nestin (#130, diluted 1:2,000) (Dahlstrand et al., 1992a; Tohyama et al., 1992) which in some sections was combined with mouse monoclonal antibodies to neurofilament (RT97, diluted 1:250) (Wood and Anderton, 1981) or to vimentin (V-6630, diluted 1:20; Sigma Chemical Co., St. Louis, MO). Other sections were incubated with mouse monoclonal antibodies to nestin (Rat-401, ascites fluid diluted 1:5) (Hockfield and McKay, 1985) which in CNS tissue was combined with rabbit antiserum to GFAP (diluted 1:200, Dako, Glostrup, Denmark). After rinsing, the sections were incubated for 45 min at 20°C with rhodamine-conjugated swine anti-rabbit antiserum (diluted 1:10; Dako) or fluorescein isothiocyanate-conjugated swine anti-rabbit antiserum (diluted 1:10; Dako), which in double labeled specimens was combined with rhodamine-conjugated goat anti-mouse antiserum (diluted 1:100; Boehringer-Mannheim, Biochemicals, Indianapolis, IN). Sections which were incubated only with secondary antiserum showed no specific labeling. The labeling with the two different antibodies to nestin was identical both in normal and injured tissues.

Electron Microscopy

Immunoelectron microscopy was performed as previously described (Risling et al., 1993). Briefly, cryostat sections were incubated for 24 h with rabbit antiserum to nestin (No. 130, diluted 1:2,000). After rinsing, the sections were incubated with 1 nm gold particle-conjugated goat anti-rabbit antiserum (diluted 1:50, Auroprobe One; Amersham Corp., Arlington Heights, IL). All antisera were diluted in 0.01 M phosphate buffer containing 0.5% IGS gelatin (Janssen Biotech, Beerse, Belgium). The sections were osmicated for 30 min and gold labeling was intensified with a silver enhancement reaction (Intense M; Amersham). The sections were then dehydrated, embedded, thin-sectioned, and contrasted. A Philips CM12 electron microscope was used.

Transgenic Mice

Transgenic mice were generated by injection of the previously described NesPlacZ/3intron construct (Zimmerman et al., 1994) into pronuclei of newly fertilized F1(B6CBA) \times F1(B6CBA) mouse eggs as previously described (Nilsson and Lendahl, 1993). Transgenic founders were identified by PCR analysis as previously described (Nilsson and Lendahl, 1993). In anaesthetized mice the dorsal funiculus was transected as described above and one or 10 days after the injury the mice were deeply anaesthetized and perfused with tyrode's solution followed by 0.2% glutaraldehyde in PBS. Spinal cord cryostat sections (14 μ m) were briefly postfixed in 0.5% glutaraldehyde in PBS and processed for X-gal histochemistry as previously described (Zimmerman et al., 1994). The sections were incubated overnight in the X-gal solution to visualize the *lacZ* expressing cells.

Cell Cultures

To prepare astrocyte brains from newborn rats were enzymatically digested with papain after removing the meninges. 5 mM L-leucine methyl ester and 0.1 mg/ml carbonyl iron was added for 3 h to the cell suspension, in order to eliminate microglial cells. The cells were then collected by centrifugation, resuspended in DME containing 10%FCS, 100 U/ml penicillin, and 100 μ g/ml streptomycin, and plated in 75 cm² tissue culture flasks (Costar Corp., Cambridge, MA). This procedure results in a mixed glial culture. To obtain separate astrocyte cultures the cell types were separated by difference in adhesiveness (McCarthy and de Vellis, 1980). After reaching confluency, the cultures were shaken at 700 rpm for 17 h, and the medium containing loosely adhering cells was discarded. The remaining cell monolayer representing predominantly astrocytes was rinsed once with PBS and then removed from the flasks with 0.17 mg/ml trypsin and 0.07 mg/ml EDTA in PBS and collected by centrifugation. The cells were resuspended in the medium and were allowed to adhere to plastic culture dishes. The medium was collected after 30 min, excluding rapidly adhering cells from the medium, resulting in >95% astrocytes in the collected medium, which was transferred to new culture dishes. For studies on the effect of mechanical trauma to astrocytes we developed an in vitro assay. In confluent astrocyte cultures growing in plastic petri dishes, an incision was

made through the cell monolayer. In some of these cultures, dorsal root ganglion (DRG) neurons from 2-d-old rat pups were seeded onto the astrocytes. The DRG neurons were cultured for 24 h and then fixed for immunocytochemistry.

Results

Nestin Expression in the Adult Intact Nervous System

We first analyzed the distribution of nestin immunoreactivity (IR) prior to injury in normal sciatic nerve, spinal cord, and optic nerve. At the light microscope level cells morphologically similar to Schwann cells in the sciatic nerve showed nestin-IR (Fig. 1 *A*). In the CNS, nestin-IR was observed in vascular cells (Fig. 1 *B*). Furthermore, sparsely scattered cells with thin, relatively short extensions in the white matter of the spinal cord (Fig. 1 *B*) and in the optic nerve (data not shown) were nestin immunoreactive. Because of their relative scarcity, one to two cells per section, we have not been able to reveal the identity of these scattered cells. By applying immunoelectron-microscopy we showed that nestin-IR in the sciatic nerve was found in myelinating, but not in non-myelinating, Schwann cells (Fig. 1 *C*) and that the vascular nestin-IR was localized to endothelial and perivascular cells (Fig. 1 *D*).

Induction of Nestin at the Site of Spinal Cord Injury

To study the expression of nestin in the injured CNS we used a well characterised spinal cord injury model where the dorsal funiculus is transected (see Materials and Methods). The effects on nestin- and GFAP-IR were analyzed 2 and 4 d, 1, 2, and 4 wk, and 13 mo after the injury. In contrast to the limited nestin-IR in the normal spinal cord, increased levels were seen already 2 d after injury. The immunoreactivity appeared to reside in glial cells in the

dorsal horns of the affected segment, in scattered cells at the site of lesion and in cells around the central canal (Fig. 2 *A*). The dorsal horns appeared not to be directly affected by the injury, since the structure of the dorsal horns was not morphologically altered, as determined by the presence of symmetrically radiating GFAP-labeled glial cells (Fig. 2 *D*) and the absence of invading blood cells in the dorsal horns (data not shown). In contrast, GFAP-labeled cells in the scar area extended processes in all directions (Fig. 2 *D*). Nestin immunoreactive cells around the central canal appeared to be located in the subventricular zone, but we cannot establish the identity of these cells (Fig. 2 *A*). 2 wk after injury the number of nestin immunoreactive cells had increased considerably, in particular in glial cells in the scar area (Fig. 2 *B*). Nestin-IR persisted for at least 13 mo after injury in the glial cells at the site of injury (Fig. 2 *C*). The GFAP-IR shifted from an initially broad area including both the dorsal horns, the scar area and regions of the surrounding spinal cord located laterally to the dorsal horns. As discussed above, cells with symmetrically radiating GFAP-positive fibers were seen, both in the dorsal horns and in the surrounding areas (Fig. 2 *D*). Later, GFAP-IR became more concentrated to the scar area and the dorsal horns (Fig. 2, *E* and *F*). When compared to the distribution of GFAP-IR it was apparent that the nestin immunoreactive cells are confined to a subset of GFAP immunoreactive cells at all timepoints after injury (Fig. 2, *A–F*). Nestin and GFAP immunoreactivity was colocalized in cells with processes extending in all directions, whereas only GFAP-IR was observed in cells with symmetrically radiating fibers (data not shown, compare Fig. 2 *A* with *D* and Fig. 2 *B* with *E*). This indicates that nestin is expressed in reactive astrocytes, but not in other astrocytes (see also Fig. 4).

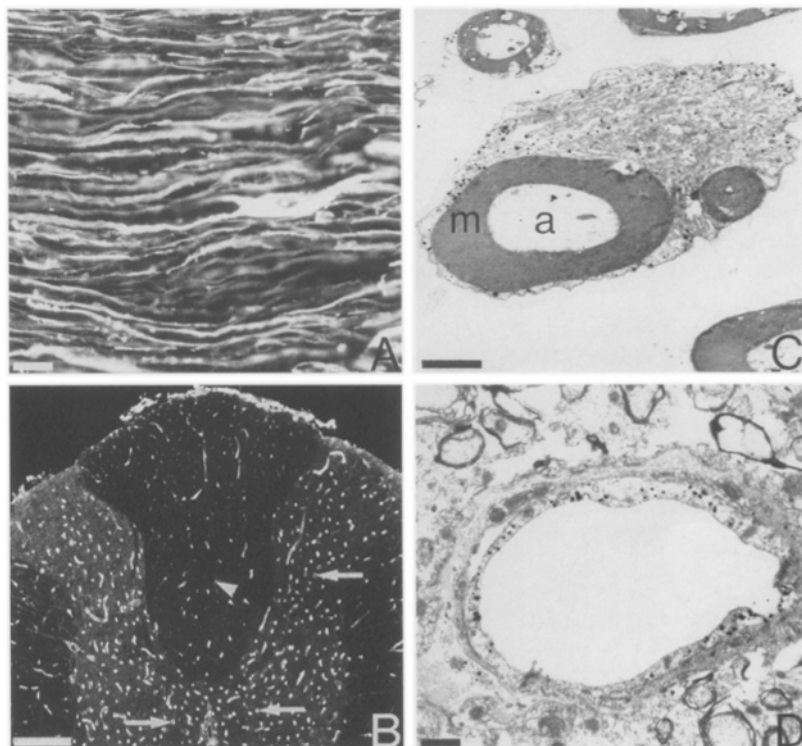


Figure 1. Nestin-IR in myelinating Schwann cells and vascular cells of the adult rat nervous system. Elongated cells in adult rat sciatic nerve show nestin-IR (*A*). In the spinal cord, cells associated with blood vessels (*small arrows*) and scattered cells with short, relatively thin extensions protruding in an apparently random fashion (*arrowhead*) show nestin-IR (*B*). Silver enhanced immunogold electron microscopy localizes the nestin immunoreactivity in the sciatic nerve to myelinating Schwann cells (*C*) and to endothelial cells and perivascular cells in the spinal cord (*D*). Abbreviations: m, myelin; a, axon. Bars: (*A*) 20 μm ; (*B*) 200 μm ; (*C* and *D*) 2 μm .

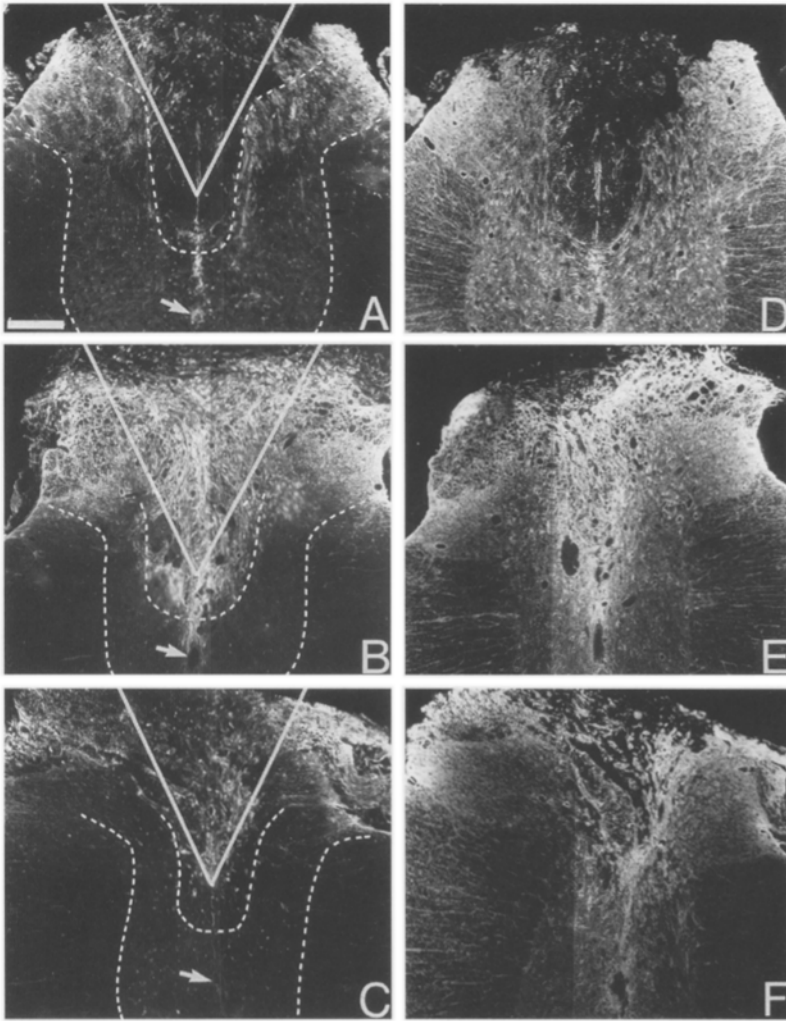


Figure 2. Nestin-IR is induced at spinal cord injuries. The dorsal funiculus was transected in adult rats and nestin-IR (A–C) and GFAP-IR (D–F) was studied 2 days (A and D), 14 days (B and E) and 13 months (C and F) after the injury. The position of the dorsal horns as well as the spinal cord grey matter is outlined with broken lines. The position of the central canal (arrow) and the approximate location of the lesion site (solid lines) are also denoted. (A) 2 d after the injury, nestin-IR is induced in the dorsal horns, in scattered cells at the lesion and in cells close to the central canal. The number of nestin immunoreactive cells at the injury increase progressively to 14 d after the lesion (B), and remain high after 13 mo (C). The induced nestin-IR corresponds closely to the area of increased GFAP-IR in adjacent sections (D–F). Bar, 200 μ m.

Rapid Nestin Induction in Degenerating Tracts in the Spinal Cord

In addition to the effects in the immediate vicinity of the injury there are also changes occurring further away from the injury, i.e., in the area of Wallerian degeneration (Ramón y Cajal, 1928). To learn about the extent of nestin induction we analyzed the distribution of nestin-IR in this area. Induction of nestin-IR was obvious in glial cells in the dorsal funiculus already at 2 d postinjury in the tract destined to Wallerian degeneration (Fig. 3 A). Induction extended at least 30 mm rostrally to the spinal cord injury. At this timepoint no morphological signs of axonal degeneration could be seen, as judged by unaltered neurofilament labeling (Fig. 3 G). In some sections nestin-IR was also seen in the cells lining the central canal (data not shown). It was evident that nestin-IR (Fig. 3 A) increased more rapidly than GFAP-IR (Fig. 3 D) in the dorsal funiculus undergoing Wallerian degeneration, indicating that nestin may be used as a sensitive molecular marker of neural degeneration. From one week after injury, when axonal degeneration was evident by loss of neurofilament labeling (Fig. 3, H and I), nestin-IR was restricted to the denervated part of the dorsal funiculus (Fig. 3, B and C). Strong nestin-IR was seen in glial cells in the degenerating dorsal funiculus at all survival times analyzed (data not

shown). The reduction in the area of nestin expression at later timepoints (Fig. 3, B and C as compared to A) is because the scar area in the Wallerian zone progressively shrinks and the nestin immunoreactive cells thus become concentrated to a smaller area. Immunoelectronmicroscopy showed that the nestin immunoreactive cells in both the immediate scar area (Fig. 4 A) and in the dorsal funiculus undergoing Wallerian degeneration (Fig. 4 B) contained numerous 8–9-nm filaments, which were present throughout the cytoplasm and extended as parallel arrays into the processes. The cells were also light in appearance compared to other cells in the white matter (data not shown). These characteristics are typical for astrocytes (Peters et al., 1991), and we therefore conclude that the nestin immunoreactive cells are astrocytes.

The IF vimentin is also upregulated in response to injury (for review see Eddleston and Mucke, 1991), and we wanted to compare the distribution of nestin and vimentin in the spinal cord injury paradigm. Vimentin-IR was widely observed, both at the site of injury (Fig. 5 B) and in the Wallerian zone (Fig. 5 D). High levels of vimentin-IR were found around the central canal (Fig. 5, B and D), which correlates with the nestin immunoreactivity pattern (Fig. 5, A and C). Cells coexpressing vimentin and nestin were also found in the scar area.

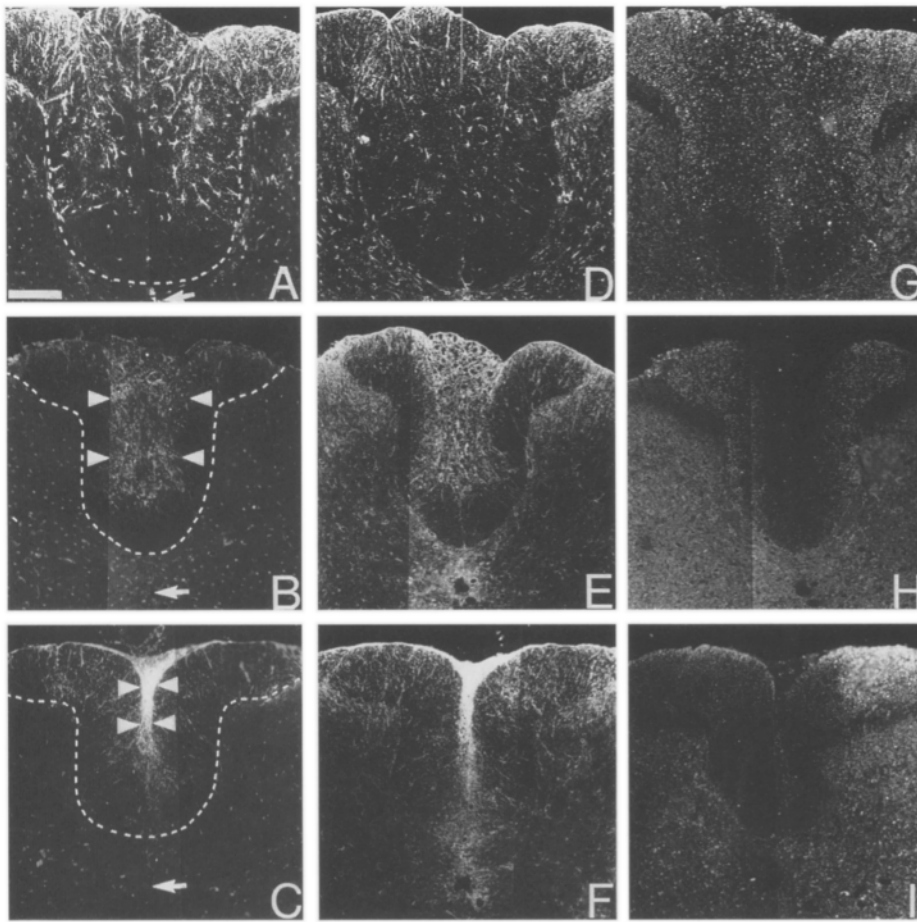


Figure 3. Rapid and longlasting induction of nestin-IR in degenerating spinal cord tracts. The micrographs show immunofluorescence with antibodies to nestin (A–C), GFAP (D–F) and neurofilament heavy chain (G–I) in sections taken 20 mm rostral to a dorsal funiculus transection 2 days (A, D, and G), 14 days (B, E, and H) and 13 mo (C, F, and I) after the injury. The dorsal funiculus is the area above the broken line and the position of the central canal is marked by an arrow. In B and C the outer limits of the scar area are outlined by arrowheads. In A the border of the scar area is not labeled since the morphology was not obviously altered at 2 d after injury (compare also Fig. 3 G). Strong nestin-IR is seen in the dorsal funiculus already after 2 d (A), before any alteration in GFAP (D), or neurofilament (G) immunoreactivity is evident. Note the difference between the nestin-IR in A as compared to Fig. 1 B. Nestin immunoreactive cells are concentrated to the central denervated part of the dorsal funiculus 14 d (B) and 13 mo (C) after the injury, closely corresponding to the area where GFAP is induced (E and F) and neurofilament immunoreactivity is abolished (H and I). Bar, 200 μ m.

Nestin Induction Occurs in Other Regions of the CNS

To study whether nestin induction appeared in other regions of the CNS in response to injury, we crushed the optic nerve five mm behind the eye in adult rats and analyzed the results at 2, 4, 7, 14, and 30 d after injury. In the non-injured optic nerve nestin-IR showed a relatively regular pattern along the nerve (Fig. 6 A). 2 d after injury, glial cells showing strong nestin-IR were seen in the nerve

where it had been compressed and throughout the distal segment (data not shown). The number of cells showing nestin-IR increased with time and after seven days a strongly nestin immunoreactive glial scar had formed (Fig. 6 B). The nestin-IR also extended to the nerve sheath (Fig. 6 B). This pattern may be a consequence of expression in fibroblasts, although nestin has not previously been shown to be expressed in fibroblasts (Redies et al., 1991; Sejersen

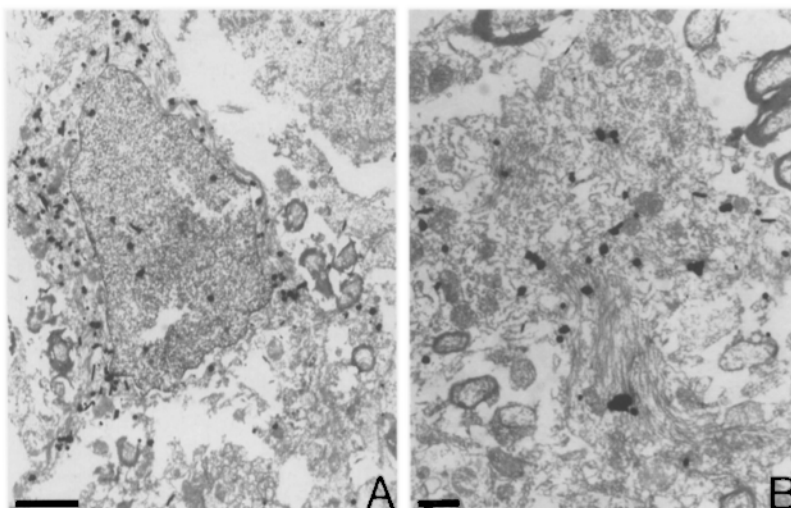


Figure 4. Nestin-IR in astrocytes in the injured spinal cord. Immunoelectronmicroscopy was used to establish which cell type exhibits nestin-IR in the injured spinal cord. Astrocytes in the scar tissue formed at the injury (A) as well as rostral to this in the degenerating dorsal funiculus (B) are strongly nestin immunoreactive. Note the presence of filaments in the cytoplasm of the cells, in particular in B. Bars: (A) 5 μ m; (B) 2 μ m.

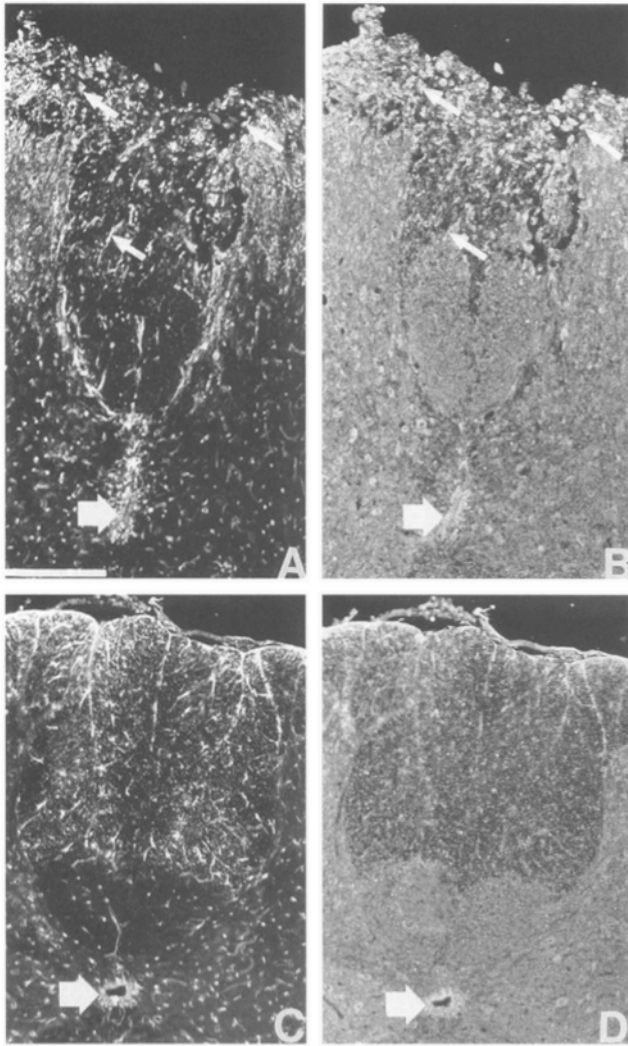


Figure 5. Vimentin-IR is induced at spinal cord injuries. The dorsal funiculus was transected in adult rats and vimentin-IR (*B* and *D*) and nestin-IR (*A* and *C*) was studied 2 d after the injury at the site of injury (*A* and *B*) and in sections taken 20 mm rostral to the dorsal funiculus transection (*C* and *D*). Vimentin-IR is observed in a wide region, including the lesion site and cells around the central canal. Cells showing both vimentin- and nestin-IR are labeled (*small arrows*). The position of the central canal is labeled (*arrow*). Bar, 200 μ m.

and Lendahl, 1993). Alternatively, the nestin-IR at the nerve sheath may come from muscle cells, in keeping with previous observations on nestin expression during muscle regeneration (Sjöberg et al., 1994b). Distal to the crush lesion, i.e., in the part of the optic nerve undergoing Wallerian degeneration, strong nestin-IR was seen in glial cells one week after injury (Fig. 6*B*). The distribution of nestin-IR corresponded closely to that of GFAP-IR in the Wallerian zone. In contrast, no GFAP-IR was seen close to the nerve sheath (Fig. 6*C*). Based on the similar immunoreactivity patterns for nestin and GFAP in the Wallerian zone, it is reasonable to assume that the nestin immunoreactive cells are astrocytes, although we have not been able to strictly prove this. The intensity of the nestin-IR was stronger at later survival times and was maximal at 14 and 30 d (data not shown).

In contrast to the dramatic increase in nestin-IR in CNS glial cells following injury, nestin expression was not altered in Schwann cells in the sciatic nerve of adult rats at all timepoints studied, both in the proximal region, at the site of injury, and in the distal region (data not shown). We did not observe any induction of nestin-IR in dorsal root ganglia, spinal cord, and retina in non-injured rats or at any timepoint (2–30 d) after transection of the sciatic and optic nerves.

Induction of Expression from Nestin Regulatory Regions in NesPlacZ/3 Intron Transgenic Mice following CNS Injury

It has previously been shown in transgenic mice that regions from the nestin gene direct expression of the reporter gene *lacZ* to CNS progenitor cells, generating an expression pattern very similar to that of the endogenous nestin gene (Zimmerman et al., 1994). We asked whether these DNA regions were activated by CNS injury. To this end, we generated a stable line of transgenic mice carrying the NesPlacZ/3intron construct. The NesPlacZ/3intron construct consists of the *lacZ* gene flanked by \sim 5 kb of rat nestin upstream sequences and the complete nestin gene, including the three introns (Zimmerman et al., 1994). Transgenic offspring to a NesPlacZ/3intron heterozygous founder male showed the previously described expression pattern in early CNS and myodermatomes for at least three generations (data not shown). To analyse the response to injury eight 8–16-wk-old offspring to the original founder male were subjected to spinal cord lesion. The five non-transgenic littermates did not show *lacZ* activity after injury (data not shown), while the three transgenic offspring did. Two of the transgenic mice were analyzed 1 d after injury and contained *lacZ* expressing cells in the area surrounding the central canal (Fig. 7*E*). The third transgenic offspring was analyzed 10 d after injury and *lacZ* expressing cells were found around the central canal, along the midline between the central canal and the scar (Fig. 7*F*), and at the site of the scar (data not shown). The *lacZ* expressing cells in the scar area appeared to be astrocytes (data not shown), while the cells between the central canal and the scar showed a morphology similar to that seen for cells expressing the endogenous nestin gene (see below, Fig. 7, *B* and *C*). Cells expressing *lacZ* were also found in the Wallerian zone in reactive astrocytes, but only infrequently around the central canal (data not shown). In Fig. 7 (*A–D*), the endogenous nestin-IR pattern before injury and at 2 d, 7 d, and 13 mo after injury is shown. Endothelial cells expressing nestin are seen in approximately equal numbers at all timepoints (Fig. 7, *A–D*), and there are also reactive astrocytes that express nestin within 48 h at the site of lesion (data not shown). In addition, there is another, more dynamic pattern of nestin expression. At 2 d after injury there is a population of nestin immunoreactive cells located around the central canal and along the midline between the central canal and the scar (Fig. 7*B*). The cells along the midline often had an elongated morphology, oriented along the dorsal–ventral axis (Fig. 7, *B* and *C*). At 7 d after injury there are still nestin immunoreactive cells around the central canal and along the midline but in addition many more cells closer to the site of injury

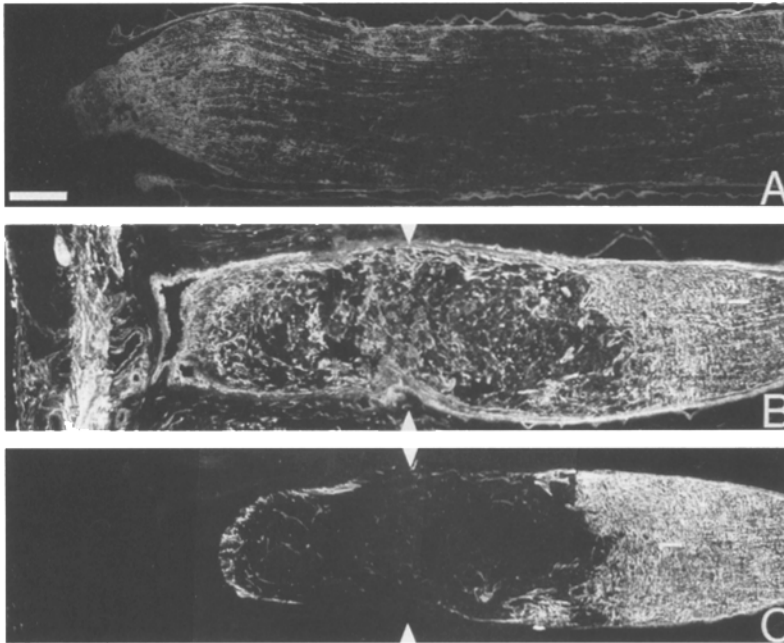


Figure 6. Increased nestin-IR in optic nerve glial cells after injury. Immunofluorescence micrographs showing adult rat retina (*left*) and optic nerve. Regularly spaced cells in the non-injured adult rat optic nerve show low levels of nestin-IR (*A*). 1 wk after the optic nerve was crushed behind the eye (*arrowheads* in *B* and *C*) strongly nestin immunoreactive cells are abundant at the lesion (*B*). Distal to the lesion (*right*) in the degenerating optic nerve nestin-IR is very pronounced (*B*). The nestin induction in the distal portion of the injured optic nerve closely resembles the pattern of GFAP-IR in an adjacent section (*C*). Bar, 200 μm .

(Fig. 7 *C*). Finally, at 13 mo after injury the nestin expressing cells that are not endothelial cells are largely confined to the area close to the injury (Fig. 7 *D*). These cells are presumably astrocytes. The *lacZ* expressing cells most likely form a subset of the cells expressing the endogenous nestin gene (compare Fig. 7, *E* with *B* and *F* with *C*).

Nestin Expression in Primary Astrocytes

To analyze whether nestin expression in astrocytes could be induced also by *in vitro* culturing we purified astrocytes and grew them at different densities. Astrocytes grown at low density showed very strong nestin-IR (Fig. 8 *A*). By contrast, denser astrocyte cultures of the same age showed weaker nestin-IR (Fig. 8 *B*). To study the direct effect of mechanical injury on astrocytes, we made an incision through the cell monolayer with a scalpel blade in confluent astrocyte cultures. Interestingly, a gradient in the intensity of nestin-IR could be seen within the injured astrocytes, such that the strongest labeling occurred in processes projecting towards the incision (Fig. 8 *C*). In contrast to this increase in nestin-IR by mechanical injury, no alteration in the level of GFAP-IR could be seen in double-labeled cultures (data not shown).

The specific increase of nestin expression after incision through the astrocyte culture provided an opportunity to directly test if a locally high nestin concentration would repel growing neurites. To test this, DRG neurons were grown on astrocyte cultures in which mechanical scars had been made, and which showed increased nestin-IR (data not shown). We found that neurites did not avoid the regions close to the scar and that many neurites traversed the scar (Fig. 9). This suggests that nestin expression is not directly associated with properties on the cell surface exerting a direct negative effect on neurite outgrowth.

Discussion

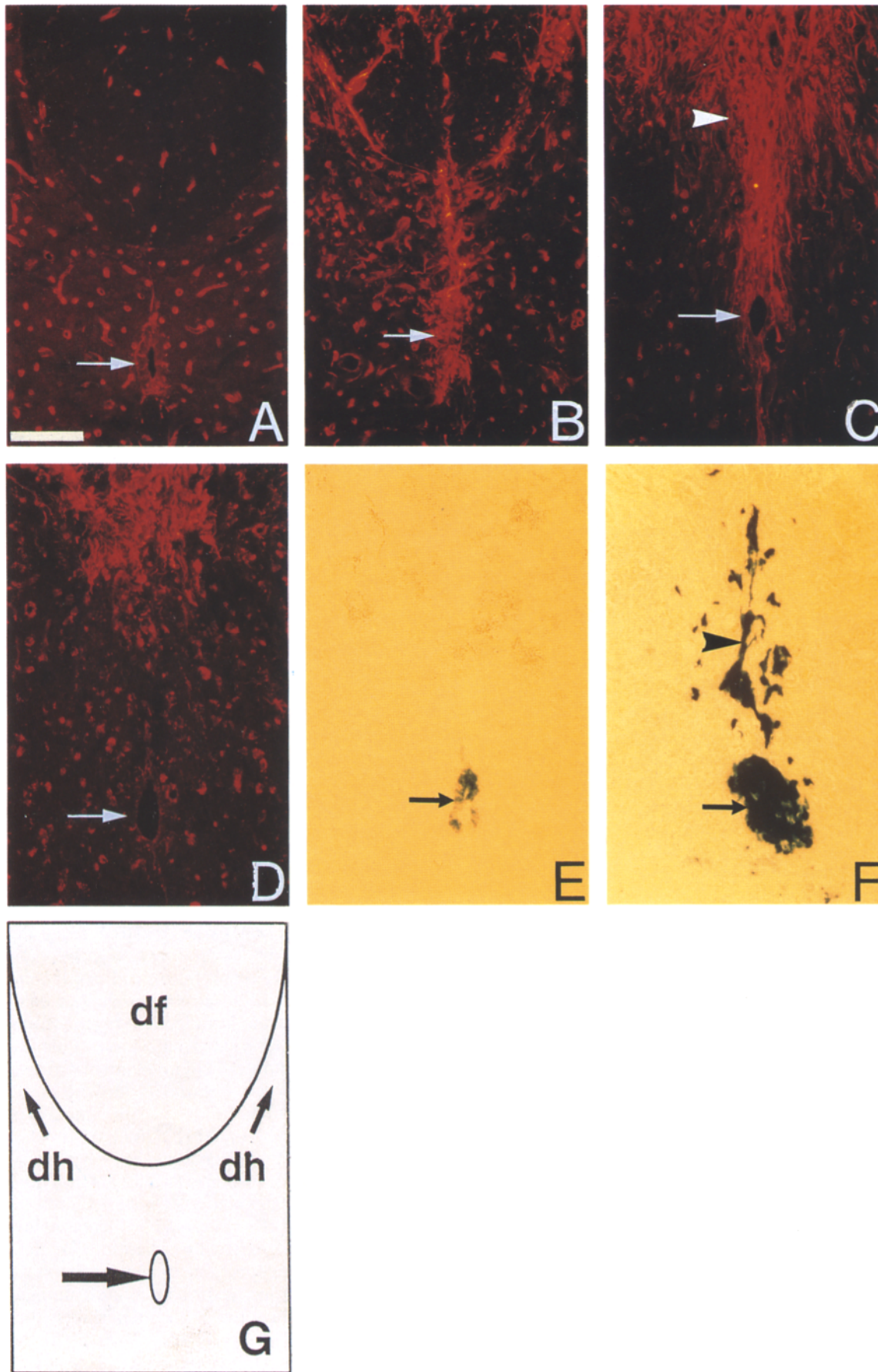
In this report we show that nestin expression is induced after injury to the nervous system. The induction appears to

be specific for the CNS, since it appears after traumatic injury to the spinal cord and optic nerve but not after injury to the sciatic nerve. The CNS specificity is further supported by a previous observation of nestin induction after grafting in cerebellum (Sotelo et al., 1994). We find that the induction of nestin expression occurs rapidly, is widespread and lasts for at least 13 mo. This dramatic transition in expression of a cytoskeletal component has ramifications for our understanding of how the glial scar is organized, how gene regulation is controlled after CNS injury and for how progenitor cells may be recruited to the site of injury.

Nestin Participates in Glial Scar Formation

It is well established that neuronal outgrowth in higher vertebrates occurs in the PNS but not in the CNS after injury. The reasons for this difference are largely unknown but the formation of glial scar tissue in astrocytes in the CNS may play a role (for reviews see Eng et al., 1992; Hatton et al., 1991; Reier et al., 1989). In this context, it is interesting to recall that in lower vertebrates such as goldfish, the optic nerve is capable of functional regeneration, which correlates with a different composition of the IF network. The predominant intermediate filament proteins in the goldfish optic nerve are keratins (Giordano et al., 1989), and there appears to be no overall upregulation of keratin mRNA in response to injury (Fuchs et al., 1994).

This may suggest that the IF composition of the glial scar is of importance for the response to injury. The prevalent view in higher vertebrates is that the glial scar after injury is composed of IF bundles generated by increased levels of GFAP and to some extent by vimentin. Our data however show that the composition of the scar is more complex and includes nestin. Nestin is strikingly upregulated in the astrocytes in the injury area and it is likely that nestin, GFAP and vimentin are actually present in the same filamentous structures in the astrocytes. This notion is supported by the finding that the patterns of nestin- and



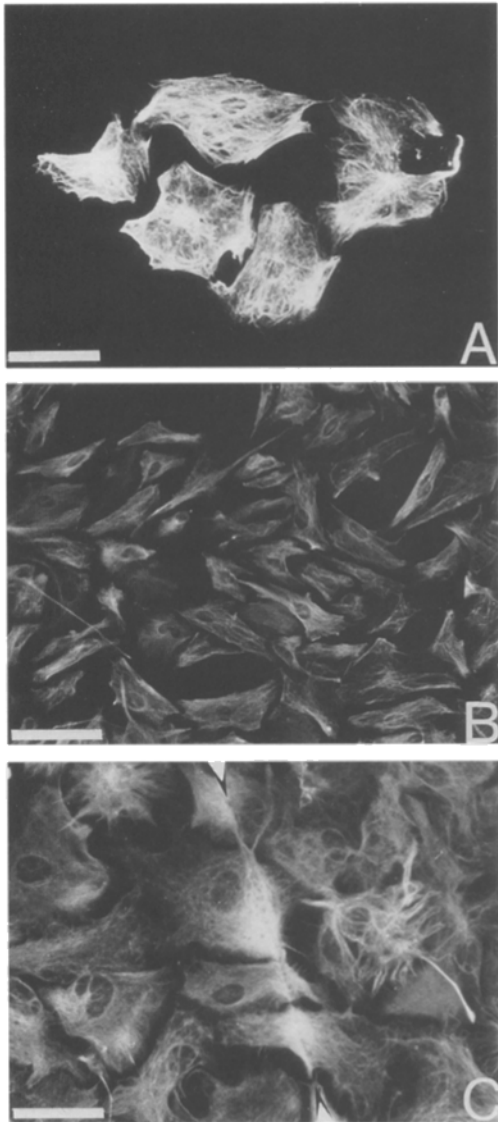


Figure 8. Nestin-IR in primary astrocytes is regulated by cell density and mechanical injury. Primary rat astrocytes seeded at low density (A) show stronger nestin-IR than astrocytes of the same age grown at high density (B). The mechanical trauma caused by an incision (arrowheads) through an astrocyte monolayer results in locally elevated nestin-IR in affected cells (C). Nestin-IR forms a gradient in the injured astrocytes and is strongest in the part of the cell closest to the injury. Bar: (A and B) 100 μm ; (C) 50 μm .

GFAP-IR are indistinguishable in an immortalized CNS cell line which expresses both IFs (Redies et al., 1991). Nestin also appears to copolymerize with the class III IFs vimentin and desmin in myogenic cells (Sejersen and Lendahl, 1993; Sjöberg et al., 1994a), and the latter two IFs copolymerize with GFAP (Sharp et al., 1982; Tölle et al., 1986). It is thus conceivable that the glial scar is built as a composite IF structure made from both GFAP, vimentin and nestin and that the high level of nestin expression significantly contributes to the formation of a dense glial scar. It should however be emphasized that an association between nestin expression and the ability of the cell to regulate neuronal outgrowth seems less likely, based on the apparent lack of repulsion of DRG neurites in a nestin-rich cellular environment.

The high levels of nestin expression in the glial scar may be part of the explanation to the apparent lack of phenotype in mice in which the GFAP gene is functionally inactivated (Gomi et al., 1994; Pekny et al., 1995). The GFAP (-/-) mice show post-traumatic reactive gliosis (Pekny et al., 1995), which may suggest that GFAP is not needed for this process, or, alternatively, that GFAP can be functionally substituted by nestin.

Nestin Expression as a Marker for CNS Injury

CNS injury represents a serious medical problem and it is important to establish easily identifiable markers that can objectively delineate the extent of the response to injury. Classically, GFAP has been the preferred marker, based on the increased levels of expression in the astrocytes after gliosis (Eng et al., 1992; Hatten et al., 1991; Reier et al., 1989). Our findings suggest that nestin is a potentially useful marker. Firstly, nestin, like GFAP, is an IF, which means that the encoded protein forms an easily recognizable cytoskeletal network. This is a general characteristic for IFs and contributes to their popularity as markers to diagnose various pathogenic conditions and tumors (for review see Osborn and Weber, 1989). Secondly, induction of nestin expression occurs rapidly, i.e., within 48 h, after injury and is found both in the area of lesion and in the Wallerian zone. In contrast, induction of GFAP is slower (for review see Eng et al., 1992; Hatten et al., 1991; Reier et al., 1989). Thirdly, nestin induction lasts for at least 13 mo, thus leaving a longlasting molecular imprint of the injury which facilitates a retrospective analysis. Finally, induction of nestin expression is more dramatic than that of GFAP. In the non-injured spinal cord and optic nerve nestin protein is expressed only at low levels, primarily in en-

Figure 7. Expression of the endogenous nestin gene and the reporter gene *lacZ* at the central canal after dorsal funiculus injury. The micrographs show nestin-IR in non-injured rat spinal cord (A) and at 2 d (B), 14 d (C), and 13 mo (D) after an incision in the dorsal funiculus. E and F shows X-gal histochemistry on sections from nestin promoter/*lacZ* transgenic mice (see Materials and Methods) 1 d (E) and 10 d (F) after a similar incision in the dorsal funiculus. Very low levels of nestin-IR are seen in the cells lining the central canal in the uninjured spinal cord (A). Shortly after an incision in the dorsal funiculus strong nestin-IR (B, 2-d postinjury) and *lacZ* expression (E, 1-d postinjury) is seen in cells close to the central canal (arrow) although this area is not directly affected by the injury. Nestin-IR and *lacZ* expression are still strong in cells residing close to the central canal 14 (C) and 10 d (F), respectively, after the injury (arrowhead). At these timepoints cells in the scar tissue formed at the injury and cells between the central canal and the lesion site also exhibit nestin-IR and *lacZ* expression, respectively. 13 mo after the injury (D) nestin-IR is weak in the cells lining the central canal, but cells in the scar tissue formed at the injury are strongly nestin immunoreactive. (G) A schematic representation of a cross section of the spinal cord, drawn in the same scale as A–F. The positions of the dorsal horns (dh), dorsal funiculus (df), and central canal (arrow) are indicated. Bar, 100 μm .

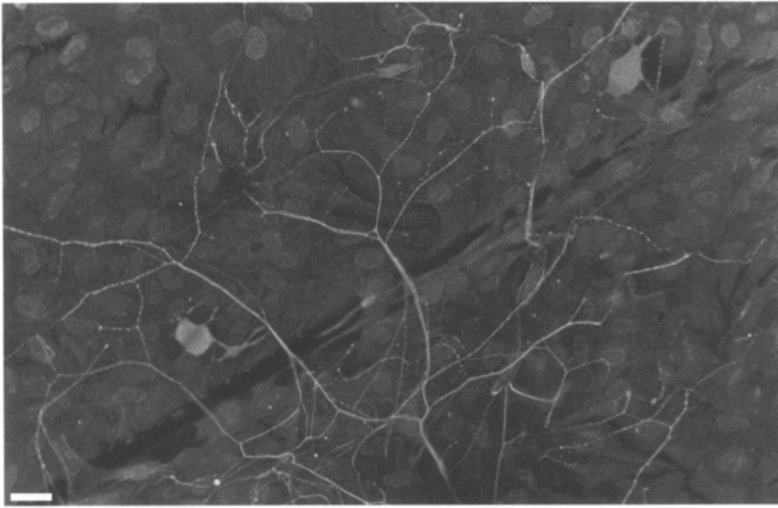


Figure 9. Culturing of DRG neurons on astrocytes subjected to mechanical injury. Primary rat astrocytes were seeded at low density and a mechanical trauma was caused as described in Fig 8. The cells closest to the scar showed elevated nestin-IR (data not shown). DRG neurons were then cultured on top of the astrocytes and the neurite processes visualized by neurofilament-IR. Note that many neurites traverse the mechanical scar. Bar, 50 μ m.

dothelial cells, while very high levels are observed in astrocytes after injury. In contrast, the GFAP protein is present at relatively high levels in astrocytes also before injury (Landry et al., 1990).

Defined Nestin Regulatory Regions Mediate an Injury Response

Nestin regulatory regions controlling the expression of the reporter gene *lacZ* in the early CNS have previously been identified (Zimmerman et al., 1994) and the data presented here demonstrate that the same regulatory regions activate the *lacZ* gene after CNS injury, both in the scar

area, around the central canal and in the region of Wallerian degeneration. The fact that the same regulatory region is capable of activating the reporter gene both during normal CNS development and after CNS injury suggests that gene regulation may be controlled by similar mechanisms in these two situations. Further dissection of the nestin promoter could narrow down the region required for CNS injury response, and critical elements most likely reside in the second intron of the nestin gene, which appears to function as an enhancer during early normal CNS development (Zimmerman et al., 1994).

Little is yet known about factors controlling nestin induction but it is possible that induction after injury is at least in part caused by an increase in expression of certain growth factors, e.g. nerve growth factor (NGF) (Ishikawa et al., 1991; Lindholm et al., 1992), basic fibroblast growth factor (bFGF) (Frautschy et al., 1991; Ishikawa et al., 1991; Koshinaga et al., 1993; Logan et al., 1992), insulin-like growth factor-1 (Lindholm et al., 1992) and ciliary neurotrophic factor (Ip et al., 1993). NGF and bFGF may be of particular interest since synergistic exposure of these two factors prolongs expression of nestin in primary cells from fetal striatum (Cattaneo and McKay, 1990). Alterna-

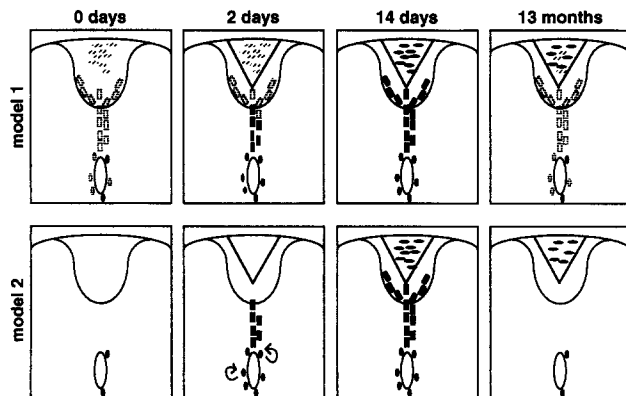


Figure 10. Two models for the dynamic spatiotemporal pattern of nestin induction in response to CNS injury. The models apply to the nestin expression patterns in a cross section of spinal cord at the site of lesion, but not to the Wallerian zone. The schematic representation of the nestin expression patterns in the two models can be compared with the experimental data in Fig. 7 (A–D). Note that nestin-positive endothelial cells and astrocytes in the dorsal horns, which express nestin at all timepoints after injury, are not shown in the two models. (*Model 1*) The four upper panels display cross sections of the area from the central canal (*oval at bottom*) to the dorsal funiculus (*upper part*) before injury (*0 days*) and at different timepoints (*2 days, 14 days and 13 months*) after lesion. The lesion is represented by a triangle in the dorsal funiculus. A number of cells around the central canal, in the area

between the central canal and in the dorsal funiculus are depicted. In this model these cells are stationary and do not migrate. Cells expressing nestin are black and cells not expressing nestin are stippled. Before injury (*0 days*) the majority of cells are not expressing nestin, except for a few cells around the central canal. In contrast, at 2-d postinjury a number of cells, both around the canal and in an area between the canal and the site of lesion have started to express nestin. The number of nestin expressing cells then increases and is maximal at 14 d. At 13 mo, nestin expression is confined to a cell population in the scar area and a few cells around the central canal. (*Model 2*) The four lower panels represent the same area and timepoints as in model 1. In this model nestin expressing cells (*filled in black*) are migratory and will migrate towards the site of injury. Note that the number of nestin expressing cells increase between 2 and 14 d after injury, suggesting that proliferation takes place in the subventricular zone (depicted by *arrows* at 2 d after injury).

tively, nestin induction may be independent of growth factor stimulation, since it occurs also in the Wallerian zone, in which the levels of growth factors do not appear to increase. Similarly, exposure of primary astrocytes to a variety of growth factors did not result in increased nestin mRNA levels (data not shown). For example, changes in cell-cell contact patterns, perhaps via cell-adhesion molecule signaling, may be involved in nestin induction. Loss of cell-cell contact could also explain the rapid upregulation of nestin in primary astrocytes, and that the highest levels of nestin protein were found when the cells were cultured sparsely. Further evidence for the importance of cell-cell contact is provided by Sotelo et al. (1994), who demonstrated that radial glial cells in cerebellum show higher levels of nestin-IR when neurons are migrating on them.

Nestin expression also appears to be controlled within the individual cell. When primary astrocytes were lesioned, the region of the cell closest to the lesion expressed higher levels of nestin-IR than more distal parts of the cell, suggesting that the injured cell is able to control the assembly of the IF network locally within the cell. This may be achieved by subcellular localization of the nestin mRNA, which has previously been observed in the neural tube, in which nestin mRNA is more abundant in the pial endfeet region of the progenitor cells (Dahlstrand et al., 1995). In support of this idea, differential localization of GFAP and keratin has been observed in radial glial cells (Holder et al., 1990) and vimentin mRNA is intracellularly localized during myogenesis (Cripe et al., 1993). In conclusion, it appears that the expression of nestin after CNS injury is subject to different levels of control, ranging from the subcellular localization of protein to the rapid and widespread induction in cells at various distances from the lesion.

Does Nestin Expression Define a Cell Population Migrating from the Subventricular Zone to the Site of Lesion in Response to CNS Injury?

Endothelial cells and reactive astrocytes at the site of injury show a stable pattern of nestin expression at all timepoints after injury. In contrast, the pattern of nestin expression in cells in the region between the central canal and the site of injury is much more dynamic. Thus, 2 d after injury most non-endothelial nestin expressing cells are found close to the central canal; two weeks after injury the majority of cells are found along a line between the central canal and the site of injury; and 13 mo after injury the majority of nestin expressing cells are astrocytes situated at the area of lesion. A similar shift in the expression pattern was seen for *lacZ* positive cells in the transgenic mice after injury (Fig. 7).

There are two possible explanations to this apparent transition in nestin expression at the site of injury, and the two models are schematically depicted in Fig. 10. First, it could be that cells are stationary and that different cell populations express nestin at different timepoints along a proximal-distal axis. Alternatively, nestin expression may persist in the same cell population and these cells migrate away from an original position around the central canal towards the site of injury. Because the number of nestin immunoreactive cells increase from two to 14 d after injury, the second model predicts that this cell population is transiently proliferating in response to injury. There are some

observations which may be interpreted to support the latter view. First, the region around the central canal in the spinal cord is the presumed location of an adult CNS progenitor cell population, since cells in the corresponding region of the brain, i.e., the subventricular zone, can proliferate and differentiate to neurons and glia postnatally (Levison and Goldman, 1993; Lois and Alvarez-Buylla, 1994; Morshead et al., 1994). Second, nestin expression is intimately associated with the progenitor cell state in many CNS regions during normal development (Dahlstrand et al., 1995; Frederiksen and McKay, 1988; Hockfield and McKay, 1985; Lendahl et al., 1990; Williams and Price, 1995) and may thus identify a similar cell type after CNS injury. Third, cells from adult striatum can be induced to proliferate and express nestin when cultured in vitro and to differentiate to neurons and glial cells when culture conditions are changed (Reynolds and Weis, 1992). Similarly, the adult CNS stem cells in the subventricular zone of the lateral ventricles in the brain express nestin in vivo (Morshead et al., 1994). These findings suggest a general correlation between nestin expression and the potential to proliferate in the CNS. Finally, the morphology and distribution of the cells two weeks after injury may suggest that the cells are migrating. Most cells have an elongated morphology and are extended in the same direction, i.e., along an axis between the central canal and the scar, as if they were migrating along a path between the central canal and the scar. In keeping with this, cells from the subventricular zone in the brain can migrate extensively (Lois and Alvarez-Buylla, 1994). Although by no means proven our data, taken together, may suggest the existence of a cell population endowed with progenitor or stem cell qualities which can be identified by virtue of nestin expression and which is recruited to the area of injury. Further analysis of such cells may be of interest to understand the plasticity in the adult CNS.

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