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Retention of embryonic features by an adult neuronal system capable of plasticity: Polysialylated neural cell adhesion molecule in the hypothalamo–neurohypophysial system

(neurosecretory system/polysialylation/immunochemistry)

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ABSTRACT **The neural cell adhesion molecule, N-CAM, changes at the cell surface during development, from a highly sialylated form [polysialic acid (PSA)-linked N-CAM, PSA-N-CAM] to several isoforms containing less sialic acid. N-CAM and its polysialic acid may serve to regulate cell apposition, thus affecting a variety of cell interactions. In the nervous system, PSA-N-CAM has until now been localized in developing tissues where it is thought to participate in the structuring of neuronal groups and tissue pattern formation. It has been proposed, however, that PSA-N-CAM may also be expressed in the adult, where it may take part in plasticity and cell reshaping. In the present study, the use of immunoblot and immunocytochemical procedures with a monoclonal antibody that specifically recognizes PSA-N-CAM revealed that the adult rat hypothalamo–neurohypophysial system, which undergoes important neuronal–glial and synaptic rearrangements in response to physiological stimuli, contains high levels of PSA-N-CAM immunoreactivity. The use of a polyclonal serum reacting with all N-CAM isoforms indicated that PSA-N-CAM is expressed together with "adult" forms of N-CAM. Light and electron microscopy demonstrated the presence of PSA-N-CAM immunoreactivity in the supraoptic and paraventricular nuclei of the hypothalamus and in the neurohypophysis; the immunoreactivity was seen in dendrites, axons, and terminals and in associated astrocytes but not in neuronal somata. We propose that the continued expression of PSA-N-CAM confers to magnocellular neurons and their astrocytes the ability to reversibly change their morphology in adulthood. In addition, our observations suggest that evidence for polysialylation may serve to identify other neuronal systems capable of morphological plasticity in the adult central nervous system.**

The neural cell adhesion molecule (N-CAM) is a cell-surface glycoprotein that serves as a ligand in the formation of cell-to-cell bonds (for reviews, see refs. 1–3); it may also be involved in cell–substrate interactions (4). Its expression is thus of critical importance in regulating the assembly of tissues. Although N-CAM is expressed transiently in many structures in the embryo, its presence becomes more limited during further development (5). Thus, in the nervous system, N-CAM is found at the earliest stage of neural tube formation and subsequently in most developing neuronal structures. N-CAM is less abundant in the adult mammalian brain, however, where it occurs with various intensity in different areas (6–9). N-CAM has also been described in the peripheral nervous system, in skeletal muscle, and in several endocrine tissues, including the hypophysis (10–12).

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N-CAM exists in several structurally distinct isoforms, which are generated from a single gene by alternative RNA splicing and polyadenylylation (for review, see ref. 13). Further N-CAM diversity arises from various posttranslational events, including glypiation (14) and glycosylation (15, 16). The most striking of these processes results in the addition of unusually high amounts of α-2,8-linked polysialic acid (PSA) on the extracellular domain of the molecule (17). The length of the sialic acid residues is developmentally regulated (17) so that, in many tissues, the highly polysialylated N-CAM (PSA-N-CAM) of high molecular mass (*ca.*220 kDa) is gradually replaced by less sialylated isoforms, the most common of which have molecular masses of 180, 140, and 120 kDa. The type and content of N-CAM thus varies considerably both as a function of tissue source and age.

The degree of sialylation of N-CAM is of particular interest since the ability of N-CAM to promote cell–cell adhesion is attenuated by PSA (18, 19). Indeed, several observations have now led to the suggestion that PSA may serve as an overall regulator of contact-dependent cell–cell interactions (20). This raises the possibility that PSA-N-CAM continues to be expressed in the adult nervous system in structures capable of morphological plasticity. The hypothalamo–neurohypophysial system, which secretes oxytocin and vasopressin, shows a remarkable capacity for neuronal–glial and synaptic plasticity in adulthood, under physiological stimulation. During parturition, lactation, and prolonged dehydration, glial coverage of oxytocinergic somata and dendrites in the hypothalamus markedly diminishes and their surfaces are left in extensive juxtaposition; concurrently, synaptic remodeling associates two or more of the neurons by creating shared synaptic terminals. Once stimulation is terminated, glial processes reappear between the neurons, and there is a reduction in synapses (for review, see ref. 21). In the neurohypophysis, where the peptides are released from neurosecretory terminals directly into the general circulation, stimulation induces glial retraction from the perivascular space, thus enlarging the neurohemal contact area (see ref. 22).

Recently, a monoclonal antibody was developed that specifically recognizes PSA-N-CAM. It was raised against the capsular polysaccharides of meningococcus group B, which share α-2,8-linked sialic acid units with PSA-N-CAM (23). Biochemical analyses (23) indicated that, in agreement with observations in the chicken (24), PSA-N-CAM is the major carrier of polysialic units in the rodent brain. In the present study, the use of this antibody permitted us to see that the

Abbreviations: anti-Men B, anti-meningococcus B antibody; FITC, fluorescein isothiocyanate; HRP, horseradish peroxidase; N-CAM, neural cell adhesion molecule; PSA, polysialic acid; PSA-N-CAM, polysialylated N-CAM; PVN, paraventricular nucleus; SON, supraoptic nucleus.

adult hypothalamo–neurohypophysial system is highly immunoreactive for PSA-N-CAM. That PSA-N-CAM continues to be expressed in this system in adulthood may explain its capacity for morphological reorganization when stimulated to release its neuropeptides.

MATERIALS AND METHODS

Materials. Tissues were obtained from four groups of Wistar rats: (*i*) males (3–4 months old), (*ii*) virgin females (2–3 months old), (*iii*) parturient females (3–5 months old), and (*iv*) lactating females (4–6 months old). Antibodies included (*i*) a mouse monoclonal IgM antibody recognizing polymers of α-2,8-linked sialic acid units, anti-meningococcus B (anti-Men B); and (*ii*) a site-directed rabbit polyclonal antibody recognizing the NH_2 terminus of N-CAM, anti-total N-CAM (see refs. 23 and 25 for further details on their production and specificities). Bacteriophage endosialidase was prepared from bacteriophage K1F propagated in *Escherichia coli*, as described in ref. 26; α-2,8-linked sialic acid polymer or colominic acid was obtained from Sigma.

Immunoblot Analysis. Under sodium pentobarbital anesthesia, brains from 10 animals from each group were dissected, and small cubes of tissue containing the supraoptic nucleus (SON), hypothalamus basolateral to the SON, cerebellum, or neurohypophysis were frozen on dry ice. The tissues were thawed in 50 mM Tris buffer (pH 8) with 1% Nonidet P-40, 1 mM $MgCl_2$, and protease and neuraminidase inhibitors (16). Extracts (10 mg of protein per ml) were made in the above buffer and boiled for 3 min in electrophoresis reducing sample buffer. The proteins (250 μg) were separated on 7% SDS/PAGE and electrophoretically transferred to nylon membranes (Nitroscreen) (4 hr at 0.5 A). After saturation of the blots with 3% defatted milk in phosphate-buffered saline (2 hr at 37°C), they were incubated overnight with the diluted antibodies (1:1000). Bound anti-Men B antibodies were revealed by incubation with immunopurified rabbit anti-mouse IgM at 1 μg/ml (4 hr at RT), followed by ^{125}I-labeled protein A (0.5×10^6 cpm/ml; 30 min at RT) and autoradiography; bound anti-total N-CAM was revealed directly by incubation of the blots with ^{125}I-labeled protein A. For enzymatic assays, tissue homogenates (100 μl) were incubated in the presence of 10^6 plaque-forming units of endosialidase (4 hr at 37°C).

Immunocytochemistry. For light microscopy, standard immunofluorescence and immunoperoxidase techniques were carried out on serial frozen sections (8–12 μm) of tissues from three animals from each group that had been fixed by perfusion with 2% paraformaldehyde in sodium phosphate buffer. Briefly, after blocking of nonspecific sites with 1% human serum albumin, sections were incubated in diluted primary antibodies for 72 hr at 4°C (1:1000 to 1:4000 for anti-Men B or ascites fluid or 1:5000 for polyclonal serum). For anti-Men B, affinity-purified rabbit anti-IgM immunoglobulins coupled to fluorescein isothiocyanate (FITC, Immunotech; Luminy, France) or horseradish peroxidase (HRP, Sigma) served as immunolabels; for anti-total N-CAM, affinity-purified FITC-conjugated sheep anti-rabbit immunoglobulins (Biosys, Compiègne, France) or sheep anti-rabbit immunoglobulins followed by rabbit peroxidase anti-peroxidase (Biosys) were used. Sections incubated with peroxidase-containing labels were treated with 0.01% diaminobenzidine and 0.01% H_2O_2. Epifluorescence was used to examine FITC-incubated sections; bright- and dark-field optics were used to examine the HRP-labeled preparations.

For electron microscopy, three virgin and three lactating female rats were anesthetized with sodium pentobarbital and fixed by perfusion with 2% paraformaldehyde and 1% glutaraldehyde in sodium phosphate buffer. Brains and hypophyses were removed, and after thorough rinsing in buffer, coronal sections (50 μm) of the neurohypophysis and portions of the hypothalamus containing the SON and paraventricular nucleus (PVN) were cut with a vibratome. The sections were incubated with anti-Men B (diluted 1:2000 to 1:4000) overnight at 4°C; immunoreactivity was revealed with HRP-conjugated anti-IgM diluted 1:50 (3 hr at RT) and diaminobenzidine/H_2O_2. No detergents were used. After light microscopic observation, blocks of selected areas were processed further for electron microscopy, which included osmium tetroxide treatment, uranyl acetate *en bloc* staining, and flat-embedding in Epon resin. Ultrathin sections were cut from the first few micrometers of each slice and examined without any further contrasting with a Philips CM10 electron microscope (for further details, see ref. 27).

Controls were performed on serial frozen sections and vibratome slices. They included incubation of sections with (*i*) anti-Men B antibody previously absorbed with colominic acid (10 μM), (*ii*) diluted mouse ascites fluid containing IgM irrelevant antibodies recognizing a proteic epitope of Men B bacteria, and (*iii*) immunolabels alone.

RESULTS

Immunoblot Analysis of N-CAM Expression in the Adult Hypothalamo–Neurohypophysial System. After reaction with the anti-Men B antibody, immunoblots performed with portions of the hypothalamus containing the SON revealed a broad band in the molecular mass range of 150–270 kDa (Fig. 1, lanes 1 and 1a). Such a band corresponded to the expected migration profile of PSA-N-CAM (16, 23). Extracts of the neurohypophysis showed a similar, although less intense, reaction (Fig. 1, lane 2). We noted no differences in extracts derived from the different groups of animals (for example, male versus female, virgin versus lactating). No other bands were detected on these gels outside of the PSA-N-CAM zone. The Men B immunoreactivity was completely abolished (Fig. 1, lane 1b) by pretreatment of SON extracts with endosialidase, which splits PSA from its carrier protein (26), and by preincubation of the antibody with sialic acid polymer (colominic acid).

For comparison, we also applied the anti-Men B antibody to gels containing extracts from the hypothalamic region

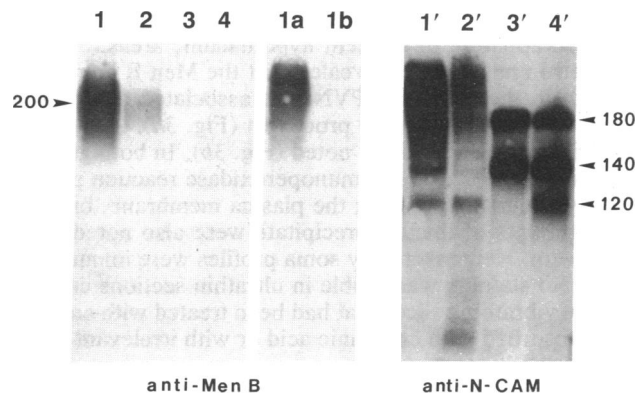

FIG. 1. Immunoblot analysis of PSA-N-CAM and total N-CAMs in the adult rat hypothalamo–neurohypophysial system. The SON (lanes 1, 1a, 1b, and 1′), neurohypophysis (lanes 2 and 2′), portions of hypothalamus basolateral to the SON (lanes 3 and 3′), and cerebellum (lanes 4 and 4′) were probed for their reactivity with monoclonal anti-Men B antibody and a polyclonal serum recognizing all N-CAM isoforms (anti-N-CAM). PSA-N-CAM, revealed by Men B immunoreactivity, was detectable only in the SON (lanes 1 and 1a) and neurohypophysis (lane 2). Several other isoforms of N-CAM were detected in all the examined structures (lanes 1′–4′). Note that the anti-Men B immunoreactivity in the SON (lane 1a) disappeared after reaction of extracts with endosialidase (lane 1b). Positions of molecular weight markers are indicated by arrows.

basolateral to the SON (Fig. 1, lane 3) and the cerebellum (Fig. 1, lane 4). In contrast to extracts of the SON or neurohypophysis, these extracts showed no detectable reaction to anti-Men B.

Incubation of gels containing extracts of the SON with antibodies recognizing all N-CAM isoforms (anti-total N-CAM) revealed a reaction not only at the position spanning 150- to 270-kDa, but also at the 180-, 140- and 120-kDa positions (Fig. 1, lane 1′). In gels containing neurohypophysial extracts, a strong reaction was also noted at the 150- to 270-kDa position in addition to one at 120 kDa (Fig. 1, lane 2′). On the other hand, no reactivity was seen at the 150- to 270-kDa position in extracts of basolateral hypothalamus (Fig. 1, lane 3′) or cerebellum (Fig. 1, lane 4); the former contained reactive polypeptides of molecular masses 180 and 140 kDa, and the latter contained reactive polypeptides of molecular masses, 180, 140, and 120 kDa.

Localization of PSA-N-CAM Immunoreactivity in the Magnocellular Nuclei. In hypothalamic sections treated with anti-Men B antibody and FITC- or HRP-conjugated secondary antibodies, a striking immunoreactivity was noted throughout the SON (Fig. 2 *A*, *C*, and *D*). Staining was also visible in the PVN, although to a lesser degree. The immunoreactive signal was particularly strong in the ventral portions of the SON and in its ventral glia lamina (Fig. 2 *A* and *C*), where neurosecretory dendrites and astrocytic processes accumulate; elsewhere, it filled the neuropil surrounding the magnocellular cell bodies, which were immunonegative (Fig. 2 *C* and *D*). Adjacent hypothalamic structures, including the optic chiasma, were not labeled above background levels (Fig. 2*A*). The specific staining disappeared in control sections that had been treated with anti-Men B preabsorbed with colominic acid or with mouse IgM irrelevant antibody. Comparison of sections obtained from the different groups of animals showed no major differences in the overall intensity of PSA-N-CAM immunoreactivity in the magnocellular nuclei. In the SON of parturient and lactating animals, however, there was some diminution of staining in the dorsal portions of the SON and, in particular, around clusters of closely juxtaposed neuronal cell bodies (Fig. 2*D*).

Incubation of sections with the anti-total N-CAM serum also resulted in positive staining of the SON (Fig. 2*B*) and PVN, distributed in a manner similar to that observed with the anti-Men B antibody. In these sections, however, staining was also apparent in adjacent hypothalamic areas.

Electron microscopy revealed that the Men-B immunoreactivity in the SON and PVN was associated mainly with astrocytic cell bodies and processes (Fig. 3*a*). Labeling of dendritic profiles was also noted (Fig. 3*b*). In both glial and neuronal elements, the immunoperoxidase reaction product usually accumulated along the plasma membrane, but variable amounts of immunoprecipitate were also noted in the cytoplasm. Neurosecretory soma profiles were immunonegative. No staining was visible in ultrathin sections cut from control vibratome slices that had been treated with anti-Men B preabsorbed with colominic acid or with irrelevant mouse IgM antibody.

Localization of PSA-N-CAM Immunoreactivity in the Neurohypophysis. Sections of the hypophysis that included the neurohypophysis and intermediate and anterior lobes showed marked differences in the intensity of anti-Men B staining of the three areas (Fig. 2*E*). The neurohypophysis was heavily labeled, and it was difficult to identify cell features with certainty. Light surface labeling of cells was seen in the intermediate and anterior lobes; in the former, there also was staining in the tissue surrounding its cells. Reaction with anti-total N-CAM produced a pattern of staining throughout the hypophysis similar to that obtained with the anti-Men B antibody. However, staining of intermediate and especially of anterior lobe cells was stronger (Fig. 2*F*).

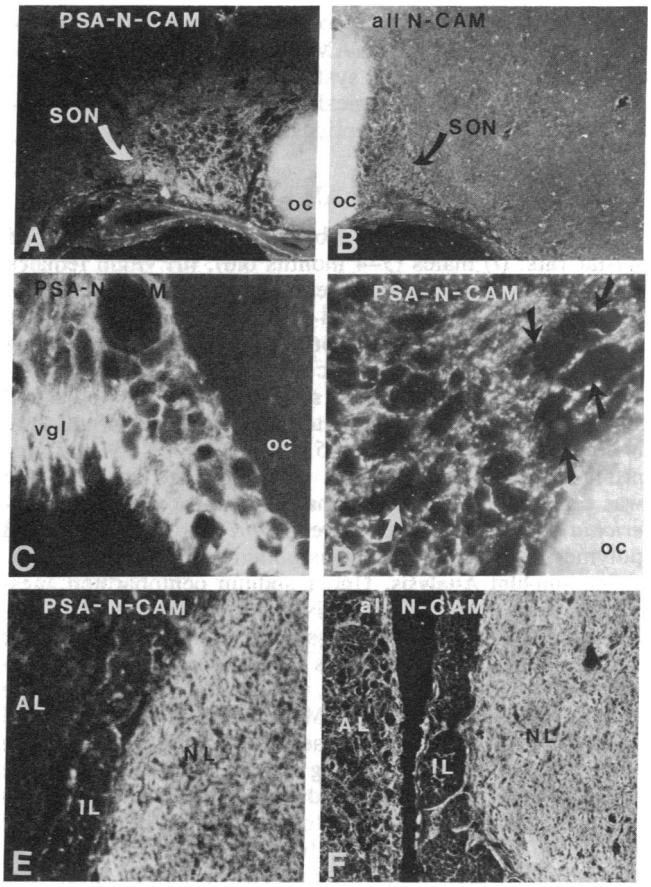

FIG. 2. Light microscopic localization of PSA-N-CAM and total N-CAMs in the hypothalamo–neurohypophysial system. After incubation of sections with the anti-Men B antibody, a striking immunoreactivity is seen in the SON (*A*, *C*, and *D*) of the hypothalamus and in the neural lobe (NL in *E* and *F*) of the hypophysis. Reaction of sections with the polyclonal serum recognizing all N-CAM isoforms (*B* and *F*) shows a distribution of immunoreactivity in the SON (*B*) and neurohypophysis (*F*) similar to that obtained with the anti-Men B antibody. However, adjacent tissues also show labeling. Note that PSA-N-CAM immunoreactivity is particularly strong in the ventral portion of the SON (*A* and *C*; vgl, ventral glia lamina); elsewhere, it occurs in the neuropil around the magnocellular somata, which remain immunonegative. In the SON of lactating animals, there is less reaction in the dorsal region of the nucleus where there are numerous clusters of closely apposed magnocellular somata (arrows in *D*). After reaction of sections of the hypophysis for PSA-N-CAM (*E*), there is strong immunoreactivity in the NL and only a slight reaction in intermediate lobe (IL) and anterior lobe (AL) cells; there is also staining in the extracellular matrix surrounding IL cells. (*A*, *B*, *D*, *E*, and *F*) Immunoperoxidase staining, dark field optics. (*C*) Immunofluorescence. The brightness of the optic chiasma (oc) in *A*, *B*, and *D* is due to dark-field illumination of its myelinated fibers; note that it is hardly visible under epifluorescence (*C*). (*A* and *B*, ×55; *C*, *E*, and *F*, ×160; *D*, ×250.)

Ultrastructural observations indicated that the anti-Men B labeling of the neurohypophysis was essentially a surface labeling of neurosecretory axonal terminals and swellings and astrocytic-like pituicytes (Fig. 3*c*). Labeling was also noted in basal lamina components (including collagen fibrils), surrounding the neurosecretory elements, and in the perivascular areas. Such staining, including that of the extracellular matrix components, disappeared in control sections.

DISCUSSION

The ability of the adult hypothalamo–neurohypophysial system to undergo significant morphological changes in response

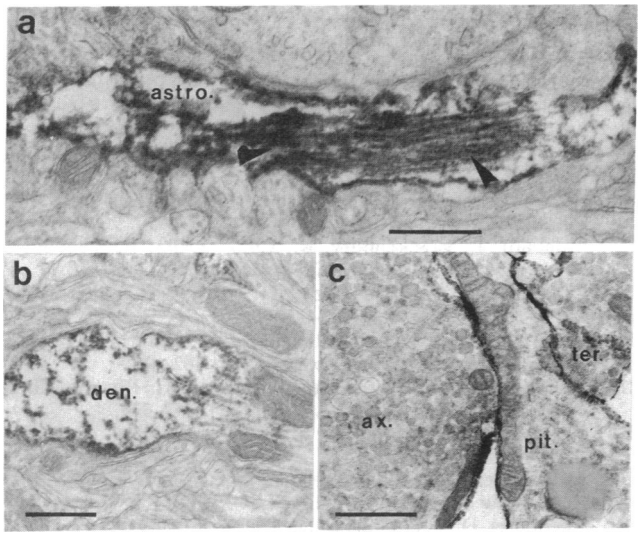

FIG. 3. Electron microscopy of PSA-N-CAM localization in the hypothalamo–neurohypophysial system. In the SON (*a* and *b*), the peroxidase reaction product resulting from Men B immunoreactivity is seen to be distributed to a varying degree along the plasmalemma and in the cytoplasm of astrocytic (*a*) and dendritic (*b*) processes. Arrowheads in *a* point to glial filaments characteristic of astrocytes. In the neurohypophysis (*c*), the immunolabel is associated with neurosecretory axons (ax.) and terminals (ter.) and astrocytic-like pituicytes (pit.). [Bars = 0.5 μm (*a* and *b*) and 1 μm (*c*).]

to physiological stimuli is now well documented (for review, see refs. 21 and 22). These changes involve a reversible reorganization of neuronal and glial elements and synaptic connections. At present, the mechanisms that permit such changes are unknown. Adhesion molecules of the N-CAM family and their sialic acid are now implicated in the control of cell–cell interactions and, in the nervous system, in the establishment of neuronal structure (see ref. 20). This led us to examine whether the hypothalamo–neurohypophysial system contains N-CAM and, in particular, its highly sialylated isoform.

Our analyses with the anti-Men B antibody that specifically recognizes PSA (23) revealed that polysialylation is indeed a major feature of this system. Our control preparations further supported such a contention, since Men B immunoreactivity was absent from tissues treated with endosialidase and from tissues treated with the antibody previously absorbed with colominic acid. We believe that this PSA is carried by N-CAM and not by other molecules, such as the sodium channel (see ref. 28), for several reasons. First, earlier biochemical studies demonstrated that N-CAM is the major carrier of PSA in the rodent and avian brain (23, 24). Second, our immunoblots, with antibodies that recognize all N-CAM isoforms (anti-total N-CAM), indicated that the hypothalamo–neurohypophysial system contains an isoform in the molecular mass range corresponding to that of PSA-N-CAM (16, 23). Finally, whereas the sodium channel is ubiquitous in the adult brain, our immunoblot and immunocytochemical analyses showed Men B reactivity restricted to the magnocellular nuclei.

Within the hypothalamo–neurohypophysial system, PSA-N-CAM immunoreactivity was localized not only in the hypothalamic nuclei containing the somata and dendrites of magnocellular neurons but also in the neurohypophysis, where their axons terminate. Labeling was seen both in male and female animals, and its intensity did not appear to vary significantly in relation to their physiological state (virgin, parturient, lactating). Although no quantification was performed, this finding is of interest, since it suggests that polysialylation is not modulated by extrinsic factors regulating the activity of the neurons but is a permanent feature of these hypothalamic neurons and their astrocytes, retained throughout the animal's life.

N-CAM molecules are expressed in a wide variety of cells, and it was of critical importance to determine which cell phenotype showed PSA-N-CAM immunoreactivity. At the light microscopic level, the immunoreactivity appeared to be associated with astrocytic elements and neuronal fibers because it was restricted to the neuropile surrounding immunonegative neurosecretory cell bodies in a manner similar to that described in developing and early postnatal neuronal tissues (29). Moreover, a strong reaction was always noted in the ventral portions of the SON and notably in the ventral glia lamina, composed essentially of neurosecretory fibers and astrocytic processes. On the other hand, no reaction was seen in clusters of juxtaposed neuronal somata that lack glial coverage (see refs. 21 and 27). Electron microscopy confirmed that in the SON and PVN, PSA-N-CAM immunoreactivity was present in astrocytic cell bodies and processes, and to a lesser extent, in dendritic profiles. It also established that in the neurohypophysis, immunoreactivity was associated both with neuronal fibers and glial elements (pituicytes). As for the subcellular localization of the antigen, our observations must be interpreted with caution. In the labeled profiles, immunoprecipitate was seen not only along the plasmalemma but also in the cytoplasm, distributed in a variable manner. A similar ultrastructural localization of N-CAM has been reported in neurons of the striatum (9). These observations seem paradoxical since we would expect to find N-CAM immunoreactivity associated only with the plasmalemma. However, a shortcoming of preembedding immunostaining is that the peroxidase immunoprecipitate diffuses variably within labeled structures and thus does not allow accurate subcellular localization of the antigen under study (see also refs. 27 and 30).

Neurosecretory axons and nerve terminals in the neurohypophysis as well as dendrites in the hypothalamus were PSA-N-CAM-immunoreactive, yet neurosecretory somata were consistently immunonegative. This may mean that PSA-N-CAM *in vivo* is distributed in a polarized fashion, which raises the question of its mode of synthesis and transport. In the neurohypophysis, we also noted some immunoreactivity in the basal lamina, which may represent secreted or membrane-released forms of the molecule participating in the formation of the extracellular matrix (4, 14).

Although we made no systematic analysis of the localization of PSA-N-CAM immunoreactivity throughout the adult rat brain, it is clear from our observations that it is present only in discrete areas. Extracts from hypothalamic structures adjacent to the SON and from a region such as the cerebellum did not contain polypeptides in the size range of PSA-N-CAM, as seen from reaction with antibodies recognizing all N-CAM isoforms, nor did they react with the anti-Men antibody. Light microscopy also showed no PSA-reactivity in tissues adjacent to the strongly immunoreactive SON and neurohypophysis. Since the hypothalamo–neurohypophysial system is capable of morphological reorganization in adulthood, it is tempting to speculate that its continued expression of PSA-N-CAM is related to its capacity for plasticity. Earlier observations would support such a contention. During the first three postnatal weeks in rodents, there is a gradual conversion of embryonic to "adult" forms of N-CAM, as different brain regions acquire their definitive structure (29). However, PSA-N-CAM immunoreactivity was still detectable in the postnatal rat brain, in regions such as the interpeducular nucleus (29), which shows potential for sprouting after lesion (31). A causal relationship between PSA expression and activity-dependent plasticity has also been demonstrated in the peripheral nervous system, in the establishment of intramuscular nerve branching (32). Investigations of

morphological plasticity in the adult central nervous system are still fragmentary and difficult to undertake, requiring complex and painstaking morphometrical techniques at the ultrastructural level. Evidence for polysialylation, obtained from biochemical or immunocytochemical means, may thus prove to be a useful marker for neuronal and/or glial changes in other neuronal systems capable of plasticity.

Finally, assuming that it is the expression of PSA-N-CAM and, in particular, its polysialic residues that confers to magnocellular neurons and their astrocytes the ability to change their morphology and connections under different conditions, by rendering them less adhesive (18, 19), the signals inducing them to do so remain to be determined. On the one hand, such signals may be proper to the glial cells. From *in vitro* studies, it appears that glial cells in different regions of the brain possess intrinsic properties that dictate the specific features of their morphology, features that in turn would influence the morphology of adjacent neurons (for review, see ref. 33). On the other hand, the signals may derive from the neurons themselves. In the hypothalamo–neurohypophysial system, there is an intermingling of neurons secreting either vasopressin or oxytocin, yet the neuronal–glial and synaptic changes in the magnocellular nuclei are specific to oxytocin neurons (27, 34, 35).

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