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Embryonic to adult conversion of neural cell adhesion molecules in normal and staggerer mice

(cell adhesion molecules/cell–cell interaction/mouse cerebellar mutants/cell surface modulation/embryonic development)

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ABSTRACT **The neural cell adhesion molecule (N-CAM) has an unusually high amount of sialic acid (28–35 g/100 g of polypeptide) and shows microheterogeneity in electrophoretic gels in its embryonic or E form. During development, the molecule undergoes conversion to several adult or A forms, which resemble the E form but which on the average have only 10% sialic acid and do not appear to be microheterogeneous. In the present study, rabbit antibodies to mouse N-CAM and two different monoclonal antibodies were used to follow the E→A conversion in normal and mutant mice. E→A conversion to three forms (M_r 180,000, M_r 140,000, and M_r 120,000) was found to occur at different rates in different parts of the brains of wild-type mice. Examination of the entire cerebellum of the granuloprival mouse mutant staggerer (*sg/sg*) showed that the E→A conversion did not occur by 21 days after birth, whereas in wild type it was almost complete at that time. There was also some delay in E→A conversion within the cerebral cortex of *sg/sg*, although phenotypically no evidence of cortical disorder has been detected. In pooled tissues from phenotypically normal-appearing littermates, (i.e., a mixture of *sg/+* and *+/+*), a slight conversion delay was also found in cerebellum and cortex. The mutants weaver, reeler, and jimpy all showed normal schedules of E→A conversion. These observations raise the possibility that a major defect in staggerer mutants relates to a failure in local surface modulation of N-CAM to produce the A forms of the molecule. Some of the failures of synapse formation and of cell survival seen in this disease may result from the anomaly, which is likely to alter the binding properties of N-CAM at critical times of development.**

A number of studies (1–4) have shown that neuron–neuron and neuron–muscle adhesion in the embryo is attributable to an unusual sialoglycoprotein, the neural cell adhesion molecule (N-CAM), that is found on the surfaces of these cells. *In vitro*, N-CAM mediates fasciculation of neurites (5), nerve–muscle binding (6), and orderly development of retinal layers in organ culture (7). Embryonic N-CAM has a high proportion of sialic acid in an unusual linkage (8) and appears as a broad diffusion zone (M_r 200,000–250,000) on NaDodSO$_4$/acrylamide gel electrophoresis (E form). After maturation (9), the molecule is altered to contain only about one-third the amount of sialic acid. In the chicken, altered N-CAM shows at least two sharp electrophoretic bands at M_r 180,000 and 150,000 (A forms).

Preliminary analysis of N-CAM binding indicates that it is second-order homophilic—i.e., N-CAM upon one cell binds directly to N-CAM upon another without intervening ligands (10). Neuraminidase treatment of N-CAM removes sialic acid both from molecules on cells and from the free molecule but does not abolish their binding capacity. Nevertheless, conversion of E form to A form (E→A conversion) represents a local surface modulation (11) that must alter the net negative charge and possibly the binding strength of the N-CAM molecule.

N-CAM function and the schedule and significance of E→A conversion have so far not been assessed *in vivo*. The existence of cerebellar mutants (12) in mice prompted us to explore these issues, particularly the possibility that a defect in E→A conversion or in cell adhesion molecules may be related to the histological disorders in these diseases. In the present paper, we describe experiments indicating that in one of these mutants, staggerer (*sg*), E→A conversion fails to occur in the cerebellar regions where severe connection defects are found.

MATERIALS AND METHODS

Mice. All mouse mutants as well as C57BL/6 (wild type) were obtained from The Jackson Laboratory and maintained in our laboratory. Because *sg* is an autosomal recessive gene, heterozygote mating (with either B6C3-a/a, *sg/+* parents or C57BL/6, *+ d se/sg + +* parents) produces on the average one *sg/sg* mutant out of four newborn animals. Between 7 and 13 days of age, homozygous staggerer mutants can be distinguished by dissecting out the brains and comparing the sizes of the cerebella; after 14 days, they can be recognized easily by their abnormal motor behavior.

Mice with reeler (*rl*) mutations were obtained by using B6C3-a/a, *rl/+* parents. Mice with weaver (*wv*) mutations were obtained by using B6CBA-A^{WJ}/A, *wv/+* parents. The recognition criteria for homozygotes were the same as for staggerer mutants. Jimpy (*jp*) mutants (B6CBA-A^{WJ}/A, Ta *jp*/Y) were compared with the other mutants, with their male littermates serving as controls. NCS (Swiss) and CD1 mice, which are not inbred strains and were also used as wild type, were obtained separately from the random-bred closed colony in the Laboratory Animal Research Center of The Rockefeller University and from the Charles River Breeding Laboratories, respectively.

Immunoprecipitation and Gel Electrophoresis. These two procedures have been described (4). Separate mouse brain areas were dissected from animals of 1, 7, 10, 21, and >60 days of age. As described previously (4, 10, 13), the tissues were homogenized into membrane vesicles, which were iodinated by using the lactoperoxidase method, extracted with detergent, then immunoprecipitated with staphylococcal protein A-Sepharose beads that had been coated with rabbit anti-mouse-N-CAM or the monoclonal antibodies 15G8 and 9E11 (4, 13). For immunoprecipitation, 1.2 mg of membrane vesicles obtained from the respective brain areas was iodinated with 1.5 mCi (1 Ci = 3.7×10^{10} becquerels) of ^{125}I; 300 μg of iodinated solubilized membrane proteins from the vesicles was then mixed with the same amount of antibody beads. Conditions were iden-

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Abbreviations: N-CAM, neural cell adhesion molecule; E and A forms, embryonic and adult forms of N-CAM; E→A conversion, conversion of E form to A form.

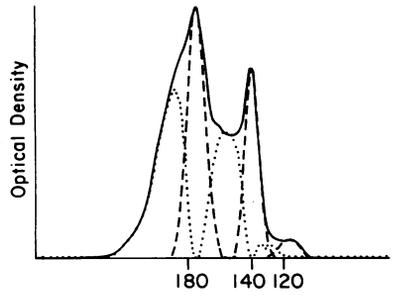

FIG. 1. Method used to analyze densitometric tracings providing data for Table 1. The tracing of N-CAM from 14-day cerebral cortex is used as an example (see Fig. 4B). Bands at M_r 180,000, 140,000, and 120,000 were simulated by Gaussian curves with means and half-height widths that best fit the tracing (---). Values left after subtracting the simulated curves from the original tracing are also shown (· · ·).

tical for labeling and immunoprecipitation in all comparative experiments.

Densitometry of Autoradiographs. Autoradiographs of the electrophoretic gels were scanned by an Optronics Photoscan P-1000 with a 100-μm window. Optical densities were recorded and processed by using a Digital PDP12 computer. A typical scan and the data reduction procedure are illustrated in Fig. 1.

To analyze a tracing (which reflected various mixtures of the E form and the three A forms), bands corresponding to M_r values 180,000, 140,000, and 120,000 were simulated by Gaussian curves. Subtraction of the areas of these curves from the original tracing left broad bands between the curves, presumably corresponding to the residual contribution of the E form. Both the percentage of the total traced area occupied by the three bands and a value of 1.0 or greater in the ratio of the band height at M_r 140,000 to that at M_r 180,000 appeared to be reliable indicators of E→A conversion. It should be noted, however, that the Gaussian fitting is only one of many possible methods of data reduction and that it tends to overestimate the amount of the A forms. The height ratios tend to correct for this error.

RESULTS

Maturation of N-CAM in Normal Mice. N-CAM purified (4) from adult (>60 days old) mouse brains neutralized the inhibition of aggregation of embryonic mouse brain cells by anti-N-CAM antibodies. Upon $NaDodSO_4$ electrophoresis, the purified antigen migrated as two prominent bands of M_r 180,000 and 140,000 and a minor band of M_r 120,000. Together, the A forms contained about 10 g of sialic acid per 100 g of polypeptide as compared to 28–35% in the E form; after neuraminidase treatment, all three bands were converted to a closely spaced doublet around M_r 120,000. These results conclusively demonstate that E→A conversion occurs in the mouse in much the same fashion as it does in the chicken (9).

The kinetics of appearance of the different A forms were examined as a function of postnatal age (Fig. 2, lanes a–g). Between 7 and 21 days of age, there was gradual conversion of the broad band around M_r 200,000–250,000 to the three distinctive bands of M_r 180,000, 140,000, and 120,000 (Fig. 2, lanes c–g). Moreover, when monoclonal antibodies (15G8) having specificity for sialic acid present in E forms were used (4), less material was precipitated as the brain matured (lanes h–k). The amounts of material precipitated by these antibodies decreased to background levels by 14 days in cerebellum, cerebral cortex (see Table 1), and in hippocampus (not shown).

We observed that a monoclonal antibody (9E11) directed against non-sialic acid determinants (4) showed no immunoprecipitation of cerebellar N-CAM by 21 days (Fig. 2, lanes l–p) but did precipitate material from adult cortex (Fig. 2, lanes q–v) as well as from hippocampus and corpus putamen (not shown). One other monoclonal antibody (6F3) behaved in a similar fashion, whereas yet another (11B6) precipitated material from all four areas; like 9E11, neither of these antibodies had reactivity with sialic acid. These findings raise the possibility that there is an antigenic difference between N-CAMs from cerebellum and cortex.

Maturation of N-CAM in Staggerer Mice. In contrast to normal cerebellum, staggerer cerebella showed mainly the E form at both 14 and 21 days of age (Fig. 3, lanes b and d). More-

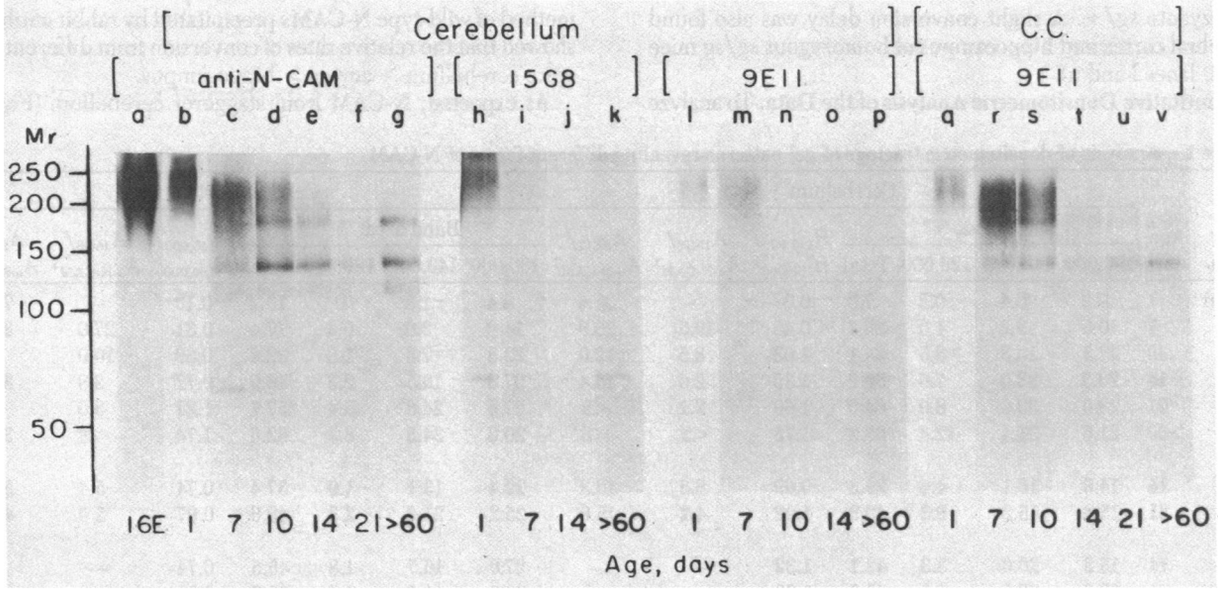

FIG. 2. Conversion of N-CAM from embryonic to adult forms in normal mice. Autoradiograph of immunoprecipitates resolved by $NaDodSO_4$/7% polyacrylamide gel electrophoresis. N-CAMs were immunoprecipitated from the membrane extracts of whole brain (lane a), cerebellum (lanes b–p), and cerebral cortex (C.C., lanes q–v). The rabbit anti-N-CAM and mouse monoclonal (15G8 and 9E11) antibodies are indicated in the figure. Ages of the mice are shown as days after birth, except for lane a, which is from 16-day-old mouse embryos (16E). M_r values are shown $\times 10^{-3}$.

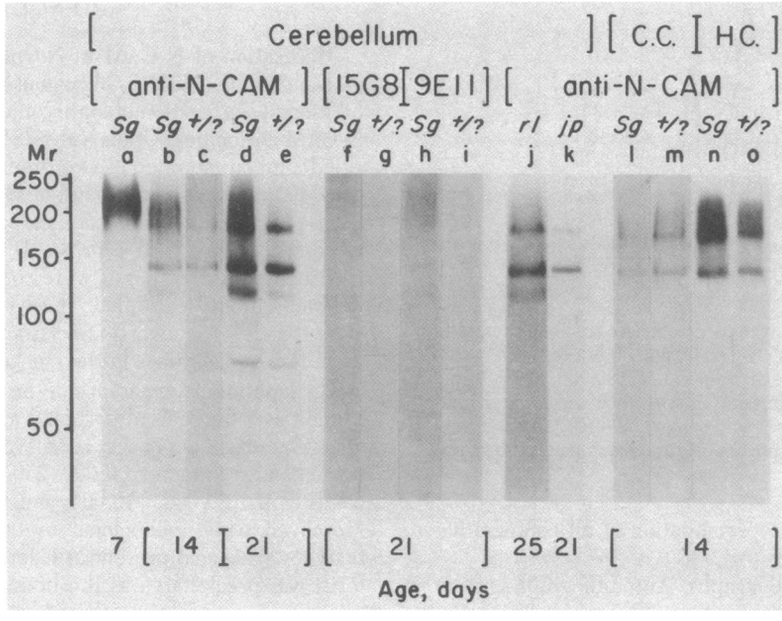

FIG. 3. Conversion of N-CAM from embryonic to adult forms in mutant mice. Autoradiograph of immunoprecipitates resolved by NaDodSO$_4$/7% polyacrylamide gel electrophoresis. N-CAMs were immunoprecipitated from membrane extracts of cerebellum (lanes a–k), cerebral cortex (C.C., lanes l and m), and hippocampus (HC., lanes n and o). Antibodies used are designated in the figure. Material from homozygous staggerer mutants (indicated *Sg*) and their littermates (+/?) are in alternate lanes and are compared to reeler (*rl*) and jimpy (*jp*) mutants (lanes j and k). Ages of the mice are shown as days after birth. M_r values are shown $\times 10^{-3}$.

over, both 15G8 and 9E11 monoclonal antibodies immunoprecipitated N-CAM from 14- and 21-day-old staggerer cerebella (Fig. 3, lanes f and h) whereas diminishing amounts of N-CAM were precipitated by these antibodies in the wild type at these ages. Other neurological mutants, reeler and jimpy (Fig. 3, lanes j and k) and weaver (not shown) resembled the wild type in that they had no delay of E→A conversion.

Pooled tissues of the phenotypically normal littermates of staggerer were examined as controls (Fig. 3, lanes marked +/?). Upon visual inspection they resembled the wild type shown in Fig. 2, but densitometric tracings (see below) showed a slight E→A conversion delay attributable to the presence of heterozygote *sg*/+. A slight conversion delay was also found in cerebral cortex and hippocampus of homozygous *sg*/*sg* mice (Fig. 3, lanes l and n).

Quantitative Densitometric Analysis of the Data. To analyze more precisely the kinetics of appearance of the different A forms of N-CAM, densitometric scans were made of the autoradiographs of normal cerebellum and cortex (for example, of lanes b–g of Fig. 2 as shown in Fig. 4*A*), and of corresponding tissues at 14 and 21 days from staggerer mutants. Typical results are illustrated in Fig. 4 and all of the data are summarized in Table 1. The tracings in Fig. 4 clearly reveal the conversion from a broad band in the E form to the three A forms.

As shown in Table 1, the M_r 180,000 band increased in area up to 21 days of age in wild-type mice, and then it decreased. Both the M_r 140,000 band and the M_r 120,000 band increased in area monotonically up to the adult period. Analysis by this method of wild-type N-CAMs precipitated by rabbit antibodies showed that the relative rates of conversion from different areas were cerebellum > cortex > hippocampus.

As expected, N-CAM from staggerer cerebellum (Fig. 4*A*,

Table 1. Analysis of densitometric tracings of gel patterns revealing different forms of N-CAM

		Cerebellum							Cerebral cortex						
		Band area, %*							Band area, %*						
Mice	Age, days	180,000	140,000	120,000	Total	$H_{140,000}/H_{180,000}$†	$A_{15G8}/A_{\alpha\text{-N-CAM}}$‡	$A_{9E11}/A_{\alpha\text{-N-CAM}}$‡	180,000	140,000	120,000	Total	$H_{140,000}/H_{180,000}$†	$A_{15G8}/A_{\alpha\text{-N-CAM}}$‡	$A_{9E11}/A_{\alpha\text{-N-CAM}}$‡
Normal	1	7.2	0.4	0.2	7.8	0.07	—	31.4	8.4	1.8	0	10.2	0.10	—	76.0
	7	10.5	9.2	1.0	20.7	0.45	19.0	26.0	14.0	3.0	0.4	17.4	0.31	27.0	31.0
	10	17.3	13.3	3.5	34.1	1.03	8.5	12.0	22.8	7.8	1.8	32.4	0.39	10.0	—
	14	20.3	23.0	7.6	50.9	1.35	2.0	10.4	27.6	16.5	2.8	46.9	0.77	2.9	32.0
	21	23.0	33.0	8.0	64.0	1.60	2.1	<2	27.8	24.5	5.4	57.7	1.27	3.0	—
	>60	21.5	34.4	12.4	68.3	1.75	<2	<2	20.0	34.3	8.3	62.6	1.74	<2	32.0
sg/*sg*	14	14.8	10.1	4.4	29.3	0.69	8.8	18.3	22.4	13.1	1.9	37.4	0.74	5.4	52.5
	21	15.8	15.2	9.3	40.3	1.02	4.2	15.0	25.2	21.6	2.8	49.6	0.97	5.0	48.3
Litter-	14	18.8	20.0	3.3	42.1	1.32	—	—	27.0	16.7	1.8	45.5	0.74	—	—
mates	21	17.6	23.5	6.5	47.6	1.33	—	—	28.7	21.5	3.5	53.7	0.96	—	—

* Area under each simulated Gaussian curve was divided by the total area.

† Ratio of the heights of the M_r 140,000 band and 180,000 band.

‡ The total area (M_r 250,000–100,000) from 15G8 or 9E11 tracings was divided by the total area of rabbit anti-N-CAM (α-N-CAM) tracing for the same preparation. The results suggest that only subpopulations of N-CAMs were precipitated by the monoclonal antibodies.

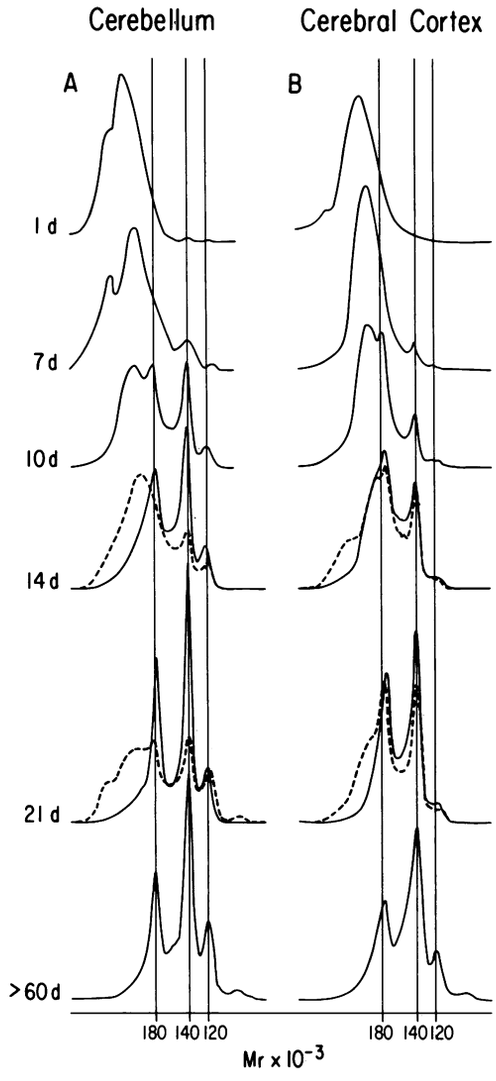

FIG. 4. Densitometric tracings of electrophoretic gel patterns of different forms of N-CAM. Corresponding age series of N-CAM from normal cerebellum (*A*) and from normal cerebral cortex (*B*) are shown (——). N-CAM from staggerer is illustrated for both tissues at 14 and 21 days of age (----). The ordinate is optical density. For purposes of comparison, the areas of the tracings were normalized to the same value.

dashed curves, 14 and 21 days) remained mainly as a broad band of higher molecular weight; the total band area was about 60% of that seen for normal cerebellum at the same age (Table 1). Although present, bands corresponding to the A forms remained relatively low in height, the peak ratios of 140,000/180,000 were lower than those of the wild type at corresponding times, and, unlike the wild type, the monoclonal antibodies precipitated material at 21 days (Table 1).

Cerebral cortex and hippocampus of *sg/sg* mice showed a slight delay in conversion as compared to wild type (Fig. 4*B*, dashed curves; Table 1), although no apparent phenotypic abnormality has been observed in these brain areas in electron microscopic studies or in behavioral observations. A slight but definite conversion delay was also found in pooled littermate cerebella and cerebral cortexes (Table 1).

DISCUSSION

N-CAM is the major neuron–neuron adhesion molecule in a variety of vertebrate species (2, 4, 13). Although N-CAM has been shown to exist in E and A forms, which differ in amount and possibly in the linkage of sialic acid (8, 9), the schedule of the conversion has not been examined in great detail. The present experiments show that, in the mouse, E→A conversion occurs mainly between the first and third postnatal week. The relative rates of conversion in different gross brain areas were: cerebellum > cortex > hippocampus. Of the three different antibodies used, the monoclonal antibody (15G8) against sialic acid present in the E form (4) most reliably indicates the conversion. It should be noted, however, that other monoclonal antibodies (9E11 and 6F3) failed to immunoprecipitate N-CAM in cerebellar tissues after 14 days, but rabbit anti-N-CAM was able to precipitate the A forms at all ages tested (see Fig. 2). This result raises the possibility that there are antigenic differences in N-CAMs from cerebellum and cortex.

The data show not only a conversion from the microheterogeneous state of the E form of N-CAM but also the existence of three distinct A forms that are not microheterogeneous. These are A_1 (M_r 180,000), A_2 (140,000), and A_3 (M_r 120,000). In all likelihood, the different A forms will have different amounts of sialic acid and possibly sialic acid attached at more than one site. The kinetics of conversion are consistent with the interpretation that A_1 and A_2 can be converted to A_3 either independently or sequentially. Further chemical studies and analyses of the functional role of each form are obviously required.

When monoclonal antibodies to mouse N-CAM became available (4), it became feasible to compare E→A conversion in the various mouse mutants having cerebellar defects of cell number, type, location, and connectivity (12, 14). The most marked common feature of these mutants, which otherwise differ in appearance, symptoms, and morphologic features, is a large reduction in the granule cell population. In staggerer, proliferation of granule cells is reduced but migration is normal; in weaver, migration of these cells is abnormal, as are the Bergman glial fibers; and in reeler, migration occurs along displaced oblique glial fibers. Staggerer shows a large reduction in Purkinje cell number, and the remaining cells have reduced arbors and no tertiary denditric spines; as a consequence, no parallel fiber synapses are made. In weaver and reeler, Purkinje cells are somewhat abnormal but only slightly deficient in number; reeler alone shows obvious defects in the cerebral cortex.

We surmised that staggerer was the most likely of the three mutants to have a defect in E→A conversion for a number of reasons: (*i*) these are recessive mutations with a likelihood of failure in the control of key enzymes, which might in turn alter cell surface modulation; (*ii*) staggerer has no detectable glial abnormalities; and (*iii*) observations in other laboratories suggested that cerebellar cells from staggerer have altered surface properties. Wheat germ agglutinin (with weak specificity for sialic acid) agglutinates normal embryonic and postnatal *sg/sg* cells but not normal postnatal cells (15). Furthermore, anti-sialic acid antibodies are bound by normal embryonic and postnatal *sg/sg* cells but not by normal postnatal cells (16). The interpretation of these findings was obscured, however, by the lack of knowledge concerning the mechanism of cell–cell adhesion.

With a knowlege of CAM properties, a clear hypothesis emerges: at least some and possibly a large number of the cerebellar defects in staggerer may be associated with a delay in E→A conversion. The present experiments prompted by this hypothesis gave results that were striking and clear cut. *sg/sg* animals showed persistence of diffusely migrating embryonic N-CAM as determined by gel patterns of specific immunoprecipitates of cerebellar tissues with anti-N-CAM antibodies. The other cerebellar mutants, reeler and weaver, as well as jimpy, showed normal schedules of E→A conversion.

Unlike reeler, homozygous staggerer appears phenotypically to show no defects in the cerebral cortex at the ultrastructural or behavioral level and the heterozygote *sg/+* appears normal in behavior. In the present study, however, *sg/sg* showed slight but definite E→A conversion delays in the cortex, as did *sg/+*, which also showed a slight delay in the cerebellum. The interpretation of such changes is difficult; for example, even in reeler, in which there are gross changes in the cerebral cortex, visual behavior appears practically normal (17). We would expect that any changes caused by the slight cortical delays would be difficult to discern; in the heterozygote that expressed the delay trait, the gene dosage effects may not sufficiently exceed thresholds to cause obvious changes in cerebellar circuitry.

It is not known whether conversion delay is a characteristic of only some or of all of the cerebellar cell types in staggerer. But the existence of the delay trait in the heterozygote and the presence of the slight delay in cerebral cortex of staggerer animals suggest that conversion delay occurs in a variety of cells. This makes it unlikely that the observed delay is merely a property of a minor or residual population of cells that remain after the extensive cell death found in the disease.

The present observations do not show a causal relationship between E→A conversion defects and the pleiotropic effects of the *sg* mutation. It is significant, however, that cell death is not inherently programmed in *sg/sg* (18). In view of the role of N-CAM in cell–cell and cell–process adhesion (3, 5), it could very well be that failure of E→A conversion allows cell and process migration to occur beyond normal limits. This failure might lead in turn to failure of cell–cell interactions at critical times and ultimately to cell death, particularly of granule and Purkinje cells.

In this connection, it is important to note that E→A conversion and even complete removal of sialic acid by neuraminidase (8, 9, 10) does not abolish N-CAM binding. Because of the large change in charge, however, it is likely to alter either the membrane distribution or the conformation of N-CAM molecules with consequent changes in binding strength. Indeed, recent experiments on reconstituted membrane vesicles (10) containing neuraminidase-treated N-CAM suggest accelerated binding, indicating an effect of reduced charge on the reaction (unpublished results). Detailed structural and physicochemical analyses of binding of the various forms of N-CAM should further clarify the physiological role of the conversion, which is a clear-cut instance of local cell surface modulation (11).

Finally, the present data suggest that one of the key defects in staggerer is either abnormal persistence of enzymatic processes that attach sialic acid to certain sites of the CAM molecule or the failure of surface sialidases either to reach appropriate levels (19) or to cleave this sugar at the proper time of development. Analysis of the relationship of these processes to the gene defect and determination of whether other cell surface glycoproteins on central nervous system neurons also undergo E→A conversion should throw further light on the significance of local cell surface modulation in cell–cell adhesion.

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