Original Article

Regulation of the chick cutaneous innervation pattern in retinoic acid-induced ectopic feathers and in the naked neck mutant

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ABSTRACT In chick skin, nerve fibers develop in a typical network formed by arcades around the base of feathers. In this study, we tried to dissociate the morphogenesis of nerve arcades and feathers, and to clarify the implication of several matricial molecules in these two developmental events. For this purpose, cutaneous nerve pattern and distribution of fibronectin, tenascin, and three epitopes of chondroitin sulfate proteoglycans (CSPGs) have been immunohistologically studied in the skin of the specific apteria of naked neck chick mutants, which lack feathers in the neck area, and in the tarso-metatarsal zone of retinoic acid-treated embryos where ectopic feathers grow. The presence of feathers was always associated with nerve arcades; no arcades were present in featherless areas. Specific immunofluorescence for tenascin and two epitopes of CSPGs revealed different distributions in the naked-neck neo-apteria as compared to control apteria. Moreover, the only difference in matricial composition in ectopic feathers concerned a CSPG isoform, bringing additional evidence that extracellular matrix molecules, and especially some (but not all) CSPGs, are involved both directly and indirectly in the cutaneous nerve pattern development.

KEY WORDS: *extracellular matrix, skin innervation, retinoic acid, naked neck, chondroitin sulfate proteoglycans*

Introduction

Bird skin is composed of feathered areas called pterylae, and bare zones named apteria. In the pterylae of chick skin, innervation forms a characteristic pattern (with a basal nerve arcade) around feathers, and develops in the embryo in parallel with the morphogenesis of these appendages (Saxod *et al.*, 1995). Previous studies showed that several matricial compounds are involved in both developments (Sengel *et al.*, 1985; Chuong, 1993; Hemming *et al.*, 1994). In order to try to attribute a more precise role to specific matricial molecules in nerve pattern formation, we have used immunohistochemistry to analyze two situations in which feather development and nerve arcade formation were potentially dissociated. The first is represented by the naked skin (neo-apteria) of the naked neck mutant. The second is created by inducing ectopic feathers on the foot scales by treatment with retinoic acid.

Results and Discussion

Skin innervation pattern of naked neck embryos

The unusual naked neck trait is caused by a single autosomal dominant gene (gene symbol *Na*) (Somes, 1990). The protein coded by the *Na* gene has not been determined yet.

As shown in Figure 1A, the *Na/Na* and *Na/na$^+$* embryos had no feathers on the dorsal part of their necks as opposed to control embryos (*na$^+$/na$^+$*). In the neo-apterium of the mutant embryo, the cutaneous nerves do not form arcades but a loose network very similar to that of a normal apterium (Fig. 1B). In feathered regions of the mutant, the nerve pattern is organized as in the pterylae of the controls, with arcades.

Matrix distribution in Na apteria

The distribution of five types of matrix molecules known to be involved in feather and nerve pattern development were analyzed in the neo-apteria of the mutant and compared to normal adjoining apteria (mid-dorsal) of control embryos (Table 1 and Fig. 2).

Among the compounds studied, three epitopes of CSPGs were particularly analyzed as these molecules have been implicated in repulsive effects on cutaneous nerve fibers (Fichard *et al.*, 1991; Hemming *et al.*, 1994; Pays *et al.*, 1997).

In the neo-apteria of the mutant compared to controls, no major difference was observed concerning the presence of fibronectin (dermis; Fig. 2F and 2K) and CS-56 CSPG epitope (dermis and epidermis; Fig. 2G and 2L).

On the contrary, the 2B9 CSPG epitope was detected in the epidermis of stage 35 Na embryos (Fig. 2M) whereas it was absent in controls (Fig. 2H), but then, the reverse situation was observed at stage 36, with 2B9 present in the epidermis of controls but absent

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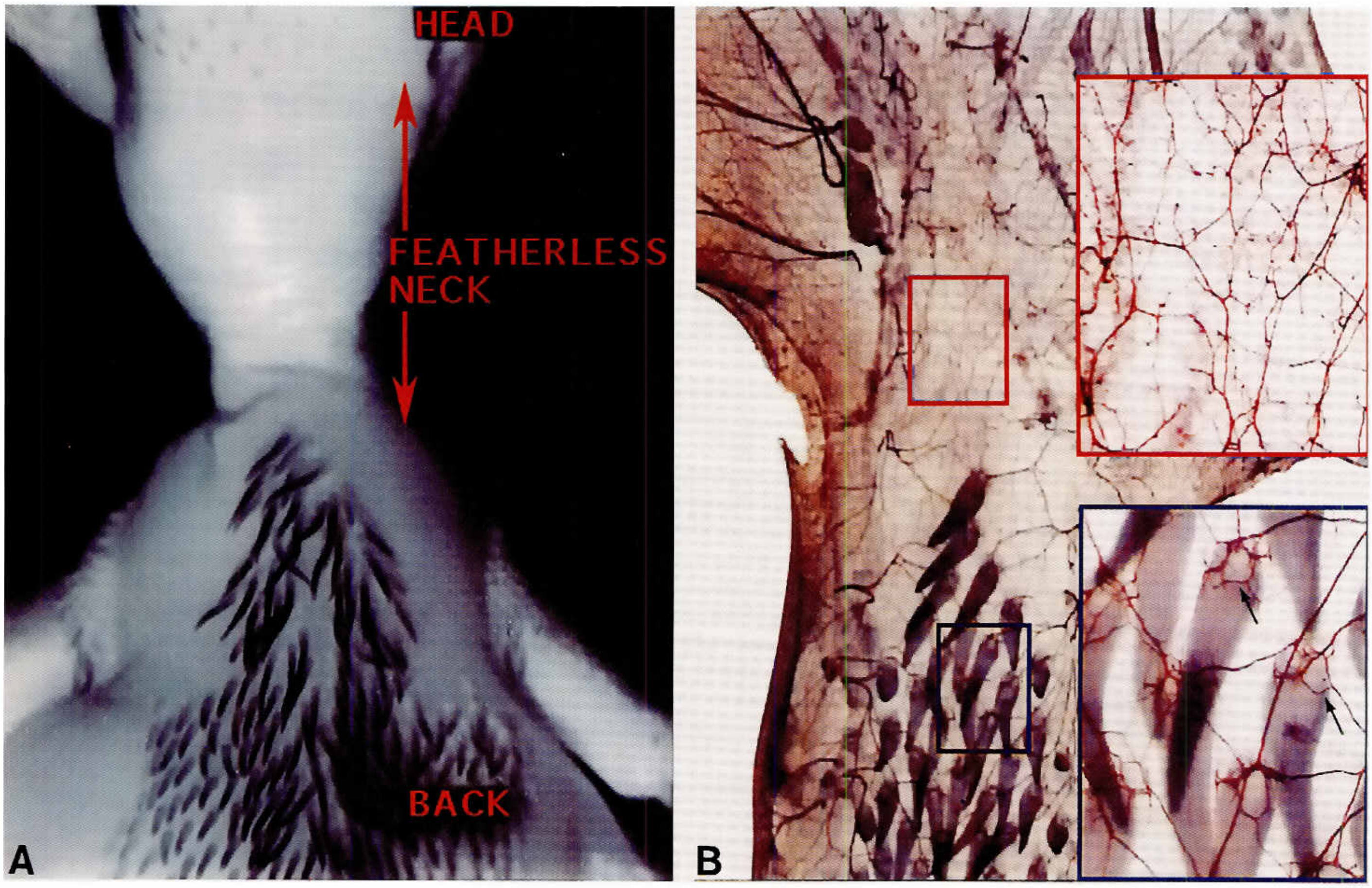

Fig. 1. Innervation in naked neck embryos. (A) *Featherless area of a stage 37 naked neck embryo.* **(B)** *Nerve pattern in a whole-mount of a stage 36 naked neck embryo. Two details are presented, showing areas with (blue) or without (red) feathers. Arrows indicate nerve arcades.*

from the mutant epidermis (Table 1). On the other hand, dermis was immunoreactive with 2B9 in both cases. The 9BA12 CSPG epitope also displayed distributions with differences limited to the epidermis. This isoform was found in the epidermis of stage 35-36 Na embryos (Fig. 2N), which was unlabeled in the corresponding controls (Fig. 2I). Finally, the most striking difference concerns tenascin which was completely absent in control apteria (Fig. 2J) but was found in the dermis and basement membrane of mutants between stages 33 and 36 (Fig. 2O). Additionally, minor differences can be noted concerning the fine distribution of some antigens. For example, the whole dermis, particularly deeply, was immunostained by 9BA12 in normal apteria at stage 35 (Fig. 2I), whereas the deep and upper dermis were preferentially labeled in Na neo-apteria (Fig. 2N). Another example concerns CS-56 immunostaining in the dermo-epidermal junction, which is stronger in mutant neo-apteria (Fig. 2L) than in controls (Fig. 2G).

These results which highlight matricial discrepancies between the two types of featherless areas, undoubtedly show that Na specific apteria cannot be considered as normal apteria.

An alternative hypothesis is that Na neo-apteria may mimick a situation in feathered areas. In order to test this hypothesis, the distribution of the five types of matrix molecules of the neo-apteria of the mutant were compared to those of the corresponding

TABLE 1

LOCALIZATION OF FIBRONECTIN, TENASCIN, CS-56, 2B9, AND 9BA12 CSPG ISOFORMS IN CONTROL APTERIA AND NA SPECIFIC NEO-APTERIA AT DIFFERENT STAGES OF DEVELOPMENT

antigen tested	stage skin	33-34 early bud	33 apteria cont	Na	34 apteria cont	Na	35 apteria cont	Na	36 apteria cont	Na
Fibronectin	E	-	-	-	-	-	-	-	-	-
	D	+	+	+	+	+	+	+	+	+
CS-56	E	+	+	+	+	+	+	+	+	+
isoform	D	+	+	+	+	+	+	+	+	+
2B9	E	+	+	+	+	+	-	+	+	-
isoform	D	+	+	+	+	+	+	+	+	+
9BA12	E	-	-	-	-	-	-	+	-	+
isoform	D	+	+	+	+	+	+	+	+	+
Tenascin	E	-	-	-	-	-	-	-	-	-
	D	+	-	+	-	+	-	+	-	+

The early bud stage of a normal pteryla is given for comparison. Presence or absence of an antigen is respectively indicated by + or -. cont, control; D, dermis; E, epidermis; Na, naked neck.

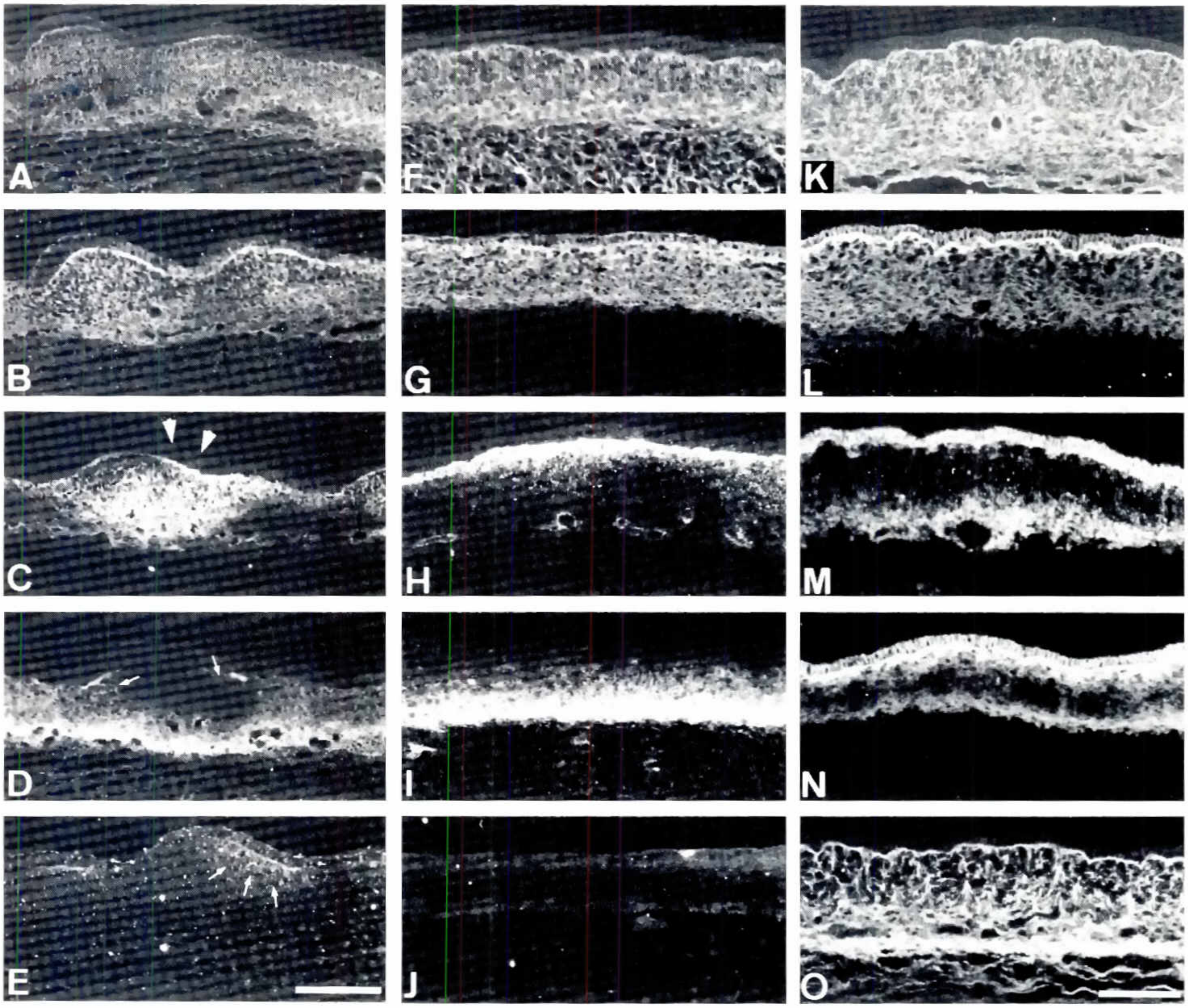

Fig. 2. Immunolocalization of matricial molecules in chick skin. **(A-E)** *Early bud stage.* **(F-J)** *Stage 35 control apteria.* **(K-O)** *Stage 35 Na specific neo-apteria. Sections were treated with the following primary antibodies:* **(A,F,K)** *anti-fibronectin,* **(B,G,L)** *CS-56,* **(C,H,M)** *2B9,* **(D,I,N)** *9BA12,* **(E,J,O)** *anti-tenascin. Small arrows and arrowheads indicate faint staining, respectively in dermis and epidermis. Bars: A-E, 100 μm; F-O, 80 μm.*

feathered region of controls at the early bud stage (Fig. 2A to 2D and Table 1). This comparison (first versus third and fifth columns of Table 1) shows that there is no difference in the overall distribution (epidermis, dermis) of these molecules. Therefore, the initial matricial status of the neo-apteria is very reminiscent of a feathered region at the first stages of morphogenesis, but dermal condensation or epidermal thickening are never formed in the neo-apteria of the mutant. However, later on, the distributions of 9BA12 and 2B9 antigens change in the Na neo-apteria; comparison with the evolution of feathers is then difficult as the distributions of the matricial molecules become extremely heterogeneous due to the development of the feathers themselves.

In conclusion, at least in early stages, Na neo-apteria rather evoke abortive pterylae, and the naked neck mutation seems mainly to affect the spatio-temporal distribution of key matricial compounds implicated in cutaneous morphogenesis, such as CSPGs (Pays *et al.*, 1997) and tenascin (Jiang and Chuong, 1992). As far as these latter components are concerned, even if some of them remained present in the skin, they display homogeneous distribution, with no patchy localization characteristic of areas containing developing feather buds. This lack of discontinuous pattern does not allow the formation of nerve arcades which develop around CSPGs rich areas (Pays *et al.*, 1997). Furthermore, as it is known that homeoproteins (Chuong, 1993; Chuong *et al.*, 1996) are associated with the induction of feathers, it could be hypothesized that the Na mutation may, in some way, affect regional homeogene expression in the anterior region of the embryo.

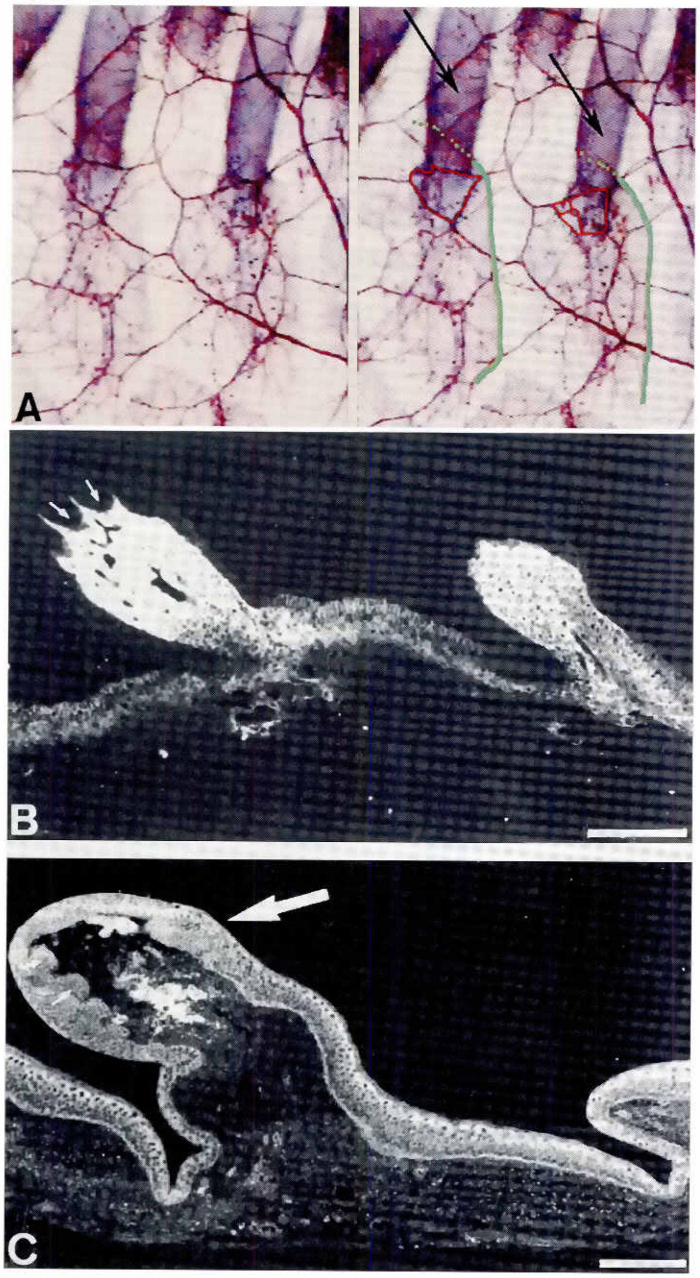

Fig. 3. RA-induced ectopic feathers. (A) *Whole-mount of tarso-metatarsal skin after E/C8 staining for nerve fibers. Ectopic feathers and their typical innervation are shown. In the right-hand picture, the main structures are highlighted: ectopic feather (arrow), scale border (green), nerve arcade (red).* **(B,C)** *Immunolocalization of the 2B9 isoform in control and ectopic feathers. Staining is abundant in the dermis of the developing control feather* **(B)**, *whereas with the same immunodetection protocol only a very restricted dermal immunofluorescence can be detected in the ectopic feather* **(C)**. *The fat arrow indicates the ectopic feather. Barb ridges (small arrows) are characteristic of the feather filament stage. Bars, 100 μm.*

Nerve pattern distribution in RA treated tarso-metatarsal skin

RA treatment produces ectopic feathers on some scutellate scales (Dhouailly and Hardy, 1978). We have previously shown that nerve arcades are found associated with these ectopic feathers (Pays *et al.*, 1997, and Fig. 3A) whereas they do not exist in the corresponding controls.

Extracellular matrix distribution in the RA-induced ectopic feathers

Four out of five matricial epitopes studied showed no major difference in distribution between ectopic and normal feathers. The 2B9 antigen however displayed a different expression pattern at the feather filament stage where the immunofluorescence was greatly reduced in the dermis of the RA-induced feathers (compare Fig. 3B and 3C). Moreover, these feathers have a normal appearance and a nerve arcade is found at their base, implying that this CSPG isoform must not be essential for feather morphogenesis and nerve pattern formation.

In summary, in Na mutants, the absence of nerve arcades is correlated with the absence of feathers and, in experimental ectopic feathers, the nerve pattern, with nerve arcades, is normal. Thus, the morphogenesis of both feathers and the nerve pattern, are tightly linked and, for the moment, cannot be dissociated. Taken together with other studies (Fichard *et al.*, 1991; Hemming *et al.*, 1994; Saxod *et al.*, 1995; Pays *et al.*, 1997) these results show that some epitopes of CSPGs (CS-56 and 9BA12 but not 2B9 isoforms) are involved in feather innervation both directly by being repulsive to neurite growth, and indirectly, by being implicated in feather morphogenesis.

Materials and Methods

Retinoic acid treatment

All trans-β-RA (125 μg), dissolved in 50 μl of absolute ethanol, was injected at E10 into the amniotic cavity of Warren chick embryos (Couvoir de Cerveloup, Vourey, France). Controls were injected with 50 μl of absolute ethanol alone. The surviving embryos were recovered at different stages varying from E12 to E18, and their tarso-metatarsal skins were removed.

Mutant animals

Naked neck embryos were kindly provided by M. Tixier-Boichard of the Institut National de la Recherche Agronomique (Jouy-en-Josas, France). At the stages used in this work, no morphological differences can be seen between homozygotes and heterozygotes.

Antibodies

- Three antibodies directed against chondroitin sulfate proteoglycans were used. CS-56 (Sigma) is a monoclonal antibody made in mouse, is specific for the glycosaminoglycanic chains of native CSPGs and binds to both the 4- and 6-sulfated moieties, but not to dermatan sulfate (Avnur and Geiger, 1984). Monoclonal antibodies 2B9 and 9BA12 (kindly provided by Dr. W. Halfter, Department of Neurobiology, University of Pittsburgh) recognize two chondroitin sulfate epitopes (Ring *et al.*, 1995).
- The monoclonal antibody E/C8, made in mouse, is directed against the neurofilament-associated protein NAPA-73 and labels all neurons (Ciment and Weston, 1982).
- The monoclonal antibody M1-B4 is made in mouse and is specific for tenascin (Chiquet and Fambrough, 1984a,b).
  E/C8 and M1-B4 were obtained from the Developmental Studies Hybridoma Bank maintained by the Department of Pharmacology and Molecular Sciences at the Johns Hopkins University School of Medicine (Baltimore, MD, USA), and the Department of Biological Sciences at the University of Iowa (Iowa City, IA, USA), under contract NO1-HD-2-3144 from the NICHD.
- Rabbit anti-fibronectin (polyclonal antibody) was obtained from DAKO.

Whole-mounts

Dorsal or tarso-metatarsal skins were removed in Ringer's solution and fixed in Carnoy's fluid. The nerve pattern was visualized with the E/C8 antibody (1:20) followed by the ABC method: a biotinylated secondary antibody horse anti-mouse was used (1:250), followed by a peroxidase-conjugated avidin-biotin complex (1:500). Each of these three steps was performed overnight at 4°C. Peroxidase activity was revealed by 0.4 g/l 3, 3'-di-amino-benzidine tetrahydrochloride (DAB), 0.25 g/l cobaltous chloride, 0.2 g/l nickel ammonium sulfate, and 0.003% hydrogen peroxide.

Immunohistochemistry

Embryos were fixed in Carnoy's fluid and embedded in Paraplast (Sigma). Deparaffinized longitudinal sections were incubated with primary antibody diluted as follows: E/C8 1:10, CS-56 1:250, 9BA12 1:10, 2B9 pure, anti-tenascin 1:100, anti-fibronectin 1:400. Specific staining was revealed by either incubation with Cy3-conjugated goat anti-mouse IgG+IgM (Jackson ImmunoResearch) diluted 1:250, or with fluorescein isothiocyanate-conjugated horse anti-rabbit IgG (H+L) (Diagnostics Pasteur) diluted 1:250 for anti-fibronectin. All incubations were performed for 1 h at room temperature. In controls, primary antibody was replaced by buffer.

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