Markers for Neural and Endocrine Cells

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4.1 The Concept of Cell Type Markers

Considerable interest has centered over the last twenty years on the search for methods permitting the distinction between different cell types in complex tissues. While classical histological staining methods have proved invaluable in studying the cellular architecture of tissues, the search for more specific techniques that afford unambiguous identification of cell types was motivated by the fact that purely histological criteria frequently prove inadequate in other than normal adult tissues. In immature and in pathological tissues the microscopical appearance of cells is often radically different from that found in the healthy adult. In addition, increasing use of isolated cells grown in tissue culture has prompted the use of criteria other than those based on cellular morphology, which is rarely identical to that of the *in vivo* counterparts of cultivated cells and which may, moreover, be influenced by tissue culture conditions.

Although considerable progress towards this goal has been achieved by the development of histochemical methods, the major recent advances have followed the introduction and widespread application of immunocytochemical technology. This has dramatically altered both the way we recognize cells and indeed even the way in which we define a given cell type. Thus in the pre-immunocytochemical era cells were originally defined by their anatomical location and cellular morphology, which were correlated to their putative function. For example, silver impregnation methods have previously been very successfully employed to illustrate the delicate arborizations of large cerebellar

neurons (Ramon y Cajal, 1905; Palay and Chan-Palay, 1974); in addition such staining methods suggested the function of such neurons in terms of their multiple synaptic connections with other cerebellar neurons. Current use of immunocytochemical methods performs the same task more easily and in a much more consistent manner (Mikoshiba et al., 1979; Reeber et al., 1981; Langley et al., 1982b; Lohmann et al., 1981; Roth et al., 1981). Similarly, astrocytes, which had early been visualized by Golgi methods as satellite cells with end-feet on blood vessels, may now be easily distinguished by the use of antibodies to the protein GFAP (**G**lial **F**ibrillary **A**cidic **P**rotein) considered to be specific for these cells (Dahl and Bignami, 1973; Eng and Bigbee, 1978). Such technology has led to a redefinition of astrocytes as cells which contain GFAP.

This specificity of expression of certain proteins by certain cell types forms the basis of current use of immunocytochemistry to identify cell types (for review see Langley et al., 1984). The precise mechanisms that lead to such specificity of expression of certain proteins are not yet clear. However it is evident that, while the genetic information contained in the cell nucleus after fertilization is potentially capable of coding for all proteins expressed by all cell types present in the adult organism, during embryogenesis regulatory mechanisms predispose the expression of certain of these proteins and determine the suppression of others. Pathological processes may nevertheless alter the normal course of events, resulting in up or down regulation of individual proteins in response to external stimuli. Moreover, cells in tissue culture have been shown to respond to external factors, including growth factors and hormones, by altered expression of "cell specific" proteins (e.g. Doherty and Walsh, 1987; Grant et al., 1988). However the mature normal cell may be defined as one in which the expression of a number of biochemical characteristics has reached a stable state. Thus, a given cell type which was previously defined only by morphological criteria may now be accorded a biochemical definition in terms of the range of proteins, glycoproteins and lipids expressed. While a large number of proteins are found to be present in many quite different cell types in the animal kingdom, a limited number may be expected to be associated with the specific nature or function of a given type and thus have a more restricted cellular distribution. This property of cell specificity of such molecules permits their use as marker substances to distinguish between different cell types. The value of these molecules as cell-type markers depends on their capacity to be expressed by a cell in both normal and abnormal (pathological or in culture) situations which may vary for certain proteins. This is particularly important when choosing a marker protein as a diagnostic tool in clinical practice. The elegance and precision of cell labeling methods have outweighed such minor disadvantages and have totally altered pathological laboratory practice for clinical diagnosis over recent years.

A second aspect of cell marker proteins should not be forgotten. It is reasonable to suppose that the exclusive or predominant expression of a given protein by a given cell type may be associated with the particular biological or physiological function of that cell. During the seventies the search for neuron specific proteins was prompted by the conviction that such proteins would be intimately associated with brain function. This is clearly the case for the enzymes involved in specific neurotransmitter metabolism or for the neurotransmitters themselves (for review see Langley et al., 1984). Extensive neuropeptide mapping studies have indeed taught us a great deal about how the brain functions (Hökfelt et al., 1980). The link between cell marker protein and function is less evident in many other cases such as for example the S-100 protein (Matus and Mughal, 1975), the function of which remains a mystery long after the initial demonstration of its cellular specificity.

This chapter will concentrate principally on aspects of cellular expression but also briefly mention the likely function of what has been more recently revealed to be a family of related glycoproteins, collectively recognized under the name of neural cell adhesion molecule NCAM.

4.2 The NCAM Family

In order to better understand the cellular distribution of the different NCAM proteins, a brief historical account of their discovery and what is currently known of their biochemistry and molecular biology will be given. The discovery of this family of marker proteins was motivated by the search for molecules implicated in the construction of the extraordinary complexity of central nervous tissue. The adult human brain contains more than 10^8 neurons, each of which forms many synaptic connections. Such connections are not formed randomly but appear to follow overall design principles that lead to very specific interconnecting patterns. The molecular mechanisms by which neurons select their target cells to produce such selective networks have intrigued neurobiologists for decades.

Three independent laboratories in two different continents applied different experimental approaches in different animal models to address this question. Over a relatively short period of time, each published reports on "brain specific molecules" which were subsequently found to have associated cell adhesive properties. Thus, Gerald Edelman's group in New York used an experimental paradigm, modified from that used in earlier searches for adhesion molecules, to select molecules involved in the reaggregation of dissociated chick retinal

cells (for reviews see Edelman, 1983, 1984a,b; Rutishauser, 1984). He succeeded in isolating and purifying a molecule involved in such neural cell aggregation and referred to it as neural cell adhesion molecule. It was soon realized that this molecule was the chick equivalent of a molecule previously identified by crossed-electrophoresis by Elizabeth Bock's group in Copenhagen from rat brain, called D2 (Jørgensen and Bock, 1974; Jørgensen et al., 1980). This was one of several brainspecific proteins identified by this group at that time (Bock et al., 1975). Curiously, amongst these figured a molecule called synaptin, which was the first description of the vesicle marker protein now called synaptophysin (see chapter 2). A third laboratory, Christo Goridis' group in Marseille, using monoclonal antibody technology introduced in the 1970's obtained an antibody recognizing a **b**rain cell **s**urface **p**rotein, BSP-2, in the mouse (Hirn et al., 1981) which was subsequently shown to be the mouse equivalent of NCAM and D2 (Faissner et al., 1984; Noble et al., 1985).

The different data obtained on these three sets of molecules can thus now be collated to build up a more complete picture of the characteristics of what has been agreed since 1985 to be the same antigen, for which the name NCAM originally given by Edelman has been adopted. Since independant studies had been performed on what were originally considered to be unrelated molecules a number of initial contradictions appear when comparing the early literature on NCAM, D2 and BSP-2; for example whether or not glial cells express this molecule. While many of these differences no longer appear to exist certain species differences regarding the NCAM polypeptide forms expressed are evident; these will be discussed in more detail below. From the vast literature on NCAM several basic characteristics of this family can be singled out.

A unique gene coding for NCAM, containing nineteen principal exons, is present in the haploid genome. A mechanism of alternative splicing is currently considered (Rutishauser and Goridis, 1986; Owens et al., 1987; Goridis and Wille, 1988) to give rise to several species of mRNA the number of which varies according to species. In the chicken brain three NCAM mRNA species of 7.4, 6.7 and 4.3 have been detected and are thought to be translated into three principal NCAM polypeptides with molecular masses of approximately 180, 140 and 120 (referred to here as NCAM 180, NCAM 140 and NCAM 120, equivalent to NCAM **l**arge **d**omain NCAM **s**mall **d**omain and NCAM **s**mall **s**urface **d**omain) (Cunningham et al., 1987). The two larger forms contain a single transmembrane region and differ essentially in the lengths of their cytoplasmic domains (Cunningham, 1986; Cunningham et al., 1987; Gennarini et al., 1984a,b; Nybroe et al., 1985; Barthels et al., 1987; Santoni et al., 1987), while the smaller isoform is anchored to the membrane by the intermediary of a phosphatidylinositol residue (He et al., 1987). The sequences external to the cell membrane of all three forms are essentially identical, containing five regions similar to those present in immunoglobulins (Barthels et al., 1987;

Cunningham et al., 1987; Williams, 1987), which has led to the classification of the NCAM gene as a member of the immunoglobulin super gene family. The homophilic binding site, in addition to a heparin binding site and a "hinge" region, are situated in the external domain of the molecule (Cole et al., 1986a,b; Cole and Glaser, 1986; Cole and Akeson, 1989; Frelinger and Rutishauser, 1986; Becker et al., 1989).

The situation appears to be rather more complex in rodents since in both the mouse (Gennarini et al., 1986; Barbas et al., 1988) and the rat (Small et al., 1987) five NCAM mRNA species are detectable of 7.4, 6.7, 5.2, 4.3 and 2.9 kb, two of which seem to encode for NCAM 120 (5.2 and 2.9 kb). These two mRNAs differ in the size of their noncoding regions, a first polyadenylation signal being used to generate the 2.9 kb mRNA and a second signal 2.3 kb downstream being used for the 5.2 kb message (Goridis and Wille, 1988; Barthels et al., 1987). By analogy with the chicken the 7.4 kb mRNA is considered to code for NCAM 180.

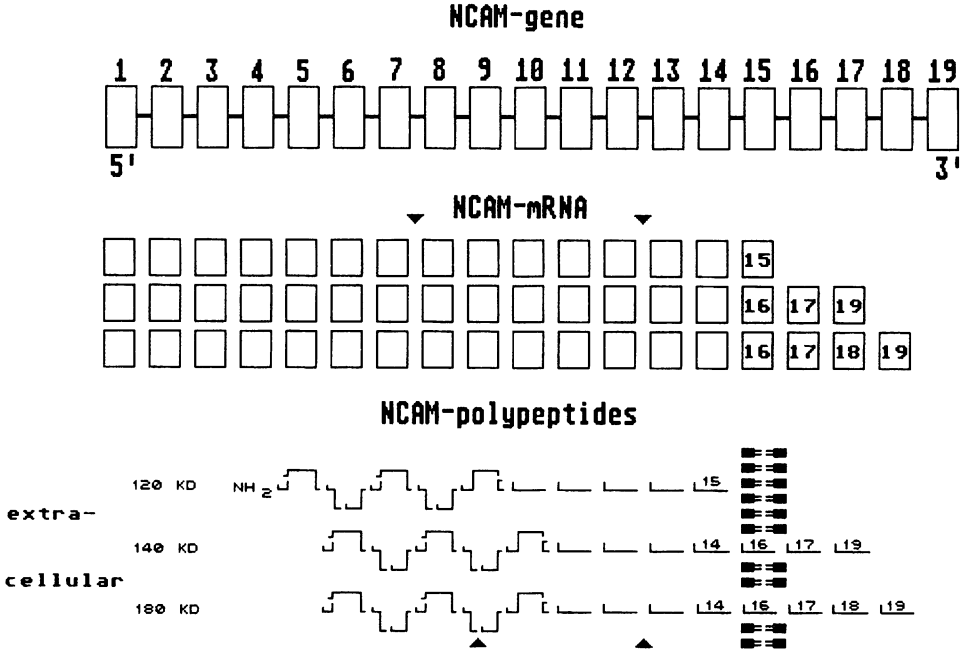

Fig. 4-1 Schematic representation of the NCAM gene, its transcription by alternative splicing and translation into the three principal NCAM polypeptides, illustrating the position of the sequences coded by different exons relative to the plasma membrane, the immunoglobulin-like domains and sites (arrowheads) of additional (tissue-specific) "minor" exons and their coded sequences.

Of the 19 major exons in the NCAM cDNA exons 1–14 are used to generate the external sequences of all three polypeptides, exon 15 codes for the anchoring sequence of NCAM 120 and exon 18 codes for the additional cytoplasmic 30–40 kD insert unique to NCAM 180 (Fig. 4.1 and Murray et al., 1986; Owens et al., 1987; Nybroe et al., 1988). Recently additional exons in the NCAM gene have been discovered, some of which code for large sequences in NCAM isoforms in certain tissues. Thus muscle NCAM mRNA contains an exon situated between exons 12 and 13 called msd (**m**uscle **s**pecific **d**omain) encoding a 37 amino acid sequence unique to this tissue (Walsh, 1988). Smaller additional exons have been found in rodent brain NCAM cDNA (Santoni et al., 1989). In particular exon π (30 bases) situated between exons 7 and 8 codes for a region in one of the immunoglobulin like domains, that may be close to the homophilic binding site and thus modify binding affinity (Santoni et al., 1989). Two more exons, one of only three base pairs, the other of 15, both situated between exons 12 and 13 code for a sequence in the hinge region of the molecule. These additional exons can give rise to minor heterogeneities in the three principal NCAM translation products, which may be fairly difficult to detect on immunoblots, since the molecular weights may differ very little from those of the principal forms. However S1 nuclease protection assays provide clearcut evidence of the existence in brain of multiple forms of mRNA coding for these additional polypeptides. Theoretically 24 different mRNA species are possible giving rise to 18 different NCAM polypeptides (Santoni et al., 1989).

The molecules are modified post-translationally by phosphorylation, sulphation and glycosylation (Lyles et al., 1984a; Nybroe et al., 1988). Glycosylation has been particularly well studied since major changes in levels of sialylation occur during normal brain development. Early so-called embryonic forms of NCAM contain high levels of polysialic acid groups, which markedly influences the adhesive binding properties of the molecule (Edelman, 1984; Rutishauser et al., 1985, 1988). NCAM isolated from adult tissue contains much less sialic acid. In addition to changes in polysialic acid, subsets of NCAM contain another carbohydrate hapten, HNK-1, consisting of 3'sulfated glucuronic acid (Noronha et al., 1986) recognized by antibodies first isolated as recognizing human natural killer cells (Schuller-Petrovic et al., 1983; Kruse et al., 1984). Thus, in summary, although three principal size classes of NCAM polypeptides have been found in both nervous and other tissues, all NCAM molecules of the same size class are not identical. Some may contain additional aminoacid sequences, others may be modified post-translationally in slightly different ways either containing different levels of polysialylation or different carbohydrate haptens (Williams et al., 1985).

4.3 Cellular Distribution of NCAM

4.3.1 Methodological Approaches

Two basic approaches have been employed to address the question of cellular NCAM expression; these may be broadly classified as morphological or biochemical.

4.3.1.1 Immunocytochemistry

The introduction by Coons et al. in 1941 of the use of flourescent probes covalently bound to antibodies and later modifications (Coons, 1961) provided the first opportunity of directly visualizing given protein tissue constituents at a cellular level. Considerable technical progress in immunocytochemical methods has subsequently eliminated many of the drawbacks of the early techniques and has led to the use of such methods as routine highly sensitive and specific staining procedures in pathological laboratories. Many techniques are currently available which differ in the probe or in the number of antibodies employed to visualize the molecule of interest. All depend for their success on the quality of the primary antibody i.e. its specificity and affinity for the antigen in question (Swaab et al., 1977). The higher the affinity of the antibody, the more it can be usefully diluted: the greater the dilution, the lower background non-specific staining will be, and thus the higher the specificity of the staining pattern.

This chapter will not attempt to review the advantages of individual methods. However it is important to remember that technical differences alone may give rise to apparently conflicting observations from different laboratories. In particular, the absence of staining may be simply due to the use of monoclonal antibodies with relatively lower affinities than commonly found with polyclonal antibodies. In addition monoclonal antibodies are sometimes more sensitive to tissue fixation procedures. Particular care should also be exercised with certain monoclonal antibodies that recognize cytoplasmic epitopes of NCAM, since cell permeabilization is a necessary prerequisite for their use.

Of the probes currently employed, fluorescent molecules such as fluoresceine isothiocyanate or rhodamine, or enzymes giving permanent preparations, such as peroxidase or alkaline phosphatase, have proved valuable for light microscopy. At the ultrastructural level peroxidase has proved to be the most

versatile. Colloidal gold particles bound either to secondary antibodies or to protein A (Langley and Aunis, 1986; van den Pol et al., 1986) are particularly useful for labeling surface of antigens of isolated or cultured cells when morphometric analysis can be performed, but are limited by penetration problems when employed on pre-embedding tissue sections. The lack of penetration into ultrathin sections has limited the routine exploitation of post-embedding immunogold methods for NCAM localization.

4.3.1.2 Biochemical Approaches

Valuable information on cellular expression of individual NCAM glycoproteins has been obtained with combined immunochemical and protein separation techniques, which has only been partially possible with immunocytochemistry since antibodies specifically recognizing each of the three NCAM isoforms are not available. In particular turnover studies have been performed on NCAM isolated by immunoprecipitation followed by gel electrophoresis of extracts from cells labeled with radioactive precursors (Hirn et al., 1983; Lyles et al., 1984b; Nybroe et al., 1986; Linneman et al., 1985). More commonly electrophoresis and immunostaining of Western blots proves to be simpler and adequate for analyzing different NCAM isoforms in different tissues (Rougon et al., 1982; Hoffman et al., 1986; Nagata and Schachner, 1986). Quantitation is possible when radioiodinated second antibodies or protein A are used to locate the protein bands. The technique of crossed-electrophoresis (Bock, 1972) and ELISA have also provided quantitative estimates of different NCAM isoforms (Bock et al., 1983). These methods have been usefully applied in biosynthetic studies of NCAM in cultured neurons, glial cells and tumor lines. *In vivo* studies on NCAM extracted from the lateral geniculate nucleus after injection of radioactive precursors into the eye (which is followed by axonal transport of newly synthesized glycoproteins along the optic nerve) has afforded comparisons with the *in vitro* situation (Nybroe et al., 1986). Cell free translation of NCAM has also been investigated using crude RNA preparations from cultured cells and tissue homogenates (Nybroe et al., 1986).

Post-translational modifications have also been studied by immunochemical techniques. The most important of these as already mentioned concerns polysialic acid (PSA) content in the so-called embryonic form and is of special interest in pathology where frequently these forms reappear. Thus after nerve injury newly synthesized NCAM is found to be highly sialylated which is probably related to the need for less stability in intercellular contacts during the period of cellular redistribution and tissue repair (Daniloff et al., 1986a). The presence of high PSA forms of NCAM can be easily suspected from Western

immunoblots by their appearance as broad diffuse bands of higher apparent Mr (200–250 kD). Pretreatment of such forms of NCAM with neuraminidase, which removes successive sialic acid residues from PSA chains, alters their anomalous electrophoretic profile from a broad smear to the descrete three banded pattern of adult brain NCAM (Rutishauser, 1984). This suggests that the three principle NCAM isoforms are present during embryonic life but are polysialylated.

Other post-translational modifications affecting the external domain of NCAM have been studied. Sub-populations of NCAM polypeptides may be distinguished by their complement of carbohydrate epitopes. The HNK-1 carbohydrate present on human natural killer cells (Abo and Balch, 1981) has been detected by successive immunoprecipitation with different antibodies on various adhesion molecules including L1, MAG and NCAM (Kruse et al., 1984; 1985; Poltorak et al., 1987). Only about 30 % of NCAM isolated from rodent brain carry this epitope. It has been suggested that this carbohydrate may itself be a ligand in cell adhesion (Künemund et al., 1988).

4.3.1.3 Hybridization Techniques

Since the isolation of clones of total or partial NCAM cDNA and their subsequent sequencing it has become feasable to examine the expression of the NCAM message either at a cellular level by the technique of *in situ* hybridization (where a specific oligonucleotide probe or a probe derived from a partial cDNA is hybridized to tissue sections) or in tissue RNA extracts by either Northern blots or S1 nuclease protection assays. This is a particularly important development since such approaches not only confirm the capacity of a cell to synthesize NCAM, but also provide valuable information on its regulation at the genetic level. Such approaches are complementary to studies employing antibodies, since in one case the message is detected while in the other its translation product, the localization of which is not always identical (Prieto et al., 1989). Furthermore the technique of S1 nuclease protection analysis provides information on the sequence of the bases in the NCAM mRNA as well as the precise nature of the exons present (Barthels et al., 1987; Santoni et al., 1989). However, in cells or tissues where NCAM turnover is slow it may prove much more difficult to detect low levels of the mRNA, while significant levels of NCAM peptides pose no problems for visualization with antibodies.

Up to the present time few reports of *in situ* hybridization studies have been published but, in contrast, mRNA analytical studies of tissue extracts are numerous (Covault et al., 1986; Murray et al., 1986; Gennarini et al., 1986) and

have provided very interesting data on developmental changes. Most early studies on NCAM distribution concentrated on embryonic development where the greatest changes in expression may be expected to occur in parallel with important tissue induction events. In this section we will concentrate mainly on NCAM expression in different cell types of adult tissues, where the phenotype is stable, before discussing some of the early modifications, which are frequently transitory or recurrent during cellular migration or initial stages of tissue formation.

4.3.2 Neurons

4.3.2.1 Immunocytochemistry

One of the earliest references to a molecule that was subsequently found to be equivalent to NCAM was published in a report of a search for brain specific antigens (Jørgensen and Bock, 1974; Bock et al., 1974; 1975; Bock et al., 1980).

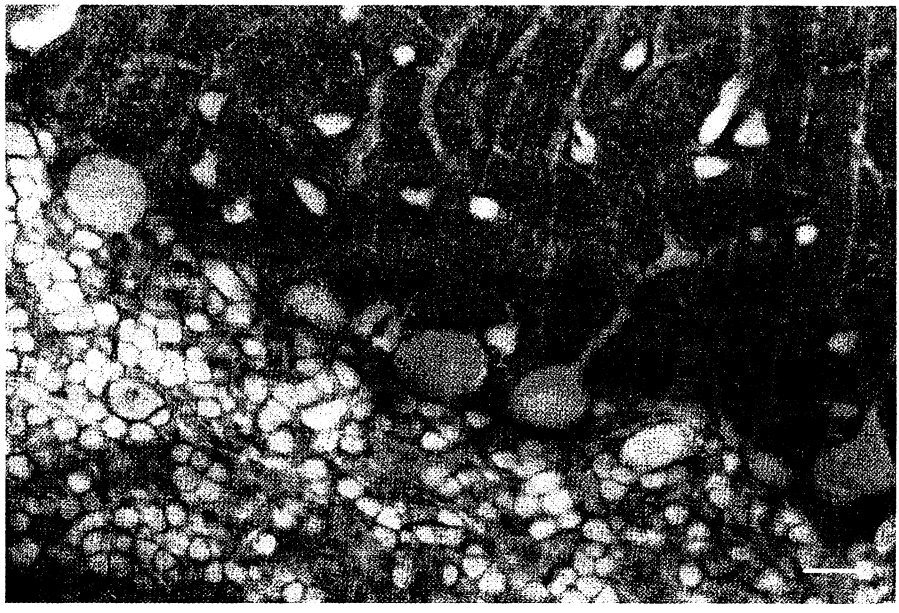

Fig. 4-2 Vibratome section of adult rat cerebellum immunoperoxidase labeled for NCAM.
Note the intense staining of the molecular layer, the unstained cytoplasm of Purkinje cells and marked staining of granule cells. Bar = 20 μm.

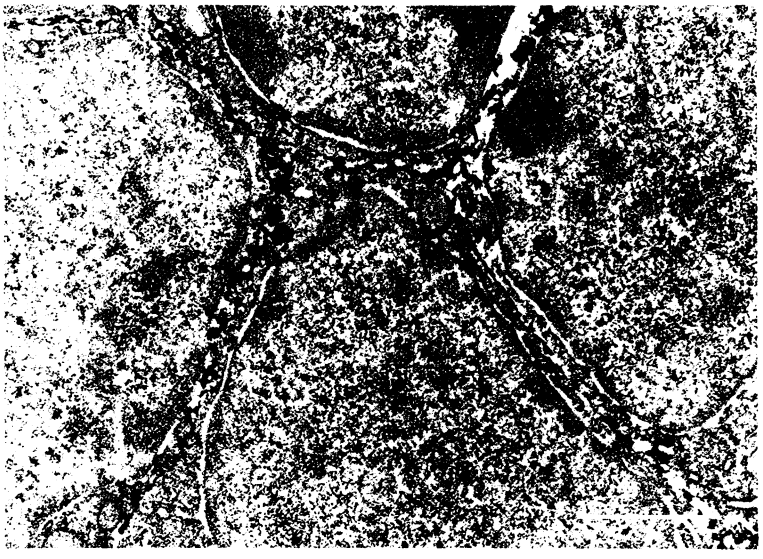

Fig. 4-3 Electron micrograph of rat cerebellar granule cells labeled for NCAM. The plasma membranes are intensely stained and cytoplasm is weakly immunoreactive. Section not counterstained. Bar = 1 μm.

The D2 antigen detected amongst several others, including protein 14–3-2 equivalent to neuron specific enolase was found to be absent from cultured astrocytes but enriched in synaptosomal fractions of rat brain. Though this antigen appeared not to be limited to nerve terminals a neuronal origin was concluded. At the same time, while much of the early work of Edelman's group concentrated on embryonic tissues, this group published direct immunocytochemical evidence of NCAM expression over the entire surface of mature sympathetic neurons, even though these were isolated from late embryonic chicks (Rutishauser et al., 1978a), with an antibody previously found to be capable of inhibiting embryonic retinal cell aggregation (Brackenbury et al., 1977; Rutishauser et al., 1978b). In contrast, a much more topographically restricted expression has been reported both in the retina (Jørgensen and Möller, 1981) and also in cerebellum where it was considered to be confined to synaptic regions of the neuronal membrane (Jørgensen and Möller, 1980). Later studies in both rodents and chickens provided convincing evidence of the widespread expression of NCAM by all types of neurons *in vivo* over their entire surface (see Figs. 4-2 and 4-3; Langley et al., 1982a, 1983; Daniloff et al., 1986b; Chuong et al., 1987). Such discrepancies are probably the result of differences in the affinity of antibodies used in different laboratories and also in different tissue fixation procedures. Immunoelectron microscopy did however

reveal interesting differences between different types of cerebellar neurons (Langley et al., 1982a; 1983). NCAM was detected in granule cells, which constitute numerically the vast majority of cerebellar neurons, intracellularly in the perikaryon, although little was detected in their axons, in addition to at their cell surface. An intracellular localization was never observed for the large cerebellar neurons such as Purkinje cells, presumably because cytoplasmic levels were below limits of detection. These data were interpreted as reflecting differences of NCAM transport or turnover in different types of neurons. The question of turnover rates of NCAM in neurons of adult tissues is an intriguing one, which has been addressed in the biosynthetic studies of Elizabeth Bock's group, which reported that NCAM turnover was extremely slow in adult tissue. In fact it was calculated to decrease 350 fold during development (Linnemann et al., 1985). This would suggest that once NCAM is transported to the cell surface of Purkinje cells or to the distant parallel fibres (axons) of granule cells in normal adult cerebellum, it is subject to very slow degradation, resynthesis and reinsertion in the membrane. In contrast, synthesis of NCAM and transport through the Golgi apparatus to the cell membrane has been shown to be a rapid phenomenon in cultured neurons, lasting only several minutes (Lyles et al., 1984a). These data explain the absence or relatively low levels of NCAM observed immunocytochemically in the cytoplasm compared with the neuronal cell membrane. Though in healthy adult nervous tissue the NCAM synthetic machinery appears to be relatively silent, it is evident that external influences which may be provoked by traumatism can rapidly stimulate synthesis (Daniloff et al., 1986a). More recent immunocytochemical studies (Daniloff et al., 1986b) have confirmed that the adult NCAM staining pattern of chick nervous system reflects that of the late embryo (Thiery et al., 1982; Crossin et al., 1985) except for a diminution in intensity. This reduced NCAM immunolabeling in adult tissue is particularly striking in myelinated fibre tracts such as spinal cord and optic nerve. Some reports suggest that the down-regulation of NCAM during development leads to its complete suppression in mature myelinated axons (Mirsky et al., 1986; Martini and Schachner, 1988). As already pointed out, caution in interpreting negative immunocytochemical data should be exercised since the detection of low antigen levels is much more dependant on technical factors. Nevertheless down regulation is confirmed by comparative analysis of both NCAM protein and NCAM mRNA levels in adults and young animals (Bock et al., 1980; Linnemann et al., 1985; Nybroe et al., 1986; Gennarini et al., 1986). It is likely that it is still present even on axons of CNS and PNS neurons in the adult though at much reduced levels and may also be associated with a slower turnover. In addition the reduced expression of NCAM in adult peripheral nerves seems also to be associated with changes in its topographical distribution on the axon surface: the membrane at nodes of Ranvier appear to contain more NCAM than is detectable on the internodal

axon (Rieger et al., 1986). These highly specialized regions which predispose the rapid saltatory conduction of impulses in myelinated nerves were shown several years previously (Langley, 1979) to be the sites of high concentrations of acidic carboxylated polyanions (which could in part include sialic acid containing glycoproteins such as NCAM). Histochemistry with ruthenium red (a former botanical stain for pectin, a carboxyl acid rich polyanion) results in staining not only of the nodal axon membrane, but also the membrane of Schwann cells, which have subsequently been shown to contain NCAM (see below).

NCAM has also been shown to be present intraaxonally at nodes of Ranvier (Rieger et al., 1986), though the function associated with such a distribution at present remains obscure. Thus, in nervous tissues, a degree of compartmentalisation of NCAM expression may occur *in vivo* after permanent stable intercellular relationships are established. In contrast, studies on neurons *in vitro* (Nègre-Aminou et al., 1988) show no differences in total NCAM membrane density between cell bodies and neurite like extensions. This parallels the situation *in vivo* for non-myelinated fibres and may reflect a lesser degree of differentiation.

4.3.2.2 NCAM Isoforms in Neurons

In surveying the literature on the nature of NCAM polypeptides expressed by neurons the reader is confronted by apparent contradictions. Many studies have been performed on neuronal cultures and it is often difficult to extrapolate from cultures to adult neurons for several reasons. Cell isolation and culture conditions may be expected to modify NCAM synthesis since this has been shown to be sensitive to traumatism influencing cell contact relationships *in vivo*. Trophic factors may at least be partly responsible for such changes (Prentice et al., 1987). In addition most *in vitro* studies have employed neurons from embryonic tissues in which the stable adult NCAM phenotype has not been attained.

It is important first to consider species differences in the relative expression of individual NCAM polypeptides. Birds appear to express all three NCAM forms but their relative amounts and developmental pattern are different from those of rodents. The small surface domain peptide NCAM 120 is very weakly expressed in embryonic chick brain (Cunningham, 1986) compared to that of rodents and then only in late development. Contradictory data on frog NCAM has been published. In one report (Sunshine et al., 1987) only NCAM 180 was detected while Levi et al. (1987) reported that NCAM 140 was the first detectable form early during embryogenesis followed by NCAM 180. The appearance of NCAM 120 was shown to be a relatively late developmental

event as in the chicken. Frog is unusual however in the expression by liver of an NCAM of 160 kD different to the brain isoforms (Levi et al., 1987).

Analysis of tissue extracts from different anatomical regions of the CNS at different developmental times have led to conclusions on cell type expression of different NCAM isoforms (Rougon et al., 1982; Chuong and Edelman, 1984). Different brain regions reflect both developmental biosynthetic trends in different compartments of a given cell type as well as the cellular composition of the tissue at a given time. Since glial cells also contain NCAM, though in lower amounts, tissue extracts correspond to a mixture of both neuronal and glial components. During CNS development neuronal proliferation and migration in general preceed glial cell multiplication and maturation. Thus total tissue NCAM at later stages will reflect a relatively greater contribution from glial cells compared to neurons. It has been found in general that NCAM 120 levels increase later than those of the other principal isoforms in developing brain. Analysis of adult spinal cord white matter demonstrates clearly that anatomical regional differences can give strong clues concerning cell type NCAM expression. Spinal cord contains relatively low amounts of NCAM 180 and 140 and larger amounts of NCAM 120 (Chuong and Edelman, 1984). Its NCAM composition may provide a better indication of astrocyte NCAM isoforms than neuronal forms, since this tissue is composed mainly of axons and glial cells and both axonal and oligodendrocyte NCAM is repressed when fibres are myelinated. Both sets of data suggest that both NCAM 180 and 140 are more typically neuronal than NCAM 120. The use of antibodies that specifically recognize NCAM 180 in immunocytochemical studies of the cerebellum indicates that this polypeptide is characteristic of post-mitotic neurons (Pollerberg et al., 1985) and suggests that this polypeptide is topographically compartmentalized. Higher concentrations of NCAM were found to exist at contact sites between cells and it may be enriched on cell extensions (Pollerberg et al., 1985; 1986). At the present time it would be unwise to generalize from these findings, even though in a quite different model, reinforcement of neuronal character which is accompanied by neurite outgrowth is also paralleled by higher NCAM 180 RNA levels and increased NCAM 180 synthesis (Doherty et al., 1988).

The capacity of mature neurons *in situ* to synthesize and axonally transport all three NCAM forms has been demonstrated for retinal ganglion neurons (Nybroe et al., 1986) and this agrees with data showing *in vitro* translation of all NCAM forms with cerebellar granule cells microsomes (i. e. post-mitotic neurons). Some reported immunochemical data of peripheral ganglia culture extracts also suggest that all three forms can be synthesized although NCAM 120 was minor, while other studies failed to detect NCAM 120 (Noble et al., 1985; Lyles et al., 1984a,b; Keilhauer et al., 1985; Nybroe et al., 1985). Such cultured cells may more closely resemble immature neurons.

4.3.2.3 "Embryonic" NCAM in Neurons

Recent immunocytochemical data have demonstrated the persistance of the carbohydrate polymer of "embryonic" NCAM with an antibody against the unusual highly sialylated NCAM (Rougon et al., 1986) in isolated regions of adult rat brain (Aaron and Chesselet, 1989; Rougon et al., 1990) in contrast to the widely held opinion that the embryonic-adult conversion is complete before adulthood. 30 % of adult frog CNS NCAM is in the highly sialylated form (Sunshine et al., 1987). Regions where high levels of PSA persist may be associated with the need for greater plasticity of neuronal contacts, which is afforded by the lower adhesive affinity of highly sialylated forms (Rutishauser et al., 1988). The relative immaturity of cultured neurons isolated from embryonic or young animals even when maintained in culture for prolonged periods is illustrated by the fact that the conversion to adult forms of NCAM is rarely observed (Nègre-Aminou et al., 1988), emphasizing the danger of extrapolating from cultures to adult tissues. Sialylation has been studied in acellular systems using lysates obtained from young and adult rats: sialylation of both NCAM 180 and 120 was observed with cerebellar granule cells, but the NCAM 140 isoform did not appear to be sialylated *in vitro* (Breen et al., 1987, see also Schlosshauer, 1989).

4.3.3 Astrocytes

The first report showing that astrocytes *in situ* express NCAM were made with monoclonal antibodies against BSP-2, before it was realized that this antigen was equivalent to NCAM (Langley et al., 1982a; Hirn et al., 1983). While clearcut evidence of immunolabeling was obtained on cerebellar tissue sections, curiously the same antibodies failed to immunolabel cultured astrocytes. This may have been due to the relative immaturity of cultured astrocytes compared with their *in situ* counterparts, the relatively low levels of astrocyte NCAM compared to neurons and the relatively low affinity of the monoclonal antibodies employed at that time. More recent immunocytochemical data with polyclonal antibodies (Noble et al., 1985) showed that cultured astrocytes indeed contain NCAM but quantitative analysis of immunolabeled cultured astrocytes (van den Pol et al., 1986) showed that membrane density was 25 fold less than that found on cultured (immature) neurons. Nevertheless, considerable variation in NCAM density was noted in neurons of the same culture. Immunoblot analysis of cultured astrocytes (Nybroe et al., 1986; Noble et al., 1985)showed a predominance of NCAM 140 with lower levels of NCAM

120, with little or no detectable NCAM 180. As suggested above for neurons, cell type characteristic NCAM isoforms can be deduced from total NCAM extracts from anatomical regions consisting mainly of one type of cell expressing NCAM. Optic nerve and spinal cord myelinated tracts contain no nerve cell bodies, axon levels of NCAM are relatively low in the adult and oligodendrocytes *in vivo* do not appear to express significant quantities of NCAM. Thus the relatively high levels of NCAM 120 and low levels of NCAM 180 found in adult rat spinal cord and optic nerve (our unpublished data) may be assumed to be mainly due to astrocytes. This agrees with the data from cultured astrocytes, but it may be argued that astrocytes in culture resemble "reactive" astrocytes.

Further indications that NCAM 120 is a predominant NCAM translation product in astrocytes are obtained from studies of mixed neuronal and astrocyte cultures of Friedlander et al. (1985) in which NCAM 120 synthesis was found to be preferentially inhibited compared to other isoforms by inhibitors of cell proliferation which affect mainly astrocytes in these cultures.

Pituicytes are the glial cells present in the neurohypophysis. While some morphological differences are notable between these cells and brain astrocytes, they resemble the latter with regard to the presence of GFA protein. Immunoelectron microscopy demonstrates that these cells, like brain astrocytes, also express NCAM (Fig. 4-5 and Langley et al., 1989a). Neurohypophysis has been found to contain little NCAM 120 (Langley et al., 1989a); it is possible that all is contributed by the pituicytes or that these cells have a different NCAM polypeptide phenotype than brain astrocytes.

Section of peripheral nerve (Daniloff et al., 1986a) or optic nerve (our unpublished data) produce a marked up-regulation of NCAM synthesis which in peripheral nerve is associated with a return to immature forms of NCAM. In optic nerve the massive astrogliosis associated with axonal degeneration is accompanied by a considerable increase in immunostaining intensity with anti-NCAM antibodies.

4.3.4 Oligodendrocytes

In contrast to astrocytes, no direct immunocytochemical evidence *in situ* has been obtained of NCAM in oligodendrocytes, the other major brain glial cell type. Nevertheless the capacity of cultured oligodendrocytes to synthesize NCAM has been unambiguously demonstrated (Trotter et al., 1989; Bhat and Silberberg, 1985, 1988), which once more emphasizes the danger of extrapolating from cultures to brain tissue. The profile of NCAM polypeptides expressed by cultured oligodendrocytes has been found to depend on their

degree of maturation. Oligodendrocyte maturation has been followed both *in vivo* and *in vitro* by the successive appearance of the glycolipid antigens 04 and 01 (Trotter et al., 1989). Using these indices it was shown that immature oligodendrocytes express both NCAM 140 and 120 while more mature cells express predominantly NCAM 120. In addition the NCAM in these cultures is highly polysialated demonstrating the relative immaturity of these cells. *In vivo* it is likely that the reduction of NCAM expression noted in culture continues further until it is almost completely or totally repressed by the time myelination occurs.

4.3.5 Schwann Cells

Even though Schwann cells are the peripheral nervous system myelinating counterparts of oligodendrocytes, marked differences exist between them. Oligodendrocytes can myelinate many axons, while the Schwann cell can myelinate a single internode. Schwann cells are surrounded by a basement membrane *in situ* and their embryonic origin is the neural crest, while oligodendrocytes like astrocytes are derived from neural tube precursor cells. Curiously, Schwann cells express proteins such as S-100 which in the CNS has been employed as an astrocyte marker. Schwann cells like both oligodendrocytes and astrocytes have been shown to express NCAM in culture, but like the latter continue to express it *in vivo* in normal adult tissues. In addition, levels of NCAM in cultured Schwann cells are influenced by interactions with neurons (Seilheimer et al., 1989). *In vivo* immunolabeling is intense when Schwann cells surround unmyelinated fibres, but it has been reported that myelinating Schwann cells no longer contain NCAM (Mirsky et al., 1986; Covault and Sanes, 1986). It is likely that down regulation occurs but low levels appear to persist in peripheral nerves at nodes of Ranvier (Rieger et al., 1986). Interestingly, the HNK-1 epitope has also been localized at these sites in optic nerve (Ffrench-Constant et al., 1986). As is the case with other neural crest derivatives, the principal isoform present in Schwann cells is NCAM 140. In crushed or sectioned peripheral nerves NCAM synthesis dramatically increases and embryonic forms reappear (Daniloff et al., 1986a). Part of this increase is probably contributed by Schwann cells.

4.3.6 Endocrine Cells

The diffuse endocrine system encompasses a wide dispersity of either isolated cells or cell collectives situated in anatomically distant regions of the body. Cells

of this system differ radically in terms of the nature of their secretory products and their physiological function, but their classification as a common series is due to data, accumulated over several years, demonstrating many shared features. These include their general secretory nature (endocrine not exocrine), certain morphological characteristics (in particular the presence of dense cored secretory granules visible in the electron microscope) and a number of biochemical parameters (see also chapter 1). Pearse (1968) was the originator of the concept of classifying neuroendocrine cells in a single series called APUD cells (amine precursor uptake and decarboxylation). An alternative terminology of neuroendocrine cells as paraneurons highlights similarities but also certain differences between them and neurons (Fujita, 1977). Certain endocrine cell types have been widely used as models for the study of secretory mechanisms, and as we have seen in chapter 2, parallels may exist with mechanisms regulating secretion at synapses in the central nervous system. Since the original APUD classification was proposed for these cells, data obtained with experimental approaches unavailable at that time have confirmed the common presence of "marker" proteins in different cell types of this series, which is one of the principal motivations behind this book. The multiplicity of common features of APUD cells led Pearse (1969) to suggest that all had a common embryonic (neural crest) origin. Subsequent advances in transplantation techniques have afforded the possibility of verifying this hypothesis. It has frequently been confirmed: chromaffin cells of the adrenal medulla may thus be viewed as modified neural crest derived sympathetic neurons in which certain neuronal characteristics such as neurite formation and neurofilament and synapsin expression are suppressed. In addition, the origin of the adenohypohysis, classically taught in medical schools to derive from the ectoderm of the stomodeum (primitive mouth), has been shown to have a neural origin, since cells destined to form this endocrine tissue are found in the neural ridge at early embryonic stages, but migrate away before formation of the neural crest (Couly and Le Douarin, 1985, 1987). Thus future hypophyseal cells may be subject to neural inductive influences before their arrival in the epithelial layer of the roof of the stomodeum. Notable exceptions to this hypothesis do however exist. The same elegant transplantation techniques demonstrating the neural origin of hypophysis have failed to confirm a similar origin for the pancreatic islet endocrine cells which are derived from cells situated in the ducts (see chapter 1; Le Douarin, 1978; Teitelman et al., 1987; Teitelman and Lee, 1987; Pearse, 1983).

4.3.6.1 Immunocytochemistry

The existence of certain common properties between neurons and neuroendocrine cells prompted us several years ago to investigate whether the same

adhesive mechanisms between cells operate in brain and the endocrine system. The presence of NCAM was first tested in adrenal medullary cells with their confirmed neural crest origin (Langley and Aunis, 1984) and later extended to a wider range of endocrine tissues, including those of non-neural origin and tumor cells (Langley et al., 1987, 1989a).

The presence of NCAM in chromaffin cells of the adrenal medulla has been demonstrated or confirmed by light microscopy by several laboratories (Jørgensen and Richter-Landsberg, 1983) and biosynthetic studies show it to be

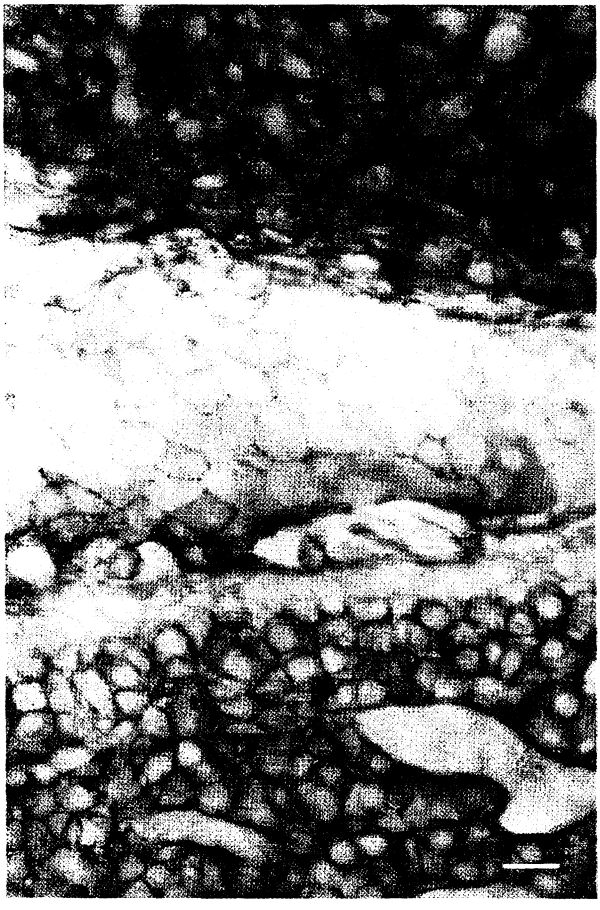

Fig. 4-4 Vibratome section of rat hypophysis immunoperoxidase labeled for NCAM.
Note the very intense staining of the neural lobe (top), the discrete surface labeling of cells in the intermediate lobe (middle) and the moderate surface labeling of anterior lobe cells (bottom). Bar = 20 μm.

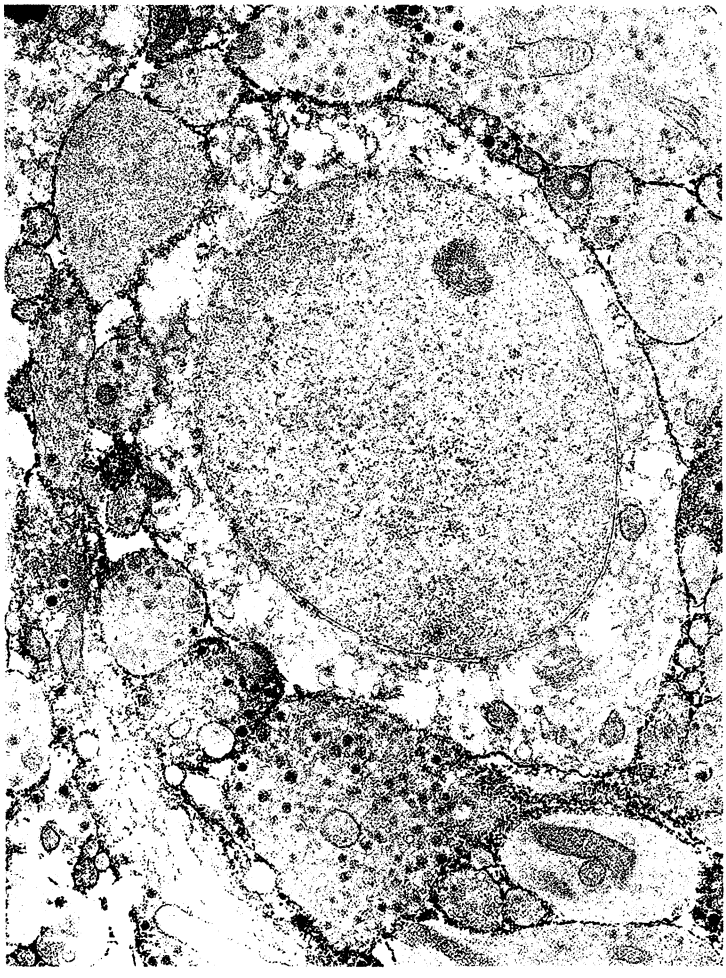

Fig. 4-5 Electron micrograph of rat neurohypophysis immunostained for NCAM, illustrating the surface staining of a pituicyte surrounded by neurosecretory cell, granule-containing, terminal swellings, which are also labeled on their surface. Section not counterstained. Bar = 1 μm.

synthesized in cultured rat chromaffin cells (Nybroe et al., 1986). Ultrastructural studies unequivocally show its membrane localization on both endocrine cell types (both adrenergic and noradrenergic) in this tissue. Little or no immunoreaction product has been detected intracellularly, which as for Purkinje cells in the cerebellum, may be the consequence of combined rapid transport and insertion into the plasmalemma after synthesis and a relatively slow turnover rate. Precise values on absolute amounts of NCAM in adrenal

medulla are not available but in the adult rat, levels are much lower than in cerebellum (unpublished observations). Some of this is present on both unmyelinated nerve fibres and the Schwann cells surrounding them.

When other endocrine tissues were examined including the three lobes of the pituitary gland, the pancreas, tumor cell lines (Langley et al., 1989a) and certain human tumors (see below), an identical surface localization of NCAM was found immunocytochemically (Figs. 4-4–4-7). However, both qualitative and quantitative differences between different endocrine tissues have been found. For instance, in addition to the pronounced surface labeling of secretory cell processes, terminal swellings and nerve terminals constituting the neuroendocrine elements of the neurohypophysis, extracellular staining was observed using polyclonal anti-NCAM antibodies (Langley et al., 1989a). Extracellular localization of this molecule has also been reported in muscle (Sanes et al., 1986; Rieger et al., 1988). Since NCAM contains a heparin binding site (Cole and Glaser, 1986; Cole et al., 1986a,b) with a relative high affinity (Nybroe et al., 1989) capable of binding it to collagen fibrils by the intermediary of laminin, it is reasonable to suppose that any NCAM lost from the cell surface would adhere to neighbouring basement membrane components. Wether the loss of

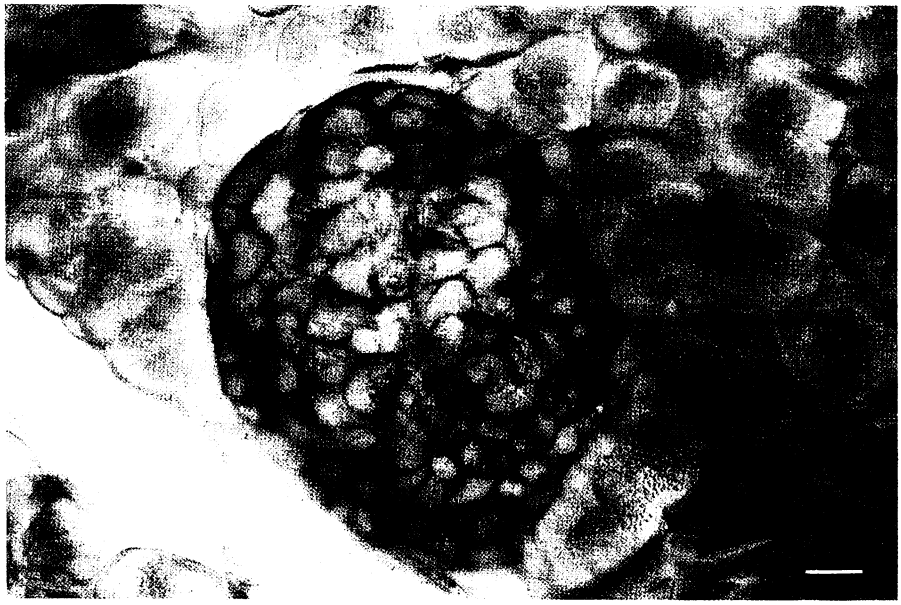

Fig. 4-6 Vibratome section of rat pancreas through an islet of Langerhans labeled for NCAM.
The surface labeling of all cells in the islet contrasts with the absence of staining in the exocrine pancreas. Bar = 20 μm.

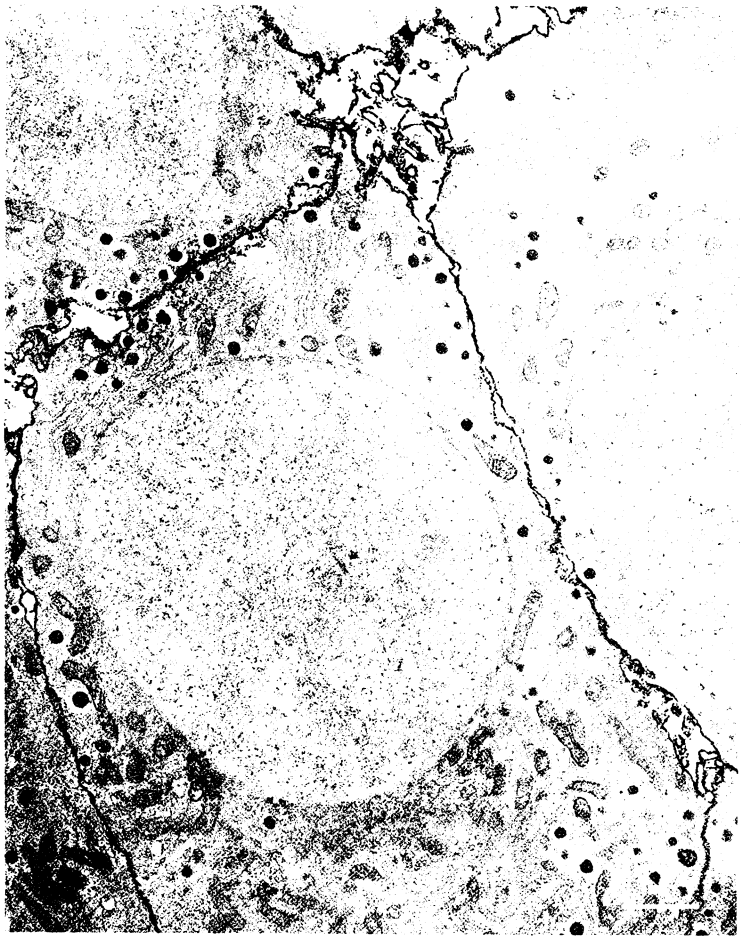

Fig. 4-7 Electron micrograph of rat endocrine pancreatic cells immunostained for NCAM.
The immunoreaction is confined to the plasma membranes. Section not counterstained. Bar = 1 μm.

NCAM from cell surfaces is a normal phenomenon in healthy tissue is not known though a soluble secreted form of NCAM with a Mr 120 kD has been shown to exist (Bock et al., 1987). However, levels of NCAM 120 are very low in the neurohypophysis and at the present time the molecular nature of NCAM found extracellularly has not been resolved.

One of the striking features of the immunstaining pattern of rat pituitary gland sections concerns variations in labeling intensity found between the three lobes constituting this tissue (Fig. 4-4). The neural lobe is the most heavily

stained and the anterior lobe is moderately labeled, while intermediate lobe endocrine cells appear very discretely labeled at their surface (Langley et al., 1989a). Apparent differences of intensity of immunolabeling should always be treated with caution in immunocytochemistry. Similar differences were however observed on immunoblots when extracts from identical amounts of adeno- and neurohypophysis were compared. It is, however, difficult to evaluate whether such overall differences in tissue concentrations reflect real differences in NCAM membrane densities in the two lobes, since their cellular composition differ radically: the anterior lobe of the hypophysis consists essentially of aggregates of cell bodies with few cell processes while the neurohypophysis, apart from a relatively small number of glial cells, which also express NCAM (Fig. 4-5), consists entirely of cell processes and terminals. Thus the membrane surface area is much larger on the basis of tissue weight in the latter compared to the former.

Pancreatic tissue is characterized by the absence of NCAM staining of exocrine cells and the marked surface labeling of all endocrine cells of the islets of Langerhans, irrespective of the nature of their secretory products (Figs. 4-6 and 4-7). Several years ago an NCAM related antigen was found to be present in rat testis localized on spermatids but not on mature spermatozoa (Jørgensen and Møller, 1983). Our recent preliminary studies on rat testis have also shown that groups of endocrine cells (Leydig cells) situated between the seminiferous tubules are immunoreactive with anti-NCAM sera. In addition, marked extracellular staining was observed in this tissue.

Molecular biological probes are currently being used to examine tissue distribution of the NCAM message. A major advantage of such techniques is their potential to study expression of individual exons in the transcript. Thus it is now possible to study not only the message for the major NCAM isoforms but also to detect the presence and analyze tissue distribution of the minor exons recently discovered. Using S1 nuclease protection assays the presence of NCAM mRNA has been confirmed in adrenal gland, neuro- and adenohypophysis and pancreas (our unpublished results).

4.3.6.2 NCAM Isoforms in Endocrine Tissues

Immunochemical analysis of NCAM polypeptide profiles of brain and endocrine tissue extracts revealed unexpected differences (Fig. 4-8 and Langley et al., 1987, 1989a). Although by immunocytochemistry with polyclonal anti-NCAM antibodies very similar cellular staining patterns are obtained with brain and endocrine tissues, the relative amounts of individual NCAM polypeptides in endocrine cells differ radically from those in brain: in addition one major exception was found amongst the endocrine tissues studied. NCAM

140 is the principal isoform expressed in rat adrenal gland, adenohypophysis and pancreatic islets but NCAM 180 is predominant in neurohypophysis. Cultured bovine adrenal medullary chromaffin cells may express measurable amounts of NCAM 180 (our own unpublished results; see also Nybroe et al., 1986) but this could be due to the increased tendency of such cells to express a neuronal phenotype, as illustrated by the appearance of neurofilament proteins in cultured chromaffin cells though these are undetectable *in vivo* (Grant et al., 1988). In addition, this could also be the result of species differences in the expression of NCAM 180 by endocrine cells. Few data exist at the moment on interspecies NCAM polypeptide expression in endocrine cells. Analysis of human tissues indicate that NCAM 140 is the predominant form present in adenohypophysis and endocrine lung tumors (Aletsee-Ufrecht et al., 1990b).

The neurohypophysis appears to represent an exception to the idea that the endocrine NCAM phenotype is NCAM 140. All three major NCAM polypeptides are found in this tissue although NCAM 120 is a minor constituent. The fact that the cell processes that form the bulk of this organ derive from perikarya situated in paraventricular and supraoptic nuclei of the hypothalamus, thus sharing similar embryonic origins with other central nervous system

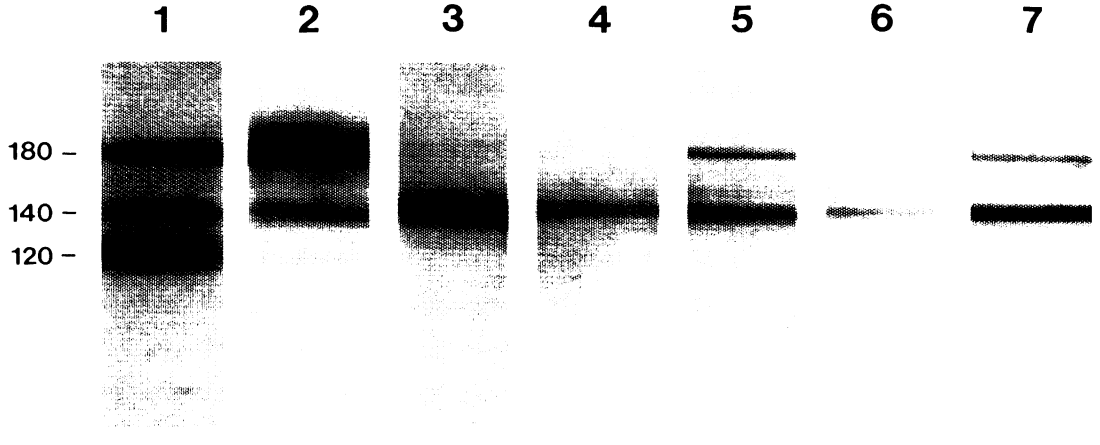

Fig. 4-8 Immunoblots showing NCAM isoforms in cerebellum (1), neurohypophysis (2), adenohypophysis (3), adrenal medulla (4), rat pheochromocytoma (5), pancreatic islets (6) and rat insulinoma cells (7).
Extracts of rat tissues and cells were resolved by polyacrylamide gel electrophoresis, transferred to nitrocellulose, and reacted with polyclonal anti-NCAM antibody. Immunoreactive bands were visualized with radiolabeled protein A and autoradiography. Molecular masses (kD) are indicated (from Aletsee-Ufrecht et al., 1988); by permission.

neurons, may be a sufficient reason to expect that their NCAM profile should more closely resemble that of CNS neurons. We have seen, however, that embryonic origin is not strictly determinant in NCAM expression. The structure of the neurohypophysis is quite different from that of other endocrine organs in that it is composed of cell processes that are very distant from their cell bodies. Studies on cultured neurones subjected to agents inducing neurite growth (Pollerberg et al., 1985, 1986) suggest that some compartmentalization of NCAM 180, perhaps due to an interaction with the cytoskeleton (Pollerberg et al., 1987), may occur between cell body and cell processes, the concentration being greater on the latter. Thus the higher levels of NCAM 180 found in neurohypohysis may be the result of such compartmentalization. In any case, these data show that all endocrine cells are not identical with regard to NCAM polypeptides, and that some may better suit the prefix "neuro-" in this context. It is interesting to note that the NCAM form that persists in adult endocrine cells is the form that is reported to be first expressed by very early embryos even before neural induction occurs (Levi et al., 1987; Jacobson and Rutishauser, 1986) and that NCAM 180 is considered by some to be the more characteristic form of differentiated post-mitotic neurons (Pollerberg et al., 1985, 1986). As is reviewed later, NCAM 140 is also the form found in certain strictly non-neural cells. Endocrine cells may thus be viewed as expressing more primitive less characteristically neuronal NCAM forms.

4.3.6.3 Endocrine Tumors

The PC12 cell line, derived from a rat adrenal medullary pheochromocytoma, has provided neurobiologists with a useful model for studying both endocrine cell biology and neuronal differentiation and maturation. Since these cells also express NCAM, their response to nerve growth factor NGF in neurite outgrowth formation has also provided the opportunity of studying NCAM expression during such phenomenon. Conflicting data have been published on the stimulatory effect of NGF (Doherty et al., 1988) or the absence of an effect (Friedlander et al., 1986) on certain forms of NCAM in PC12 cells. These differences may be the result of using different sublines of these cells. NCAM is essentially localized on the surface of PC12 cells as has been found for other endocrine and neural cells, and NCAM 140 is the predominant though not exclusive isoform present. Walsh's group observed both increased levels of NCAM 180 and NCAM mRNA of 7.2kb (the message considered to encode NCAM 180) after NGF treatment (Prentice et al., 1987), suggesting that this isoform may be more concentrated on neurite-like extensions. Elevated levels of this form have also been noted in another experimental endocrine tumor, a

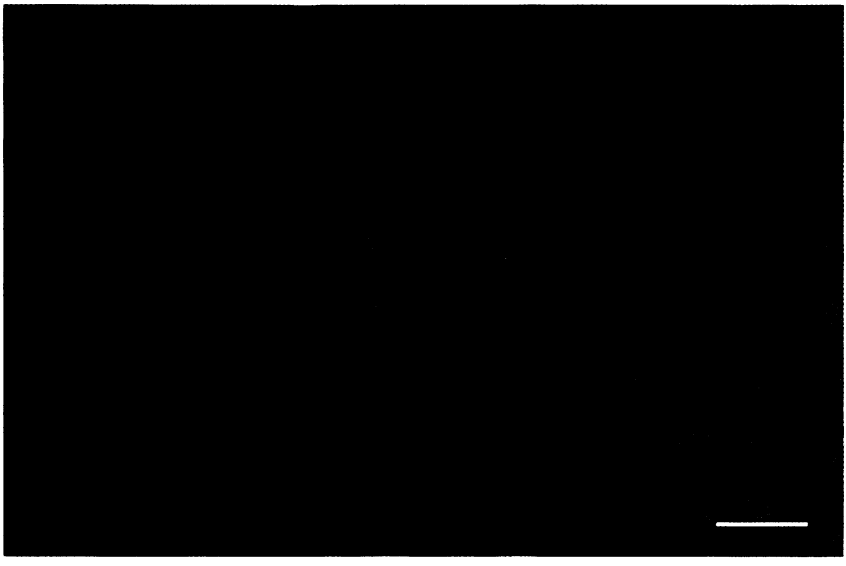

Fig. 4-9 Cultured rat insulinoma (RIN A2) cells labeled for NCAM by immunofluorescence. Note the pronounced surface labeling. Bar = 20 μm.

rat insulinoma (Fig. 4-9), compared with their normal counterparts (Langley et al., 1987). Rat insulinoma cells in culture tend to produce thin cell process unlike normal pancreatic β cells although these are less pronounced than those of NGF treated PC12 cells. Thus increased NCAM 180 expression may not be a directly related to the malignant transformation, but associated with cell morphological changes.

It is interesting to note that the expression of another CAM first identified in PC12 as **N**GF **i**nducible **l**arge **e**xternal (NILE) glycoprotein (McGuire et al., 1978) and subsequently shown to be equivalent to the cell adhesion molecule NgCAM of chick (Friedlander et al., 1986) and L1 in rodents (Bock et al., 1985) parallels that of NCAM 180 (Pigott and Kelly, 1986): it too is upregulated after NGF treatment. In fact the expression of L1 and NCAM 180 in many tissues is similar and may in part be due to an association between these two molecules (Thor et al., 1986) particularly on cell processes. Antibodies against L1 have been found to strongly stain neurohypophysis (Langley et al., 1989b;), which also contain high levels of NCAM 180: in contrast adenohypophysis and pancreatic islets are weakly or not at all stained for L1 nor do they contain NCAM 180. Chromaffin cells of the adrenal medulla represent an enigma, since only 20% appear to express L1 strongly in the rat adrenal which contains very little NCAM 180.

Two main classes of human endocrine tumors have been recently studied with regard to their NCAM expression; lung tumors and hypophyseal tumors. We have studied the small cell lung carcinoma (SCLC) on cell lines established from several patients (Fig. 4-10, Aletsee-Ufrecht et al., 1990a). These cell lines grow best in suspension culture but can be grown on coverslips, facilitating immunocytochemistry, where they retain a rounded cell morphology and their endocrine phenotype as exemplified by the presence of synaptophysin and neuron specific enolase. Immunoblot analysis has shown that NCAM 140 is the predominant if not exclusive NCAM form expressed. Other lung tumors examined included mesotheliomas, squamous cell carcinomas (i. e. non-endocrine tumors) and "large cell carcinoma". NCAM was found only in the latter: it is likely that these tumors are derived from SCLC. NCAM levels were found to vary between different SCLC patients, and even between two lines derived from tumors of the same patient at different times. Levels of other cell markers were also found to vary but in different directions. It can be concluded that the regulation of NCAM synthesis varies during the course of the disease and that such regulation parallels neither that of vesicle or souble proteins.

We have also recently examined human pituitary tumors for the presence of NCAM (Aletsee-Ufrecht et al., 1990b) including prolactinomas, growth hormone producing adenomas, and inactive adenomas, which were each characterized on the basis of clinical features, immunohistochemical data or

Fig. 4-10 Cultured human small cell lung carcinoma (SCLC) cells immunofluorescently labeled for NCAM showing marked surface labeling. Bar = 20 μm.

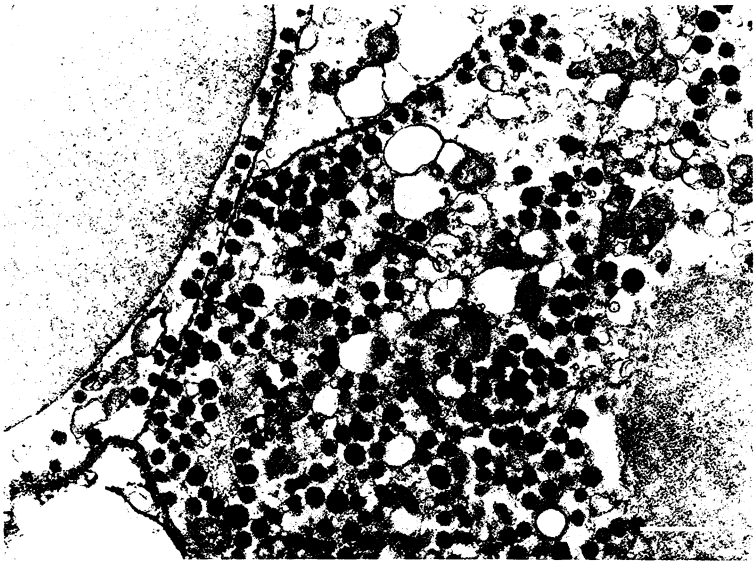

Fig. 4-11 Electron micrograph of human growth hormone producing adenoma immunostained for NCAM, illustrating the intense surface labeling of these cells. Section not counterstained. Bar = 1 μm (from Aletsee-Ufrecht et al., 1990b, by permission of Elsevier, Amsterdam).

serum hormone analyses. NCAM 140 was detectable on immunoblots of all but prolactinomas. Highly sensitive immunocytochemical methods confirmed a surface localization of NCAM in all these tumors (Fig. 4-11) even in prolactinomas, although staining intensity was much less in prolactinomas, confirming reduced levels in this tumor.

The presence of NCAM in certain human tumors may also be responsible for the staining observed with other anti-tumor antibodies of previously undetermined antigen specificity. From a number of monoclonal antibodies developed against a variety of tumors, some have been shown to recognize the NCAM molecule (Patel et al., 1989). In addition the staining of tumors with anti HNK-1 (Leu7) antibodies (chapter 7; Willison et al., 1986) could be due to a subset of NCAM molecules carrying the HNK-1 carbohydrate hapten.

4.3.7 Fibroblasts

Fibroblasts represent a further ambiguous class of cells on which apparently contradictory data have been published. Earlier reports produced no evidence

of NCAM in fibroblasts. Fibroblasts in normal peripheral nerve in endo-, peri- and epineural situations are NCAM negative in adult tissue and also at all developmental ages studied (Seilheimer and Schachner, 1988; Martini and Schachner, 1988; Seilheimer et al., 1989). Fibroblasts isolated from skin and brain have not been found to contain NCAM. In contrast, immunocytochemistry demonstrates NCAM expression by fibroblast-like cells which accumulate in nerve stumps after crush lesions or section of peripheral nerves and by perisynaptic fibroblasts in denervated muscle (Gatchalian et al., 1989). Fibroblasts isolated from both peripheral nerve and skeletal muscle and subsequently maintained in culture also have the capacity to express NCAM (Gatchalian et al., 1989). The isoform expressed is NCAM 140, which is the same as that found in peripheral nerve Schwann cells. Mesenchymal cells possibly related to fibroblasts in regenerating newt limbs have also been found to be NCAM positive (Maier et al., 1986). The capacity to express NCAM may thus be confined to particular subsets of fibroblasts or be induced either in culture or in pathological situations in which increased NCAM synthesis is known to occur: NGF activity also increases in peripheral nerves after injury.

4.3.8 Muscle

The considerable number of studies devoted to both adult and developing skeletal muscle since the initial discovery of NCAM in embryonic muscle (Grumet et al., 1982) have provided valuable insight into the regulation of NCAM synthesis and its molecular biology. We will not deal in detail here with muscle and the reader is referred to a recent review (Walsh, 1988) for a more detailed analysis. A brief discussion however of NCAM in muscle is pertinent in the context of its potential as a neural cell marker. It was the first tissue outside the nervous system in which NCAM expression was found to persist into adulthood. While NCAM is detectable in all primitive germ cell layers in early embryos, defined tissue inductive events lead either to its suppression or to its continued expression (Edelman, 1984). During skeletal muscle histogenesis, down-regulation of NCAM during myotube maturation and innervation is associated with a dramatic change in its localization on the surface membrane of the muscle cell. The wide distribution over the entire myotube surface is replaced in the adult by restricted expression over specialized contact regions, the neuromuscular junctions. This adult topographical distribution can be reversed to that found in immature muscle by denervation.

Analysis of the polypeptide nature of muscle NCAM illustrates how different tissues can process these molecules in specific ways. Initial immunoblot studies

on muscle suggested a molecular mass for this antigen similar to that of brain NCAM 140. Further investigations showed that muscle NCAM mRNA contains an additional exon, not present in brain, encoding an amino acid sequence unique to muscle, and it was suggested that the isoform present in differentiated myotubes is equivalent to a modified NCAM 120, which, like this brain isoform, is anchored to the plasma membrane by phosphatidyl inositol (Moore et al., 1987).

4.3.9 Other Non-Neural Tissues

A rare but unusually malignant kidney tumor (Wilms tumor) represents a particularly interesting example of NCAM re-expression in pathological tissue. NCAM is detectable during early development of the kidney, but it is subsequently down-regulated and is absent from the adult gland (Roth et al., 1987). In Wilms tumour NCAM has been found to be not only strongly expressed, but as the highly sialylated "embryonic" form (Roth et al., 1988). The malignant transformation may thus be interpreted in terms of a reversion of the normal differentiation and maturation process leading to a cell with more primitive characteristics. Elevated levels of high PSA containing NCAM undoubtedly influences the contact relationships and "social behaviour" of these cells, properties which many years ago had been suggested to be linked to cell surface sialic acid content (Langley and Ambrose, 1964).

NCAM has been detectable in a few other non-neural tissues. Several adhesion molecules have been fond in rodent intestine with different distributions (Thor et al., 1986). NCAM appears to be confined to mesenchymal and neuroectodermally derived parts of the intestine. In particular it was found to be located in the outer muscle layers, not only in Auerbach's plexus but also in mesenchymal parts, and it was present in the submucosa, the muscularis mucosa, the lamina propria surrounding the crypts and in the inner part of villi. Surprisingly, all three NCAM isoforms were reported to be present, thus reflecting the CNS pattern rather than that found in peripheral nervous or non-nervous tissues. Individual NCAM containing cellular elements were unfortunately not identified in this study. Thus it is not known if intestinal endocrine cells contain NCAM. Some staining in the muscularis mucosa and in the lamina propria may be due to neurons or their processes.

Chicken skin has been found to contain NCAM (Chuong and Edelman, 1985a,b) which is similar or identical to that of brain except in its polypeptide profile: only NCAM 140 is detectable in skin (Murray et al., 1986). This persists into adulthood and is subject to the embryonic-adult post-translation modification described for the brain antigen. It is associated with the morphogenesis

of chicken feathers, a process which continues throughout adult life. A cyclic pattern of both NCAM and LCAM (equivalent to the calcium dependent cell adhesion molecule E-cadherin) expression is found in growing and regenerating feather buds. In addition to membrane expression, NCAM is also located in certain extracellular regions after cell disappearance. NCAM mRNA has also been detected by the blot hybridization technique in embryonic heart, breast muscle, gizzard, lung and kidney (Murray et al., 1986). In non-neural tissues NCAM 180 mRNA has been only detectable in association with nervous elements such as the nerve plexuses in developing lung and the large enteric ganglia of gizzard. Most data suggest that when NCAM is present in non-neural tissues NCAM 140 is the isoform generally found.

4.4 Expression of NCAM During Development

Attempts to relate NCAM function to tissue structure and in particular to tissue inductive events have strongly motivated studies of its expression throughout embryonic development. Major "decisions" which determine the course of histogenesis are made relatively early in embryonic life. Radical changes in tissue levels of cell adhesion molecules which either stabilize intercellular contacts or promote cellular migration may be expected to coincide with this period of intense activity of cell sorting and reorganisation. The pattern of NCAM expression is indeed extremely plastic during early embryonic life (Edelman, 1983, 1984b). This is also true in adult tissues subject to inductive changes (Chuong and Edelman, 1985a,b). It is beyond the scope of this chapter to review in detail such complex changes in NCAM regulation during nervous tissue and endocrine tissue development. Readers are referred to the excellent studies by Edelman's group. In pioneering studies Thiery and coworkers (1982) reported the very early appearance of NCAM in embryonic tissues. NCAM is detectable in all three primitive germ layers, the endoderm, the mesoderm and the ectoderm, and has been shown to be present in those regions concerned with primary developmental axes (neural plate, neural tube, notochord, somites) and in those regions in which later inductive events occur such as the neural crest, optic, otic and pharyngeal placodes, cardiac mesoderm, mesonephric primordium and limb buds. When tissue inductive events occur, NCAM expression may be either suppressed permanently or temporarily or maintained according to the nature of induction.

A number of studies have addressed the problem of changing levels of NCAM during nervous tissue development. Levels vary between different

brain regions or tissues during development (Bock et al., 1983; Linnemann et al., 1985). Levels are reduced more in myelinated tracts than in regions containing cell bodies (Daniloff et al., 1986b). Cerebellar development is a relatively late event compared with rat forebrain, occurring during the first three weeks of postnatal life. NCAM levels are low before birth but increase dramatically during the period of parallel fibre (granule cell axon) formation and synaptogenesis corresponding to a period of dramatic increase of neuronal surface area (Jørgensen and Honegger, 1983). Cerebellar NCAM subsequently decreases to a fairly constant level. This may be correlated with data from biosynthetic studies on cultures of rat brain cells obtained at embryonic day 17 and at postnatal day 25, which demonstrate a 50 % reduction in overall level (Nybroe et al., 1986). Analysis of NCAM mRNA levels show that the levels of the transcription product are reduced during brain development three times more than that of the translation products (Gennarini et al., 1986). This may be interpreted in terms of a slower NCAM turnover as development proceeds or a higher NCAM mRNA stabilization. A slower turnover rate of NCAM has been suggested after insertion into plasma membranes after axonal transport in the optic system (Garner et al., 1986). Interestingly, even within the cerebellum a differential distribution of individual NCAM polypeptides has been found during development (Pollerberg et al., 1985). While NCAM is detectable by immunocytochemistry with polyclonal antibodies recognizing all NCAM polypeptides at earliest times in the germinative layer of the cerebellum, NCAM 180 is not detectable in this zone. This polypeptide appears to be confined, in the molecular layer, to post-mitotic neurons. Regional differences have also been noted using specific probes capable of distinguishing between NCAM 180 and NCAM 140 messages (Murray et al., 1986). The NCAM 180 message has been detected as early as the third day of embryonic life in chick spinal cord but was not found in neuroepithelium. Although NCAM 140 message was detectable in embryonic heart, breast-muscle, skin, gizzard lung and kidney, NCAM 180 mRNA was found to be absent from these tissues and only detectable in tissues containing neural elements (such as the large enteric ganglia in gizzard or the nerve plexuses in oesophagus and lung buds). Very recent *in situ* hybridization studies confirm these earlier findings (Prieto et al., 1989).

A particularly relevant example of developmental NCAM up- and down-regulation in the context of this chapter is found in cells which constitute the adrenal medulla. The endocrine chromaffin cells are derived, as are sympathetic ganglia, from NCAM positive precursors in the neural crest (Thiery et al., 1982). When these cells migrate away from the neural crest towards their future site NCAM expression is suppressed. At the same time substrate adhesion molecules are upregulated (Duband et al., 1986). The reaggregation of these cells during the histogenesis of the adrenal gland coincides with

renewed expression of NCAM 140, and is accompanied by down-regulation of fibronectin receptors. These data have been interpreted in terms of the stabilizing effect of NCAM on intercellular contacts, which would be counter-productive during the migration phase, but which are critical during formation of the adrenal medulla. In addition, since the cortical cells surrounding the centrally situated groups of adrenal medullary chromaffin cells do not express NCAM, a mechanism of "cell sorting" may operate in defining the particular tissue architecture of this endocrine gland (see Friedlander et al., 1989).

4.5 Post-Translational Developmental Modifications

The dramatic modifications in the expression of NCAM that occur during development or in post-traumatic or pathological situations underlined earlier can take a number of different forms. We have seen that tissue levels of total NCAM, and accordingly membrane density, is dependant on endogenous environmental stimuli. Such factors may also differentially influence the transcription of individual mRNA species coding for the different NCAM isoforms independently of each other. It is not yet known if these various NCAM mRNAs differ in stability or translation efficiency. In addition post-translational modifications represent a major aspect of these glycoproteins. NCAM is both phosphorylated and sulfated in its transit through the Golgi apparatus (Lyles et al., 1984b). No significant alterations in phosphorylation are thought to accompany nervous tissue development, and it would appear that they are unlikely to have major functional significance. Moreover, activators of protein kinase C, which is involved in the phosphorylation of many proteins, stimulates the synthesis of certain members of the immunoglobulin supergene family but do not modify NCAM synthesis (Doherty et al., 1988). In contrast the level of sulfation decreases during development (Lyles et al., 1984b; Linnemann et al., 1985).

The post-translational modification which has received most attention concerns glycosylation. Expression of the HNK-1 carbohydrate epitope and more recently another carbohydrate epitope called L3 have been studied (Kruse et al., 1984, 1985; Keilhauer et al., 1985; Kücherer et al., 1987) but the degree of sialylation of NCAM has been investigated in most detail. From a functional viewpoint, since the sialic acid content profoundly affects the adhesive binding capacity of NCAM, such modifications assume as much importance as NCAM membrane density. Sialylation of NCAM is unusual in as far as forms of NCAM with high sialic acid levels (as much as 30% of the

molecule) contain this sugar in the form of the α-2,8 **p**olysialic **a**cid (PSA, Cunningham et al., 1983; Finne et al., 1983). At the very earliest stages of embryonic development PSA levels appear to be low. Then NCAM with high PSA content is produced, and subsequently PSA content is reduced as development proceeds but certain zones with high PSA NCAM persist in the adult (Sunshine et al., 1987; Aaron and Chesselet, 1989; Rougon et al., 1990). Since the appearance of the three principal NCAM isoforms is not simultaneous during development (NCAM 120 appears somewhat later than the other two) it is not clear if all forms are poly-sialylated to the same degree. Data from explant cultures of mouse and chick tissues suggest that the conversion from high to low PSA NCAM is not due to processing of the embryonic into the adult form, but by *de novo* synthesis of NCAM with lower sialic acid content and that this is due to intracellular regulation of sialyl transferase (Friedlander et al., 1985). More recently (Breen et al., 1987, 1988) found that an endogenous Golgi sialytransferase was capable of sialylating NCAM 180 and NCAM 120 *in situ*, though curiously obtained no evidence of NCAM 140 sialylation under their conditions. In addition highest tissue activities of this enzyme coincide with highest tissue levels of NCAM PSA.

In pathological tissues increased PSA NCAM levels (Daniloff et al., 1986a) has been linked to the process of cellular reorganisation. In contrast in developing cerebellum no correlation was found between reduction in PSA levels and the migratory status of cerebellar granule neurons (Nagata and Schachner, 1986).

At present no direct data exist on NCAM sialylation in neuroendocrine tissues. In the adult it is likely to be relatively low since immunoblots reveal the presence of discrete NCAM bands. Some data suggest that in the neurohypophysis PSA levels may be higher (Langley et al., 1989a).

4.6 Concluding Remarks on NCAM as a Neuronal and Neuroendocrine Cell Marker

The original concept that NCAM is confined in the adult to neurons and moreover with restricted localization to specialized regions of the neuronal membrane has been gradually eroded over the past few years. Clearcut evidence has demonstrated its expression by other neural cells in both PNS and CNS and also by endocrine cells independant of whether their embryological origin is neuroectodermal or not. In addition the presence of NCAM in adult muscle and certain other non-nervous tissues of adult chicken including skin and lung confirms the wider than neural distribution of this molecule. The

cellular expression of NCAM cannot thus be taken as an absolute criterion of neuronal character. NCAM 140 may be regarded as the more typical isoform of neuroendocrine cells and may provide an additional parameter to characterize putative neuroendocrine cells in non-nervous tissues. For example the expression of NCAM 140 by SCLC contrasts with its absence in other lung tumors such as mesotheliomas or adenomas, confirming the endocrine nature of this tumor.

In contrast NCAM 180 appears to be more characteristic of post-mitotic mature neurones than of other neural cells in their terminally differentiated state. Strictly non-neural cells do not appear to express this isoform, although the capacity of paraneurons to synthesize NCAM 180 does not seem to be permanently lost. As has been shown for pheochromocytoma PC12 cells, stimuli which predispose differentiation towards a neuronal phenotype may up-regulate synthesis of NCAM 180. In quantitative terms, however, the expression of NCAM is heavily biased towards the nervous system. Levels found even in neural crest-derived tissues are much lower than those of the CNS. In addition, levels of NCAM have been found to vary between different types of endocrine tumor. For instance, human prolactinomas appear to contain less than growth hormone producing or inactive adenomas of the hypophysis. This may itself be of use to distinguish between tumors particularly if the prolactinoma is inactive.

Nevertheless it is clear that the use of NCAM as a marker for neural and neuroendocrine cells should be treated with caution. No single marker protein fulfills the criterion of absolute specificity. Together with a battery of other marker proteins NCAM isoforms can aid cellular characterization and diagnosis of tumors. Polysialylation of NCAM polypeptides represents an unusual post-translational modification probably unique to members of this family of proteins. Its reappearance or continued presence in adult tissues seems to be associated with either altered intercellular contact phenomena following a pathological process or with the need to retain a higher degree of plasticity in cell contacts in normal tissues. Its presence in Wilms tumor suggests it would be a good marker in this tissue and prompts its search in other tumors. A recent report (Moolenaar et al., 1990) showed the presence of high levels of NCAM sialylation in a cell line derived from an endocrine lung tumor. It is possible that high PSA NCAM forms may be generally associated with the dedifferentiated state of malignantly transformed neural and neuroendocrine cells.

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