The Nerve Growth Factor-Inducible Large External (NILE) Glycoprotein and Neural Cell Adhesion Molecule (N-CAM) Have Distinct Patterns of Expression in the Developing Rat Central Nervous System

Lora Beasley and William B. Stallcup
Cancer Research Center, La Jolla Cancer Research Foundation, La Jolla, California 92037

The nerve growth factor-inducible large external (NILE) glycoprotein and the neural cell adhesion molecule (N-CAM) have both been implicated in the process of nerve fiber fasciculation. To evaluate the respective roles of the 2 molecules in fiber tract formation, we used immunohistochemical means to compare their distributions in the developing rat central nervous system. In the spinal cord, hindbrain, forebrain, retina, and cerebellum, N-CAM was present on undifferentiated cells in germinal zones as well as on differentiating cells and in nerve fiber tracts. In contrast, NILE was restricted to the developing fiber tracts in all these areas. No fiber tracts were found that were obviously lacking one or the other of the 2 molecules during the period of tract development. However, in all cases except that of the cerebellar molecular layer, nerve fiber tracts appeared to lose NILE and retain N-CAM after the major phases of tract development were completed. The fact that NILE is restricted to nerve fiber tracts during relatively short but crucial phases of tract development suggests that NILE plays a very specific role in the formation of fiber bundles. The more ubiquitous N-CAM molecule may have a more general role in neural histogenesis.

The orderly development of the nervous system is dependent in part on the ability of neural cells to recognize and interact with one another according to very specific patterns. It is widely believed that cell-surface molecules mediate cell-cell recognition and adhesion, and work in a number of laboratories has led to the identification of an expanding array of molecules that may be involved in these processes. One such molecule is the nerve growth factor-inducible large external (NILE) glycoprotein, a 230,000 Da cell-surface molecule first identified on PC12 cells (McGuire et al., 1978). Antibodies prepared against NILE have been used to demonstrate that an immunologically cross-reactive family of NILE-like glycoproteins, ranging from 215,000 to 230,000 Da, is expressed by neuronal cell lines and neurons in primary cultures prepared from the rat nervous system (Salton et al., 1983a, b; Stallcup et al., 1983; Stallcup and Beasley, 1985b). Using fluorescence immunohistochemistry with frozen tissue sections, we have shown that NILE is difficult to detect in immature neurons and on the cell bodies of differentiating neurons, but is readily detectable on axonal projections in developing nerve fiber tracts of the rat nervous system (Stallcup et al., 1985a). NILE may play an important role in the formation of these fiber tracts, as evidenced by the finding that antibody against NILE is effective in blocking neurite fasciculation in cultures of embryonic rat brain (Stallcup and Beasley, 1985a).

In terms of its relationship to other components involved in neuronal recognition, NILE has recently been shown by immunological comparisons to be very similar or identical to the neuron-glia cell adhesion molecule (Ng-CAM) and to the L1 adhesion molecule. The high-molecular-weight forms of Ng-CAM and L1 (>200,000 Da) cross-react strongly with antibodies against NILE and comigrate with NILE on SDS-PAGE (Bock et al., 1985; Friedlander et al., 1986; Sajovic et al., 1986). In spite of these molecular similarities, there is not yet general agreement as to the role that NILE, Ng-CAM, and L1 play in histogenesis. For example, Ng-CAM was initially thought to be involved only in neuron-glia interactions (Grumet et al., 1984a), but is now believed to be involved in both neuron-glia and neuron-neuron interactions (Grumet et al., 1984b; Hoffman et al., 1986). L1 has been found to mediate neuron-neuron but not neuron-glia interactions (Keilhauer et al., 1985). Furthermore, L1 has been implicated in axon fasciculation in postnatal cerebellar cultures (Fischer et al., 1986), while NILE appears to mediate fasciculation of axons in embryonic brain cultures but not in cultures of postnatal cerebellum (Stallcup and Beasley, 1985a). It is possible that these discrepancies result more from differences in the antibodies used for the studies than from differences in the glycoproteins, but until it is resolved, the question of NILE:Ng-CAM:L1 identity requires further study. It is clear, however, that the NILE:Ng-CAM:L1 class of molecules is distinct from N-CAM, which is also involved in neuronal cell adhesion and in neurite fasciculation (Edelman, 1983; Rutishauser, 1983). Immunochemical, electrophoretic, and proteolytic peptide comparisons have confirmed the separate identities of the 2 classes of adhesion molecules (Faissner et al., 1984a; Grumet et al., 1984a; Rathjen and Rutishauser, 1984; Stallcup and Beasley, 1985b).

Since we have found that antibodies against NILE and against N-CAM have nonidentical effects on fasciculation in different types of primary culture systems (Stallcup and Beasley, 1985a), it becomes important to assess the respective contributions made by NILE and N-CAM to the process of fiber tract formation in
different areas of the developing nervous system. One means of addressing this problem is to compare the appearance and distributions of NILE and N-CAM in fiber tracts. Theoretically, fasciculation in any given fiber tract might involve (1) preferential expression of one or the other of the 2 molecules, (2) sequential expression of the 2 molecules, or (3) parallel expression of the 2 molecules.

Although some comparisons of Ng-CAM to N-CAM and of L1 to N-CAM have been published (see Discussion), a direct comparison of NILE to N-CAM expression has not yet been presented. Since the precise relationship of NILE to Ng-CAM and L1 has not yet been firmly established, and since it is important to obtain a more comprehensive comparison of NILE to N-CAM expression in mammals to complement the available data on Ng-CAM and N-CAM in the chick, we have studied the appearance and distribution of NILE and N-CAM immunoreactivity in several parts of the developing rat nervous system. We have not only tried to compare the schedules for expression of NILE and N-CAM in the different regions, but to establish how exclusively the 2 molecules are expressed in fiber tracts, as opposed to other structures. The results of our study reinforce previous conclusions concerning the similarity of NILE to Ng-CAM and L1 and further illustrate the differences that exist between the patterns of NILE and N-CAM expression in the CNS.

Materials and Methods

Antisera. Preparation of rabbit antiserum against the NILE glycoprotein has been previously described. This antibody has been used to investigate the biochemistry, distribution, and function of NILE (Stallcup et al., 1983, 1985; Stallcup and Beasley, 1985a, b). The antiserum specifically immunoprecipitates NILE-related glycoproteins of 215,000-230,000 Da from a variety of neuronal cell types. Immunofluorescence experiments with the antibody show that NILE is selectively expressed by neurons in both primary cultures and frozen sections. Anti-NILE antibody was used at a 1:25 dilution in the present series of experiments. Rabbit antibody against mouse N-CAM was the generous gift of Dr. Gerald Edelman (Rockefeller University). This antibody is highly cross-reactive with rat N-CAM (Chuong et al., 1982). It was used at a 1:50 dilution. Monoclonal antibody against the D1.1 ganglioside has been previously described (Levine et al., 1984, 1986; Stallcup et al., 1984). The D1.1 ganglioside provides a convenient marker for localizing germinal cells of the rat CNS. Tissue culture supernatant from D1.1 hybridoma cultures was used at a 1:10 dilution for immunohistochemistry. Fluorescein-labeled goat antibodies against rabbit and mouse immunoglobulins were purchased from TAGO. They were used at 1/50 dilutions.

Immunohistochemistry. Timed pregnant Sprague-Dawley rats were obtained from Zivic Laboratories. Their embryos and pups were used for our studies. Embryonic tissues were fixed by immersion for 6 hr at 4°C in 1% paraformaldehyde/0.1% glutaraldehyde buffered with 0.1 M sodium phosphate, pH 7.2. Tissues were then stored overnight in the same fixative containing 20% sucrose. Eleven and 12-day-old embryos were fixed whole, while the brains from older embryos were removed for fixation. Spinal cords of these older embryos were exposed prior to fixation by removing the overlying tissue. Postnatal animals were perfused through the heart with cold 1% paraformaldehyde/0.1% glutaraldehyde, pH 7.2. Brains were removed and postfixed overnight in the same fixative containing 20% sucrose. Fixed tissues were embedded in OCT compound, and 15 μm sections were cut on a LabTek II cryostat microtome. Spinal cords were sectioned in a transverse orientation, while brains were cut horizontally. Sections were mounted on gelatin-coated slides.

For immunofluorescent staining, we used adjacent sections to compare the distributions of NILE, N-CAM, and D1.1. All washes and antibody dilutions were made with PBS, pH 7.4, containing 2% normal goat serum and 0.1% Triton X-100. Sections were incubated overnight at 4°C with primary antisera, washed twice, and incubated for an additional hour at room temperature with fluorescein-labeled secondary antiserum. Following 2 more washes, slides were rinsed in distilled water, coverslipped in glycerol, and examined with a Nikon Optiphot microscope equipped for epifluorescence. Kodak Tri-X pan film was used for the photography.

Results

Immunofluorescent staining with anti-NILE and anti-N-CAM was compared in the developing rat spinal cord, hindbrain, forebrain, retina, and cerebellum. In some cases monoclonal antibody against the D1.1 ganglioside was used to locate germinal zones of the CNS (Levine et al., 1984, 1986; Stallcup et al., 1984).

Spinal cord

Staining with anti-NILE antibody first appeared in the spinal cord on embryonic day eleven (E11) as a very thin rim of fibers in the outer margin of the ventral cord (Stallcup et al., 1985). By E12, NILE-positive fiber tracts were clearly visible in the ventral and lateral funiculi of the cord (Fig. 1c). Germinal cells in the interior regions of the cord were negative for NILE. Antibody against N-CAM also stained fiber bundles in the developing funiculi, but, in addition, this antibody showed that N-CAM was present on germinal cells in the ventricular zones of the cord (Fig. 1b).

At E14, the dorsal, ventral, and lateral funiculi were intensely stained with anti-NILE antibody (Fig. 1, f, h). Germinal cells lining the central canal (indicated by staining with anti-D1.1 antibody in Fig. 1d) did not express NILE. Cells in the intermediate zones were also negative for NILE. In contrast, anti-N-CAM brightly labeled both the fiber tracts and cells in the intermediate zones of the E14 spinal cord (Fig. 1e). At higher magnification, it was apparent that germinal cells in the ventricular zone also expressed N-CAM (Fig. 1g). At E18 these relative patterns of NILE and N-CAM distribution remained much the same, although the funiculi were more extensive and the germinal zones were smaller than at E14 (not shown).

In the spinal cord of the newborn rat, N-CAM was still easily detectable in the funiculi, as well as on cells of the more interior zones (Fig. 1, i, k). In contrast, the funiculi were only weakly labeled by anti-NILE antibody at this stage of development (Fig. 1, j, l).

Brain

Hindbrain

The patterns of anti-NILE and anti-N-CAM staining in the developing rhombencephalon and mesencephalon were very similar to those observed in the spinal cord. At E12, 1 d after the first appearance of NILE in the hindbrain, anti-NILE labeling was confined to fiber tracts of the marginal zones of the rhombencephalon and mesencephalon (Fig. 2c). Cells in the ventricular portions of these areas were not labeled by anti-NILE. Anti-N-CAM intensely stained the fiber tracts in the marginal zones and, more faintly, the germinal cells of the ventricular zones (Fig. 2b).

At E14, the differences between the staining patterns of the 2 antibodies were more marked. NILE was detected in fiber tracts of the marginal and intermediate zones of the rhombencephalon (Fig. 2f) and mesencephalon (not shown). Cells of the marginal zones, located by staining with anti-D1.1 antibody (Fig. 2d), were negative for NILE. In contrast, cells in the marginal zones were labeled by anti-N-CAM, as were cell bodies and fibers in the intermediate and marginal zones (Fig. 2e).
in the case of the spinal cord, the presence of N-CAM on both cell bodies and fibers made it difficult to identify the fiber tracts in sections of hindbrain stained with anti-N-CAM. These fiber tracts were clearly visible in anti-NILE-stained sections because NILE was mostly absent from the cell bodies interspersed among the fibers.

**Forebrain**

At E14, NILE was seen in fiber tracts in the marginal zone of the diencephalon (Fig. 2i), but was not present in the germinal zone identified by staining with anti-D1.1 (Fig. 2g). N-CAM, however, was present in both the fiber zone and germinal zone (Fig. 2h).

NILE first appeared in the telencephalon at E15 on fibers of the deep white tract (Stallcup et al., 1985). As the cortex developed, this tract was overlaid by cells of the growing cortical plate. Figure 2k shows anti-NILE staining of the E18 deep white tract at the point where fibers enter the ganglionic eminence to form the internal capsule. Figure 2m shows the deep white tract at higher magnification in the posterior telencephalon. Anti-N-CAM stained not only the NILE-positive deep white tract, but also cells on both sides of the tract (Fig. 2j, l).

As noted in the case of the spinal cord, although staining with anti-N-CAM was still seen during the first postnatal week, NILE became progressively more difficult to detect in the hindbrain and forebrain after birth. Tracts that were positive for NILE at E18 were only weakly stained by anti-NILE on postnatal days 1 and 2 (P1 and P2; not shown).

**Retina**

In the retina, NILE could first be detected on the developing optic nerve at E13. At E14, this staining with anti-NILE in the optic nerve was more pronounced, and a small amount of staining could be found on the axons of the retinal ganglion cells,
which lie on the inner surface of the retina and merge to form the optic nerve. By E15, both this inner fiber layer of the retina and the optic nerve were very clearly labeled by anti-NILE (Fig. 3c). During this period from E13 to E15, N-CAM was present on the innermost fiber layer and in the optic nerve, but was also distributed on cell bodies throughout the width of the retina (Fig. 3b). These staining patterns are shown at higher magnification in Figure 3, d-f for the case of the E16 retina. Except for the NILE-positive axons of the retinal ganglion cells, the rat retina was largely undifferentiated at this stage and was stained throughout with both anti-D1.1 (Fig. 3, a, d) and anti-N-CAM (Fig. 3, b, e). As noted above for the brain and spinal cord, the
bright staining of cell bodies with anti-N-CAM made it difficult to identify the fiber zones of the retina. Three weeks postnatally, the layers of the mature retina were clearly visible (Fig. 3g). N-CAM was still present on cell bodies in the 2 nuclear layers and was especially evident in the plexiform layers and fiber zones (Fig. 3h). Except for some staining in the outer plexiform layer, anti-NILE showed very little reactivity with the fiber zones of the mature retina (Fig. 3i).

Cerebellum

At P2, NILE was detected only on fibers in the white matter of the developing cerebellum (Fig. 4c). Staining with anti-N-CAM, however, was seen almost throughout the cerebellar cortex (Fig. 4b). The lone exception appeared to be the cells of the developing external granule cell layer (EGL). Very similar patterns were seen with the 2 antibodies at P4. NILE was still restricted to the external granule cell layer (EGL). Very similar patterns were seen with the 2 antibodies at P4. NILE was still restricted to the external granule cell layer (EGL). Very similar patterns were seen with the 2 antibodies at P4. NILE was still restricted to the external granule cell layer (EGL). Very similar patterns were seen with the 2 antibodies at P4. NILE was still restricted to the external granule cell layer (EGL). Very similar patterns were seen with the 2 antibodies at P4. NILE was still restricted to the external granule cell layer (EGL). Very similar patterns were seen with the 2 antibodies at P4. NILE was still restricted to the external granule cell layer (EGL). Very similar patterns were seen with the 2 antibodies at P4. NILE was still restricted to the external granule cell layer (EGL). Very similar patterns were seen with the 2 antibodies at P4. NILE was still restricted to the external granule cell layer (EGL). Very similar patterns were seen with the 2 antibodies at P4. NILE was still restricted to the external granule cell layer (EGL). Very similar patterns were seen with the 2 antibodies at P4. NILE was still restricted to the external granule cell layer (EGL). Very similar patterns were seen with the 2 antibodies at P4. NILE was still restricted to the external granule cell layer (EGL). Very similar patterns were seen with the 2 antibodies at P4. NILE was still restricted to the external granule cell layer (EGL). Very similar patterns were seen with the 2 antibodies at P4. NILE was still restricted to the external granule cell layer (EGL). Very similar patterns were seen with the 2 antibodies at P4. NILE was still restricted to the external granule cell layer (EGL). Very similar patterns were seen with the 2 antibodies at P4. NILE was still restricted to the external granule cell layer (EGL).

By P7, the molecular layer had begun to form (Fig. 4d), and this fiber layer was stained by both anti-N-CAM and anti-NILE (Fig. 4, e, f). In contrast to NILE, however, N-CAM was still widespread in the cerebellar cortex. Anti-N-CAM staining was very bright on cells of the internal granule cell layer, and was now apparent on cells of the EGL as well. These patterns of anti-NILE and anti-N-CAM staining were seen more clearly at P10, when both the P10 and molecular layer were larger (Fig. 4, g-i). N-CAM was present on the NILE-positive fibers of the molecular layer, and in addition was expressed by cells of both the internal and external granule cell layers.

In contrast to the other areas of the brain that we examined, the molecular layer of the cerebellar cortex retained NILE immunoreactivity into maturity. The distributions of NILE and N-CAM in 6-month-old cerebellum are shown in Figures 4, j-l. Anti-NILE and anti-N-CAM staining were very similar at this time, although N-CAM appeared to be more abundant than NILE in the granule cell layer.

Discussion

In the introduction we briefly summarized data that indicate that both NILE and N-CAM are involved in fasciculation and fiber tract formation (see also Stallcup and Beasley, 1985a; Stallcup et al., 1985). Three possible models were outlined for the expression of NILE and N-CAM in any given fiber tract: (1) A fiber tract might express preferentially either NILE or N-CAM. (2) A fiber tract might express the 2 molecules sequentially, with the expression of one following the early expression of the other. (3) A fiber tract might express the 2 molecules in parallel, the expression of one coinciding with the expression of the other. Theoretically, it would be possible for different patterns of NILE and N-CAM expression to occur in different fiber tracts. We observed, however, that the relationship between NILE and N-CAM expression was very similar in different regions of the rat CNS, including the spinal cord, hindbrain, forebrain, retina, and cerebellum. The pattern of expression did not conform precisely to any one of the 3 models outlined above, but displayed features of both the sequential and parallel modes of expression. Three recurrent themes were noted.

1. In all regions examined, undifferentiated germinal cells expressed N-CAM but not NILE. Primary germinal zones lining the ventricles, a secondary germinal zone in the EGL of the cerebellum, and undifferentiated cells in the developing retina were identified using antibody against the D1.1 ganglioside.
Figure 3. NILE and N-CAM in the retina. a-c, Embryonic day 15. a, Anti-D1.1. b, Anti-N-CAM. c, Anti-NILE.  ×140. d-f, Embryonic day 16. d, Anti-D1.1. e, Anti-N-CAM. f, Anti-NILE.  ×280. g-i, Postnatal day 23. g, Phase. h, Anti-N-CAM. i, Anti-NILE.  ×280. G, Retinal ganglion cells and axons; IP, inner plexiform layer; IN, inner nuclear layer; OP, outer plexiform layer; ON, outer nuclear layer; P, photoreceptor layer.
cell bodies are positive for these molecules. The most consistently noted cells of this type are immature, postmitotic granule neurons that are beginning their migration from the cerebellar external granule cell layer (Faissner et al., 1984b; Grumet et al., 1984b; Rathjen and Schachner, 1984; Daniloff et al., 1986). Neuronal soma of the cortical plate and subplate of the developing mouse telencephalon have also been reported to be transiently positive for L1 (Fushiki and Schachner, 1986).

Regarding the loss of NILE immunoreactivity from fiber zones in the more mature nervous system, we have previously suggested a correlation between myelination and the disappearance of staining with anti-NILE antibody (Stallcup et al., 1985). In particular, we observed that unmyelinated fiber tracts of the CNS, such as the olfactory nerve and cerebellar molecular layer, continued to stain with anti-NILE antibody in the adult rat. Similar observations have been made for Ng-CAM in the chick CNS (Daniloff et al., 1986). Tracts that became myelinated were observed to lose Ng-CAM immunoreactivity, while unmyelinated tracts were still Ng-CAM-positive in the adult. The latter areas included the molecular layer of the cerebellum, the olfactory nerve, and the gray matter of the dorsal and ventral horns of the spinal cord. L1 immunoreactivity is also retained in the molecular layer of the adult mouse cerebellum (Rathjen and Schachner, 1984). It is not known whether the loss of NILE:Ng-CAM:L1 immunoreactivity in most parts of the adult CNS represents an actual decrease in the amount of these molecules or a process, such as myelination, resulting in the masking of antigenic components so that they are not accessible to antibody. The fact that staining of myelinated tracts is still obtained with anti-N-CAM might be taken as evidence that accessibility to
N-CAM in the dynamics of fiber tract development. Observations indicate that NILE plays a more specialized role than restricted to axonal surfaces rather than cell bodies, and usually first appears on axons involved in fiber tract formation, is largely required for further structuring and fine-tuning of processes in are localized more exclusively on axonal projections, may be N-CAM-mediated interactions a degree of flexibility that en- mature cells or nerve fibers. This type of modulation may give cells appear to have less N-CAM on their surfaces than more of changes in the level of expression. For example, germinal with the anti-N-CAM antibody we used does not allow us to surface molecule can produce differences in cell-cell adhesive- ed out that modulation of the structure and amount of a cell- surface molecule may be with the cell adhesion molecules Ll and N-CAM and the shared carbo- hydrate epitome L2 during development of the mouse neocortex. Dev. J. Neurosci. 6: 739-758. Faissner, A., J. Kruse, C. Goridis, E. Bock, and M. Schachner (1984a) Involvement of the nerve growth factor-inducible large external glycoprotein (NILE) which is involved in cell adhesion. EMBO J. 3: 461-465. Rathjen, F., and M. Schachner (1984) Immunocytochemical and biochemical characterization of a new neuronal cell surface component (L1 antigen) which is involved in cell adhesion. EMBO J. 3: 1-10. Rutishauser, U. (1983) Developmental biology of a neuronal cell adhe- sion molecule. Nature 310: 549-554. SaJovic, P., E. Kouvelas, and E. Trenkner (1986) Probable identity of N-CAM and the high-molecular-weight component of L1 antigen. J. Neurochem. 47: 541-546. Salton, S., C. Richter-Landsberg, L. Greene, and M. Shelanski (1983a) Nerve growth factor-inducible large external (NILE) glycoprotein: Studies of a central and peripheral neuronal marker. J. Neurosci. 3: 441-454. Salton, S., M. Shelanski, and L. Greene (1983b) Biochemical properties of the nerve growth factor-inducible large external (NILE) glycoprotein. J. Neurosci. 3: 2420-2430. Stallcup, W., and L. Beasley (1985a) Involvement of the nerve growth factor-inducible large external glycoprotein (NILE) in neurite fasciculation in primary cultures of rat brain. Proc. Natl. Acad. Sci. USA 82: 1276-1280. Stallcup, W., and L. Beasley (1985b) Polymorphism among NILE-related glycoproteins from different types of neurons. Brain Res. 346: 287-293. Stallcup, W., L. Arner, and J. Levine (1983) An antiserum against the PC12 cell line defines cell surface antigens specific for neurons and Schwann cells. J. Neurosci. 3: 53-68. Stallcup, W., L. Beasley, and J. Levine (1984) Cell surface molecules that characterize different stages in the development of cerebellar internurons. In Molecular Neurobiology, Cold Spring Harbor Symp. Quant Biol. 48: 761-774. Thiery, J.-P., L. Duband, U. Rutishauser, and G. Edelman (1982) Cell adhesion molecules in early chicken embryoogenesis. Proc. Natl. Acad. Sci. USA 79: 6737-6741. Thiery, J.-P., A. DeLouvée, M. Grumet, and G. Edelman (1985) Initial appearance and regional distribution of the neuron–glia cell adhesion molecules in the chick embryo. J. Cell Biol. 106: 442-456.

The Journal of Neuroscience. March 1987, 7(3) 715