A Monoclonal Antibody against a Laminin–Heparan Sulfate Proteoglycan Complex Perturbs Cranial Neural Crest Migration In Vivo

Marianne Bronner-Fraser and Thomas Lallier

Developmental Biology Center and Department of Developmental and Cell Biology, University of California, Irvine, California 92717

Abstract. INO (inhibitor of neurite outgrowth) is a monoclonal antibody that blocks axon outgrowth, presumably by functionally blocking a laminin–heparan sulfate proteoglycan complex (Chiu, A. Y., W. D. Matthew, and P. H. Patterson. 1986. *J. Cell Biol.* 103:1382–1398). Here the effect of this antibody on avian neural crest cells was examined by microinjecting INO onto the pathways of cranial neural crest migration. After injection lateral to the mesencephalic neural tube, the antibody had a primarily unilateral distribution. INO binding was observed in the basal laminae surrounding the neural tube, ectoderm, and endoderm, as well as within the cranial mesenchyme on the injected side of the embryo. This staining pattern was indistinguishable from those observed with antibodies against laminin or heparan sulfate proteoglycan. The injected antibody remained detectable for 18 h after injection, with the intensity of immunoreactivity decreasing with time. Embryos ranging from the neural fold stage to the 9-somite stage were injected with INO and subsequently allowed to survive for up to 1 d after injection. These embryos demonstrated severe abnormalities in cranial neural crest migration. The predominant defects were ectopic neural crest cells external to the neural tube, neural crest cells within the lumen of the neural tube, and neural tube deformities. In contrast, embryos injected with antibodies against laminin or heparan sulfate proteoglycan were unaffected. When embryos with ten or more somites were injected with INO, no effects were noted, suggesting that embryos are sensitive for only a limited time during their development. Immunoprecipitation of the INO antigen from 2-d chicken embryos revealed a 200-kD band characteristic of laminin and two broad smears between 180 and 85 kD, which were resolved into several bands at lower molecular mass after heparinase digestion. These results indicate that INO precipitates both laminin and proteoglycans bearing heparan sulfate residues. Thus, microinjection of INO causes functional blockage of a laminin–heparan sulfate proteoglycan complex, resulting in abnormal cranial neural crest migration. This is the first evidence that a laminin–heparan sulfate proteoglycan complex is involved in aspects of neural crest migration in vivo.

NEURAL crest cells are a highly migratory population of cells that give rise to a variety of derivatives including neurons and Schwann cells of the peripheral nervous system, pigment cells, adrenomedullary cells, and some skeletal and connective tissue (19). The mechanisms underlying the migration and differentiation of this population have been the focus of numerous recent experiments. The role of the extracellular matrix (ECM)[1] in the process of neural crest cell movement has been of particular interest, since ECM molecules like fibronectin (FN) and laminin (LM) have been found to promote neural crest migration in vitro (24, 29) and have been detected along neural crest migratory pathways in vivo (15, 23, 28). Antibodies against the cell binding region of FN (26) and against integrin (6, 7), a cell surface receptor for FN and LM (12), have recently been shown to interfere with normal cranial neural crest migration. These data strongly suggest the importance of a functional interaction between FN and its receptor in cranial neural crest migration. Although laminin is present along neural crest pathways and has been implicated as an important molecule in neurite outgrowth, little is known about the role of laminin, if any, in neural crest migration in situ.

Some interesting parallels between neural crest migration and neurite outgrowth have recently been described. Keynes and Stern reported that motoraxons eminating from the ventral neural tube preferentially move through the anterior half of each somite in the trunk of the avian embryo (14). Subsequently, it was noted that trunk neural crest cells follow a spatially and temporally similar pathway of migration through the anterior somite to that of the motoraxons (8, 27). In tissue culture, antibodies against integrin have been shown to disrupt both neural crest migration (6) and neurite outgrowth (3, 32) on fibronectin and laminin substrates. These

1. *Abbreviations used in this paper*: ECM, extracellular matrix; FN, fibronectin; HSPG, heparan sulfate proteoglycans; INO, inhibitor of neurite outgrowth; LM, laminin.

experiments suggest that neural crest cells and growing axons both share some common cell surface receptor molecules, responsiveness to extracellular matrix components, and pathways of migration

The role of the ECM in the outgrowth and regeneration of peripheral neurites has been the subject of many recent studies in tissue culture (1, 17, 21, 30). When plated on the ECM deposited by a variety of different cultured cell lines, neurons respond rapidly with extensive neurite outgrowth (21). Laminin and heparan sulfate proteoglycans are major components of the neurite outgrowth promoting factors produced by various nonneuronal cells (17). In addition, purified laminin provides a particularly good substrate for axon outgrowth (17, 21). However, antibodies that block neurite outgrowth on purified laminin do not usually block outgrowth on the natural neurite-promoting complex (17, 21). This suggests that the active complex in naturally occurring matrices may contain other isoforms of laminin or laminin in which some epitopes are altered or masked by associated molecules such as heparan sulfate proteoglycan.

Matthew and Patterson (23) have isolated the monoclonal antibody INO (inhibitor of neurite outgrowth) which recognizes and functionally blocks the neurite promoting complex in its native form, in which laminin is associated with heparan sulfate proteoglycan. In adult rat tissues, this complex was observed on the surface of glial cells in the peripheral but not central nervous system, and within the basement membranes surrounding most tissues (8). In a recent study examining the distribution of laminin and heparan sulfate proteoglycans (HSPG), we have noted that the patterns of LM and HSPG immunoreactivity overlap in avian embryos during neural crest migration (Krotoski, D., C. Domingo, R. Perris, and M. Bronner-Fraser, manuscript in preparation). This is consistent with the notion that laminin and heparan sulfate proteoglycan exist as a complex in the avian embryo as well. The present study examines the possible functional role of this laminin–heparan sulfate proteoglycan complex in cranial neural crest migration in situ by functionally perturbing this complex using the INO antibody.

Materials and Methods

Monoclonal Antibodies

INO (kindly provided by Dr. W. Matthew, Harvard University), anti-laminin, and anti-heparan sulfate proteoglycan (2; kindly provided by Dr. D. Fambrough, The Johns Hopkins University) hybridoma cells were grown in DME supplemented with 20% fetal calf serum and non-essential amino acids. Before injection, the antibody-containing culture medium supernatant was concentrated 20–30-fold as described previously (6) using Amicon Corp., Danvers, MA, microcentricon filters which pass molecules under 30 kD. As a control, culture medium lacking monoclonal antibodies was concentrated in the manner described above and is referred to as concentrated medium.

Preparation of the Embryos for Microinjection

White Leghorn chicken embryos were used for antibody injection. Embryos ranging in age from stages 8–10 according to the criteria of Hamburger and Hamilton (11) were used for most experiments in which INO was injected into the mesencephalon. To examine the effects of INO on slightly older embryos, stage 11–13 were used. For injections into the trunk, embryos between stages 12 and 17 were used as hosts. The eggs were washed with 70% ethanol and a window was made over the embryo. India ink (Pelikan, Hanover, FRG) was injected under the blastodisc to aid in visualization. After injection, the window over the embryo was closed with adhesive tape and the embryo was returned to the incubator until the time of fixation.

Microinjection of Antibodies into Chicken Embryos

For injections into the mesencephalon, concentrated medium containing INO, LM, or HSPG antibodies was injected lateral to the mesencephalic neural tube. The antibodies were backfilled into glass micropipettes with openings of 20–30-μm diam. A micromanipulator was used to position the pipette tip within the mesencephalon. The antibody suspension (~4 nl in volume) was expelled with a pulse of pressure and the injection micropipette was withdrawn. For injections into the trunk region, antibody was microinjected into the somites as described previously (5). To mark the level of injection, a tungsten needle with carmine on its tip was inserted into and withdrawn from one somite rostral to the injected somite and on the opposite side of the embryo. The carmine marker remained near the injection site.

Histology and Tissue Sectioning

Cryostat Sections. Embryos were fixed overnight in methanol at 4°C, hydrated in PBS for 1 h, placed in 0.5% sucrose in PBS containing 0.01% azide for 2–4 h, and into 15% sucrose in PBS at 4°C overnight. They were then placed in 7.5% gelatin (Sigma Chemical Co., St. Louis, MO; 300 Bloom) and 15% sucrose in PBS at 37°C for 2–3 h, oriented, and frozen in Tissue Tek OCT (Miles Laboratories, Naperville, IL) in liquid nitrogen. 10-μm frozen sections were cut on a Histostat cryostat (Reichardt Scientific Instruments, Buffalo, NY).

Paraffin Sections. Embryos were fixed for 1.5 h in Zenker's fixative, washed in running water, and placed in 70% ethanol. The embryos were then dehydrated and embedded in paraffin. 10-μm serial sections were cut on a Leitz microtome and mounted on albuminized slides.

Immunofluorescent Staining

Distribution of the Injected INO. Cryostat sections from embryos fixed from 2–24 h after injection were stained for 1 h at room temperature with a highly fluorescent antibody against mouse Ig's (Antibodies, Inc., Davis, CA). Sections were examined on an Olympus Vanox epifluorescence microscope and data were recorded photographically.

Identification of Neural Crest Cells. After fixation and paraffin embedding, sections were stained with HNK-1 antibodies which serve as a marker for migrating neural crest cells (8, 33). Sections were deparaffinized in histosol, hydrated through a graded series of ethanols, and washed in PBS. They were then incubated with culture medium from HNK-1 hybridoma cells for 3 h at room temperature or overnight at 4°C. After washing in PBS, slides were incubated with a rabbit antibody that recognizes mouse IgMs for 1 h, followed by an hour incubation with a fluorescein-conjugated goat antibody against rabbit IgGs. The slides were washed in PBS, coverslipped, and observed with an Olympus epifluorescence microscope. Data were recorded photographically.

Purification of Antibodies

INO, anti-laminin, and anti-heparan sulfate proteoglycan antibodies were purified from culture medium supernatant from hybridoma cells by precipitation with 50% ammonium sulfate. The sample was then centrifuged, resuspended in distilled water, and dialyzed against 0.1 M PBS at 4°C for 48 h. The dialysate was placed over a protamine-sulfate sepharose column and eluted with 1.1 M NaCl/0.1 M PBS.

Preparation of Small Fragments of the INO Antibody

Small fragments of IgM antibody were prepared as described by Matthew and Reichardt (22). INO was purified as described above and equilibrated on a P-2 column (Bio-Rad Laboratories, Richman, CA) with Tris-buffered saline (pH 8.0). The void volume was collected and the concentration was adjusted to 1 mg/ml in Tris buffer. The whole IgMs were digested with 0.01 mg/ml trypsin (TPCK; Millipore Corp., Bedford, MA) for 5 h at 37°C. B-Mercaptoethanol was then added to the mixture (final concentration of 10 mM) for 5 min at 37°C, followed by soybean trypsin inhibitor (final concentration of 0.1 mg/ml) for 5 min at 25°C. The small IgM fragments were dialyzed four times against 1 liter of 0.1 M PBS at 4°C for 48 h. Fragments were sized on a Biogel P-300 column and only fragments smaller than 150 kD were used for the microinjection experiments. Of these, 90% were 55 kD

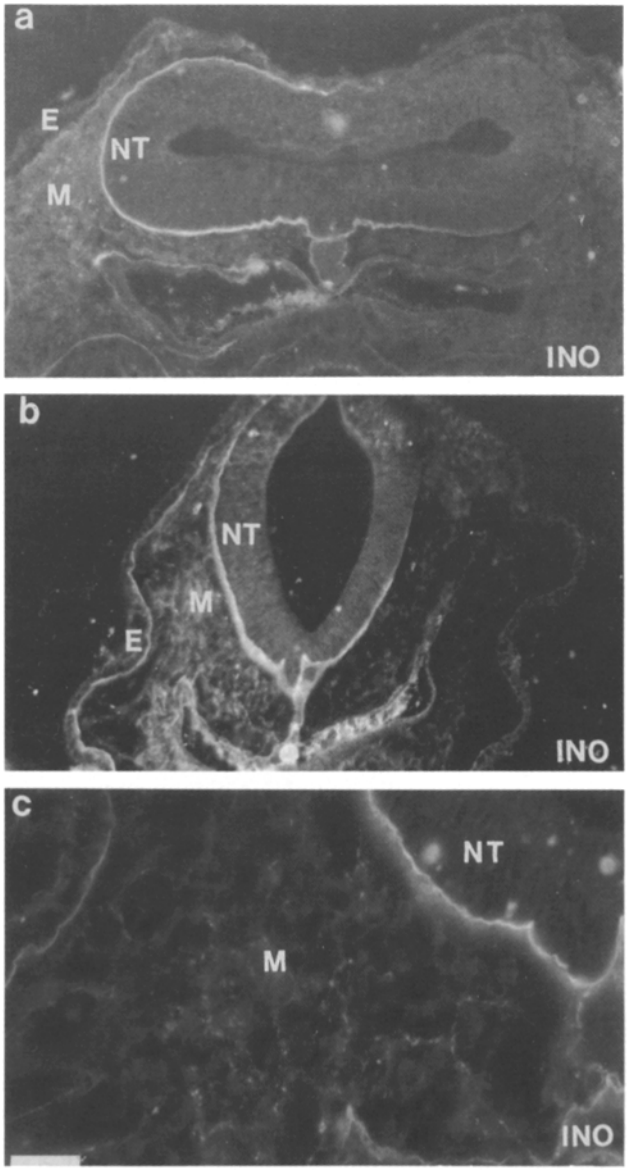

Figure 1. Fluorescence photomicrographs of transverse cryostat sections through the mesencephalon of chick embryos 2–3 h after injection of INO antibody lateral to the mesencephalic neural tube (*NT*). (*a* and *b*) INO was observed in the basement membrane surrounding the neural tube, under the ectoderm (*E*) and the endoderm. The INO antibody distribution was primarily unilateral, with little immunoreactivity detectable across the embryonic midline. (*c*) Higher magnification of the same embryo shown in *b* illustrates that fibrillar INO immunoreactivity was observed within the cranial mesenchyme (*M*). INO, antibody that inhibits neurite outgrowth. Bar: (*a*) 50 μm; (*b*) 77 μm; (*c*) 29 μm.

or less. Thus, by virtue of their size, 90% of the fragments were monovalent. Approximately 500 μg/ml of small fragments were used for injection.

Immunoprecipitation of the INO Antigen from 2-d Chicken Embryos

Chick embryos with 10–17 somites were minced by trituration and incubated in INO for 2 h at 4°C. The sample was then lysed using a Bronwill (Rochester, NY) Biosonk probe sonicator for 30–60 s. The INO samples were incubated with FMP-Fractogel beads (Bioprobes, Tustin, CA) conjugated with rabbit anti-mouse IgM (Zymed, Berkeley, CA) for 8 h at 4°C. The beads were then washed extensively with 20 mM Tris buffer, pH 8.1, containing 150 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$. The antigen was eluted in a minimal volume of 100 mM glycine, pH 3.0, and immediately neutralized with 2 M NaOH. The eluants were then concentrated using Centricon microconcentrators (Amicon Corp.). A portion of the INO eluant was subjected to heparinase digestion. Heparinase (Sigma Chemical Co.) was diluted in 50 mM Tris, pH 7.0, containing 100 mM NaCl and 100 μg/ml BSA. Five units of heparinase were added to 10 μl of the INO eluant (1 mg/ml) and incubated for 5 h at 37°C (20, 31).

Analysis of the antigens was performed using SDS-PAGE using 8% gels in a vertical mini-slab gel system (Hoefer Scientific Instruments, San Francisco, CA), with a 4% stacking gel utilizing the buffer system described by Laemmli (13). Polyacrylamide gels were then fixed in a solution of 10% ethanol/5% acetic acid and stained using a Silver stain kit (National Diagnostics Inc., Somerville, NJ) until bands appeared within the gel. Excess surface staining was removed using Argent-X silver stain bleach (National Diagnostics). This tended to lower background and gave sharper bands on the gel. Antibodies against laminin and heparan sulfate proteoglycan have been characterized elsewhere (2).

Results

Distribution of INO after Injection

INO was injected lateral to the mesencephalic neural tube in embryos with up to nine somites. The distribution pattern of the antibody and the longevity of detectable immunoreactivity were determined as a function of time after injection. Embryos were examined from 2 to 24 h after injection.

At all times examined, the distribution of antibody appeared to be primarily unilateral, confined to the injected side. In embryos fixed 2–3 h after injection, immunoreactivity was observed surrounding all basal laminae on the injected side, including the basal surface of the neural tube, ectoderm, endoderm, and notochord (Fig. 1, *a* and *b*). Some immunoreactivity was noted on the ventral-most portion of the neural tube on the uninjected side. This indicates that a small amount of antibody did cross the midline. In addition to staining in basal laminae, some INO immunoreactivity was noted within the cranial mesenchyme. This staining pattern was non-uniform and fibrillar in appearance (Fig. 1 *c*), suggesting that the INO was not in a lamellar organization but rather in a loose matrix surrounding mesenchyme cells. INO immunoreactivity was detected in the prosencephalon, mesencephalon, and rhombencephalon, suggesting that the injected antibody was able to diffuse rostral and caudal to the site of injection.

To compare the distribution of INO with that of laminin and heparan sulfate proteoglycan, stage 8–10 embryos were injected with antibodies against LM or HSPG, and allowed to survive for 2–3 h. Like INO, the distribution of LM (Figure 2 *a*) or HSPG (Fig. 2 *b*) was primarily unilateral. Binding of both antibodies was observed on the basal surface of the neural tube, ectoderm, endoderm, and notochord, as well as in fibrils within the cranial mesenchyme. These patterns were indistinguishable from that observed for injected INO antibody.

The pattern of INO immunoreactivity remained the same as described above in embryos fixed at progressive times after injection, but the intensity of immunoreactivity decreased. By 18 h after injection, only very low levels of immunoreactivity were detectable on the basal surface of the neural tube, ectoderm, and endoderm.

The distribution and longevity of INO immunoreactivity

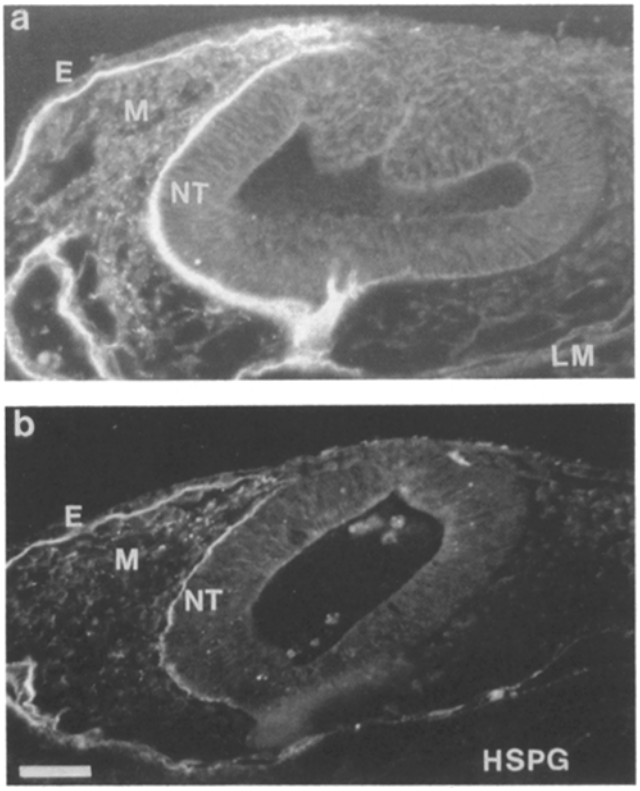

Figure 2. Fluorescence photomicrographs of transverse cryostat sections through the mesencephalon of chick embryos 2–3 h after injection of control antibody lateral to the mesencephalic neural tube (*NT*). *a* illustrates the distribution of anti-laminin antibodies after injection. The distribution is unilateral and similar to that of INO. Staining was observed around the neural tube, ectoderm, and endoderm, as well as within the cranial mesenchyme; (*b*) illustrates the distribution of anti-heparan sulfate proteoglycan antibodies after injection. Staining was observed unilaterally around the neural tube, ectoderm, endoderm, and within the cranial mesenchyme. *LM*, anti-laminin; *HSPG*, anti-heparan sulfate proteoglycan. Bar, 50 μm.

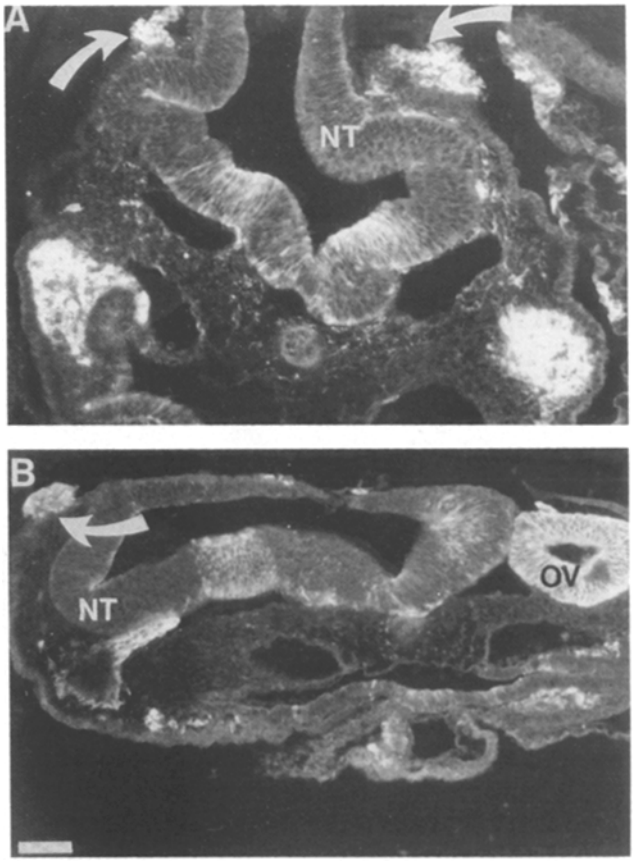

Figure 3. Fluorescence photomicrographs of transverse paraffin sections through the cranial region of embryos and fixed within the first day after injection. Neural crest cells can be recognized by their immunofluorescence after staining with the HNK-1 antibody. (*a*) An embryo, injected at the 6-somite stage and fixed 19 h later, with ectopic aggregates of neural crest cells (indicated by arrows) observed bilaterally adjacent to the neural tube (*NT*). The neural tube was open and deformed; (*b*) Another embryo injected at the 6-somite stage and examined 20 h later. In the rhombencephalon at the level of the otic vesicles (*OV*), an ectopic aggregate of neural crest cells (indicated by the arrow) was observed adjacent to the neural tube. Bar: (*a*) 50 μm; (*b*) 58 μm.

was examined in embryos having 10 or more somites. After injection into the mesencephalon, the pattern and persistence of INO staining was identical to that described above for younger embryos. When injected into the trunk somites, INO also had a primarily unilateral distribution around the neural tube, dermamyotome, and ectoderm. In addition, some fibrillar staining was noted in the sclerotome. The pattern of distribution was identical to that previously reported for laminin (15, 22). INO immunoreactivity remained detectable for approximately 18 h after injection of antibody into the trunk.

Effects of INO on Neural Crest Migration In Situ

Embryos were injected with INO and allowed to survive for up to 24 h after injection to determine the effects of the antibody on cranial neural crest migration in vivo. The results are summarized in Table I. 68 embryos were injected with the antibody. Of these 74% had abnormalities in neural crest

Table I. Effects of INO, LM, and HSPG Antibodies on Cranial Neural Crest Migration In Vivo

	Type of Antibody				
Type of defect	INO	INO fragments	Anti-LM	Anti-HSPG	Conc. medium
Total number	68	10	39	28	34
Percent affected	74	70	10	4	12
Percent NC in lumen of NT	25	20	4	4	6
Percent ectopic NC cells	40	30	0	0	6
Percent neural tube anomalies	31	40	0	4	3

NC, neural crest. NT, neural tube. Percentages are expressed as percent total *n* of embryos in each category. Some embryos had defects in more than one category.

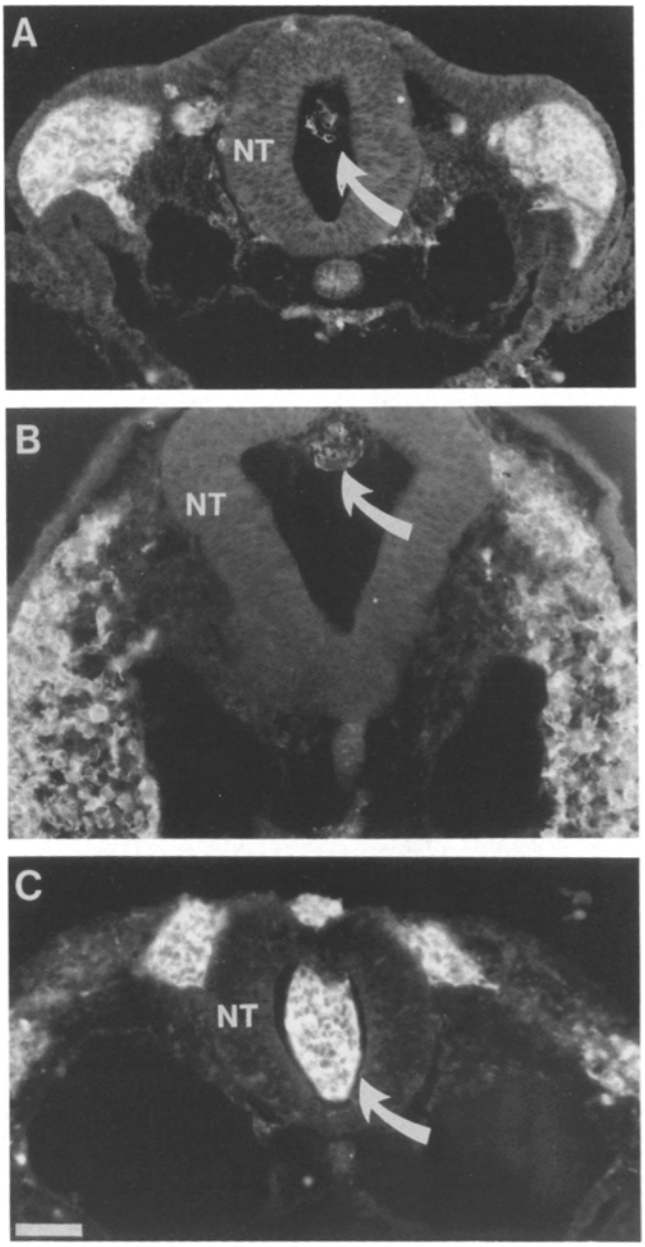

Figure 4. Fluorescence photomicrographs of transverse paraffin sections through the cranial region of embryos injected with INO antibody and fixed within the first day after injection. Neural crest cells can be recognized by their immunofluorescence after staining with the HNK-1 antibody. (*a*) A section at the level of the otic vesicles in the rhombencephalon illustrating neural crest cells (indicated by the arrow) which have remained within the lumen of the neural tube. This embryo was at the neural fold stage at the time of injection and was fixed 19 h later; (*b*) another embryo, injected with INO at the three-somite stage and fixed 18 h later. At the level of the mesencephalon, neural crest cells (indicated by arrow) appeared to be trapped within the lumen of the neural tube; (*c*) an embryo injected with small INO fragments. At the level of the otic vesicles, neural crest cells (indicated by the arrow) have remained within the neural tube. Bar, 50 μm.

and/or neural tube development. Although the antibody was injected at the level of the mesencephalon, defects were noted both in the mesencephalon and in the rhombencephalon. This is not surprising since the injected antibody diffused into the rhombencephalon. The predominant defect was displaced aggregates of neural crest cells external to the neural tube, which will be referred to as ectopic neural crest cells, observed in 40% of the 68 embryos injected with INO. 31% of the embryos had neural tube anomalies, and 25% had neural crest cells within the lumen of the neural tube. Many of the embryos had multiple defects, e.g., both ectopic neural crests and neural tube deformities (observed in 13% of the embryos) or ectopic cells and cells within the lumen of the neural tube (6% of the embryos). This is illustrated in Fig. 3, *a* and *b*, which shows two embryos with ectopic aggregates of neural crest cells external to the neural tube, as well as severely malformed neural tubes. However, the majority (70%) of the affected embryos had only one of the three types of defect. In most but not all cases, the ectopic cells were observed unilaterally on the injected side or above the dorsal neural tube in serial sections through the entire head (Fig. 3 *b*). In a few cases, ectopic aggregates were seen bilaterally (Fig. 3 *a*). Other embryos had a build-up of neural crest cells within the lumen of the neural tube, as illustrated in Fig. 4. In these cases, an aggregate of neural crest cells could be seen protruding into the lumen of the neural tube. Such defects were observed at the level of the mesencephalon (Fig. 4 *b*) or at the level of the otic vesicles in the rhombencephalon (Fig. 4 *a*). Although most experiments were performed with concentrated medium containing INO antibodies, similar results were obtained for embryos injected with purified INO antibody. In contrast to a previous study in which antibodies to integrin, a FN and LM receptor, were microinjected into the mesencephalon (7), no apparent reduction in the numbers of neural crest cells on the injected side was noted relative to the uninjected side in any of the 68 INO-treated embryos. Since the effects of INO were sometimes bilateral, it remains possible that both the injected and uninjected side were equally affected. It should be noted that many neural crest cells in the injected embryos appeared to migrate normally in the presence of the INO antibody. INO may, therefore, only affect subpopulations of neural crest cells, or may affect cells for a limited time.

In another series of experiments, INO was injected into the trunk somites during the time of initial neural crest migration and motoraxon elongation. In contrast to the striking effects observed with cranial neural crest cells, we were unable to detect defects in either trunk neural crest migration or motoraxon outgrowth when using either concentrated INO medium or 1 mg/ml of purified antibody (Fig. 5).

Effects of Antibodies against Laminin, Heparan Sulfate Proteoglycan, and Monovalent Fragments of INO on Cranial Neural Crest Migration In Situ

The monoclonal antibody INO is an IgM, and, therefore has numerous functional binding sites. To control for possible effects caused by large antibody molecules that may be unrelated to functional blockage of the INO epitope, embryos were injected with two IgM antibodies, an antibody against chick laminin and against a chick heparan sulfate proteoglycan. Of the 39 embryos injected with anti-laminin antibodies, four demonstrated abnormalities in neural crest migration, i.e., neural crest cells within the lumen of the neural tube (see Table I). This level of abnormality probably represents a baseline in the embryos used for this study, since

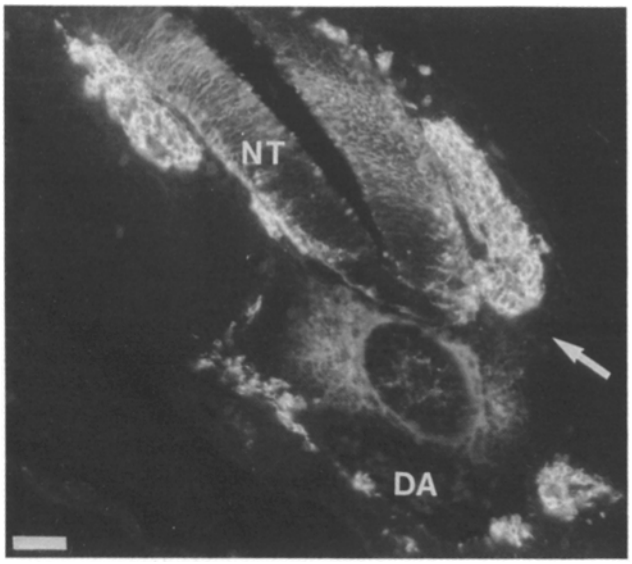

Figure 5. Fluorescence photomicrograph of a transverse paraffin section through the trunk of an embryo injected with INO. The embryo had 26 somites at the time of injection and the antibody was injected at the level of the 17th somite (limb bud region). Both the injected side (indicated by the arrow) and the uninjected side had similar patterns of neural crest cell distribution both within the sclerotome and at the level of the dorsal aorta (*DA*). NT, neural tube. Bar, 50 μm.

injection of concentrated hybridoma medium lacking monoclonal antibodies (referred to as concentrated medium; see Materials and Methods) resulted in about the same degree of embryonic defects (Table I). However, 90% of the embryos were normal (Fig. 6, *c* and *d*). Similarly, injection of antibodies against heparan sulfate proteoglycan resulted in abnormalities in only 1 of the 28 embryos (Fig. 6, *a* and *b*; Table I).

Since INO is an IgM and is multivalent, we wished to eliminate the possibility that injected antibody was causing its effects by cross-linking. Small fragments of the INO antibody were produced and injected lateral to the mesencephalic neural tube. Ten embryos were used for injection of small IgM fragments (see Table I). 70% of these embryos had defects that were indistinguishable from those observed after injection of the intact INO IgM molecule (Fig. 4 *c*). This suggests that the INO antibody renders its effects by interfering with the ability of cells to functionally bind to a laminin–heparan sulfate proteoglycan substrate and not by nonspecific cross-linking.

Time Course of Neural Crest Sensitivity to INO

For all the experiments described above, embryos ranging in age from the neural fold stage to the 9-somite stage were used as hosts. Embryos at all these ages exhibited ectopic neural crest cells outside of or within the neural tube as well as neural tube deformities. In embryos ranging from the neural fold to the 9-somite stage, no correlation was found between the embryonic stage at the time of injection and the morphology of the resultant defects. However, the percentage of affected embryos decreased markedly at the 9-somite stage and no abnormalities in neural crest migration were noted in embryos having more than 9 somites (n = 18 embryos). Since the antibody distribution and persistence were comparable to those of younger embryos, this suggests that embryos are sensitive to antibody perturbations for only a limited time during their development (Table II).

Identification of the INO Antigen in Chicken Embryos

INO is a monoclonal antibody that is thought to recognize a rat laminin–heparan sulfate proteoglycan complex. Immunoprecipitations were performed to investigate the nature of the INO antigen in the chicken embryo. After immunoprecipitation, the antigens were separated on an 8% SDS-polyacrylamide gel and bands were detected using silver stain (Fig. 7). In 2-d chicken embryos, a major band was detected with an approximate molecular mass of 200 kD (Fig. 2, lane *1*). A band of this molecular mass was also detected in immunoprecipitations with the antibody against laminin (2; and authors' unpublished observation), suggesting that this may represent a subunit of laminin. In addition to the 200-kD band, two broad smears were detected from 180 to 150 kD and from 130 to 85 kD (Fig. 7). After heparinase digestion (Fig. 7, lanes *2* and *3*), a band at 200 kD as well as a fainter band at 180 kD remained. The broad smears, however, disappeared and numerous low molecular mass bands between 22 and 44 kD became discernable. These may represent heparan sulfate proteoglycan core proteins or their breakdown products. In all of these preparations, bands were observed that probably correspond to known components of the immunoprecipitation procedure including IgMs (70, 25, 22 kD), IgGs (55 and 22 kD), and BSA (68 kD).

Discussion

The data presented in this study demonstrate that the INO antigen in chick embryos is a laminin–heparan sulfate proteoglycan complex that is present along cranial neural crest migratory pathways. In the adult rat, the INO antigen has been observed in regions that promote neurite regeneration in the peripheral nervous system (10). By microinjecting antibodies that functionally perturb cell binding to this complex, the present results indicate that the laminin–heparan sulfate proteoglycan complex probably plays a role in the normal development of the cranial neural crest and neural tube. After injection into the mesencephalon, INO antibody caused ectopic aggregates of neural crest cells external to the neural tube as well as an accumulation of neural crest cells within the lumen of the neural tube. In addition, many embryos had neural tube deformities. The distributions of the INO antigen, laminin, and heparan sulfate proteoglycan are all consistent with the idea that INO may either prevent normal migration of some neural crest cells from the neural tube, or may affect a subpopulation of already migrating neural cest cells as they progress through the cranial mesenchyme. The LM–HSPG complex is observed around the basal surface of the neural tube from which neural crest cells emigrate as well as in the cranial mesenchyme through which they migrate.

The staining pattern of INO observed in the mesencephalon is remarkably similar to that previously observed for fibronectin (15). Thus, both fibronectin and the LM–HSPG complex have temporal and spatial distribution patterns that are consistent with playing a role in cranial neural crest migration. Previous studies from this laboratory have shown that antibodies against integrin, a fibronectin and laminin

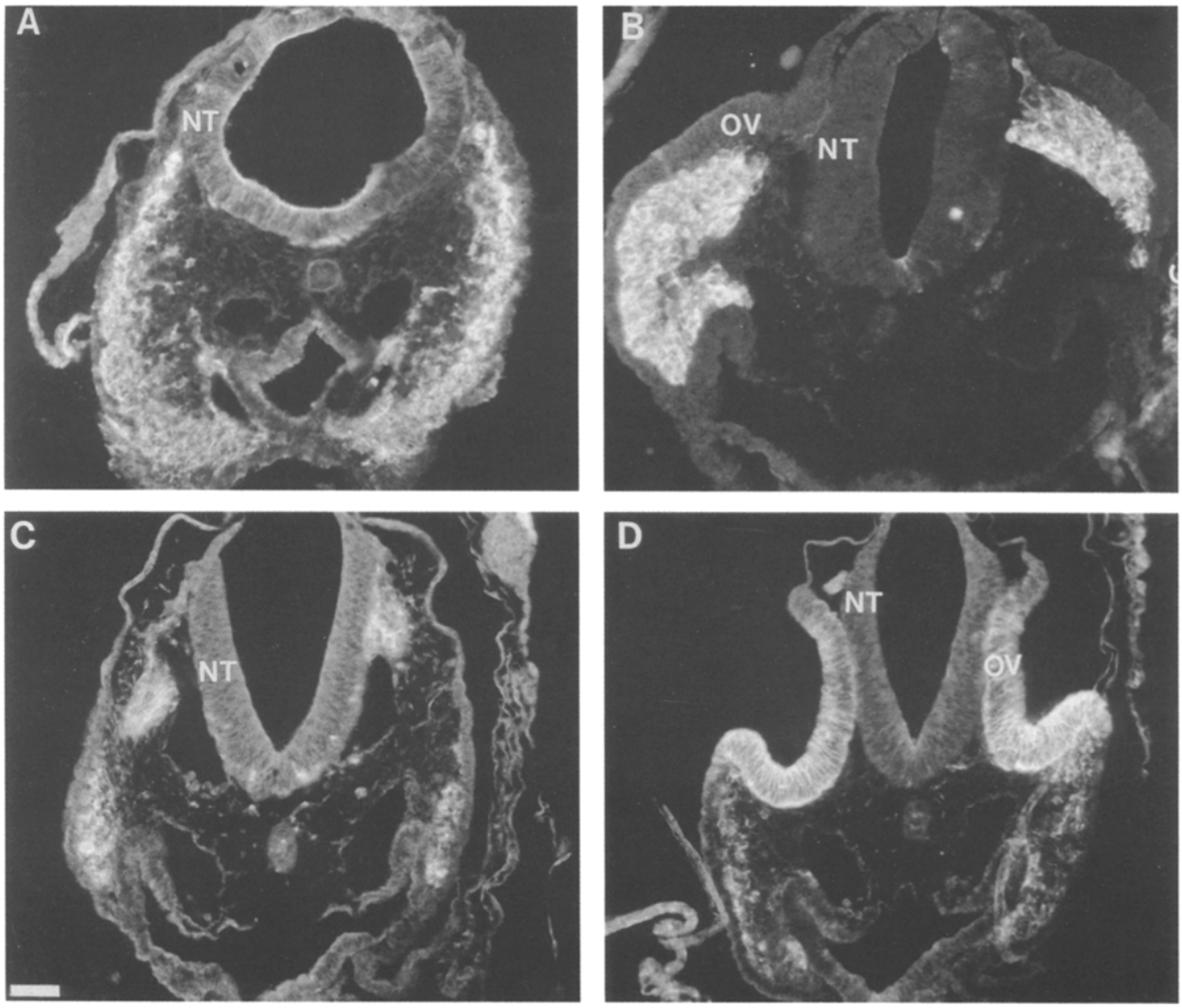

Figure 6. Fluorescence photomicrograph of transverse paraffin sections through embryos injected with control antibodies. Neural crest cells can be recognized by their immunofluorescence after staining with the HNK-1 antibody. (*a*) A section through the mesencephalon of an embryo injected with the antibody against heparan sulfate proteoglycan at the 7-somite stage and fixed 18 h later. Both injected and control sides have identical patterns of neural crest cells distribution, and no ectopic neural crest cells or neural tube (*NT*) anomalies were observed; (*b*) a section through the rhombencephalon of the embryo picture in *a* at the level of the forming otic vesicles (*OV*). (*c*) A section through the anterior rhombencephalon of an embryo injected with the antibody against laminin. This embryo was injected at the 9-somite stage and fixed 19 h later. No neural crest or neural tube anomalies were noted. (*d*) A section through the rhombencephalon of the embryo pictured in *c* illustrating the pattern of neural crest cell distribution at the forming otic vesicles. Bar: (*a* and *d*) 50 μm; (*b*) 37 μm: (*c*) 47 μm.

Table II. Temporal Sensitivity of Cranial Neural Crest Cells to INO Injection

Embryonic age	Total number	Percent affected	Type of defect		
			NC in lumen of NT	Ectopic NC cells	Neural tube anomalies
				%	
Neural fold	3	100	67	33	33
3-Somite	4	100	25	50	75
4-Somite	4	75	25	75	50
5-Somite	8	75	25	38	50
6-Somite	19	68	26	47	32
7-Somite	6	83	0	67	33
8-Somite	9	56	44	22	11
9-Somite	9	33	11	11	22
10–14-Somites	18	0	0	0	0

NC, neural crest. NT, neural tube. Percentages are expressed as percent total *n* of embryos in each category. Some embryos had defects in more than one category.

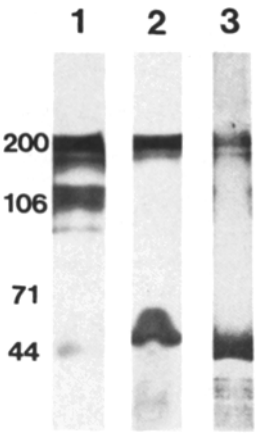

Figure 7. Immunoprecipitation of the INO antigen. Lane *1*, 2-d chicken embryos immunoprecipitated with INO without heparinase digestion. A strong band was observed at 200 kD as well as two broad smears from 180 to 150 kD and 130 to 85 kD. Lanes *2* and *3*, 2-d (lane *2*) and 3-d (lane *3*) chicken embryos immunoprecipitated with INO and then digested with heparinase. Bands were observed at 200 kD and at 180, but the broad smear disappeared and numerous lower molecular mass bands appeared.

receptor (12), also inhibit migration of some cranial neural crest cells (6, 7). The primary defects caused by integrin antibodies were a reduction in the numbers of neural crest cells on the injected side and a build-up of neural crest cells within the lumen of the neural tube. Synthetic peptides that interfere with cell binding to fibronectin (4) and antibodies against the cell binding region of fibronectin (26) have been reported to have similar effects. These experiments sum to suggest an important function for fibronectin in cranial neural crest migration. However, antibodies to integrin are equally likely to block neural crest cell adhesion to laminin as to fibronectin. The present study has shown that microinjection of INO, which presumably blocks the interaction of some neural crest cells with a laminin–heparan sulfate proteoglycan complex in its native configuration, also perturbs cranial neural crest migration. The defects observed after INO injection are qualitatively different than those observed after injection of antibodies against the integrin receptor or against fibronectin. The dominant defect with INO was ectopic cells external to the neural tube, whereas this defect was not seen in embryos treated with integrin antibodies. The dominant defect with integrin antibodies was a reduction in neural crest migration, which was absent in INO-treated embryos. Both antibodies did, however, result in some embryos with neural crest cells within the lumen of the neural tube. One interpretation of these observations is that laminin–heparan sulfate proteoglycan complex and fibronectin may play different roles in cranial neural crest migration. For example, different subsets of cells may respond better to fibronectin than to LM–HSPG. Alternatively, fibronectin and the laminin–heparan sulfate proteoglycan complex may act at different places or times in a complicated cascade of cell–matrix interactions.

The effects of the INO antibody are qualitatively and quantitatively similar to those previously observed after injection of the HNK-1 antibody (9), which recognizes a carbohydrate moiety on the surface neural crest cells (33), as well as on numerous cell adhesion molecules (16). Microinjection of HNK-1 antibody into the mesencephalon resulted in ectopic neural crest cells within and external to the neural tube, as well as neural tube deformities. In addition, simultaneous injection of HNK-1 and integrin antibodies resulted in perturbations that appeared to be additive, suggesting that the two molecules act at different sites. Curiously, the HNK-1 antibody alters the adhesion of neural crest cells to laminin but not fibronectin substrates in vitro. This may suggest that those HNK-1 antigens on the surface of neural crest cell are involved in neural crest cell binding to laminin. In light of this observation, it is interesting to note that the effects of INO and HNK-1 antibodies are identical.

Cranial neural crest cells only appear to be sensitive to INO during a limited period of their development, i.e. from the neural fold stage to the 9-somite stage. In previous studies using CSAT (Bronner-Fraser, M., unpublished observation) or HNK-1 (9) antibodies, a similar time period of cranial neural crest cell sensitivity to injected antibodies was observed. This time period corresponds to the time during which cranial neural crest cells are initially emigrating from the neural tube and in the early stages of their migration. It is, therefore, possible that INO interferes with either the initiation or the early stages of cranial neural crest migration. One scenario is that INO may interfere with the departure of premigratory neural crest cells from the neural tube. Such an interference in emigration could result in cells not contacting their proper substrate. This could lead to displaced neural crest cells within or adjacent to the neural tube, or presumptive neural crest cells remaining inside and thereby deforming the neural tube. In our experimental paradigm, INO only affects a subpopulation of neural crest cells, since many of the cells appear to migrate normally. This could result from either inherent differences between populations of neural crest cells or from limited temporal interactions between neural crest cells and LM–HSPG.

The finding that INO can block neurite outgrowth in vitro (23), neuronal regeneration in situ (30), and neural crest migration in situ suggests that neurons and neural crest cells share some common cell surface receptors and responsiveness to extracellular matrix molecules. The INO antigen is present in only the peripheral, but not the central nervous system (10). Neural crest cells are progenitors to most of the neurons and glia of the peripheral nervous system, with the remainder of components arising from the ectodermal placodes (19). In the trunk, it has been shown that peripheral neurites and neural crest cells share the same migratory pathway; both neural crest cells and motoraxons preferentially move through the anterior half of the trunk somites (8, 14, 27). Thus, neural crest cells and neurites may use similar guidance cues. In adult animals, the INO antigen has been consistently found in regions that support neuronal regeneration, such as the basal lamina surrounding Schwann cells and in the transitional region between the peripheral and the central nervous systems (10). The distribution of INO during embryogenesis is not yet known. However, the distribution of laminin that is likely to occur naturally within a LM–HSPG complex suggests that INO is present in regions of active neural crest cell migration and neurite outgrowth. To date, functional perturbation of neurite outgrowth from embryonic neurons in situ has not been reported. Here no abnormalities in neural crest migration or motoraxon outgrowth were noted when INO was injected into the trunk somites at similar concentrations to those that resulted in functional perturbations in the cranial region. This may indicate that the guidance cues in the head and in the trunk are different. Alternatively, it remains possible that higher antibody concentrations or different sites or methods of antibody delivery are necessary to achieve functional perturbations in this region of the embryo.

It has previously been shown that laminin is the active component of the neurite-promoting factors produced by various nonneuronal tissues that are able to support neurite outgrowth (18). The form of laminin present in these neurite promoting factors is likely to be in the form of a laminin–heparan sulfate proteoglycan complex (10, 18, 23). The present results indicate that this complex is also present in avian embryos and is functionally important in some aspects of cranial neural crest migration. The finding that both the laminin–HSPG complex and fibronectin may play roles in neural crest development highlights the fact that multiple interactions may be important during complicated morphogenetic events. It is likely that many cell surface and cell substrate molecules are involved in the guidance of cell movement. A goal of future experiments will be to delineate the numerous interactions involved in neural crest migration and to examine the possible synergism between candidate molecules.

We thank Dr. Scott Fraser for helpful comments on the manuscript, and Georgia Guillory and Carmen Domingo for excellent technical assistance.

This work was supported by U.S. Public Health Service Grant HD-15527 and by Basic Research Grant 1-896 from the March of Dimes. M. Bronner-Fraser is a Sloan Foundation Fellow.

Received for publication 13 July 1987, and in revised form 17 December 1987.

References

1. Adler, R., M. Manthorpe, S. D. Skaper, and S. Varon. 1981. Polynornithine-attached neurite promoting factors (PNPFs). Culture sources and responsive neurons. *Brain Res.* 206:129–144.
2. Bayne, E. K., M. J. Anderson, and D. M. Fambrough. 1984. Extracellular matrix organization in developing muscle; correlation with acetylcholine receptor aggregrates. *J. Cell Biol.* 99:1486–1501.
3. Bozyczko, D., and A. F. Horwitz. 1986. The participation of a putative cell surface receptor for laminin and fibronectin in peripheral neurite extension. *J. Neurosci.* 6:1241–1251.
4. Boucaut, J. C., T. Darribere, T. J. Poole, H. Aoyama, K. M. Yamada, and J. P. Thiery. 1984. Biologically active synthetic peptides as probes of embryonic development: a competitive peptide inhibitor of fibronectin function inhibits gastrulation in amphibian embryos and neural crest migration in avian embryos. *J. Cell Biol.* 99:1822–1830.
5. Bronner-Fraser, M. E. 1984. Latex beads as probes of a neural crest pathway: the effects of laminin, collagen, and surface charge on bead distribution. *J. Cell Biol.* 98:1947–1960.
6. Bronner-Fraser, M. 1985. Alterations in neural crest migration by a monoclonal antibody that affects cell adhesion. *J. Cell Biol.* 101:610–617.
7. Bronner-Fraser, M. 1986. An antibody to a receptor for fibronectin and laminin perturbs cranial neural crest development in vivo. *Dev. Biol.* 117:528–536.
8. Bronner-Fraser, M. 1986. Analysis of the early stages of trunk neural crest migration in avian embryos using monoclonal antibody HNK-1. *Dev. Biol.* 115:44–55.
9. Bronner-Fraser, M. 1987. Perturbations of cranial neural crest migration by the HNK-1 antibody. *Dev. Biol.* 123:321–331.
10. Chiu, A. Y., W. D. Matthew, and P. H. Patterson. 1986. A monoclonal antibody that blocks the activity of a neurite regeneration-promoting factor: studies on the binding site and its localization in vivo. *J. Cell Biol.* 103:1383–1398.
11. Hamburger, V., and H. L. Hamilton. 1951. A series of normal stages in the development of the chick embryo. *J. Morphol.* 88:49–92.
12. Horwitz, A. F., K. Duggan, R. Greggs, C. Decker, and C. Buck. 1985. The CSAT antigen has properties of a receptor for laminin and fibronectin. *J. Cell Biol.* 101:2134–2144.
13. Hudson, L., and F. C. Hay. 1980. Practical Immunology. Blackwell Scientific Publications, Oxford.
14. Keynes, R. J., and C. D. Stern. 1984. Segmentation in the vertebrate nervous system. *Nature (Lond.).* 310:786–789.
15. Krotoski, D., C. Domingo, and M. Bronner-Fraser. 1986. Distribution of a putative cell surface receptor for fibronectin and laminin in the avian embryo. *J. Cell Biol.* 103:1061–1072.
16. Kruse, J., R. Mailhammer, H. Wenecke, A. Faissner, I. Sommer, C. Goridis, and M. Schachner. 1984. Neural cell adhesion molecules and myelin-associated glycoprotein share a common carbohydrate moiety recognized by monoclonal antibodies L2 and HNK-1. *Nature (Lond.).* 311:153–155.
17. Lander, A. D., K. Tomaselli, A. L. Calof, and L. F. Reichardt. 1983. Studies on extracellular matrix components that promote neurite outgrowth. *Cold Spring Harbor Symp. Quant. Biol.* 48:611–623.
18. Lander, A. D., D. K. Fuji, and L. F. Reichardt. 1985. Laminin is associated with the "Neurite Outgrowth-Promoting Factors" found in conditioned media. *Proc. Natl. Acad. Sci. USA.* 82:2183–2187.
19. LeDouarin, N. M. 1982. The Neural Crest. Cambridge University Press, New York.
20. Lories, V., H. De Boeck, G. David, J. J. Cassiman, and H. Van Den Berghe. 1987. Heparan sulfate proteoglycans of human lung fibroblasts: structural heterogeneity of the core proteins of the hydrophobic cell-associated forms. *J. Biol. Chem.* 262:854–859.
21. Manthorpe, M., E. Engvall, E. Ruoslahti, F. M. Longo, G. E. Davis, and S. Varon. 1983. Laminin promotes neuritic regeneration from cultured peripheral and central neurons. *J. Cell Biol.* 1882–1890.
22. Matthew, W., and L. Reichardt. 1982. Development and application of an efficient procedure for converting mouse IgM into small active fragments. *J. Immunol. Methods.* 50:239–253.
23. Matthew, W. D., and P. H. Patterson. 1983. The production of a monoclonal antibody that blocks the action of a neurite outgrowth-promoting factor. *Cold Spring Harbor Symp. Quant. Biol.* 48:625–631.
24. Newgreen, D. F., and J. P. Thiery. 1980. Fibronectin in early avian embryos: synthesis and distribution along the migration pathways of neural crest cells. *Cell Tissue Res.* 211:269–291.
25. Newgreen, D. 1984. Spreading of explants of embryonic chick mesenchyme and epithelia on fibronectin and laminin. *Cell Tissue Res.* 236: 265–277.
26. Poole, T. J., and J. P. Thiery. 1986. Antibodies and synthetic peptides that block cell-fibronectin adhesion arrest neural crest migration in vivo. *In* Progress in Developmental Biology. H. Slavkin, editor. Alan R. Liss, Inc., New York. 235–238.
27. Rickmann, M., J. W. Fawcett, and R. J. Keynes. 1985. The migration of neural crest cells and the growth of motor axons through the rostral half of the chick somite. *J. Exp. Morphol. Embryol.* 90:437–455.
28. Rogers, S. L., K. J. Edson, P. C. Letourneau, and S. C. McLoon. 1986. Distribution of laminin in the developing peripheral nervous system of the chick. *Dev. Biol.* 113:429–435.
29. Rovasio, R. A., A. Delouvee, K. M. Yamada, R. Timpl, and J. P. Thiery. 1983. Neural crest cell migration: requirements for exogenous fibronectin and high cell density. *J. Cell Biol.* 96:462–473.
30. Sandrock, A. W., and W. D. Matthew. 1987. An in vitro neurite-promoting antigen functions in axonal regeneration in vivo. *Science (Wash. DC).* 237:1605–1608.
31. Shively, J. E., and H. E. Conrad. 1976. Formation of anhydrosugars in the chemical depolymerization of heparin. *Biochemistry.* 15:3932–3942.
32. Tomaselli, K. J., L. F. Reichardt, and J. L. Bixby. 1986. Distinct molecular interactions mediate neuronal process outgrowth on nonneuronal cell surfaces and extracellular matrices. *J. Cell Biol.* 103:2659–2672.
33. Tucker, G. C., H. Aoyama, M. Lipinski, T. Tursz, and J. P. Thiery. 1984. Identical reactivity of monoclonal antibodies HNK-1 and NC-1: conservation in vertebrates on cells derived from the neural primordium and on some leukocytes. *Cell Differ.* 14:223–230.