Neural Differentiation, NCAM-mediated Adhesion, and Gap Junctional Communication in Neuroectoderm. A Study In Vitro

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Abstract. We studied the development of NCAM and gap junctional communication, and their mutual relationship in chick neuroectoderm in vitro. Expression of NCAM, as detected by monoclonal and polyclonal antibodies, and development of junctional communication, as detected by extensive cell-to-cell transfer of 400–500-D fluorescent tracers, occurred in cultures from stage-2 embryos onward. Both expressions presumably required primary induction. The differentiating cells formed discrete fields of expression on the second to third day in culture, with the NCAM fields coinciding with the junctional communication fields delineated by the tracers. Other neural differentiations developed in the following order: tetanus toxin receptors, neurofilament protein, and neurite outgrowth. Chronic treatment with antibody Fab fragments against NCAM interfered with the development of communication, suggesting that NCAM-mediated adhesion promotes formation of cell-to-cell channels. Temperature-sensitive mutant Rous sarcoma virus blocked (reversibly) communication and the subsequent development of neurofilament protein and neurites, but expression of NCAM continued.

THE cells in many organized tissues are endowed at their junctions with specialized membrane channels which provide a direct communication between the cytoplasms (Loewenstein, 1987). These cell-to-cell channels form clusters recognized by electron microscopy as "gap junctions." The channels appear early in development and are pervasive in the embryo. This has led to the hypothesis that junctional communication plays a role in cellular differentiation, namely in the transmission of morphogenetic signals (Furshpan and Potter, 1968; Loewenstein, 1968). The hypothesis gained support from the discovery that the communication becomes compartmentalized in the course of development (Lo and Gilula, 1979); the compartment borders, as delineated by tracer diffusion, coincide with developmental borders, as defined by fate maps or cell lineage (DeLaat et al., 1980; Warner and Lawrence, 1982; Weir and Lo, 1982). Moreover, the developmental fate of early embryo cells was shown to be altered by communication blockade (Warner et al., 1984).

The cell-to-cell channels are collaborative products; each cell in a (homologous) cell junction contributes a symmetric half. From probings of electrical leakage along the cell-to-cell pathway (Loewenstein and Kanno, 1964), it is clear that the hookup between the channel halves is tight, implying close range attractive molecular interactions between them

(Loewenstein, 1981). But are these the only attractive forces that matter for the establishment of communication, or are other adhesive forces between cell membrane surfaces required in addition? So far, cogent information in this regard is available only for the lowest organisms: sponges. In the sponge cells, adhesion by specific glycoprotein surface molecules (Humphreys, 1965) was found to be a requisite for communication (Loewenstein, 1967). Such adhesion presumably is necessary for membrane apposition (approximation) or for channel stabilization in these cells.

Here we studied this question for an adhesion molecule of nerve cells: NCAM. This homophilic glycoprotein ligand (Edelman, 1986; Rutishauser and Goridis, 1986) emerges after primary neural induction in frog embryo (Jacobson and Rutishauser, 1986), is expressed throughout nervous system development (Rutishauser et al., 1978; Balak et al., 1987), and appears to play a role in a variety of cell–cell interactions (Rutishauser, 1986) including the initial stages of neuromuscular synaptogenesis (Rutishauser et al., 1983). We used a culture of chick embryo neuroectoderm, which undergoes neural differentiation (Keane et al., 1984). This in vitro system allowed us to determine NCAM expression and junctional communication in individual cells, and to analyze the topographical relationship between these expressions, without having to contend with the three-dimensional complexities of embryos. It also allowed us to manipulate NCAM function and junctional communication to examine their mutual interaction. We blocked NCAM function with anti-

body Fab fragments against this molecule and blocked junctional communication by means of temperature-sensitive mutant Rous sarcoma virus.

The in vitro neuroectoderm system develops three further neural differentiations: tetanus toxin receptors, neurofilament protein, and neurite extensions. These expressions could be conveniently traced in individual cells, offering us the opportunity to explore their relationship with junctional communication and NCAM-mediated adhesion.

Materials and Methods

Embryo and Cultures

Virus- and pathogen-free fertilized eggs of white Leghorn chicken (SPAFAS, Inc., Norwich, CT) were incubated at 37.5°C (58% relative humidity). The eggs were cracked into a dish containing CA^{2+}-, Mg^{2+}-free Tyrode (CMF)[1] solution, and the neuroectoderm from embryo stages 2–5 was isolated by microdissection and enzyme treatment as outlined in Fig. 1. The neural plate region, as fate mapped by Rudnick (1944), was dissected free; the tissue was treated with 0.25% collagenase (type II; Worthington Biochemical Corp., Freehold, NJ) and 0.25% pancreatin (Sigma Chemical Co., St. Louis, MO) in CMF for 5 min at 37°C, transferred to Tyrode's solution containing 1% FCS, and the neuroectoderm was pulled away from the other germ layers with microforceps; the neuroectoderm was then broken up into small pieces by repeated pipetting. The pieces were pooled from three to five embryos and cultured on 35-mm dishes (Nunc, Roskilde, Denmark) in DMEM supplemented with 10% FCS, at 35.5°C (5% CO_2 and 95% air). As shown before, the procedure yields pure neuroectoderm by criteria of scanning electronmicroscopy (Keane et al., 1979) and of immunohistochemistry (Keane et al., 1984). In the experiments on stage-1 embryos, the entire presumptive neural plate region was used for the isolation procedure.

The embryos were staged according to Hamburger and Hamilton (1951).

Antibodies

Mouse monoclonal antibodies PP and 5E (10 μg/ml), which recognize all known polypeptide and carbohydrate forms of NCAM (Watanabe et al., 1986; Frelinger and Rutishauser, 1986) were used. For indirect immuno-

1. *Abbreviations used in this paper:* CMF, Ca^{2+}-, Mg^{2+}-free Tyrode solution; DMEM, Dulbecco's modified Eagle's medium.

staining the secondary antibody was goat anti-mouse, labeled with rhodamine or FITC. For inhibition of NCAM function, Fab fragments (0.5 mg/ml) of polyclonal rabbit antisera against affinity-purified NCAM were used. For immunostaining here the secondary antibody was FITC-labeled goat anti-rabbit IgG. Tetanus toxin (5 μg/ml) (a gift of Dr. R. O. Thompson, Wellcome Research Laboratories, Kensington, England) was used in conjunction with horse anti-tetanus toxin and rhodamine- or FITC-labeled goat anti-horse IgG, and rabbit antiserum to neurofilament protein (1:200 dilution) (a gift of Dr. S.-H. Yen, Albert Einstein School of Medicine, NY), in conjunction with rhodamine- or FITC-labeled goat anti-rabbit IgG. The use of Fab fragments instead of divalent antibodies alleviated problems of agglutination of cells and redistribution or internalization of antigens.

Immunostaining

Living cells were incubated with either antibody PP (1:500), 5E (1:1,500), or Fab fragments of polyclonal rabbit antisera against NCAM (0.5 mg/ml) in DMEM for 30 min at 37°C, washed three times in DMEM, and then treated with the appropriate secondary antibody for 30 min at 37°C. Cells were then washed three times in DMEM, before the probings of junctional communication (in DMEM plus serum). Where communication was not probed, the stained tissues were fixed in 10% buffered formalin (10 min at room temperature) and mounted on glass slides in 50% glycerol in Dulbecco's phosphate buffered saline (DPBS).

For labeling of the intracellular neurofilament protein, the cultures were fixed in 10% buffered formalin, permeabilized with 95% ethanol at −20°C, incubated with anti-neurofilament antibodies, washed three times in DMEM, treated with secondary antibodies, washed again, and mounted on glass slides as above.

For staining of tissue sections we used the monoclonal 5E and PP anti-NCAMs as primary antibodies. Sections were first treated with rabbit serum (1:500) in DMEM for 10 min at 37°C (blocking solution), washed three times in DMEM, and incubated with 5E (1:1,500) or PP (1:500) for 30 min at 37°C. Sections were then washed in DMEM, treated with FITC goat anti-mouse antiserum (1:200) in DMEM, washed, and mounted in 50% glycerol in DPBS.

For cryosectioning the embryos were fixed in 10% buffered formalin (pH 7.2) at room temperature for 10 min. The embryos were infiltrated with 30% sucrose in DPBS for 1 h at 4°C and embedded in OCT compound (Miles Scientific, Miles Laboratories, Inc., Naperville, IL). To facilitate proper embryo orientation for sectioning, the anterior, posterior, and Hensen's node portions were marked with carbon particles. Cryostat sections (12 μm; 2800E cryostat; Reichart-Jung, Atlanta, GA) were placed on acid-washed slides coated with poly L-lysine, and mounted in 50% glycerol in DPBS.

The immunostained tissues were viewed in a fluorescence microscope (Nikon Diaphot or Leitz Fluovert).
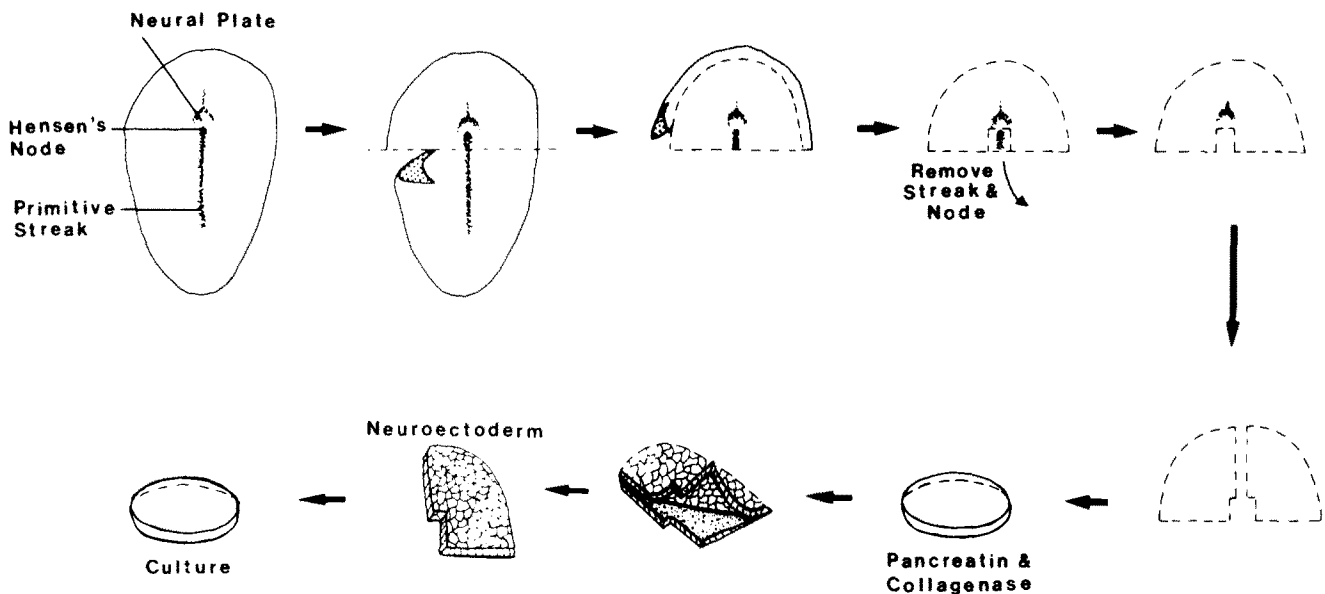

Figure 1. Schematic representation of the isolation procedure of neuroectoderm as described in the text (stage-4 embryo).
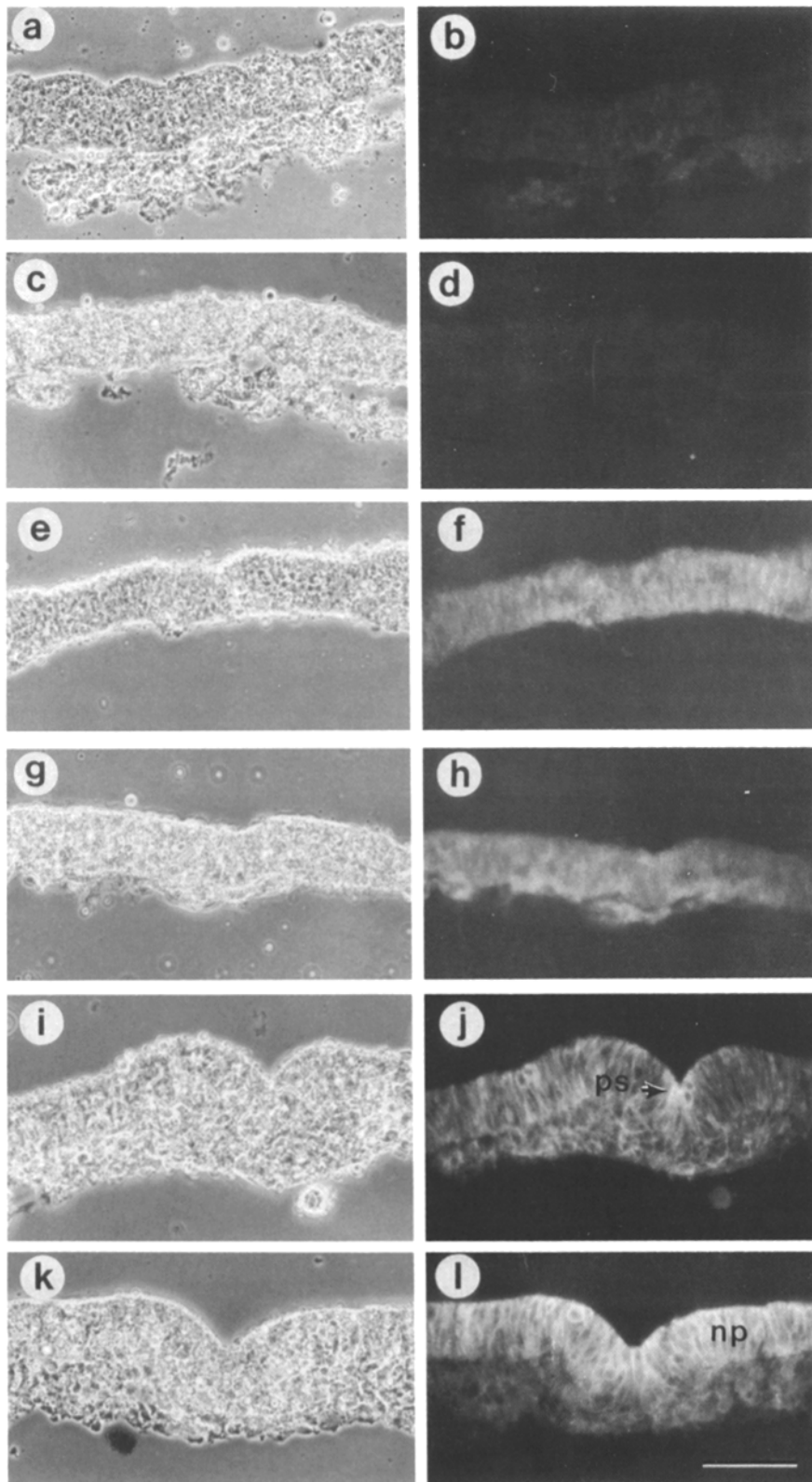
Figure 2. NCAM expression in the chick embryo during gastrulation. Transverse sections through embryos of stages 1-5. (*Left*) Phase-contrast photomicrographs; (*right*) fluorescence photomicrographs; staining with monoclonal NCAM antibody PP. Stage 1, NCAM expression is not above background; (*a* and *b*), staining with primary antibody plus the fluorescein-conjugated secondary antibody; (*c* and *d*), staining with secondary antibody alone. Stage 2 (*e* and *f*), NCAM staining appears in ectoderm and endoderm. Stages 3 (*g* and *h*) and 4 (*i* and *j*), NCAM stains in all primary germ layers, particularly intensely in the primitive streak (*ps*). Stage 5 (*k* and *l*), the neural plate (*np*) has become stratified and NCAM stains intensely there. Bar, 50 μm.

Probing of Junctional Communication

Lucifer Yellow CH or carboxyfluorescein (Molecular Probes Inc., Junction City, OR) was microinjected into living cells by iontophoresis or pneumatic pressure. The micropipette was inserted into the test cell under phase contrast optics; the illumination for fluorescence excitation was then switched on and the fluorescent tracers were microinjected. The spread of the fluores-

cence was continuously observed by eye through the binoculars of the microscope. In parallel, the fluorescence was monitored by means of a television camera (DAGE MTI 66 SIT) coupled to the phototube of the microscope, and videotaped for analysis. The video gain was constant in order to maintain equal detection sensitivity for fluorescence spread. We evaluated tracer spread from the injected cell at 5 min after injection: if at least two first-order neighbors were fluorescent at that time, we scored this result as

Table I. Neural Differentiation In Vitro

Day*	NCAM	Tetanus toxin receptors	Neurofilament protein	Neurite outgrowth
1	+	−	−	−
2	+ +	+	−	−
3	+ + +	+ + +	+	+
4	+ + + +	+ + + +	+	+ + +
5	+ + + +	+ + + +	+ + +	+ + + +

* Time in culture of explants from embryo stage-4-5.

junctional transfer; if second- or higher-order neighbors were fluorescent, we scored it as "extensive transfer." When communication and NCAM expression were probed conjointly, the yellow/green fluorescence of Lucifer Yellow or of carboxyfluorescein was set apart from the red fluorescence of the rhodamine-labeled NCAM antibody by the use of appropriate excitation and barrier filters.

For determinations of electrical coupling, rectangular (hyperpolarizing) current pulses (2.5×10^{-9} A, 200-ms duration, 1 per s) were passed between the interior of one cell and the grounded medium, and the resulting steady-state membrane voltages were determined in this cell and a first-order neighbor. The two microelectrodes (0.5 M potassium sulfate) were connected to balanced bridge circuits to pass current in both directions across the junctions and to measure potentials. We balanced the bridge circuits before cell impalement and measurements were accepted on condition that the balance was maintained after withdrawal of the electrodes from the cells. Currents and membrane potentials were monitored continuously (see Socolar and Loewenstein, 1979). Cells were considered as electrically coupled, when the current injected into one cell (i_1) resulted in a voltage change (V_2) of ≥ 0.5 mV in the first-order neighbor.

Virus Infection

For reversible blocking of junctional communication, neuroectoderms from stages 1 or 4 were infected with temperature-sensitive mutant Rous sarcoma virus (*ts* NY68), at 10^7-10^8 focus forming units/ml (Keane et al., 1984). The virus was a gift of Dr. H. Hanafusa, Rockefeller University, New York.

Results

Neural Differentiation in the Embryo and In Vitro

In Situ. NCAM was expressed from stage 2 onward in the embryo. Both the epiblast and hypoblast cell layers stained with the monoclonal PP antibodies against NCAM at stage 2, and all primary germ layers were stained at stages 3 and 4, most intensely in the primitive streak (Fig. 2). By stage 5, when the notochord forms, the neural plate stood out by its strong staining from mesoderm and endoderm. The same staining patterns were obtained with the monoclonal 5E and polyclonal anti-NCAM reagents. There was no detectable NCAM expression at stage 1, before primary induction.

Other neural differentiations appeared in the following order in the embryo: tetanus toxin receptors (at stage 12-13), neurofilament protein (at stage 17-18, 16-20 h after the toxin receptors), neurite extensions (stage 18-19, 24-32 h after the toxin receptors).

In Vitro. The neuroectoderm isolated from the embryo attached within 24 h to the culture dishes forming islands of growing cells. Isolates from embryo stage 4 or 5 continued to differentiate: NCAM expression increased, tetanus toxin receptors and neurofilament protein appeared one after the other, and neurites eventually grew out, as summarized in Table I.

NCAM was the first neural differentiation detected in the cultures (Fig. 3). It was present on day 1 in cultures from embryo stage 2, that is, as early as in the embryo, and continued to be present in cultures from later embryo stages. For example, in cultures from stage 4-5, the expression began as a weak and patchy immunostain on day 1, which progressively became stronger over the next 3 d. By day 2-3, the NCAM-expressing cells had formed discrete fields. These same fields then also expressed the tetanus toxin marker, as shown by double immunolabeling (Fig. 4, *b* and *c*). An intricate network of nerve cell processes began to appear on day 3-4. Nerve cells, exhibiting NCAM and tetanus toxin markers prominently and eventually also neurofilament protein markers, were then the predominant cell type (Fig. 3, *f* and *h* and Fig. 4, *e* and *f*). Between the aggregates, flat fibroblast-like cells formed a monolayer; most of these cells represent neural crest cells that undergo melanogenesis and astrocyte precursors that bind antibodies to glial fibrillary acidic protein after 2 wk of culture (Keane et al., 1984) (delamination and shedding of mesenchymal cells are complete well before our isolation of the neuroectoderm, Kochav et al., 1980).

Isolates from embryo stages 2 or 3 showed essentially the same features, but the various neural expressions developed more slowly.

Junctional Communication In Vitro

We probed junctional communication mainly with the 443-D fluorescent dye Lucifer Yellow and accessorily with the 376-D carboxyfluorescein. We chose these probes because the 300-400-D molecular size range is likely to be pertinent to intercellular morphogenetic signaling (Crick, 1970). A portion of the cells in the isolates from postinduction embryo stages were capable of transferring the injected tracers to one another. This capacity of junctional communication increased with time; the number of communicating cells increased and the degree of communication increased. To evaluate this communication development, we used two simple criteria: (*a*) the presence of tracer in at least two first-order neighbors of the injected cell within 5 min of the injection, a criterion for evaluating the frequency of communication; (*b*) the presence of tracer also in at least second-order neighbors, a criterion for the degree of communication. The two-cell requirement in *a* reduced the risk of including dividing cells in the scoring of cell-to-cell transfer. (As it turned out, the risk was small; >98% of the transfer-positive injection trials [350 trials] showed transfer to more than one neighbor.) The second-order neighbor requirement in *b* provided a standard for junctional permeability higher than that in first-order neighbor transfer. We scored the percentage of the injection trials that satisfied criterion *a*, and the percentage of these transfer-positive trials that satisfied criterion *b*; the scores will be referred to as "transfer incidence" and "extensive-transfer incidence", respectively.

Fig. 5 (*solid symbols*) gives the basic features of the communication, as indexed by these scores, in isolates from embryo stage 4. On day 1, transfer was rare and inextensive; the transfer incidence was 10% and the extensive-transfer incidence, 0%. On day 2 the transfer incidence had risen sevenfold and the extensive-transfer incidence, to 25%. By day 4, the time when neurites started to grow out, the transfer incidence began to decline. Cells with neurites were invariably transfer negative.
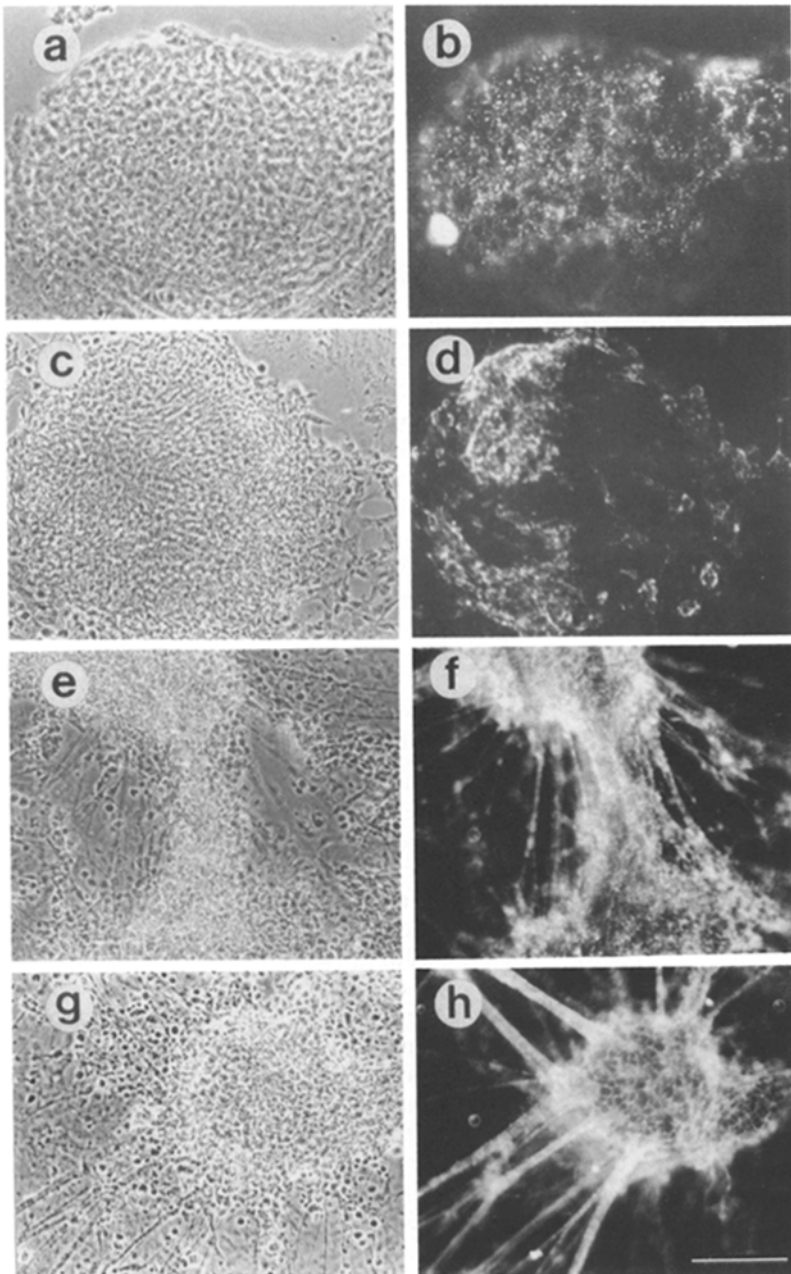
Figure 3. NCAM expression in neuroectoderm culture. Photomicrographs of stage-4 isolates various days in culture, stained with monoclonal NCAM antibody PP. (*Left*) Phase contrast; (*right*) fluorescence mode. Day 1 (*a* and *b*), NCAM shows up as a dotted fluorescence. Day 2 (*c* and *d*), cell masses have grown out, and NCAM is seen in discrete regions (expression fields). Day 4, (*e* and *f*), cells have extended neurites that stain intensely with the NCAM antibody. Day 6 (*g* and *h*), neurites have formed bundles (*h*). Bar, 50 μm.

We verified these communication features for the 376-D carboxyfluorescein. The geometric mean diameter of this molecule (by Corey-Pauling-Kollum models) is similar to that of Lucifer Yellow (~11 Å) but it binds less to cytoplasm. Carboxyfluorescein, in fact, gave transfer incidences closely similar to those of Lucifer Yellow (50 trials, data not shown).

NCAM Is Not Expressed and Extensive Junctional Tracer Transfer Does Not Develop in Preinductive Cultures

Isolates from the preinductive embryo stage 1 showed no sign of NCAM even after 2 wk in culture and, as shown before (Keane et al., 1984), they failed to develop tetanus toxin receptors, neurofilament protein or neurite growth.

Such isolates also failed to develop extensive junctional transfer. They presented a certain baseline communication, a low incidence tracer transfer to first order-neighbors ($\leqslant 20\%$), not unlike that in postinductive cultures on day 1. But communication did not develop beyond that; the transfer incidence did not rise above the 20% level over 6 d in culture, and the extensive-transfer incidence stayed 0 (Fig. 5, *A* and *B*, *open symbols*; and Fig. 7 *II*).

As part of the baseline communication, the cells presented electrical coupling. In the preinductive isolates 70% of the cells were electrically coupled to first-order neighbors, from day 2 onward (total of 56 measurements, on day 2, 3, and 6). The mean membrane potential in these isolates was −37 mV ± 17 SD ($n = 78$) for electrically coupled cells and −22 mV ± 18 SD ($n = 34$) for cells in which coupling was not detected. There was no obvious correlation between membrane
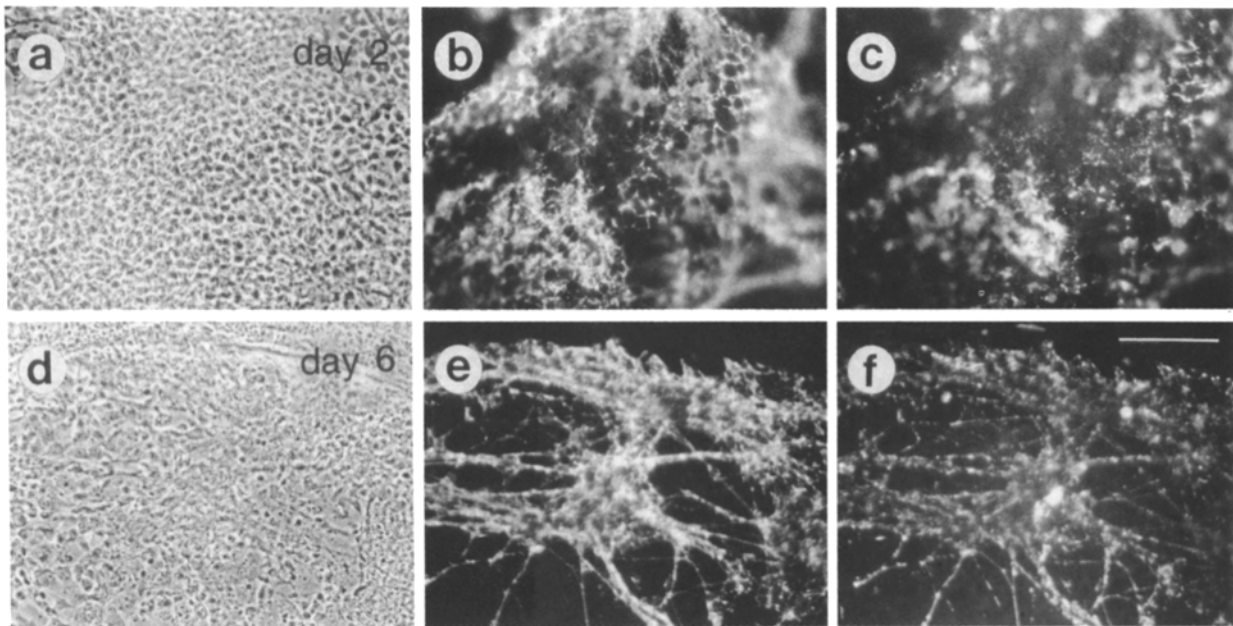
Figure 4. Development of neural markers in stage-4 isolates. Culture day 2, NCAM (*b*) and tetanus toxin receptors (*c*) stain with their respective immunoreagents in the same field shown in phase contrast in *a* (photomicrographs). Day 6, neurites stain with NCAM- (*e*) and neurofilament immunoreagents (*f*); *e* and *f* are photomicrographs of the same region shown in phase contrast in *d*; the same neurites exhibit both markers. Bar, 50 µm.

potential and electrical coupling; there were cells with high membrane potential among the noncoupling cells, and vice versa (membrane potentials ranged from −5 to −85 mV in noncoupling cells and from −6 to −80 mV in coupling cells).

Fields of Junctional Communication Correlate with Fields of NCAM Expression

The presence of Lucifer Yellow transfer correlated with NCAM expression. From day 2 on, when the NCAM-

positive cell areas were clearly identified by immunostaining, the transfer incidence in these areas was ∼60%, and by day 4, all NCAM-positive cells (without neurites) were transfer-positive (Fig. 6 *A*, *solid triangles*). Moreover, communication in NCAM-positive areas was always extensive (Fig. 6 *B*). The Lucifer Yellow tracer spread not only to second-order neighbors (as indexed by the extensive-transfer incidence) but frequently to higher-order ones, outlining fields of communication (Fig. 7 *I*, *d*). By contrast, transfer was rare in NCAM-negative areas and when it occured at all,
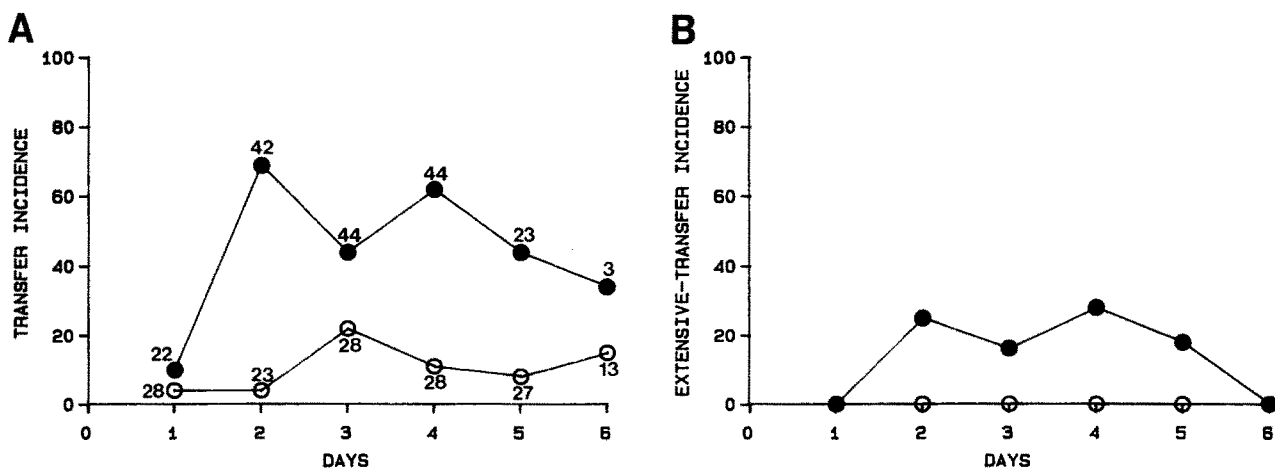

Figure 5. Junctional transfer of Lucifer Yellow in neuroectoderm cultures: (○) stage-1 isolates and (●) stage-4 isolates, during the first 6 d in culture. Plotted are: (*A*) the transfer incidence, that is, the percentage of injection trials resulting in Lucifer transfer to at least two first-order neighbors (see Materials and Methods); and (*B*) the extensive-transfer incidence, that is, the percentage of these transfer-positive trials resulting in transfer to at least second-order neighbors. The number of injection trials are given next to each datum point of transfer incidence, in this and other figures of this type.
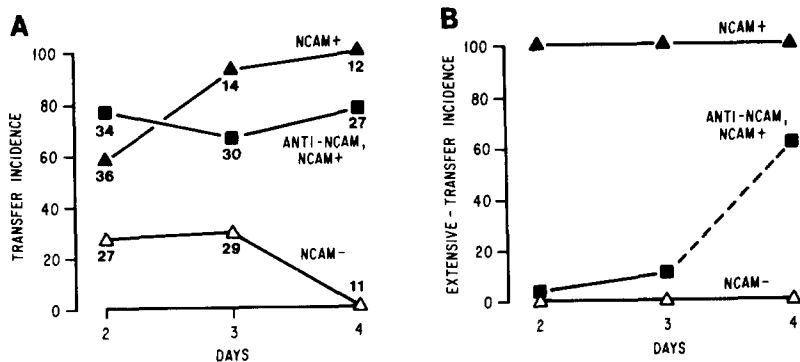
Figure 6. Junctional communication in NCAM-positive and NCAM-negative neuroectodermal cells (stage-4 isolates). *A* shows the transfer incidence and *B* shows the extensive-transfer incidence in NCAM-positive (▲) and NCAM-negative cells (△). NCAM-positive cells were identified by means of red fluorescent immunostaining (monoclonal mouse anti-NCAM plus goat anti-mouse antibody) immediately before the probings of communication with yellow fluorescent Lucifer. The graph also shows the effect on the transfer incidences of long-term anti-NCAM treatment (■, polyclonal rabbit anti-NCAM Fab, 0.5 mg/ml). For identification of NCAM-positive cells here the immunostaining was by FITC-labeled goat anti-rabbit IgG.

it was limited to first- or second-order neighbors (Fig. 7 *I*, *c* and *d*).

The correlation between communication and NCAM expression was most strikingly shown by experiments in which the Lucifer Yellow tracer was injected into cells at the border of a field of NCAM expression. The tracer then generally stayed within the confines of that territory: it spread far into the field, say, to 20th-order neighbors in 3-d-old cultures, but not to NCAM-negative cells (Fig. 7 *d*), not even when these were first-order neighbors of the injected cell. The borders of the Lucifer Yellow-transfer fields and the NCAM expression fields appeared to coincide rather precisely.

This coincidence was harder to show from the NCAM-negative side of the border because the transfer was so infrequent there. But it could be corroborated: among the NCAM-negative cells probed at the border, there were some which transferred Lucifer Yellow to NCAM-negative cells; these probings showed a corresponding lack of border trespassing.

The topographic relation was analyzed by superposition on the television monitor of the communication and NCAM fields delineated by the Lucifer Yellow fluorescence and the NCAM immunostain. Lucifer Yellow was injected on either side of the NCAM field border, into regions where the border was sharply defined in the microscope. Table II summarizes the results of 34 border probings from the NCAM-positive side and 9 from the NCAM-negative side, in transfer-positive cells. On both sides the coincidence of communication and NCAM boundaries was high: there was apparent trespassing of the border in only 2 out of 43 transfer-positive cases. Both apparent trespassings (one in the probings from the NCAM-positive side and one in those from the NCAM-negative side; Table II, day 2) occurred at regions where the boundary was ill-defined due to cell overlap. While such blurred boundaries were avoided at the site of injection, they were not always avoidable in probings on the NCAM-positive side when the tracer spread far afield.

In summary, the stage-4 neuroectoderm cells developed a territorial communication during the first few days in culture, where fields of communication, as traced by Lucifer Yellow cell-to-cell transfer, closely corresponded with fields of NCAM expression.

Anti-NCAM Blocks Development of Extensive Communication

For the preceding analysis of field borders, the cells had been briefly exposed for immunostaining to monoclonal anti-NCAM IgG immediately before the tests of junctional communication. Evidently the antibody binding to NCAM did not disrupt communication here. But would antibody binding affect communication upon long-term exposure to polyclonal antibody? Cell adhesion can be blocked, for at least some time, by anti-NCAM treatment (Rutishauser et al., 1978), and so the number of new channels formed would decrease if NCAM adhesion is necessary for channel formation (a reasonable possibility if NCAM is essential for cell-cell approximation). Since the cell-cell channel protein has a half-life of the order of 5 h (mouse liver; Fallon and Good-enough, 1981), one would expect the number of channels to decrease by adhesion-blocking anti-NCAM treatments lasting more than about half a day. Such a decrease would be expected to show up more sensitively in the extensive-transfer incidence than in the transfer incidence (the former is more sensitive to changes in junctional permeability in the high permeability range).

We examined this point by exposing isolates from embryo stage 4 to polyclonal (monovalent) anti-NCAM Fab fragments, 0.5 mg/ml medium, from the first day on in culture. In a test run using retinal cells, we found that this Fab concentration sharply reduced the cell adhesion in a simple rotation-aggregation assay (data not shown). In the neuroectoderm culture this anti-NCAM treatment almost entirely suppressed the development of extensive junctional transfer for at least 3 d. Fig. 6 *B*, (*solid squares*) shows this for probings of identified NCAM-positive cells. During this time the (first-order) transfer incidence was 65–75% (91 injection trials; Fig. 6 *A*, *solid squares*), not very different from the controls (*solid triangles*).

Anti-NCAM treatments inhibit adhesion only transiently (Rutishauser et al., 1978; Rutishauser, 1986), and even the high concentration of monovalent Fab used in the present experiments was unable to permanently overcome the multivalent binding between cells in the retina-cell aggregation assay. Based on our premise, the inhibitory effect on communication, therefore, would be expected to be transient, too. In fact, the effect subsided after 3-4 days of the anti-NCAM Fab treatment; by day 4 the extensive-transfer incidence had risen (Fig. 6 *B*).

Similar effects have been obtained by treating liver cell cultures with antibodies against CAM, an adhesion molecule of hepatocytes found by Öbrink and his colleagues (Ocklind and Öbrink, 1982). Here anti-CAM transiently blocked the
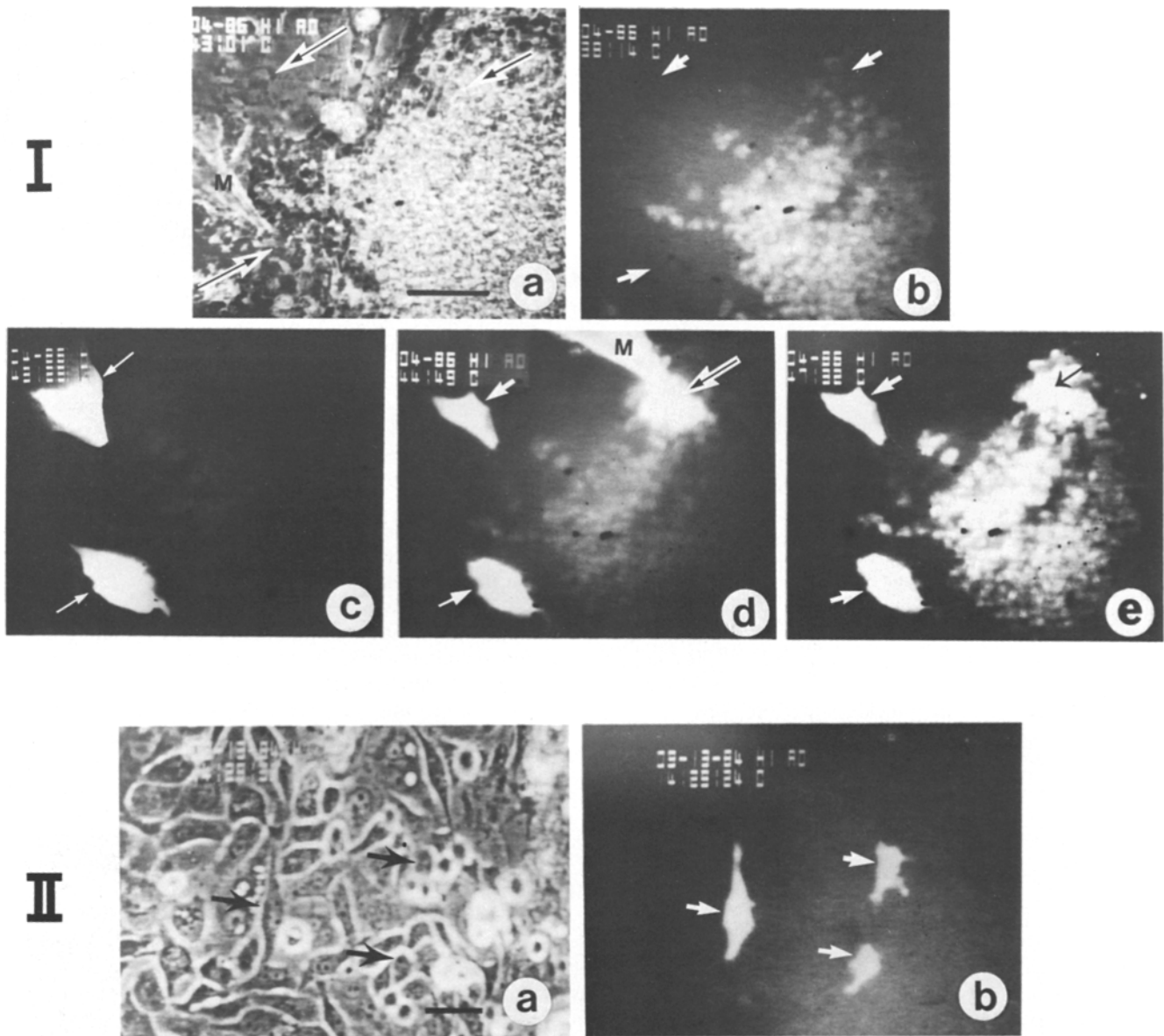
Figure 7. (*I*) Coordinate expression of NCAM and junctional transfer in stage-4 neuroectoderm culture (day 3). (*a*) Phase-contrast video picture of the region tested. (*b*) Red fluorescence video picture showing a field of NCAM expression as stained with the red fluorescent rhodamine-labeled antibody reagent. Two NCAM-negative cells (*left arrows*) were then microinjected with Lucifer Yellow (yellow fluorescent); *a* shows one of the micropipettes (*M*) still in position. (*c–e*), Yellow fluorescence video pictures (the red fluorescence of the NCAM immunostaining has been filtered out): (*c*) the picture, 4 min after injection, shows the Lucifer Yellow confined to the two injected NCAM-negative cells; (*d*) an NCAM-positive cell (*right arrow*) was then injected with Lucifer Yellow, and the picture, 30 s after the start of the injection, shows the spread of the tracer wide into the field (the Lucifer Yellow–filled micropipette (*M*) is still in position); (*e*) 3 min after the start of the injection (pipette withdrawn) the tracer had spread extensively, to more than 20th-order neighbors within the NCAM field, but not to NCAM-negative cells, not even to those close to the injection site at the upper border of the field. The communication field corresponds with the NCAM field (compare with *b*). (The transfer to the two groups of cells in the upper left quadrant, seemingly isolated from the main body of fluorescent cells was probably mediated by fine processes or by deeper lying out-of-focus cells.) The NCAM-negative cells were larger than NCAM-positive ones; a common feature in cultures 3 d old, or older. In the fluorescence mode, the size of the Lucifer Yellow–containing NCAM-negative cells is exaggerated by the video system by "bleeding over"; because these cells did not transfer Lucifer Yellow, their fluorescence is very intense, locally saturating the video light signal and causing bleeding over to adjacent video pixels. Bar, 50 µm. (*II*) Preinductive stage-1 neuroectoderm exhibits no junctional transfer. Three cells (*arrows*) in the region were injected with carboxyfluorescein (day 3 in culture). (*a*) Phase contrast; (*b*) the fluorescence image 5 min after the injection shows the tracer confined to the injected cells. Bar, 20 µm.

formation of junctional communication between aggregating rat liver cells, retarding the development of carboxyfluorescein-transferring junctions (Machida, K., B. Öbrink, and W. R. Loewenstein, unpublished results).

Anti–NCAM Blocks Neural Differentiation

The treatment with anti–NCAM Fab also inhibited the development of neurofilament protein and neurite outgrowth.

Table II. Border Analysis of NCAM- and Communication
Fields in Stage-4 Cultures. Probings of Junctional Lucifer
Yellow Transfer on Either Side of the NCAM Field Border

	NCAM-positive side		NCAM-negative side	
Days in culture	Transfer-positive cases	Transfer-positive cases with transfer confined to NCAM-positive field	Transfer-positive cases	Transfer-positive cases with transfer confined to NCAM-negative field
2	21	20	7	6
3	13	13	2	2

Tabulated are the number of cases among those exhibiting junctional transfer (transfer positive) in which the transfer was confined either to the NCAM-positive or the NCAM-negative field. All probings were done directly at the border of an NCAM-expression field, as identified by immunostaining.

When stage-4 isolates were treated with anti-NCAM Fab as in the preceding experiments, they did not express these neural markers for at least five days. When the Fab was washed out on day 6, the two markers appeared within 48 h, demonstrating the reversibility of the inhibition (Fig. 8).

The Fab concentration in the culture medium was always in excess in these experiments, and so the cells did not stain directly for NCAM. That NCAM, nevertheless, was present on the cells was shown by means of an antibody recognizing the rabbit anti-NCAM Fab (Fig. 8 b).

NCAM Is Expressed during Block of Communication by src Protein

Does blockade of junctional communication affect NCAM production? We used Rous sarcoma virus to produce long-term blockade of communication. The viral src gene encodes the protein pp60^{v-src}, a protein tyrosine kinase (Bishop, 1982; Hunter and Cooper, 1985) that reversibly blocks junctional communication (Atkinson et al., 1981; Azarnia and Loewenstein, 1984 a, b). Isolates from embryo stages 1 and 4 were infected with a viral mutant (tsNY68) whose pp60^{v-src} protein kinase is active at 35°C (permissive temperature) and inactive at 41°C. This allowed us to block communication over long periods of time and to lift the block by simply shifting the temperature of the cultures. Block of communication in cultures of chick embryo cells and other avian and mammalian types of cells ensues within 10-20 min of a temperature downshift under these conditions, and the block is reversed about as fast upon temperature upshift (Atkinson et al., 1981; Azarnia and Loewenstein, 1984a; Rose et al., 1986).

We infected the cultures immediately after explantation (day 1) and kept them at the permissive temperature for 6 d. Fig. 9 illustrates a typical block and its reversibility in a stage-4 culture. Whereas there was little or no junctional transfer of Lucifer at the permissive temperature (Fig. 9 left, c), transfer was extensive 3 h after raising the temperature to the nonpermissive level (right, c). NCAM, on the other hand, was expressed throughout the permissive-temperature period and after it (Fig. 9, right and left, b); and from day 2 on at the permissive temperature the expression level was well above that of uninfected cells on day 1.

Although present at all times, NCAM appeared to be somewhat reduced by the src action. In areas where the expression was faint, it was then difficult to video record the
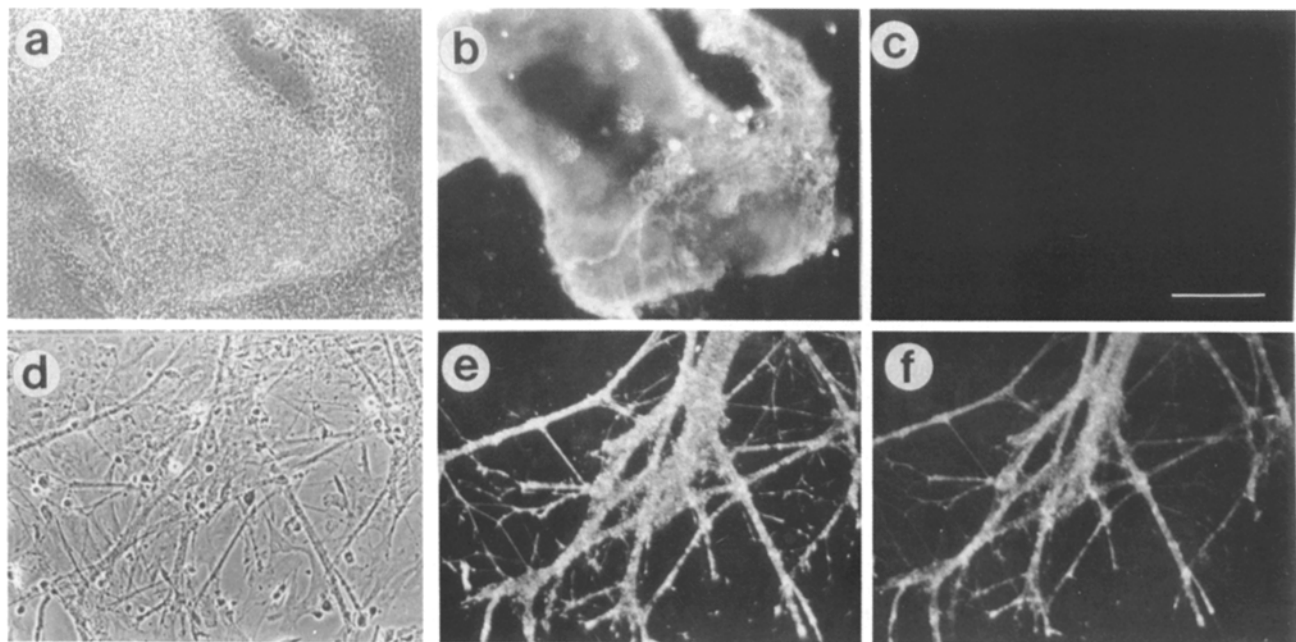

Figure 8. Reversible block of neurofilament protein expression and neurite outgrowth by chronic treatment with (rabbit) anti-NCAM Fab fragments (0.5 mg/ml). Top row shows photomicrographs of a stage-4 culture on day 6 of the Fab treatment, when neurofilament- and NCAM (PP) immunoreagents were applied. Neurites have not grown out (a, phase contrast) (compare with Fig. 3 h); and neurofilament protein is not detected by the immunostain (c). NCAM is expressed (b); as NCAM does not stain directly with the NCAM antibodies (PP or 5E) in the presence of the high concentrations of anti-NCAM Fab, the NCAM is detected with the aid of an FITC-conjugated goat anti-rabbit IgG which recognizes the rabbit Fab. Bottom row shows a stage-4 culture-treated with the anti-NCAM Fab for 6 d, 48 h after the wash out of the anti-NCAM Fab. Neurites have extended (d, phase contrast) and stain intensely for NCAM (e) and neurofilament protein (f). Bar, 50 μm.
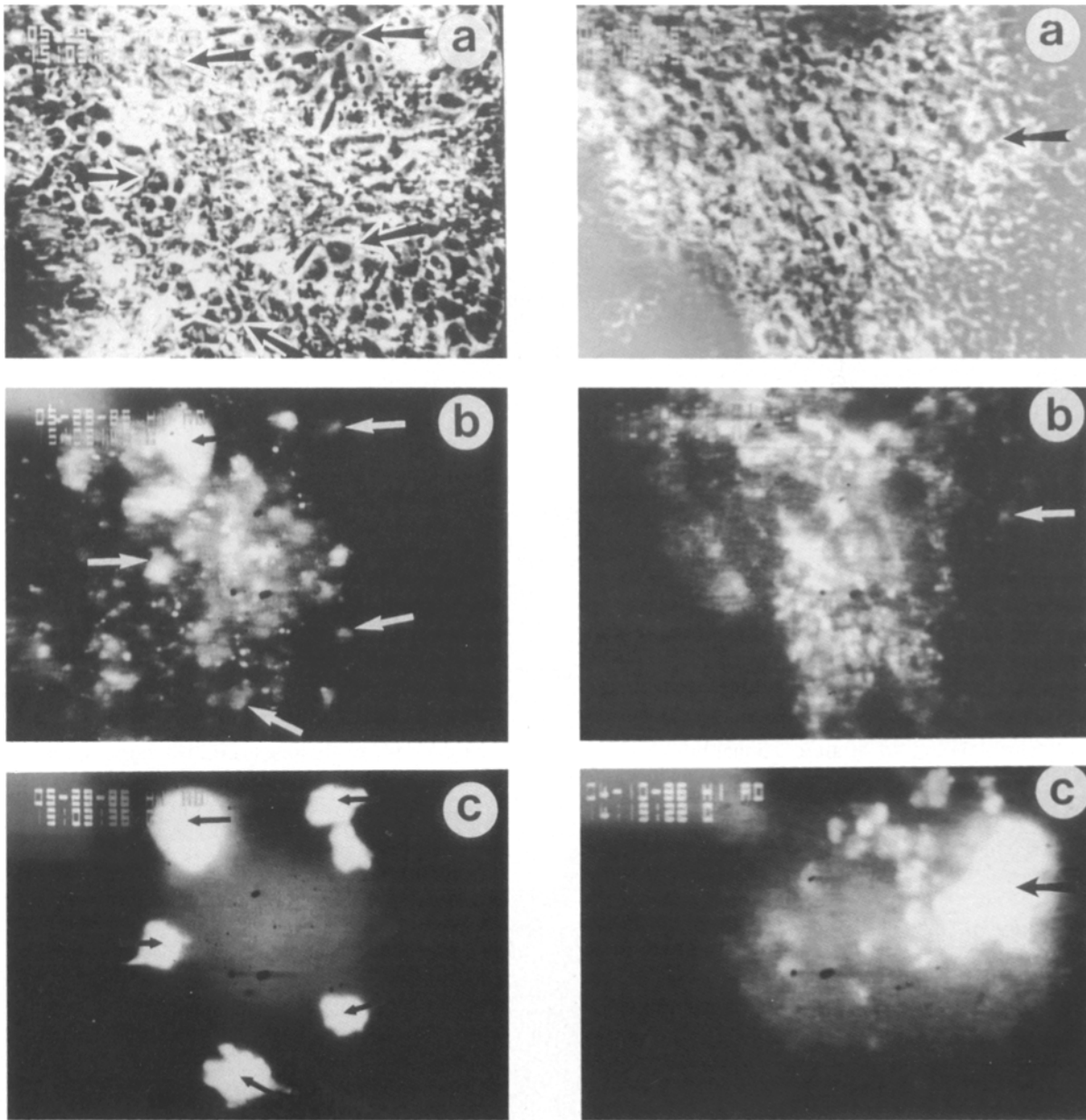
Figure 9. Reversible block of junctional transfer by temperature-sensitive Rous sarcoma virus (tsNY68), and NCAM-expression in stage-4 cultures. The cells were infected on day 1 in culture and maintained at 35°C, the permissive temperature, for 6 days. The left column shows a culture on day 6 (35°C) and the right column shows a culture on the same day, but 3 h after raising the temperature to 41°C, the nonpermissive level. (*a*) Phase contrast. (*b*) NCAM immunostain fluorescence; (*c*) Lucifer Yellow fluorescence. (*Left*) Lucifer Yellow was injected into five cells (*arrows*); only one cell (*right upper quadrant*) exhibits transfer, namely to a single neighbor (a sister cell from an incompleted division?). All three video pictures, *a–c*, show the same microscope field. In many cells the NCAM immunostain is patchy and therefore does not completely outline the cells. However, Lucifer Yellow outlines the entire cell. In the video picture the size of the Lucifer Yellow fluorescent image of the cells is highly exaggerated with respect to the phase contrast image, due to bleeding over of the intense light to adjacent video pixels. Each of the fluorescent outlines in *c*, except for the upper right-hand one, corresponded exactly to one cell, as was clear from direct observation through the microscope done in parallel with the video imaging. (*Right*) One cell is injected (*arrow*), showing transfer to an extensive field of cells. The injected cell belongs to a NCAM-positive region. The immunofluorescence of that cell shows up clearly in the photographic reproduction (*b*), but not so in all other cells of that region where it was too faint to be videorecorded with high enough intensity. However, all cells in that region were immunofluorescent, as seen directly through the microscope. Here, too, the three pictures display the same microscope field.

NCAM immunofluorescence with high enough intensity for good photographic reproduction. In Fig. 9 *b*, for example, many cells in the area around the injection site (*arrows*) were so weakly immunofluorescent that they did not show up in the reproduction. The NCAM-immunofluorescence, however, was clearly visible by direct microscopic observation over the entire area of Lucifer spread in Fig. 9 *right*, *c*. Thus, while this figure illustrates the blocking effect of *src* on junc-
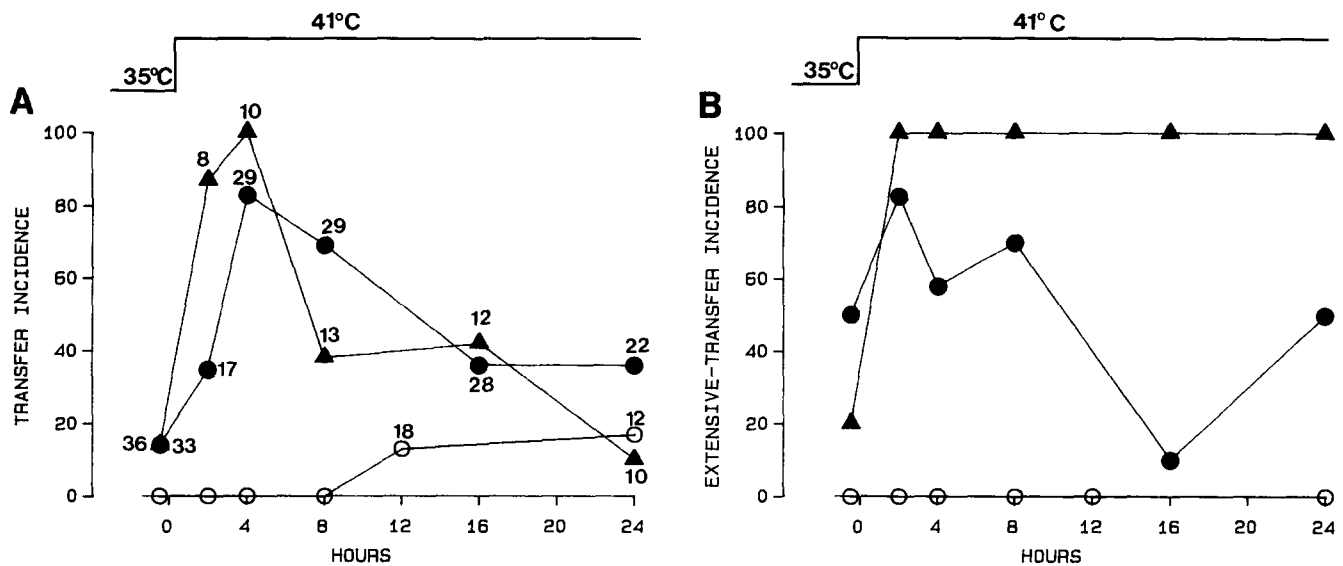
Figure 10. Reversal of virus-induced block of junctional transfer. The cultures were infected with the temperature-sensitive virus on day 1 in culture and kept at 35°C, the permissive temperature, for 6 d. Transfer of Lucifer Yellow was tested in parallel cultures on day 6 at that temperature and at various times after shifting to 41°C, the nonpermissive temperature. (*A*) Transfer incidence, and (*B*) extensive-transfer incidence in (○) stage-1 cultures; (●) stage-4 cultures not immunostained; (▲) NCAM-positive cells of stage-4 cultures immunostained.

tional communication and the persistence of NCAM during the block, it is not useful for correlating the field of NCAM expression with that of junctional communication.

Fig. 10 summarizes the results of all junctional probings, including those in stage-1 cultures. Stage-1 cultures showed no transfer at all at the permissive temperature (Fig. 10, *open circles*); even the first-order transfer incidence was zero (compare with Fig. 5). In stage-4 cultures, both indices of junctional transfer were <20% at that temperature. This was so in random ("blind") samplings (Fig. 10, *solid circles*), as well as in identified NCAM-positive cell regions (*solid triangles*). Upon shifting to the nonpermissive temperature, the transfer incidence and extensive-transfer incidence rose within 4 h to 100% in the NCAM-positive regions (Fig. 10); the same communication levels exhibited by cultures not infected with the virus (compare with Fig. 6, *solid triangles*).
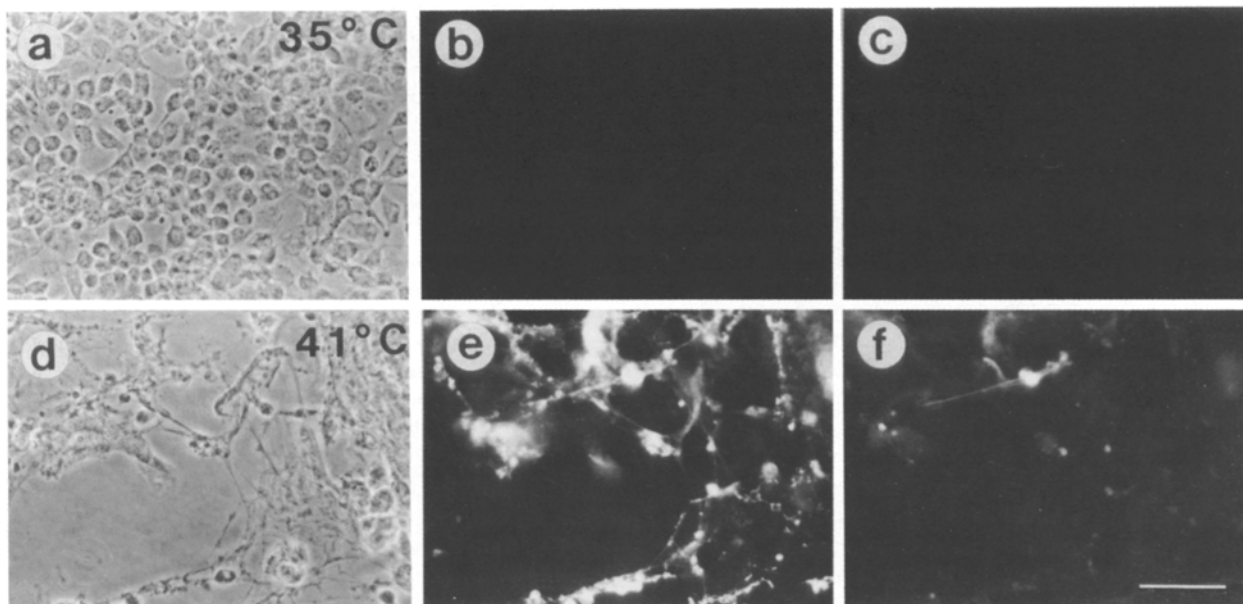

Figure 11. Reversible block of tetanus toxin receptors, neurofilament protein, and neurite outgrowth by temperature-sensitive Rous sarcoma virus. The cells were infected on day 1 in culture and maintained at the permissive temperature, 35°C, for 6 d. (*a-c*) Photomicrographs taken on day 6 at 35°C, after staining for tetanus toxin receptors and neurofilament protein. The cells do not exhibit neurites (*a*), tetanus toxin receptors (*b*), or neurofilament protein (*c*). (*d-f*) Photomicrographs taken 5 h after shifting to the nonpermissive temperature, 41°C. (*d*) Neurites have extended and the cells exhibit tetanus toxin receptors (*e*) and neurofilament protein (*f*); at this early time, the neurofilament protein is not yet as strongly expressed as the tetanus toxin receptors. Bar, 50 μm.

The rise in junctional-transfer incidence was transient; 4–8 h after shifting to the nonpermissive temperature level, the incidence began to decline (Fig. 10 A; the spread of tracer from the few NCAM positive cells that still showed transfer after 8 h, was invariably extensive; Fig. 10 B, *solid triangles*). This decline was associated with neural differentiation (see below), and may be analogous to the decline that takes place in untransformed cultures during neural differentiation (see Fig. 5, day 4 onward), culminating with the complete shut off of transfer in cells extending neurites.

The junctional transfer in day-4 cultures not infected with the virus was not sensibly changed by the shifts in temperature. Neither the transfer incidence nor the extensive-transfer incidence was different at the two temperatures there (data not shown), demonstrating, as did earlier work with virus-transformed cells (Atkinson et al. 1981; Azarnia and Loewenstein, 1984a, 1987) that the effects on junctional transfer in the cultures transformed by the temperature-sensitive virus were not due to the temperature change per se.

Expressions of Neurofilament Protein and Neurite Outgrowth Are Reversibly Blocked, Along with Junctional Transfer, by src Protein

The development of the other neural markers was blocked at the permissive temperature, as shown before (Keane et al., 1984). Tetanus toxin receptors, neurofilament protein and neurite processes were absent at the permissive temperature when junctional communication was blocked. After the shift to nonpermissive temperature and the consequent lifting of the junctional blockade, they appeared in the same order as they did in the absence of the blockade (see Table I), but at faster pace: tetanus toxin receptors and neurofilament protein were detectable within 5 h of the temperature upshift (Fig. 11).

Discussion

The neuroectoderm mimicked in culture two aspects of general embryonic development: (a) neural cells differentiated, sequentially expressing NCAM, tetanus toxin receptors, neurofilament protein (and neurite extensions); and (b) the cells established territorial communication, with high junctional permeabilities within the communication fields and low permeabilities at the borders, reminiscent of embryonic communication compartments. This made it possible to analyze in vitro, with good spatial resolution, the topological relationships between communication and the neural differentiations—a task not easily manageable in the embryo.

The expression of communication, as determined with the 400–500-D tracers, and the expressions of the various neural markers had closely corresponding topographies. In the case of NCAM, where the correspondence with communication was analyzed in most detail, the cell fields marked by the NCAM immunostain coincided rather precisely with the fields delineated by the Lucifer Yellow tracer. There was furthermore a close temporal correlation between the onset of NCAM expression and that of junctional communication.

These findings opened two possibilities concerning the developmental relationship between NCAM and junctional communication: (a) communication, that is, cell-to-cell channel formation, is promoted by NCAM-mediated cell adhesion—a plausible relationship if such adhesion is critical in

cell approximation; or (b) NCAM expression is induced by signals conveyed through cell-to-cell channels—a possibility in the venue of the hypothesis of a role of junctional communication in cellular differentiations. To weigh these alternatives, we interfered with the NCAM function on one hand, and with communication on the other.

The results obtained were consistent with mechanism a: chronic anti–NCAM treatment prevented development of extensive communication. The role of NCAM-mediated adhesion in neuroectoderm, thus, may be like the role of the glycoprotein-mediated adhesion in sponges, where a specific glycoprotein ligand on the cell surfaces (see Moscona, 1968; Burger et al., 1978) is essential for cell-to-cell channel formation (Loewenstein, 1967). Such glycoprotein-mediated adhesions may be obligatory preludes for cell–cell channel formation, if the adhesions are necessary for the approximation of the cell membranes; or the adhesions may be concurrent with channel formation, if they stabilize the membrane approximation. Models for such adhesion-dependent or adhesion-assisted channel formation have been proposed by one of us (Loewenstein, 1981).

Although not excluded, mechanism b appears less likely in view of the results obtained with Rous sarcoma virus. The temperature-sensitive virus caused profound depression of communication at the permissive temperature, yet NCAM expression was not prevented. The expression of NCAM may have been reduced, as in the case of rat cerebellar cells transformed by the virus (Greenberg et al., 1984). But the important point here is that NCAM, which has a half-life at the membrane of 16–20 h (Rutishauser, U., unpublished results), continued to be expressed throughout the 6 d of junctional blockade.

However, in contrast to NCAM, other neural differentiations in the cells were prevented. Tetanus toxin receptors, neurofilament protein, and neurite outgrowth, which were all expressed by the cells at the nonpermissive temperature or in untransfected cells, failed to appear at the permissive temperature when communication was blocked. (Moreover, the neurofilament protein and neurites also failed to be expressed during the block of extensive communication produced by chronic anti–NCAM treatment.) In these differentiations, then, junctional communication (whose block is coordinately reversed with the block of differentiation) might be involved, although we do not exclude the possibility of a more direct action of the viral *src* protein on differentiation.

The reversal of the junctional block in stage-4 cultures had a special feature: the restored transfer capacity declined 4–8 h after shifting to the nonpermissive temperature level (Fig. 10). This is very different from the behavior of various cell lines derived from avian or mammalian embryos, transformed by temperature-sensitive Rous sarcoma virus (including the same virus strain), where the junctional transfer stays at the maximum for days at the nonpermissive temperature (Azarnia and Loewenstein, 1984c; Rose et al., 1986). It also differed from the behavior of stage-1 neuroectoderm cells, where the transfer incidence actually increased (up to the level of untransformed 6-d-old cultures) after the temperature upshift (Fig. 10, *open circles*; compare with Fig. 5 A). This special behavior seems to reflect the developmental potential of the stage-4 cells. Unlike the other transformed cells mentioned, stage-4 cells can undergo neural differentiation; and the decline in junctional transfer coincides with that differentiation, namely with expression of tetanus toxin

receptors and neurofilament protein. There was a similar correlation between declining junctional transfer and neural differentiation in untransformed stage-4 cells, but at lower speed (Fig. 5). What took days to develop in the untransformed state, took only hours upon reversal of the transformed state on day 6. Once the viral *src* protein activity is turned off, the neural development goes *tempo presto*, as if the developmental process had been dammed up, so to speak. Finally, as development proceeds to neurite outgrowth, the capacity of junctional transfer is completely lost in either case, foreshadowing the habitual state of adult neurons (excepting those with electrical synapses).

Only cultures from postinductive embryo stages were capable of developing a high degree of communication, and this salient feature also applied to NCAM development. Presumably these neuroectoderm differentiations require primary induction, as does nervous-system development in the embryo in general.

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