STUDIES OF THE RAT CNS PLASMA MEMBRANE-ASSOCIATED NONPERMISSIVE/INHIBITORY FACTOR ON CELL ADHESION AND NEURITE OUTGROWTH

by

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ABSTRACT

In higher vertebrates, lesions in the central nervous system (CNS) are irreversible due to the lack of regenerative growth from the injured axons. Evidence has shown that, in addition to neurotrophic factors, there exist some negative neurite outgrowth inhibitors. Recent studies have identified collapsin in embryonic and adult chick brain, myelin-associated neurite outgrowth inhibitory proteins from rat CNS, inhibitory glycoproteins of posterior somite and optic tectum in the chick, and myelin-free membranes from injured brain tissues.

Using cultures of neuroblastoma X glioma hybrid NG108-15 cells as an in vitro neurite outgrowth assay we have identified a plasma membrane-associated nonpermissive substrate protein factor from adult rat CNS. The purified plasma membrane fraction was prepared by homogenizing tissues in isotonic buffer, followed by ultracentrifugation on a stepwise sucrose density gradient. Upon extraction under alkaline conditions of 0.1 M NH_4OH, a solubilized protein fraction was obtained for coating dishes as substrate for cell cultures. Precoating with the solubilized plasma membrane proteins from rat CNS prevented cell adhesion and differentiation of NG108-15 cells, which remained undifferentiated as clusters of round cells after 24 hours in

culture.

Using a cell number bioassay of NG108-15 cells, nonpermissive substrate activity in plasma membranes from newborn rat brain, brainstem and spinal cord was observed to increase gradually during early postnatal development. A substantial amount of nonpermissive activity was found in the CNS of early postnatal rats even before the process of myelination, which occurred on postnatal day 12-15. In addition, nonpermissive activity was also observed in plasma membranes from demyelinated CNS tissues of rats after postnatal treatment of 5'-azacytidine. Furthermore, the plasma membrane from adult rat grey matter was also a highly nonpermissive substrate, though slightly less than that of the white matter. All these findings indicate that the rat CNS plasma membrane-associated nonpermissive activity is not highly related to myelination, and appears to differ from the reported myelin-associated neurite outgrowth inhibitory proteins.

This solubilized inhibitory protein was partially purified by precipitation with 0~50% saturation of ammonium sulphate, followed by gel filtration on Sepharose CL-4B, or by column chromatography on DEAE-cellulose, or by non-denaturing preparative polyacrylamide gel electrophoresis. Upon analytical SDS-polyacrylamide electrophoresis of partially

purified materials, the plasma membrane-associated inhibitory principle appeared to be a protein of molecular weight 50~60 KDa. The activity was relatively heat-stable but labile at acidic pH and sensitive to trypsin digestion. The physicochemical properties and molecular mass of this plasma membrane-associated nonpermissive protein distinguish it from other known neurite outgrowth inhibitor factors.

These results indicate that the alkaline extract of plasma membranes of adult rat CNS is highly nonpermissive for neuritogenesis, and a membrane-associated protein is responsible, partly or totally, for the inhibitory activity in the developing and differentiated CNS.

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LIST OF ABBREVIATIONS

Units of Measurement

g	gram
mg	milligram
μg	microgram
ml	millilitre
μl	microlitre
mm	millimetre
min	minute
hr	hour
M	molar
mM	millimolar
mA	milliampere

General Terms

5'-AZ	5'-azacytidine
BDNF	brain derived neurotrophic factor
BSA	bovine serum albumin
CAM	cell adhesion molecule
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate
CNPase	2', 3'-cyclic nucleotide-3'-phosphohydrolase
CNS	central nervous system
CNTF	ciliary neurotrophic factor
dBcAMP	N^6, 2'-O-dibutyryladenosine 3': 5'-cyclic

	monophosphate
DEAE	diethylamonioethyl
DMEM	Delbeco's Modified Eagles Medium
DRG	dorsal root ganglia
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EGTA	ethylene glycol-bis(β-aminoethyl ether) N,N,N'-tetraacetic acid
FCS	fetal calf serum
FGF	fibroblast growth factor
xg	unit of gravitational force
GAP-43	growth associated protein-43
IM	injured tissue membrane
KDa	kilodaltons
MAG	myelin associated glycoprotein
Mr	molecular weight
NGF	nerve growth factor
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PLP	proteolipid protein
PMSF	phenylmethyl-sulphonyl fluoride
PNA	peanut agglutinin
PNS	peripheral nervous system
SDS	sodium dodecyl sulphate
Tris	Tris(hydroxymethyl)aminomethane

1. INTRODUCTION

1.1 Development and myelination of the mammalian central nervous system

Neuroepithelial cells of the neural tube are thought to give rise to the neurons, astrocytes, oligodendrocytes and ependymal cells that form the vertebrate CNS (Jacobson, 1978). During CNS development, glial precursors proliferate in subventricular zones and then migrate through the CNS to adopt their final destinations and differentiate into various types of mature glial cells (Smart, 1961; Lewis, 1968; Privat & Leblond, 1972; Paterson et al., 1973; Paterson, 1983). Oligodendrocyte precursors originate at the ventral ventricular zone dorsal to the ventral midline region in the embryonic rat spinal cord (Noll & Miller, 1993). The precursor cells, oligodendrocyte-type-2 astrocyte (O-2A), are bipotential, and differentiate in a later stage of development into oligodendrocytes and type-2 astrocytes (fibrous astrocyte) in culture. Type-1 astrocytes have a fibroblast-like morphology and are found in cultures of both CNS white matter and grey matter. Type-2 astrocytes have a process-bearing morphology resembling neurons or oligodendrocytes and are seen in significant numbers only in cultures of white matter and probably correspond to fibrous (fibrillary) astrocytes (Raff et al., 1983).

Myelin is a product of the oligodendrocyte, which can often direct their processes formed from plasma membranes to distant target axons (Davison and Peters, 1970; Raine, 1977). These extensions seek out an axon, establish a connection and proceed to envelop the axonal cylinder with a continuous wrapping of compacted membrane. Formation of myelin follows a set pattern (Raine, 1977; Caley & Butler, 1974). Once contact is made between the oligodendrocyte and axonal membrane, the glial membrane begins to wrap around the axonal membrane until a complete circuit is accomplished and the extracelluar faces adhere. At this point, a compaction process is initiated within the cytoplasmic space which results in the association of the two apposing bilayers and the creation of a major dense line (so-called because of its appearance in electron micrographs). Concurrent compaction of the outer leaflets of the oligodendrocyte plasma membrane forms the intraperiod line, which is the less-electron dense extracellular counterpart to the major dense line. The resulting multilayers are thus composed of repeating units. The highly ordered spiral array of membrane and aqueous compartments can, depending on the species, achieve an appreciable thickness on 5-20 multilayers.

In general, myelination occurs in a caudal to rostral direction in rodent CNS over the period from birth to approximately 30 days of age. However, myelin protein

synthesis continues in the adult, and a significant level of mRNA for both myelin basic protein (MBP) and proteolipid protein (PLP) is found in adult rats. It is observed that during development, myelin constituents are synthesized by the oligodendrocytes at two intervals separated by a five day time lag. The early appearance of galactosylceramide (GalC) and Wolfgram protein (W1) occurs just after differentiation of the progenitor cell when the oligodendrocyte is still undergoing cell division. A postmitotic late appearance of MBP and PLP occurs shortly before the oligodendrocyte begins to myelinate axons.

Myelin is a lipid-protein membrane construct contained within the CNS and PNS of vertebrates. Acting primarily as an electrical insulator, myelin controls and increases the speed of signal transmission along the axon from the nerve cell body to the synaptic junction. The membrane encircles the axon at a number of discontinuous points or internodes and forms the nonconducting counterpart to the Nodes of Ranvier. Myelin is very rich in lipids (70% dry weight) (Norton et al., 1984), among which galactocerebroside (GC) has been identified as a specific marker for the oligodendrocyte (Raff et al., 1978). In addition to lipids, rodent CNS myelin contains 30% proteins, which consist mainly of proteolipid protein (PLP; 50% of total protein), myelin basic protein (MBP; 30-50% of total protein), 2', 3'-cyclic nucleotide-3'-phosphohydrolase

(CNP; 5% of total protein), myelin-associated glycoprotein (MAG; <1% of total protein), and several enzymes. Other minor components of the myelin have not been fully characterized.

Proteolipid protein (PLP), also called lipophilin because of its propensity to bind lipid (Mosarello, 1976), is an intrinsic membrane protein with an approximate molecular mass of 30 KDa and constitutes the single most abundant protein of myelin (Boggers & Moscarello, 1982). Studies have shown that the PLP sequence is highly conserved between species. For example, rat and human PLP are essentially identical, differing at position 95 (Ser in rat, Ala in human), while only four substitutions appear in the bovine protein. In genetic studies of actively myelinating animals, the transcriptional rate of PLP as well as MBP is coincident with the appearance of compact myelin within the CNS (Milner et al., 1985). This observation emphasizes the important relationship between myelin compaction and the appearance of the major myelin proteins.

Myelin basic protein (MBP), comprising 30-40% of the total protein in the CNS myelin (Deber & Reynolds, 1985; Smith, 1992), actually refers to a family of structurally related polypeptides, in rodents, 4 major forms with Mr of 14, 17, 18.5 and 21.5 KDa. MBP has a high isoelectric point (> 10), and can bind acidic proteins or phospholipids through

ionic interactions (Smith, 1992). The exact physiological role(s) of MBP has yet to be defined, though it has been suggested that it is responsible for the maintenance of the compact structure of myelin sheath through protein-lipid and protein-protein interactions (Smith, 1992). Labelling studies have shown that MBP is contained exclusively within the cytoplasmic spaces of myelin (Harris & Findlay, 1983), and can be readily isolated in its lipid-free form by acid extraction of chloroform/methanol-solubilized myelin. Determination of functional role(s) of MBP within myelin is further complicated by the observation that, depending on the species, MBP is present in several molecular forms (i.e., proteins of differing molecular weights) (De Ferra et al., 1985; Takahashi et al., 1985). At the present time, there is no known function unique for these MBP isoforms.

The isolation of myelin is generally based on ultrcentrifugation in discontinuous sucrose gradients taking advantage of the uniquely low buoyant density of the membrane. Both of sedimentation procedures (Bejamins et al., 1973; Norton and Posuslo, 1973; Zimmerman et al., 1975) and techniques utilizing the flotation principle (Cruz et al., 1985; DeVies et al., 1983; Fujimoto et al., 1976; Konat et al., 1985; Waehneldt & Mandel., 1972) have been described for the isolation of CNS myelin. The myelin fraction isolated by density gradient centrifugation is generally a mixture of

membranes related to the process of myelin formation, consisting mostly of myelin lamellae besides oligodendroglial plasma membranes and other regions of transition to myelin, paranodal glial membranes, newly formed myelin and possibly some axolemma still attached to myelin. In an attempt to separate these different myelin particles, a number of investigators has subfractionated the crude myelin fraction and obtained biochemically and morphologically distinct subfractions of light and heavy myelin (Bejamins et al., 1976; Zimmerman et al., 1975), myelin-like fraction (Agrawal et al., 1970; Sabri et al., 1975), myelin-related fraction (Waehneldt & Mandel, 1972; Waehneldt et al., 1977), and plasma membranes (Agrawal et al., 1974; Poduslo, 1975; Sun et al., 1988). The higher lipid content of the myelin membranes permits their separation from other cellular membranes. Procedures to separate relatively pure myelin from plasma membranes have been reported for rat brain tissues (Agrawal et al., 1974; Sun et al., 1988) and oligodendroglia (Poduslo, 1975; Polak & Szuchet, 1988).

1.2 Nerve regeneration in the central nervous system after injury

Invertebrates and lower vertebrates are quite capable to regenerate axons in their central nervous system (CNS). If the brain or spinal cord of a fish or axolotl is damaged, the

cut axons will begin to regenerate within a few days, will regrow back to their original targets and will form functional connections there: not only will axons regenerate, but also they show great accuracy in their ability to find and remake appropriate connections with their original target (Davis et al., 1990; Gaze & Jacobson, 1963; Sperry, 1963; Attardi & Sperry, 1963). Frogs are at an intermediate stage, being able to regenerate axons in their optic nerves, but not in most of the rest of their CNS (Lyon & Stelzner, 1987). Regeneration in the optic nerve may have been preserved because it is still a developing structure, new retinal ganglion cells being born throughout the life of an animal, and producing axons which have to grow through the optic nerve to the brain (Gaze et al., 1979; Beach et al, 1979).

The ability to regenerate axons has been gradually lost during evolution, and in mammals is almost completely absent. Why is this useful repair function lost? One reason may be due to evolutionary changes in properties of the microenvironment of the CNS. It is interesting and possibly significant that the loss of regeneration in the CNS in vertebrates coincides with the loss of the ability to regenerate other body parts. Axolotls, for example, will regenerate whole limbs if they are removed as well as axons in the CNS (Brokes & Kintner, 1986; Keeble & Maden, 1989). Adult frogs, however, cannot regenerate limbs, and neither can they

regenerate most of the axons of their CNS, although tadpoles can do both. The ability to regenerate cannot be of much evolutionary benefit, so it is theoretically possible that some chance genetic change could have led to its loss. It is also conceivable that some benefit accrues from restricting the regenerative ability of animals, although it is difficult to see what that benefit might be.

In higher vertebrates, neurite outgrowth in the CNS ceases at the end of the developmental period. Lesions in the differentiated CNS are irreversible due to the almost complete lack of regenerative growth from the injured axons (Cajal, 1928). In obvious contrast, peripheral axons regenerate well (Sunderland, 1972). There seem to be three possible mechanisms responsible for the failure of axons to regenerate in the CNS: (1) the relative impenetrabilities of astrocytic tissue; (2) the decrease in vigour of axon growth from neurons as they become mature; and (3) the presence of inhibitory factors.

1.3 Factors influencing nerve regeneration in higher vertebrate CNS

1.3.1 The relative impenetrabilities of astrocytic tissue

There are three types of glial cells around the CNS

neurons in higher vertebrates: astrocyte, oligodendrocyte and microglia. Nerve cell bodies are surrounded by glial cells. There are between 10 and 50 times more glial cells than neurons in the central nervous system of vertebrates. Glial cells are probably not essential for processing information, but they are thought to have important roles in the nervous system. When a region of the CNS is damaged there is immediate death of cells in the damaged area, and axons passing through this region are cut. The axons distal to the cut degenerate, as does their myelin, and this debris is slowly removed by microglia. Astrocytes in and around the damaged area become reactive, undergoing cell division, and increase in content of cytoskeletal materials, leading to a glial scar, so called because of its white scar-like appearance microscopically. The glial scar consists of many fine, closely apposed, interweaving astrocytic processes, interspersed with varying numbers of oligodendrocytes. The CNS environment surrounding an axon, which is trying to regenerate, therefore varies with time after damage. Initially there is much axonal and other debris, including degenerating myelin in the white matter. Later, the environment becomes a glial scar. Regeneration of axons does not succeed and astrocytes and oligodendrocytes are present throughout the process. Either or both of these processes may be responsible for providing the inhibition of growth after CNS injury.

The ultrastructural appearance of damaged CNS has been studied. Axon tips in contact with axons are quite different in appearance to axons that have gotten stuck in scar tissue in a peripheral neuroma. In the peripheral neuronal neuroma, the axons are swollen and bloated with cytoskeletal material, vesicles and other membranous organelles, while axons in contact with astrocytes appear as if they have received a normal physiological stop signal, and are trying to form a synapse: the axon endings are not swollen, do not contain much cytoskeletal material, and may even contain structures that look like synaptic vesicles (Liuzzi & Lasek, 1987). There is also evidence that tissues composed primarily of astrocytes can block the regeneration of axons. Glial scars in the CNS contain very few oligodendrocytes, but axons will not penetrate them; and such glial scars can be transplanted into peripheral nerves, where they will also block the regrowth of PNS axons (Reier & Houle, 1988). Further, optic nerves which are free of oligodendrocytes, either by transplanting from late embryonic animals which have not yet developed oligodendrocytes, or by obtaining them from the mutant oligodendrocyte-deficient rat, are still not very conducive to axonal regeneration (Giftochristos & David, 1988; Reier & Houle, 1988; Marciano et al., 1990; Berry et al., 1989). Despite of the in vivo evidence that astrocytic tissues can block axon regeneration, it has been difficult to reproduce this inhibitory effect in vitro; indeed, monolayer cultures of

astrocytes provide a particularly advantageous surface for the growth of axons from most neurons (Noble et al., 1984; Tomaselli et al., 1988). A method for culturing astrocytes as a more physiological three-dimensional tissue has shown that this tissue is not penetrated by axons regenerating from postnatal neurons, although the more vigorous axons from the embryonic neurons are able to do so (Fawcett et al., 1989).

Astrocytic tissues are clearly inhibitory to axon growth, but the mechanism by which they become inhibitory is less clear, although there are some clues. Astrocytes, like oligodendrocytes, produce molecules which are inhibitory to axon growth cones (Keynes et al., 1990). The astrocytic growth cone collapsing activity is probably due to the same molecules which exclude axons from the posterior half of each embryonic somite (Davis et al., 1990). There is also in vitro evidence that the glial scar surrounding an area of damage produces substances which are inhibitory to axon growth when neurons are placed onto tissue sections of the damaged brain (Rudge & Silver, 1990). A possible mechanism for the inhibitory effect of astrocytic tissues centres on the proteases and protease inhibitors secreted by many cells. Invasive malignant tumour cells secrete large quantitaties of proteases, and if these are blocked, the cells cease to be invasive (Ossowski, 1988). Static tissues, such as astrocytes, secrete a preponderance of protease inhibitors

(Gloor et al., 1986). Axon growth cones are a specialised form of invasive cell process, and also secrete proteases (Pittman & Patternson, 1987). If one of these, for example serine protease, is inhibited, the growth of axons from embryonic neurons into astrocytic tissue is reduced (Fawcett & Housden, 1990).

1.3.2 The decrease in vigour of axon growth from neurons as they become mature.

The neuron usually goes through two main phases in its existence. First it has to migrate to its correct site, and produce an axon which has to grow accurately through the complex terrain of the developing nervous system to form the right connections with its targets. If the neuron lives through the crisis of neuronal cell death, it then settles down to a very different second function, that of processing and transmitting information. This second phase does not call for axon growth in the sense of active elongation led by a growth cone, although the axon may have to stretch as the organism grows. All that is normally required is some ability to form and retract connections at the target neurons, in order to subserve the processes of neuronal plasticity. To some extent each neuron seems to process its own program which tells it how far its axon needs to grow during development. This is reflected both in the rate of axonal growth, and in

the actual length of processes. If a variety of neurons are removed from an embryo during their normal period of axon growth, and are then examined in tissue culture, their rate of axon outgrowth correlates well with the distance between the neurons and its normal target (Davies, 1989). That there is a program which determines length of outgrowth is shown by cross-species transplant experiments, in which neurons from large animals (human) are implanted into smaller ones (rat). Embryonic human striatal neurons have been implanted into adult rat, and grow their axons for a very much greater distance than the equivalent embryonic rat cells implanted into the same site (Wictorin et al., 1990). In view of the developmental changes in neuronal function, which comes with maturity, and the finishing of the normal axonal growth programme, it is perhaps surprising that any adult axons are able to regenerate, but in fact more or less all axons do retain some regenerative ability into adulthood. However, regeneration from adult neurons is less vigorous than axon outgrowth from the same neurons examined during development. Transplants of many types of embryonic neurons into favourable sites in an adult brain can make extensive connections with the host, axons from the transplant growing for considerable distances. Transplants of adult or even early postnatal brain are generally much less successful, because most of the neurons die, and because those few that survive do not generally grow axons (Bjorklund & Stenevi, 1984). The same

pattern is seen if one examines the reciprocal connections, from host brain to transplant. Embryonic transplants into early postnatal brain may receive extensive axonal connections from the host, while transplants into adult animals are much less well innervated by host axons (McLoon & Lund, 1983). The same sort of observations can be made in tissue culture: a tissue culture model of a glial scar is penetrated hardly at all by axons from postnatal neurons, but quite readily by axons from embryonic neurons (Fawcett et al., 1989). The mechanism of the decline in axon growth remains to be elucidated.

There is evidence that the axonal cytoskeleton in regenerating axons may differ from that in embryonic axons: the microtubule associated protein (MAP1) is expressed during embryogenesis but not in regeneration, and regenerating but not embryonic frog retinal axons grow in spirals, probably due to some cytoskeletal properties (Grant & Tseng, 1986; Woodhams et al., 1989). Most of the cytoskeletal genes expressed in development, however, are re-expressed during regeneration (Miller et al., 1989; Hoffman & Cleveland, 1988). In general, it seems likely that some neuronal genes are permanently turned off after the axon growth program has run its course. In some pathways regenerating axons have different cell surface adhesion molecules on their surface compared to developing ones, and some of these adhesion molecules may not

be appropriate for the damaged tissue surrounding the regenerating axon (Cohen et al., 1987; Hall et al., 1987; Grant et al., 1986).

The growth-associated protein-43 (GAP-43) is an axonal phosphoprotein which is expressed at high levels during development and is reinduced by regeneration in the PNS (Skene & Willard, 1981; Hoffman, 1989; Van Der Zee et al., 1989). Consequently it is believed to be a key molecule in the regulation of axonal growth (Stene & Willard, 1981; Benowitz et al., 1981). However, the induction of GAP-43 expression is not sufficient to endow these neurons with the capacity to regrow their axons (Ng et al., 1988; Tetzlaff et al., 1991; Doster et al., 1991; Verhaagen et al., 1993).

1.3.3 Inhibitory factors to neurite outgrowth in mammalian CNS

The ability of neurons to regenerate their axons can be influenced by the same trophic factors that are needed to keep the neuron in the adult animal (Lindsay, 1988). It is possible that manipulation of the neurotrophic and neurotropic factors surrounding a damaged neuron might be sufficient to revitalise its axon growth. For example, Schwann cells in peripheral nerves are surrounded by basement membranes containing laminin. This extracellular matrix protein is the

most potent substrate for neurite growth (Edgar et al., 1984), and it acts synergistically with neurotrophic factors such as NGF, which are known to be produced at injury sites (Abrahamson et al., 1986). Since laminin is virtually absent in the adult CNS of higher vertebrates, it has been argued that the pattern of expression of laminin might determine whether regeneration can occur. However, the levels of both laminin and NGF are elevated at lesion sites in the CNS. In addition, Schwann cells in peripheral nerves produce a variety of neurotrophic factors and even increase the production after denervation. The hypothesis that there is a difference in the production of trophic factors between the PNS and CNS being responsible for their different regeneration capabilities seems plausible. However, identified neurotrophic factors, such as nerve growth factor (NGF), ciliary neurotrophic factor (CNTF), brain-derived neurotrophic factor (BDNF), and fibroblast growth factors (FGF-1 & FGF-2), are present in the developing and adult CNS. Also, increases in laminin and other neurotrophic factors have been found at CNS lesion sites (Barde et al., 1982; Korsching et al., 1985; Liesi, 1985; Whittemore et al., 1985, 1986, 1987; Needles et al., 1986; Shelton & Richardt, 1986). These results strongly argue against the hypothesis that the lack of CNS regeneration is mainly due to an absence, or insufficient production, of neurotrophic factors by denervated glial cells (Cajal, 1928).

Why are the reexpressed neurotropic and neurotrophic factors unable to trigger functional regeneration as in the PNS? Recent studies by several research groups have suggested that this failure of regeneration in the higher vertebrate CNS is not due to neurons being innately incapable of regenerating their axons, but in instead due to the environment of the mature nervous tissue being in some way inhibitory to axonal growth. There are three main types of experiments that demonstrate this hypothesis. The first is to observe the behaviour of axons that regenerate well in the PNS trying to grow in the CNS; an example of this is the axons of the sensory neurons in the dorsal root ganglia. These axons bifurcate near the cell, one branch going to peripheral sensory organs, and the other into the spinal cord. The peripheral branch will regenerate to the periphery when cut, and the central branch will also regenerate in the dorsal root (which has the structure of peripheral nerve) if this is damaged, but regeneration ceases when axons reach the spinal cord (Bignami & Dahl, 1976; Carlstedt et al., 1987; Liuzzi & lasek, 1987). A second type of experiment is to take CNS tissue (optic nerve) and graft it into a peripheral nerve. Here it acts as a barrier; axons will regenerate up to the graft, but will not grow into it (Weinberg & Spencer, 1979; Aguayo et al., 1978; Anderson & Turmaine, 1986; Hall & Kent, 1987). The third type of experiment is to graft a tissue that is permissive to axonal regeneration (a length of peripheral

nerve) into the CNS. Neurons close to the graft will, in this case, regenerate their axons for considerable distances through it (Benfey & Aguayo, 1982; David & Aguayo, 1981; Richardson et al., 1984; Blakemore, 1984; So & Aguayo, 1985). The conclusion to be drawn from these experiments is that: there seem to be no obvious differences between neurons from the CNS and PNS in their ability to grow back after injury, but interactions between axons and their immediate environment play a determinant role in the success or failure of regeneration. Neurons in the CNS are able to regenerate their axons, if the CNS glial environment is changed to that of the PNS. The mature CNS glial environment is a nonpermissive environment for axons to regenerate via some unknown mechanisms.

Recent studies have shown the existence of neurite growth inhibitory signals. Some in vitro observations could not be explained simply by a lack of trophic factors or growth-promoting substrates. Also biological and biochemical studies have led to the isolation of membrane-bound proteins that have inhibitory activity, from various tissues, including the developing and adult CNS. In addition, certain neurotransmitters have been shown to arrest growth cones. Also multifunctional molecules that can exert both positive and negative effects have been found.

1.3.3.1 Inhibitory glycoproteins in chick embryo somites

In early embryonic development, neural crest cells migrate out from dorsal neural tube to form the ganglia of the PNS (Davies & Lumsden, 1990). Later, motor axons grow out from the spinal cord. Crest cells and motor fibres are found exclusively in the anterior part of the forming sclerotomes (Keynes & Stern, 1984). Neither N-CAM, N-cadherin, fibronectin, nor laminin are differentially distributed in the anterior and posterior somites; however, tenascin has been found to be associated with the migrating neural crest cells themselves (Stern et al., 1989). Interestingly, lectin peanut agglutinin (PNA) staining is sharply localized to the posterior half of each somite (Davies et al., 1990; Stern et al., 1986). In coculture experiments, cells from the posterior positions of somites appear to arrest neurite growth (Stern et al., 1986). By using lectin-affinity chromatography, two molecules of molecular weight 48 KDa and 55 KDa have been identified from posterior somitic tissue (Davies et al., 1990). These proteins are membrane bound and have to be solubilized with detergent. A rabbit antiserum raised against the 48 KDa and 55 KDa components eliminated the growth cone collapsing activity (Davies et al., 1990). Proteins that cross-react with the antibodies are found in grey, but not white matter of adult chicken brain (Keynes et al., 1990). These studies showed that a negative signal,

probably associated with the 48 KDa and 55 KDa PNA-binding membrane glycoproteins, is present in the posterior halves of sclerotomes. This signal is present before any contact with neural crest cells or axons and appears to influence cell migration and axon growth in the forming PNS. The exact identities of these factors remain to be investigated.

1.3.3.2 A molecule with an inhibitory activity on neurite growth involved in map formation in the retinal tectal system

Retinal axons project in a topographic manner onto the optic tectum: the temporal retinal fibres terminate in the anterior part, and the nasal fibres in the posterior part of the tectum. This model system for neuronal pathfinding has been studied extensively in vitro and in vivo (Starling, 1991; Udin & Fawcett, 1988). In tissue culture experiments, embryonic chick retinal fibres extend preferentially on tectal, as compared with retinal, cell monolayers. In similar experiments, retinal axons respond differentially to tectal cells from different positions along the anterior-posterior axis of the tectum. That temporal axons preferentially extend on cells from more anterior tectal regions is in agreement with the normal distribution of these fibres in vivo (Bonhoeffer & Huf, 1982). This finding is consistent with the assumption of a gradient of a molecule present in the tectum and recognized by temporal retinal axons. Nasal axons show no

preference for monolayer from any part of the tectum.

The above findings have been confirmed in a new assay system using membrane preparations, instead of cell monolayers (Walter et al., 1987b). Using this assay, temporal retinal axons again have a strong preference for anterior, rather than posterior, tectal membrane. This preference is lost after heat treatment of the posterior tectal membrane, but, interestingly, not after treatment of the anterior tectal membranes (Walter et al., 1987a). This suggests that the observed preference is not the result of an attraction of the temporal fibres to the anterior tectal membranes but rather their avoidance of posterior membranes, probably due to the presence of a heat-sensitive inhibitory activity. Similar inhibitory activities could be detected in retinal membrane preparations from fish and mouse (Godement & Bonhoeffer, 1989; Vielmetter & Steurmer, 1989). Biochemical studies have shown that an activity that causes growth cone collapse and avoidance in posterior membranes can be removed by phosphatidylinositol-specific pospholipase C (PI-PLC), indicating that the relevant molecule is attached to the membrane with a glycolipid anchor. Studies on specific binding of the active molecule to PNA lectin and enhanced incorporation into lipid vesicles assign the activity to a 33 KDa glycoprotein (Stahl et al., 1990). This protein is more abundant in posterior in tectal membranes and disappears on or

about embryonic day 15 (E15), when the inhibitory activity disappears from the posterior tectal membranes. After incorporation into lipid vesicles, the purified molecule is active and induces growth cone collapse (Stahl et al., 1990). Analysis of the behaviour of frog retinal growth cones that are in contact with dissociated tectal cells in vitro indicates the presence of a similar neurite growth inhibitory activity on the surface of radial glial cells (Johnston & Gooday, 1991). But whether this activity is related to the 33 KDa glycoprotein from chick tecum is currently not known.

1.3.3.3 Oligodendrocyte and CNS myelin-associated inhibitors of neurite growth

In established primary cultures from optic nerves of young rats, glial precursor cells, astrocytes, immature oligodendrocytes and mature myelin-producing oligodendrocytes could be distinguished. When central or peripheral neurons from newborn animals are added to these cultures, neurons, irrespective of their origin, attach to most cells in these cultures, but spare one type of glial cell; the web of growing neurite soon form "windows" around the mature oligodendrocytes. The oligodendrocyte surfaces not only inhibit the movement of neuronal growth cones, but also the spreading and locomotion of other cell types. When frozen sections of spinal cord are used as culture substrata, cells

become attached mostly to the grey matter, indicating that the CNS white matter is a highly nonpermissive substratum (Carbonetto et al, 1987; Savio & Schwab, 1989). The inhibitory property of oligodendrocytes persists even in the presence of neurotrophic factors and has been observed for a variety of cultured neurons, as well as neuroblastoma and PC12 cells. Spreading and migration of 3T3 fibroblasts are also inhibited (Schwab & Caroni, 1988).

In a video time-lapse study, DRG growth cones growing on a laminin substrate in the presence of NGF were compared with respect to their interactions with individual astrocytes and oligodendrocytes (Bandtlow et al., 1990). Contact with oligodendrocytes always leads to rapid arrest of neurite growth, whereas contact with astrocytes results in an unchanged or slightly reduced growth velocity. Interestingly, the contact of the tips of the growth cone filopodia with the processes of oligodendrocytes is sufficient to arrest these growth cones, suggesting that second messengers may be involved. Growth arrest is long lasting (hours), but is strictly local, i.e. other neurites arising from the same cells continue to grow normally. These findings indicate that oligodendrocytes of the CNS actively inhibit neurite outgrowth by a contact-mediated mechanism.

Since oligodendrocytes are the myelin producing cells of

the CNS, Caroni and Schwab (1988a) attempted to isolate the inhibitory components from spinal cord myelin. Polypeptide bands from preparative SDS-polyacrylamide gel electrophoresis of myelin proteins are cut out, eluted, and reconstituted into lipid vesicles. Liposomes containing eluted proteins of Mr 250 kDa and 35 kDa (called NI250 and NI35, respectively) inhibit fibroblast spreading and neurite outgrowth. Similar results are obtained for reconstituted polypeptides of the same molecular sizes extracted from membranes of oligodendrocyte-containing CNS cell cultures. In contrast, liposomes containing similar proteins from PNS myelin are permissive substrates. Immunological and biochemical studies suggest that NI250 and NI35 are closely related; NI250 seems to be a complex containing NI35 (Bandtlow & Schwab, 1991). Neither activity, nor the corresponding protein bands, can be found in goldfish or trout CNS, a result that accords with the known growth-promoting surface properties of oligodendrocyte-like cells isolated from goldfish optic nerves (Bastmeyer et al., 1991; Caroni & Schwab, 1988a) suggesting that fish do not possess the relevant inhibitory molecules. This property could explain the capacity for fibre regeneration in the CNS of lower vertebrates.

In the presence of neutralizing antibodies against NI35 and NI250, DRG neurites can grow over oligodendrocytes in culture and into optic nerve explants (Bandtlow et al., 1990;

Caroni & Schwab, 1988b). For these in vivo experiments, two independent paradigms have been chosen: application of the inhibitor-neutralizing antibody IN-1 (Schnell & Schwab, 1990), and elimination of oligodendrocyte precursor cells by X-irradiation of newborn rat spinal cord and optic nerve (Savio & Schwab, 1990). In young rats with bilateral thoracic spinal cord transections, completely interrupting the corticospoinal tract (CST), the capacity of the CST to regenerate has been studied in control animals and in animals treated with the inhibitor neutralizing antibody IN-1 or in oligodendrocyte- and myelin-free rats. In the control animals, CST fibres become sprouted for distance of about 1mm at the lesion site, but there is no elongation beyond this distance. In contrast, some CST fibres become elongated up to 10~20 mm in the experimentally treated animals, and in the majority of cases extended for at least 4~7 mm (Savio & Schwab, 1990; Schnell & Schwab, 1990).

These studies suggest that NI-35/250 can exert boundary functions for late growing CNS tracts and, in target areas, can restrict access of fibres to particular regions and layers. Their presence in the adult CNS suggests a possible important function: the stabilization of the CNS against sprouting in unwanted regions, particularly in the white matter.

1.3.3.4 Plasma membrane-associated neurite growth inhibitors

The surfaces of neighbouring axons are generally a good substrate for neurite growth both in vitro and in vivo (Wessels et al., 1980). Tissue culture experiments have shown that rat sympathetic fibres and rat retinal fibres mix freely when tissue explants of the same type are placed close to each other in a tissue culture dish (Bray et al., 1980). However, when a retinal explant is placed close to a sympathetic explant, the outgrowing fibres do not mix, and, after some time in culture, form separate territories. Time-lapse video microscopy indicates that this selective fasciculation is the result of a mutual inhibition between retinal and sympathetic fibres (Kapfhammer et al., 1986).

Later studies have shown that the observed inhibition between heteronymous sets of growth cones and neurites is a relatively common phenomenon in the vertebrate nervous system. It appears to be present between most combinations of PNS and CNS neurons, but, importantly, is absent between all homonymous pairs studied (Kapfhammer & Raper, 1987). Growth cone collapse specifically induced by heteronymous neurite surfaces has since been found in several other systems. Examples in the CNS include the avoidance of nasal retinal axons by temporal retinal growth cones (Bonhoeffer & Huf,

1986; Raper & Grunewald, 1990), the avoidance of tectobulbar neurite by temporal retinal growth cones (Kroger et al., 1991), and in the PNS, the avoidance of DRG axons by preganglionic sympathetic growth cones (Moorman & Hume, 1990).

Clearly, a variety of inhibitory molecules on neuronal cell surface can be expected to account for the inhibitory activities associated with different nerve fibres that act only on particular growth cones. As a first step to purify these molecules, Raper & Kapfhammer (1990) developed an assay that allows the rapid testing of solubilized biochemical fractions of inhibitory activity. This assay is based on the collapse of growth cones after its exposure to an inhibitory cell surface molecule. This collapse reaction is very similar to growth cone behaviour after contact with an inhibitory neurite. Based on the observation that DRG cones are inhibited by CNS neurites (Kapfhammer & Raper, 1987), purification of the inhibitory activity for DRG growth cones from E10 chick brain has been attempted. The activity can be solubilized by the detergent CHAPS and has been greatly enriched through several steps of column chromatography (Raible & Raper, 1990; Raper et al., 1992; Raper & Kapfhammer, 1990). The purified activity is inhibitory to growth cones of sensory and retinal ganglion cells.

This membrane-associated growth cone collapsing

component, designated as collapsin, has recently been partially purified from adult chick brain membranes as a 100 KDa glycoprotein and further been cloned and sequenced. Collapsin can induce the collapse and paralysis of neuronal growth cones in vitro; it is effective at concentrations of ~10 pM. The C-terminal half of collapsin contains a single immunoglobulin-like domain and an additional highly basic region. The N-terminal half of collpsin shares significant homology with fasciclin IV, a growth cone guidance protein in grasshopper. Recombinant collapsin causes sensory ganglion growth cones to collapse but not retinal ganglion cell growth cones (Luo et al., 1993).

Purified myelin-free membranes from kainic acid-injured adult rat brain tissues, though not from normal brain tissues, have been observed to be highly inhibitory for neurite initiation. The inhibitory activity present in injured tissues has been solubilized by detergent, observed to be sensitive to glycosaminoglycan lyase digestion, and characterized as a proteoglycan complex with an apparent molecular weight of 160~220 KDa (Bovolenta et al., 1993). These findings indicate that the plasma membrane, in addition to myelin, of the mammalian CNS contains highly nonpermissive molecule(s) inhibiting neurite outgrowth.

After injury to the adult avian or mammalian spinal cord,

there is little evidence of anatomical regeneration or functional recovery (Cajal, 1928; David & Aguayo, 1981; Shimizu et al., 1990). Embryonic CNS, however, exhibits greater repair abilities after damage. For example, chick embryos subjected to a complete transection of the thoracic spinal cord up to and including embryonic day (E) 12, of the 21-day developmental period, are capable of extensive anatomical repair and functional recovery. The repair is so extensive as to be anatomically and physiologically indistinguishable from untransected controls (Hasan et al., 1991; Shimizu et al., 1990). In this preparation, repair is mediated, at least in part, by the regeneration of axotomized brainstem-spinal fibres (Hasan et al., 1990; Hasan et al., 1991). Conversely, embryos transected after E12 show rapidly diminishing repair such that a transection on E15 resulted in no anatomical or functional recovery (Hasan et al., 1991; Hasan et al., 1992; Shimizu et al., 1990). These findings suggest that an initial permissive period for spinal cord repair is followed by a subsequent restrictive period. Ethell et al (1993) have demonstrated that the plasma membrane of embryonic chick spinal cord undergoes a developmental transition from permissive to nonpermissive substrates for neuritogenesis, and that the transition period occurs around embryonic day 13 of the 21-day developmental period. Cell surface plasma membranes were prepared from homogenates of embryonic chick spinal cord segments, and fractionated from

myelin by sucrose density gradient centrifugation. The plasma membrane proteins solubilized by ultrasonication were subjected to an in vitro assay using clonal NG108-15 cells to monitor effects of permissive and nonpermissive substrates. The chick spinal cord of early embryonic days was permissive, and the permissiveness decreased with development as the spinal cord and brain of late embryonic chick became highly nonpermissive.

1.3.4 Other factors with activity to arrest growth cones

1.3.4.1 Multifunctional properties of tenascin and related molecules

Tenascin is a multimeric ECM protein occurring in many organs, including the PNS and CNS. The subunits contain several fibronectin type III, as well as epidermal growth factor (EGF) repeats (Pearson, 1988; Reichardt & Tomaselli, 1991). Purified tenascin as a substrate for neurons or non-neuronal cells possesses poorly adhesive characteristics (Chiquet-Ehris-mann et al., 1988; Halfter et al., 1989; Lochter et al., 1991). Within the fibronectin repeats, domains with differential effects on neuronal migration and outgrowth seem to exist (Husmann et al., 1992). Tenascin can also mediate adhesion of neurons to astrocytes (Grumet et al., 1985; Kruse et al., 1985). Tenascin as a culture substrate

for neurons shows a dual effect: adhesion of cell bodies and cell spreading is relatively poor, but, neurite outgrowth is enhanced (Chiquet, 1989; Lochter et al., 1991). Surprisingly, the addition of soluble tenascin inhibits neurite growth on several types of substrates (Lochter et al., 1991). The oligodendrocyte-associated, tenascin-related ECM molecules J1 160/180 (Janusin) show similar dual functions as culture substrates for neuronal adhesion and outgrowth, which depend on divalent cations and state of aggregation (Pesheva, 1989, 1991). However, J1 160/180 repulses growth cones at substrate boundaries without leading to growth cone collapse (Schachner, 1992).

1.3.4.2 Glia of the midline and the optic chiasm

The dorsal midline of the spinal cord separates the dorsal columns and is not crossed by any fibres. Studies have suggested that the glial structure of the roof plate may play an active role in inhibiting the fibres, correlating with the immunohistochemical presence of keratan/chondroitin sulphate proteoglycans (Snow et al., 1990). Recently a 320 KDa proteoglycan has been purified that inhibits neurite outgrowth in vitro and is localized to the midline of the chick spinal cord and hindbrain (Cole & McCabe, 1991). There is, however, no direct demonstration of an inhibitory action of the spinal cord roof plate. The molecular basis of this phenomenon is

yet unclear.

1.3.4.3 Epidermis and Merkel cells

An inhibitory activity exists in chick epidermis, which is virtually free of sensory nerve fibres (Saxod, 1978). Using time-lapse video microscopy, Verna (1985) has demonstrated that sensory growth cones avoid epidermal cells and that this avoidance is present at a distance of up to 100 μm from the epidermal explant, suggesting the presence of a secreted inhibitory factor. In a subsequent study, this inhibitory activity could be abolished by culture of the epidermal explant in tunicamycin, indicating the involvement of sugar moieties (Fichard et al., 1990). The proteoglycan specific inhibitor β-D-xyloside and antibodies to chondroitin sulphate have also been shown to be effective in reducing the inhibitory activity from chick epidermis (Fichard et al., 1991). These results indicate that a chondroitin sulphate proteoglycan is likely involved in this inhibitory activity.

1.3.4.4 Neurotransmitters and electrical activity can arrest growth cones

Besides membrane-associated molecules, some neurotransmitters have been shown to arrest growth cones. For example, serotonin and dopamine exert a highly selective and

cell-specific growth inhibition on specifically identified neurons of the mollusc Helisoma (Haydon et al., 1984, 1987; McCobb et al., 1988). Similar responses have been described for the growth cones of dendrites of mammalian hippocampal pyramidal cells upon exposure to glutamate, whereas the axonal growth cones of these neurons are relatively unaffected (Mattson et al., 1988). The affected growth cones respond by an arrest of filopodial activity, followed by a transient collapse of growth cone structure. Other observations have shown that stimulation of embryonic D_1 dopamine receptors mediates a subset of chick retinal neurons (Landford et al., 1988). At the present time, however, the cellular mechanisms responsible for these responses are not certain.

2. RATIONALE OF THE INVESTIGATION

Neurons in the CNS of higher vertebrates fail to regenerate after injury. The microenvironment of the CNS has an active nonpermissive effect on cell adhesion and neurite outgrowth. Recent evidence suggests that myelin of the oligodendrocyte is responsible for the inhibitory effect. Two myelin proteins of Mr 35 KDa and 250 KDa have been identified as the potent myelin-associated inhibitors on neurite outgrowth (Caroni & Schwab, 1988).

Recently, other membrane associated inhibitory proteins have also been identified in neural and other tissues, including collapse of axonal growth cones and thus retraction of neurites. Plasma membranes from chick embryonic brain have been found to contain a component that causes collapse of growth cones of dorsal root ganglia neurons in culture (Raper & Kapfhammer, 1990). This membrane associated growth cone-collapsing component, designated as collapsin, has recently been partially purified from adult chick brain membranes as a 100 KDa glycoprotein and has been cloned and sequenced (Lou et al., 1993). Furthermore, purified myelin-free membranes from kainic acid-injured rat brain tissues, though not from normal brain tissues, have been observed to be highly inhibitory for neurite initiation (Bovolenta et al., 1993). These findings indicate that the plasma membrane, in addition to myelin, of

the mammalian CNS contains highly nonpermissive molecules inhibiting neurite outgrowth.

Several studies have shown that nerve fibres can grow down the spinal cord if lesions are made in the early postnatal period in rats or hamsters or opossum (Bernstein & Stelzner, 1983; Bregman et al., 1989; Kalil & Reh, 1982; Treherne et al., 1992). Hasan et al (1991) and Shimizu et al (1990) have shown a close correlation between failure of regeneration of spinal cord descending tracts and the appearance of myelin in the chick embryo: successful anatomical and functional regeneration of descending spinal tracts occurs up to E12, but not E13, which is exactly when myelin formation starts in the spinal cord. Ethell et al (1993) have shown that the embryonic chick spinal cord undergoes a developmental transition from permissive to nonpermissive substrates for neuritogenesis.

In order to elucidate the mechanism of the inhibitory effect of higher vertebrate CNS for neurite outgrowth, it is imperative to isolate the molecule(s) involved in the inhibitory effect, to compare their biological and chemical properties, and to determine their functional significance during development and in the adult stage.

3. AIMS OF THE PRESENT STUDIES

3.1 To establish a rapid and reproducible bioassay for nonpermissive effects on neurite outgrowth, using the clonal NG108-15 cell as a model.

3.2 To characterize the physicochemical properties of the nonpermissive inhibitory activity in plasma membranes from adult rat CNS.

3.3 To elucidate the developmental profile of the nonpermissive substrate activity in plasma membranes of the rat CNS during early postnatal growth.

3.4 To observe the effect of demyelination on the nonpermissive activity in plasma membranes of the rat CNS.

3.5 To partially purify the nonpermissive protein factor from plasma membranes of the adult rat CNS.

4. MATERIALS AND METHODS

4.1 MATERIALS

Tissue culture supplies, including Dulbeco's modified Eagle's medium-high glucose (DMEM), fetal bovine serum (FBS), Ham's F12 nutrient medium (F12), and penicillin-streptomycin and L-glutamate were purchased from GIBCO Canada (Calgary, Alberta, Canada). Spectrapor dialysis tubing, DE-Cellex, protein molecular weight standards for SDS-PAGE, Coomassie Brilliant Blue R-250, bis-acrylamide, ammonium persulfate, and sodium dodecyl sulphate were purchased from Bio-Rad Laboratories (Mississauga, Ontario, Canada). Bovine serum albumin (BSA) was from Miles Laboratories, Konkabee, IL, USA. Bovine myelin basic protein (MBP) was purchased from Sigma Chemical Co., St. Louis, MO, USA.

All other laboratory reagents were obtained either from Fisher Scientific Co. (Winnipeg, Canada) or Sigma Chemical Co. (St. Louis, MO, U.S.A.).

4.2 METHODS

4.2.1 Assay of nonpermissive substrate activity with NG108-15 cells in culture.

In this study, a cell culture bioassay using the neuronal cell line of NG108-15 has been established for monitoring the nonpermissive activity of plasma membranes from rat brain and spinal cord. The clonal NG108-15 cell, upon induction of differentiation via factors that increase intracellular cAMP, expresses neuronal properties analogous to those observed in cultured primary neurons. NG108-15 cells have been observed to (1) generate action potentials in response to electrical and chemical stimuli, (2) form presynaptic terminals, (3) make synaptic-like cell-cell contacts with other NG108-15 cells, and (4) form functional synapses with culture myotubes (Han et al., 1991; Nelson et al., 1976; Nirenberg et al., 1983)

Neuroblastoma X glioma hybrid NG108-15 cells were maintained in Medium D, containing 90% Dulbecco's modified Eagle's medium (DMEM), 10% fetal calf serum (FCS) ,$1x10^{-6}$ M hypoxanthine, $1x10^{-4}$ M aminopterine, 100 units/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamate, at 37°C in a humidified atmosphere of 10% CO_2-90% air. For routine assay, cells were dislodged, collected by centrifugation at 500xg for 5 minutes, and resuspended in appropriate volumes of prewarmed medium at 37°C. To enhance morphological differentiation, NG108-15 cells were induced by subculturing in the presence of 1 mM dBcAMP in serum-free, chemically defined medium, consisting of 75% DMEM, and 25% F-12 nutrient mixture, supplemented with insulin (25 μg/ml), transferrin 50 μg/ml),

and oleic acid-bovine serum albumin (10 μg/mg albumin/ml).

Effects of permissive and nonpermissive substrates were monitored semiqualitatively by counting the number of adhering cells at specific time intervals after cell plating. For assays, samples of plasma membranes were resuspended and extracted in 0.1 M NH_4OH, followed by centrifugation to obtain solubilized membrane proteins. Circles of 5~10 mm in diameter were marked with a felt pen at the bottom of the 34- or 54- mm culture dishes, and coated with a drop (10~20 μl) each of solubilized membrane protein samples and left at 4°C overnight in a humidified atmosphere. After removal of membrane samples and three washings, with 2~3 ml PBS each, NG108-15 cells at 20,000 cells/ml were plated in serum-free, chemically defined medium in the presence of 1 mM dBcAMP. After 2~5 hours, attached cells were stabilized by direct addition of a dense fixative solution (1%, v/v, glutaradehyde in 5%, w/v, polyvinylpyrrolidone), which also displaced the culture medium together with the unattached cells from the attached cells at the bottom, followed by two washings with excess PBS. The number of cells within the marked circles precoated with membrane proteins or control with bovine serum albumin (BSA) was counted with the aid of an ocular graticule. The substrate permissiveness was expressed as a percentage of the cell number in the membrane-coated area divided by the cell number in a BSA-coated control area (Ethell et al., 1993).

4.2.2 Preparation of rat CNS myelin and plasma membranes

Sprague-Dawley rats (250-350g) of both sexes were sacrificed by decapitation. The brain, brainstem and spinal cord were separately dissected, and meninges and blood vessels carefully removed under a dissecting microscope. Pooled brain, brainstem or spinal cord tissues were separately processed for isolation of myelin and plasma membranes.

Grey and white matter: rat brain tissue enriched in grey matter was obtained from superficial neocortex layers, whereas white matter-enriched tissue was dissected from the corpus callosum.

Procedure for isolation of CNS myelin and plasma membranes by the floatation technique of sucrose density gradient centrifugation was adapted from Henn (1980), with slight modifications (Ethell et al., 1993). The frozen spinal cord, brainstem or brain tissues were thawed on ice and homogenized in 0.32 M sucrose in 25 mM Tris-HCl, pH 7.2, containing protease inhibitors of 1 mM phenylmethysulfonyl fluoride (PMSF), 10 mM EDTA and EGTA. The homogenate was centrifuged at 500xg for 10 minutes to pellet crude nuclei and cellular debris, and the supernatant was again centrifuged at 54,000xg for 60 minutes to pellet crude plasma membranes. The pellet was resuspended in the homogenization buffer,

containing 50% (W/V) sucrose. Preparation of purified myelin and plasma membrane was obtained by centrifugation at 54,000xg for 180 minutes in a discontinuous step gradient of 11%, 25.5%, 35.5% and 50% (W/V) sucrose, by resuspending the crude myelin and plasma membranes in the homogenization buffer, containing 50% sucrose, as the bottom cushion layer of the sucrose step gradient. The enriched fraction of myelin was obtained as a major band at the interface between 11% and 25.5% of sucrose; the enriched fraction of plasma membranes was obtained as a major band at the interface between 25.5% and 35.5% of sucrose, and recovered by diluting the solution to approximately 0.32 M sucrose with phosphate-buffered saline (PBS), pH 7.2, followed by centrifugation at 60,000xg for 30 minutes. Plasma membrane pellet was further purified by subjecting again to the second discontinuous gradient of 11%, 25.5%, 35.5% and 50% (w/v) sucrose at 54,000xg for 180 minutes, by resuspending the plasma membrane pellet in the 50% sucrose buffer as the bottom cushion layer. The purified plasma membrane band at the interface between 25.5~35.5% sucrose was recovered by centrifugation after diluting the solution to 0.32 M sucrose with PBS, and stored at -70°C or lyophilized as dry powder for future use.

4.2.3 Alkaline extraction of rat CNS plasma membranes

Purified rat spinal cord plasma membranes were extracted

with 10 volumes of 0.1 M NH_4OH, containing protease inhibitors, at 4°C for 1~2 hours. After centrifugation, supernatants were collected and diluted with PBS to appropriate protein concentrations for coating culture dishes for assays of permissive and nonpermissive substrate effects on cultures of NG108-15 cells.

4.2.4 Protein determination.

For general determination of protein concentrations, the method of Bradford (Baradford, 1976) was used. For samples containing interfering substances, protein concentrations were determined by the method of Lowry et al (1951), as modified by Bensadoun and Weinstein (1976).

Distribution of protein in fractions after gel filtration on Sepharose CL-4B was monitored with a LKB 4050 spectrophotometer by absorbance at 278 nm.

4.2.5 Demyelination of neonatal rats with 5-azacytidine

To obtain demyelination in rats, one group of 10 neonatal rats were treated by systemic injections of 5-azacytidine (5'-AZ, mitotic inhibitor) subcutaneously daily at 3.5 μg/g body weight, and another group of neonatal rats simultaneously injected with the same volume of phosphate-buffered saline

(PBS) as controls. The injection was carried out from postnatal day 1 to 15. At the end of treatment, both experimental and control rats were dissected, and tissues of brain, brainstem and spinal cord were processed for isolation of plasma membrane and myelin as described above.

4.2.6 Ammonium sulphate precipitation

Ammonium sulphate was added gradually to the supernatant to obtain 50% saturation. The mixture was stirred at 4°C for 1 hour and the precipitate was pelleted by centrifugation at 15,000xg for 1 hour. The 0~50% ammonium sulphate precipitate was redissolved in 0.25% NH_4HCO_3 and dialysed extensively against the same buffer at 4°C overnight. The dialysed solution was lyophilized for storage.

4.2.7 Gel filtration on Sepharose CL-4B

An alkaline extract of solubilized proteins of plasma membranes was applied onto a column of Sepharose CL-4B, equilibrated in 20 mM sodium phosphate buffer, pH 7.4, containing 1 mM PMSF, 1 mM DTT and 0.05% CHAPS. After elution, fractions were monitored by the cell number assay of NG108-15 cells for nonpermissive substrate activity, and active fractions were appropriately pooled.

4.2.8 DEAE-cellulose ion-exchanger chromatography

Solubilized membrane proteins were applied onto a DEAE-cellulose column equilibrated in 20 mM Tris-HCl, pH 8.2. The column was washed and the adsorbed proteins were eluted by increasing concentrations of NaCl in the same buffer. The inhibitory membrane protein fraction was eluted with 0.2 M NaCl. After dialysis in 0.25% NH_4HCO_3 overnight to remove salts, the active fractions were lyophilized and subjected to the bioassay of NG108-15 cells.

4.2.9 Preparative polyacrylamide gel electrophoresis.

Membrane proteins were fractionated on a 7.5% separating gel of non-denaturing polyacrylamide gel electrophoresis at pH 8.9. One "marker" lane was cut and stained with Coomassie Brilliant Blue R-250. Protein bands to be analyzed were carefully aligned with the remaining unstained gel and segmented according to the stained bands of the "marker" gel. Each gel segment was homogenized in 5 volumes of 0.25% NH_4HCO_3, pH 8.2, and extracted for 2~3 hours. The gel extracts were then centrifuged and the supernatants lyophilized. Salts were removed by subjecting the supernatants through mini-columns of Sephadex G-25. Lyophilized samples were redissolved in PBS and subjected to the NG108-15 cell assay for nonpermissive substrate activity.

4.2.10 Analytical SDS-polyacrylamide gel electrophoresis.

Analytical polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecyl sulphate (SDS) was performed on a 10% separating gel with a 4% stacking gel (Laemmli, 1970). For comparison, 5 μg of sample proteins were loaded onto each lane. After electrophoresis, the gel was stained with 0.25% Coomassie Brilliant Blue R-250 in 45% methanol and 9% glacial acetic acid, and destained by diffusion in 10% methanol and 7% glacial acetic acid for visualization of protein bands.

Molecular weight of the nonpermissive protein was estimated by SDS-polyacrylamide gel electrophoresis. Molecular weight markers of phosphorylase B (97K), bovine serum albumin (66K), ovalbumin (45K), carbonic anhydrase (29K), soybean trypsin inhibitor (20K), and lysozyme (14K) and the solubilized membrane protein samples were pre-treated by heating at 100°C for 3 minutes in the presence of 1% sodium dodecyl sulphate and 5% β-mercaptoethanol. Electrophoresis was carried out in a 10% separating gel of polyacrylamide containing 1% SDS, and electrophoresed at a current of 10 mA/cm of gel surface. The relative mobilities (Rf values) of the protein markers were plotted against their molecular weights. The molecular weight of the nonpermissive protein was estimated by plotting its electrophoretic mobility (Rf value) to compare with those of the protein markers.

5. RESULTS

5.1 Bioassay for nonpermissive substrate activity using the hybrid NG108-15 cell line

For successful quantification and isolation of biological active principles, a relatively simple and specific assay is essential. Extracts of purified plasma membranes from adult rat CNS have been observed to be able to inhibit cell adhesion and the neurite outgrowth on NG108-15 cells. A bioassay based on the ability of inhibiting cell adhesion and neurite outgrowth on these cells has been developed for monitoring the nonpermissive activity in plasma membranes and various fractions of plasma membrane proteins at different steps of purification. This bioassay using NG108-15 cells is relatively sensitive and simple, but significantly less time consuming than that of using primary cultures of rat spinal cord neurons.

The neuroblastoma X glioma hybrid NG108-15 cell line was derived from a Sendai-virus-induced fusion of a mouse neuroblastoma cell line (N18 TG-2) and a rat glioma cell line (G6 BU-1). This hybrid cell line is one of the most characterized and studied cell lines of neural origin. This cell line has been shown to synthesize, store, and secret acetylcholine (McGee et al., 1977), and form functional

synapses with striated muscle cells in vitro (Nelson et al., 1976). In addition, NG108-15 cells possess a number of receptors for neuroactive compounds: opiate receptors (Klee & Nirenberg, 1974; Klee et al., 1975), muscarinic acetylcholine receptors (Traber et al., 1975), as well as receptors for prostaglandins PGE1 (Hamprecht & Schultz, 1973). On the basis of all these neuronal characteristics, it is evident that the NG108-15 cell line behaves as neuronal cells in culture.

5.1.1 The nonpermissive substrate activity of rat CNS plasma membrane on NG108-15 cells

Substrate effects of purified plasma membrane from adult rat brain, brainstem and spinal cord were examined by precoating culture dishes with alkaline extracts (0.1 M NH_4OH) of the membranes, followed by cultures of NG108-15 cells in serum-free, chemically defined medium in the presence of 1 mM dBcAMP. For comparison, the plasma membrane from sciatic nerve was also assayed similarly, by coating dishes under the same conditions for cultures of NG108-15 cells. After 5 hours, cells grown on the control BSA-coated area became adhered to the substrate with short neurites of half- to one-cell diameter (Figure 1a). However, cells grown on substrate of plasma membrane alkaline extracts all remained as undifferentiated round cells with no sign of substrate adhesion (Figure 1b). On the contrary, cells grown on

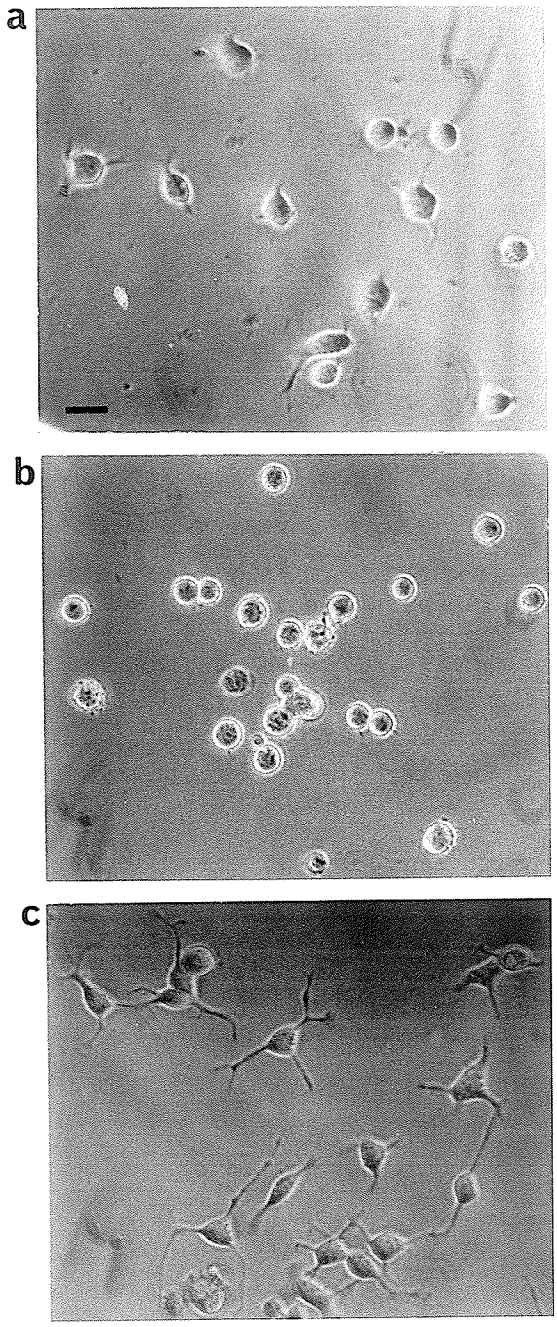

Figure 1. Effects of precoating plasma membrane proteins from adult rat spinal cord and sciatic nerve on cell morphology of NG108-15 cells in culture. Spinal cord and sciatic nerve membranes were prepared by sucrose density gradient centrifugation, and solubilized proteins were obtained by extracting the membranes with 0.1 M NH_4OH, followed by centrifugation. The nonpermissive substrate effects of membrane proteins were measured by precoating culture dishes at 100 μg/ml. Phase-contrast photomicrography of NG108-15 cells were taken at 5 hours for BSA control (a), spinal cord plasma membrane extract (b), and sciatic nerve plasma membrane extract (c). Scale for cells = 50 μm.

substrate of alkaline extracts of sciatic nerve plasma membranes exhibited significant morphological differentiation, having a flattened morphology with neurite of generally one- to three- cell diameter after 5 hours in culture (Figure 1c). The alkaline extract of spinal cord plasma membrane is a highly restrictive substrate for cell adhesion and differentiation of NG108-15 cells, while the alkaline extract of sciatic nerve plasma membrane is a highly permissive substrate under similar conditions.

After 24 hours in culture, almost all NG108-15 cells grown on spinal cord plasma membrane extracts remained spherical, showing little substrate adhesion, neuronal differentiation or process outgrowth, with one or two exceptions of becoming attached (Figure 2a), while control cells on a BSA substrate became highly differentiated with neurites of one- to two-cell diameter (Figure 2c). Only after 48 hours, cells grown on spinal cord plasma membrane extracts became reaggregated as small clusters, attached to the substrate and showed some degree of differentiation, as evidenced by the appearance of a few short neurites, although they maintained a generally round morphology (Figure 2b); whereas cells on BSA substrate differentiated further, some with neuritic branches of long varicose processes (Figure 2d).

5.1.2 Dose-response curve of the rat CNS plasma membrane

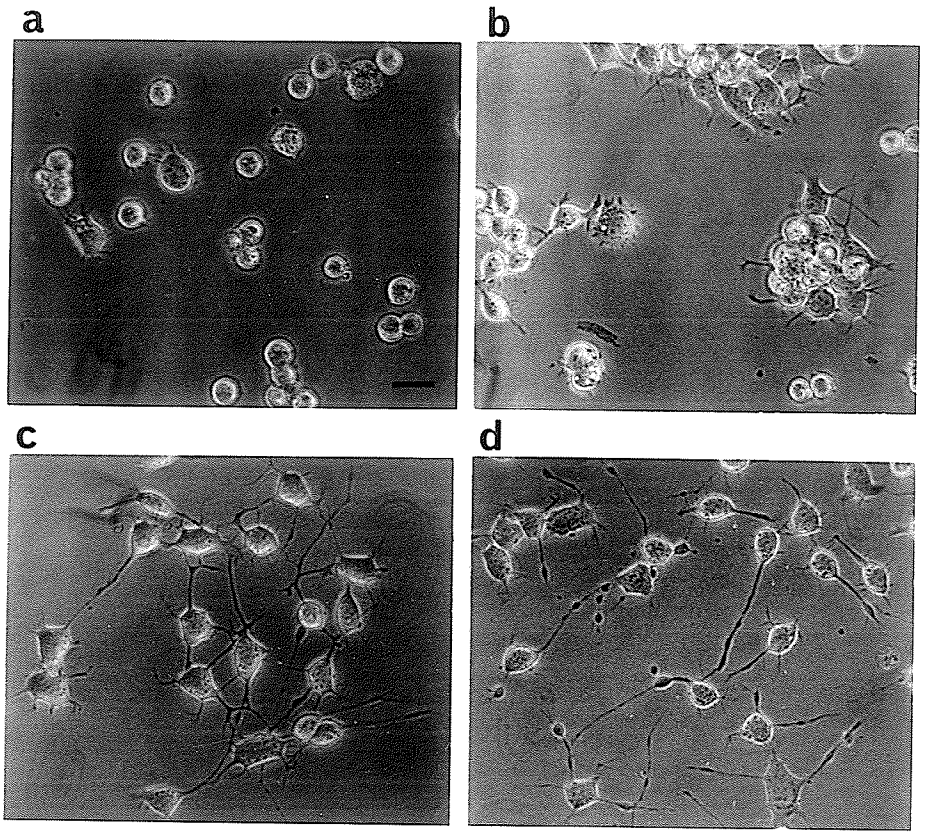

Figure 2. Effects of precoating plasma membrane proteins from rat spinal cord on NG108-15 cells in culture after 24 hours and 48 hours. Solubilized plasma membrane proteins were obtained by extracting membranes with 0.1 M NH_4OH, followed by centrifugation, and precoated culture dishes at 100 μg/ml. Phase-contrast photographs of cells were taken at 24 hours (a, c) and 48 hours (b, d) for spinal cord plasma membrane extract (a, b) and BSA control coating (c, d). Scale for cells = 50 μm

nonpermissive substrate activity on the NG108-15 cells

The nonpermissive effect of spinal cord plasma membrane was further examined by plating equal numbers of NG108-15 cells onto dishes heterogeneously coated side by side with extracts of rat spinal cord plasma membranes and BSA controls at various concentrations. The substrate effect of the rat brain plasma membrane was then analyzed and compared semiquantitatively by expressing the number of cells adhering on the coated area of plasma membrane proteins at different concentrations and expressed as a percent of those attaching to the adjacent control substrate area of BSA-coating. The permissive activity of the brain plasma membrane extract, as measured in a percentage of the number of adhering cells, increased inversely in proportion to the protein concentration of the extract, being 8, 30, 59 and 91% for 50, 25, 12.5 and 6.25 μg of protein/ml, respectively (Figure 3). This cell number assay, as a semiquantitative measurement of permissive/nonpermissive activity (%), is expressed as:

$$\text{Permissive activity (\%)} = \frac{\text{Number of cells adhered on membrane protein coated area X 100}}{\text{Number of cells adhered on BSA coated area}}$$

Substrate effects of alkaline extracts of rat CNS plasma membranes under different experimental conditions were

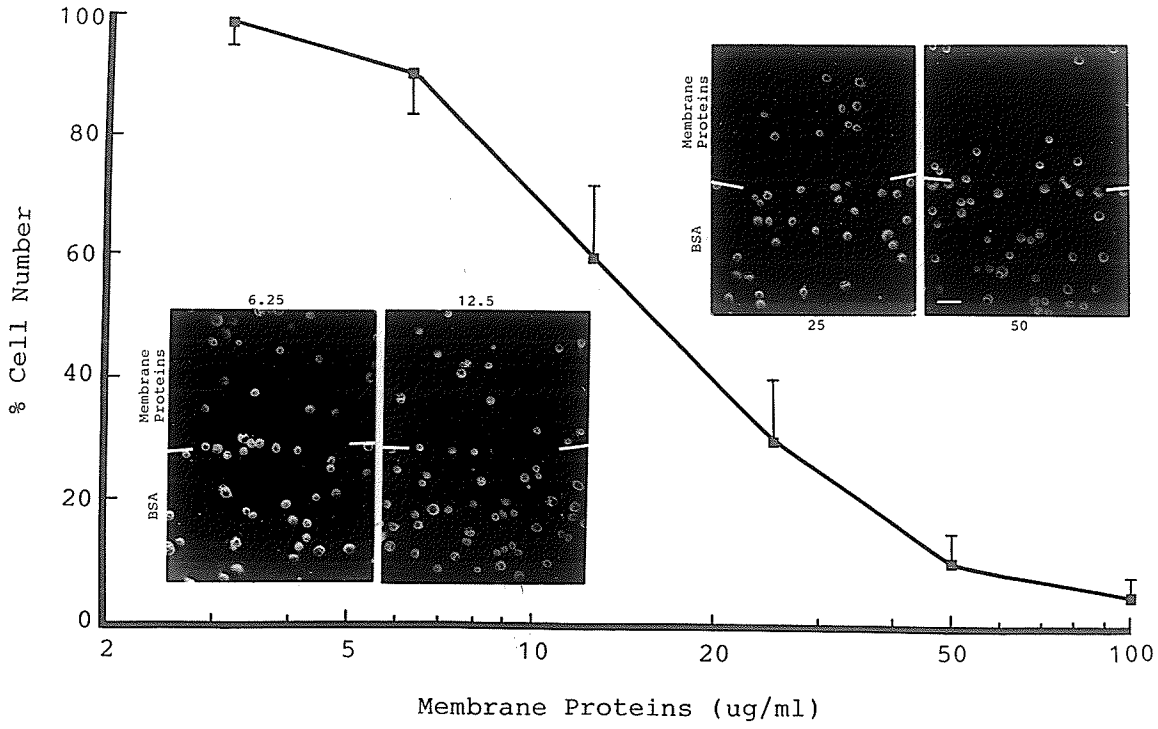

Figure 3. Dose-response curve of nonpermissive activity of precoating plasma membrane proteins from adult brain on cultures of NG108-15 cells. The nonpermissive substrate activity was monitored by cell number in triplicates at protein concentrations of 3.125, 5.25, 12.5, 25, 50 and 100 μg/ml. Phase-contrast photomicrographs of NG108-15 cells were taken at 2 hours after cell plating. BSA = bovine serum albumin. (Magnification = 100 X)

analyzed and compared. One unit of nonpermissive substrate activity was expressed as the amount of membrane protein that exhibited 50% inhibition (half-maximal) of cell adhesion. The alkaline extract of crude plasma membrane of adult rat brain at 16.5 μg/ml expressed 1 unit of nonpermissive activity (Figure 3).

The optimal plating density of NG108-15 cells for this bioassay system was observed to be 20,000 cells/ml: 2 ml for 35 mm dishes and 5 ml for 54 mm dishes.

5.2 Profile of the nonpermissive substrate activity in plasma membrane of the rat CNS during early postnatal development

5.2.1 Growth of the rat CNS during postnatal development

After dissection, the postnatal rat CNS of brain, brainstem and spinal cord at various stages of development were weighed. Figure 4 shows the growth rate in weight of the postnatal rat CNS. The spinal cord increased at a significantly faster rate than those of the brain and brainstem, both of which grew at very similar rates from postnatal day 1 to 21.

5.2.2 Developmental profile of nonpermissive substrate activity on plasma membranes from rat CNS during early

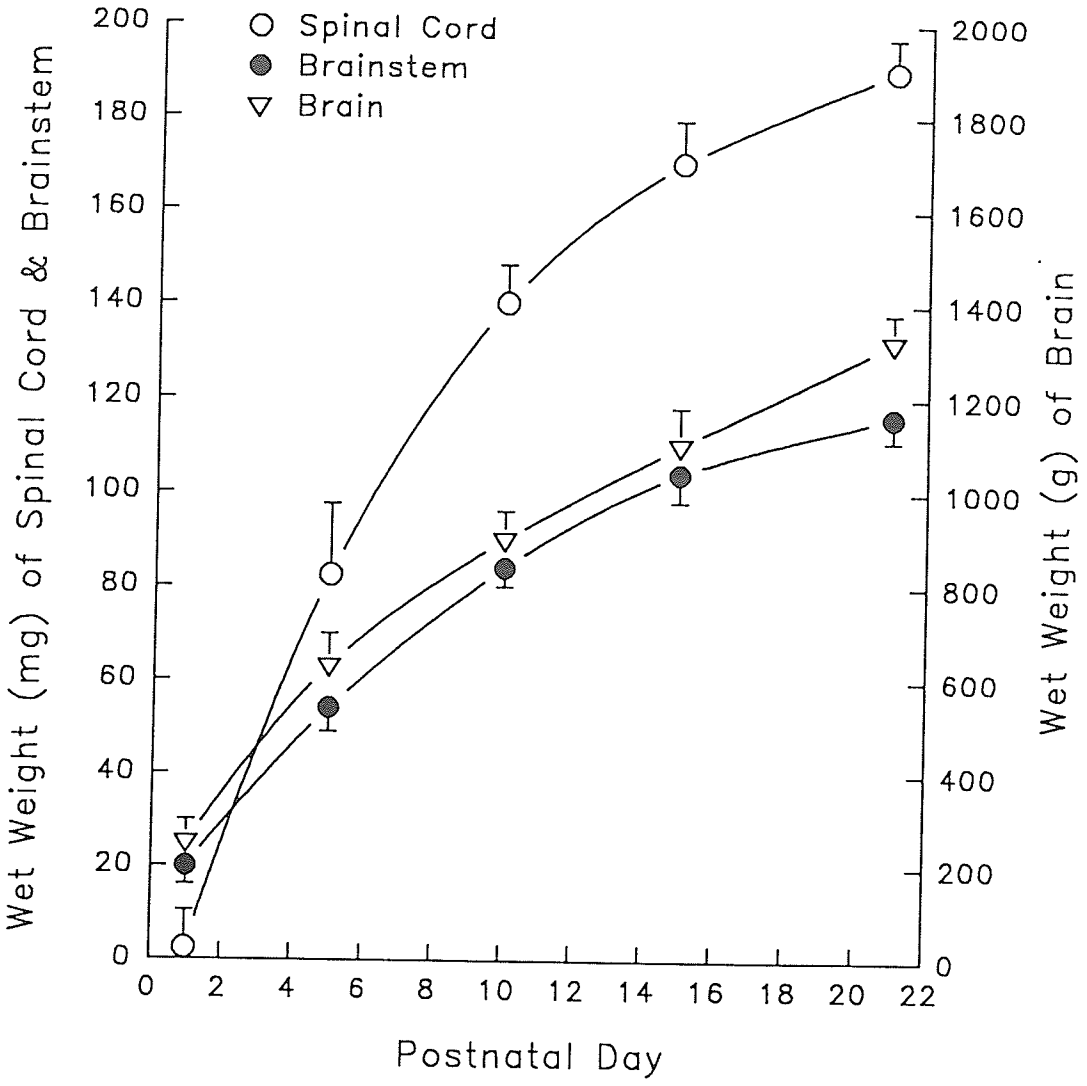

Figure 4. Growth of the rat CNS during early postnatal development. Growth rates of postnatal rat brain, brainstem and spinal cord were monitored in weight increases at different developmental stages from postnatal day 1 to 21. Wet weights for spinal cord and brainstem were in milligrams (mg), and wet weights for brain in grams (g). Values are means ± S.D. of 5-6 rats.

postnatal growth

The solubilized proteins from isolated rat CNS plasma membrane had nonpermissive activity on NG108-15 cells as early as postnatal day 1 (Figure 5). The activity increased gradually from day 5 and became levelled at day 15 till day 21 for both brain and brainstem, while the activity in spinal cord was not significantly increased from day 1 to 10, and thereafter increased gradually till day 21 (Figure 5). The order of relative potency of nonpermissive activity during postnatal development from high to low was: brain > brainstem > spinal cord. The relative activity was expressed as units/mg protein. One unit is defined as the amount of protein/ml for precoating dishes to obtain 50% inhibition of cell adhesion from a dose-response curve.

The protein patterns of plasma membranes from brain, brainstem and spinal cord of postnatal rats at various developmental stages were examined by analytical SDS-PAGE, as shown in figure 6. There were no significant differences in protein patterns of plasma membranes from brain, brainstem and spinal cord of postnatal rats from day 1 to 21, except one or two minor bands (Figure 6A, B & C). Figure 6D shows the protein pattern of spinal cord myelin preparation. There were little myelin basic protein (MBP) bands in myelins at postnatal day 1, 5 and 10. On the contrary, there were

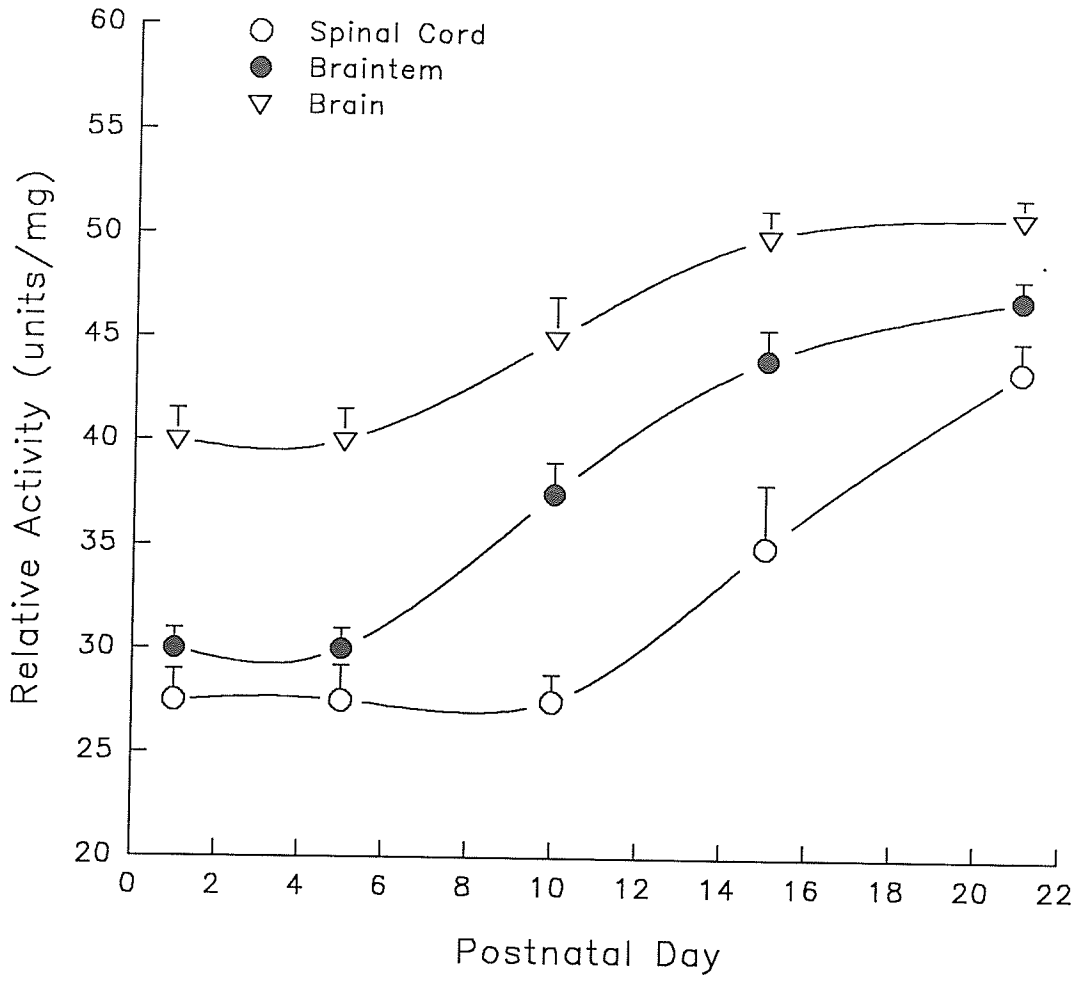

Figure 5. Nonpermissive substrate activity of plasma membrane proteins from neonatal rat brain, brainstem and spinal cord during early postnatal development of day 1, 5, 10, 15 and 21. Nonpermissive substrate activity is expressed as units/mg membrane protein. One unit is defined as the amount of protein per ml for precoating dishes to exhibit a nonpermissive activity equal to 50% of the maximal activity obtained from a dose-response curve of a crude standard. Values are means ± S.D. of 4-6 rats.

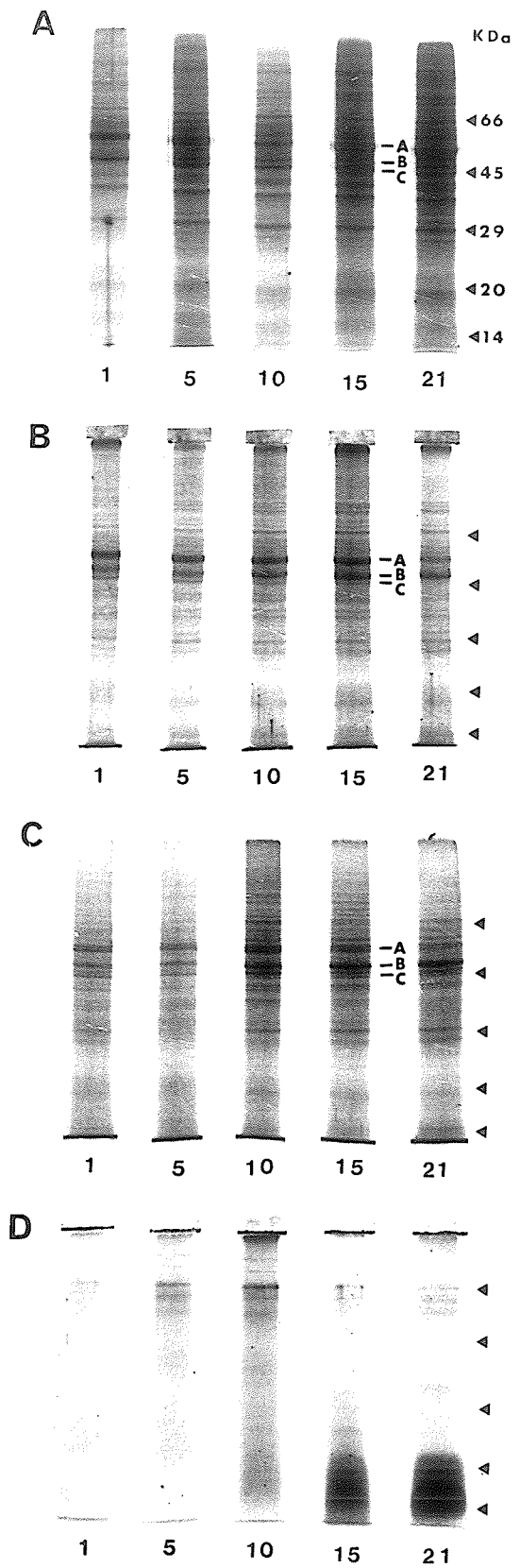

Figure 6. Analysis of protein pattern of purified plasma membrane and myelin fractions from postnatal rat CNS by 10% SDS-polyacrylamide gel electrophoresis. (A). Brain plasma membrane; (B). Brainstem plasma membrane; (C). Spinal cord plasma membrane; (D). Spinal cord myelin. Samples were from rats of postnatal day 1, 5, 10, 15 and 21. Molecular weight standards: bovine serum albumin (66K), ovalbumin (45K), carbonic anhydrase (29K), soybean trypsin inhibitor (20K), and lysozyme (14K).

dramatic increases in amount of MBP bands, of molecular weights 14~20 KDa, in myelin samples obtained from day 15 and 21 rats (Figure 6D). These results indicate that myelination becomes highly active at postnatal day 15.

In another series of experiments, the nonpermissive activity of the brain, brainstem and spinal cord was studied in more detail at postnatal day 12, 14, 16, 18 and 26, as shown in Figure 7. The relative activity (units/mg) of the brain increased gradually from day 12 to 16 and became levelled afterwards, while the activities in brainstem and spinal cord increased slowly from day 12 to 16, but markedly between day 16 to 18, and then gradually reached the similar levels of approximately 50 units/mg for the whole CNS at day 26.

Figure 8 shows the protein patterns of brain and spinal cord plasma membranes and myelins from postnatal rats upon analytical SDS-PAGE. As shown in Figure 8A & B, there were no significant differences in protein patterns between plasma membranes from the brain and spinal cord, except for one or two minor bands. Figure 8C & D show the patterns of MBP (Mr 14~20 KDa) from brain and spinal cord myelin fractions increased gradually from postnatal day 12 to 26, indicating that the myelination process was progressing gradually during this period of development.

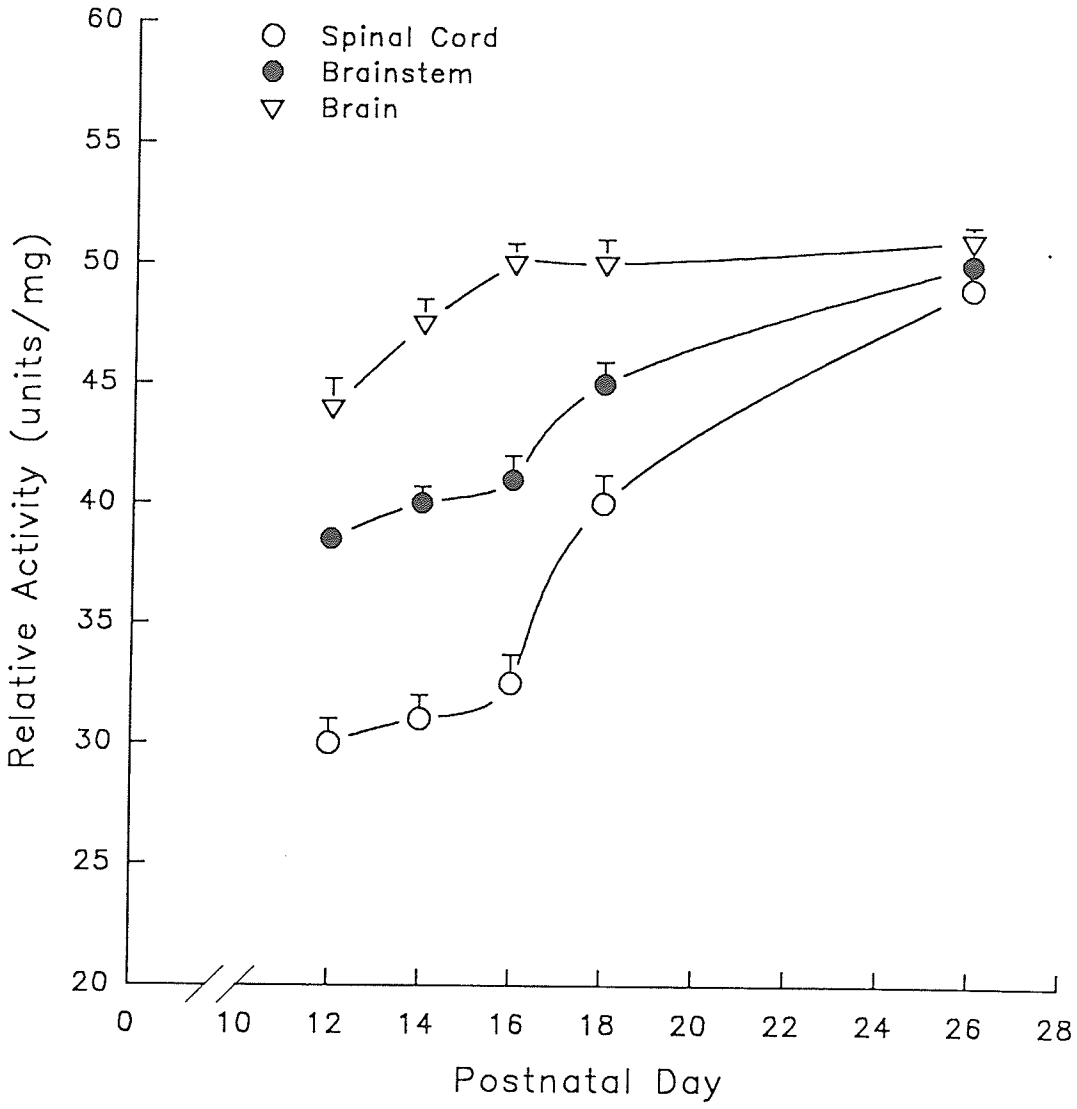

Figure 7. Nonpermissive substrate activity of plasma membrane proteins from brain, brainstem and spinal cord during postnatal development of day 12, 14, 16, 18 and 26. Nonpermissive substrate activity is expressed as units/mg membrane protein. One unit is defined as the amount of protein per ml for precoating dishes to exhibit a nonpermissive activity equal to 50% of the maximal activity obtained from a dose-response curve of a crude standard.

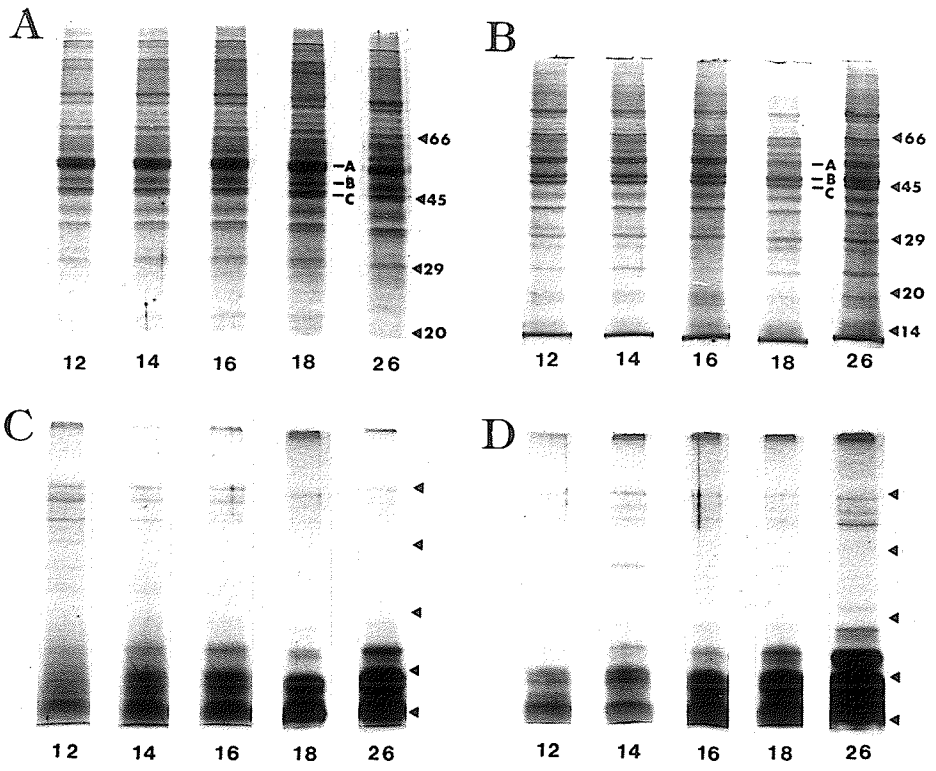

Figure 8. Analysis of protein pattern of purified plasma membrane and myelin fractions by 10% SDS-polyacrylamide gel electrophoresis. (A). Brain plasma membrane; (B) Spinal cord plasma membrane; (C). Brain myelin; (D). Spinal cord myelin. Samples were from rats of postnatal day 12, 14, 16, 18 and 26. Molecular weight standards: bovine serum albumin (66K), ovalbumin (45K), carbonic anhydrase (29K), soybean trypsin inhibitor (20K), and lysozyme (14K).

5.2.3 Nonpermissive activity in rat CNS plasma membranes after 5'-azacytidine treatment

Gliogenesis in the rat optic nerve is disrupted by neonatal treatment with the mitotic inhibitor 5'-azacytidine (5'-AZ). The rate of myelination and number of oligodendrocytes are markedly reduced in treated animals (Yamate & Ransom, 1985). For comparison, nonpermissive substrate activity was monitored on NG108-15 cells by using solubilized plasma membrane proteins, at various dilutions of the same concentrations, obtained from 5'-AZ treated and control rat brain, brainstem and spinal cord plasma membranes, as shown in figure 9. Results using the cell number assay of NG108-15 cells show that the CNS (brain, brainstem and spinal cord) of 5'-AZ treated rats retained substantial amounts of nonpermissive activity, though significantly less than that of the untreated controls (Figure 9).

Figure 10 shows the protein patterns of plasma membrane and myelin from 5'-AZ treated rat brain and spinal cord, as determined by analytical SDS-PAGE. Comparison of 5'-AZ treated and untreated control brain and spinal cord plasma membranes showed no significant differences in protein patterns (Figure 10A); but protein patterns of the myelin fractions of 5'-AZ treated rats were markedly different from those of the untreated controls (Figure 10B). The 5'-AZ

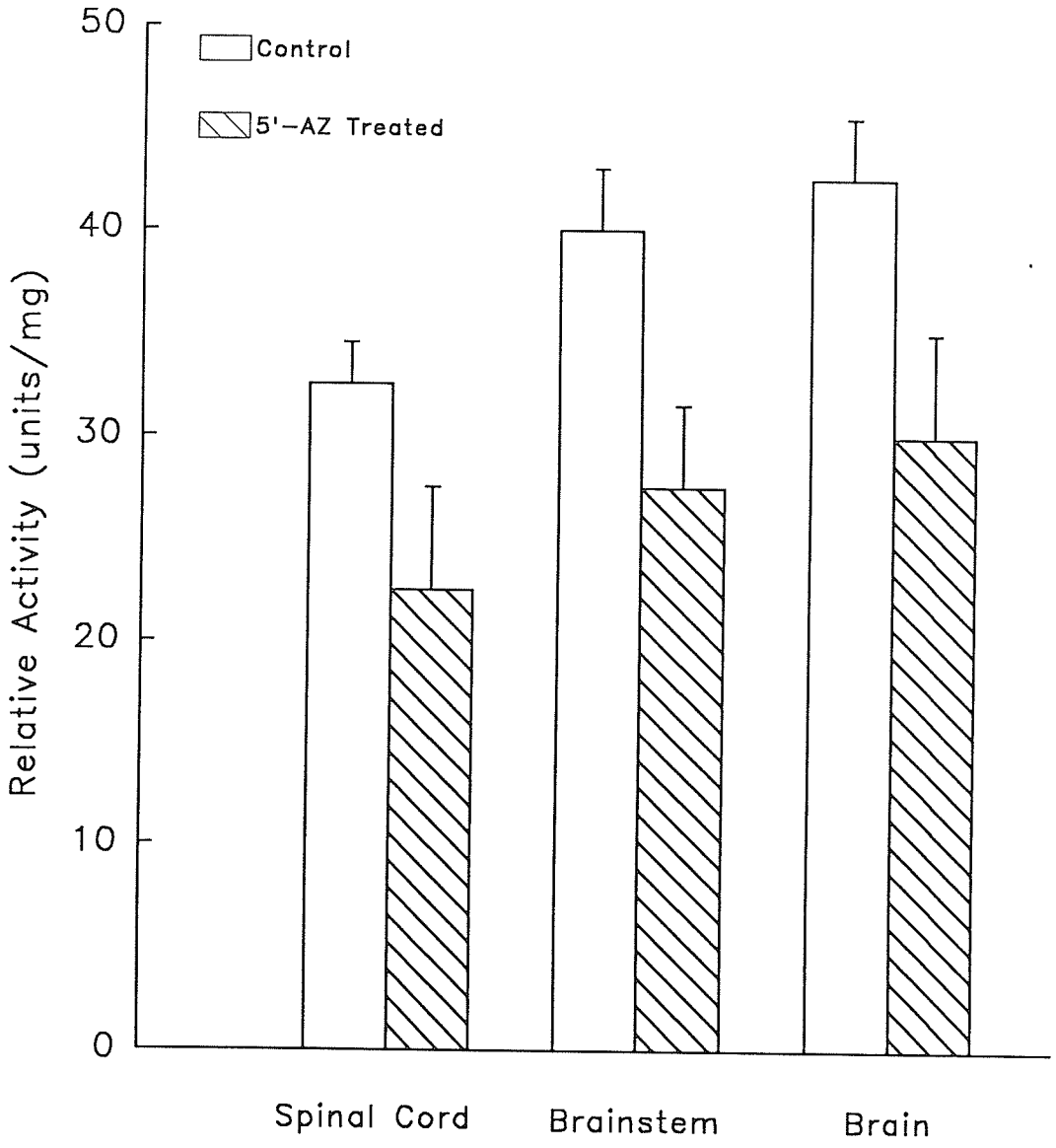

Figure 9. Comparison of nonpermissive substrate activity in CNS plasma membrane proteins between 5'-AZ treated and untreated control rats. Postnatal rats were treated by daily injections of 5'-AZ or PBS (control) for 15 days. One unit is defined as the amount of protein per ml for precoating dishes to exhibit a nonpermissive activity equal to 50% of the maximal activity obtained from a dose-response curve of a crude standard.

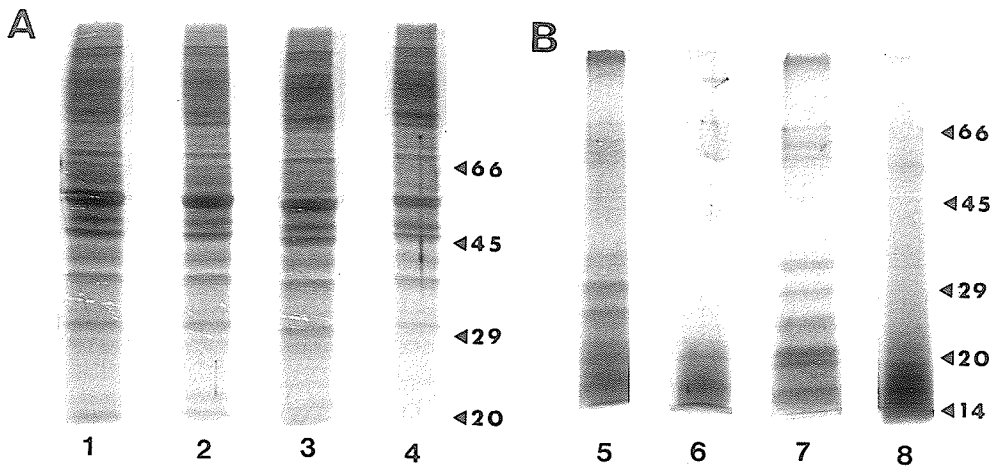

Figure 10. Analysis of protein pattern of purified fractions of plasma membrane (A) and myelin (B) from 5'-AZ treated and untreated control rats by SDS-polyacrylamide gel electrophoresis. 1. Brain plasma membrane (5'-AZ); 2. Brain plasma membrane (control); 3. Spinal cord plasma membrane (5'-AZ); 4. Spinal cord plasma membrane (control); 5. Brain myelin (5'-AZ); 6. Brain myelin (control); 7. Spinal cord myelin (5'-AZ); 8. Spinal cord myelin (control). Samples were from postnatal day-15 rats. Molecular weight standards: bovine serum albumin (66K), ovalbumin (45K), carbonic anhydrase (29K), soybean trypsin inhibitor (20K), and lysozyme (14K).

treated myelin contained little MBP of Mr 14~20 KDa but several prominent protein bands of Mr 25~35 KDa, which were totally absent in control myelin fractions, which contained essentially mostly MBP bands of Mr 14~20 KDa (Figure 10B).

5.2.4 Nonpermissive substrate activity of plasma membranes from adult rat brain white and grey matters

Substrate effect of alkaline extracts of plasma membrane from the white and grey matters was also examined and compared similarly by using this semiquantitative cell number assay, as shown in Figure 11. The alkaline extract of white matter plasma membrane was a highly nonpermissive substrate but only slightly more potent than that of the grey matter plasma membrane, which was also relatively highly nonpermissive (Figure 11).

The above results indicate that myelin is not totally responsible for the nonpermissive activity in the rat CNS, because the nonpermissive activity from brain or spinal cord plasma membranes exists before myelination, as early as postnatal day 1, while myelination occurs around postnatal day 12 to 15 in the rat. Similarly, the elimination of oligodendrocytes, therefore the process of myelination, by 5'-AZ treatment during early postnatal development could not abolish, though considerably reduced the nonpermissive

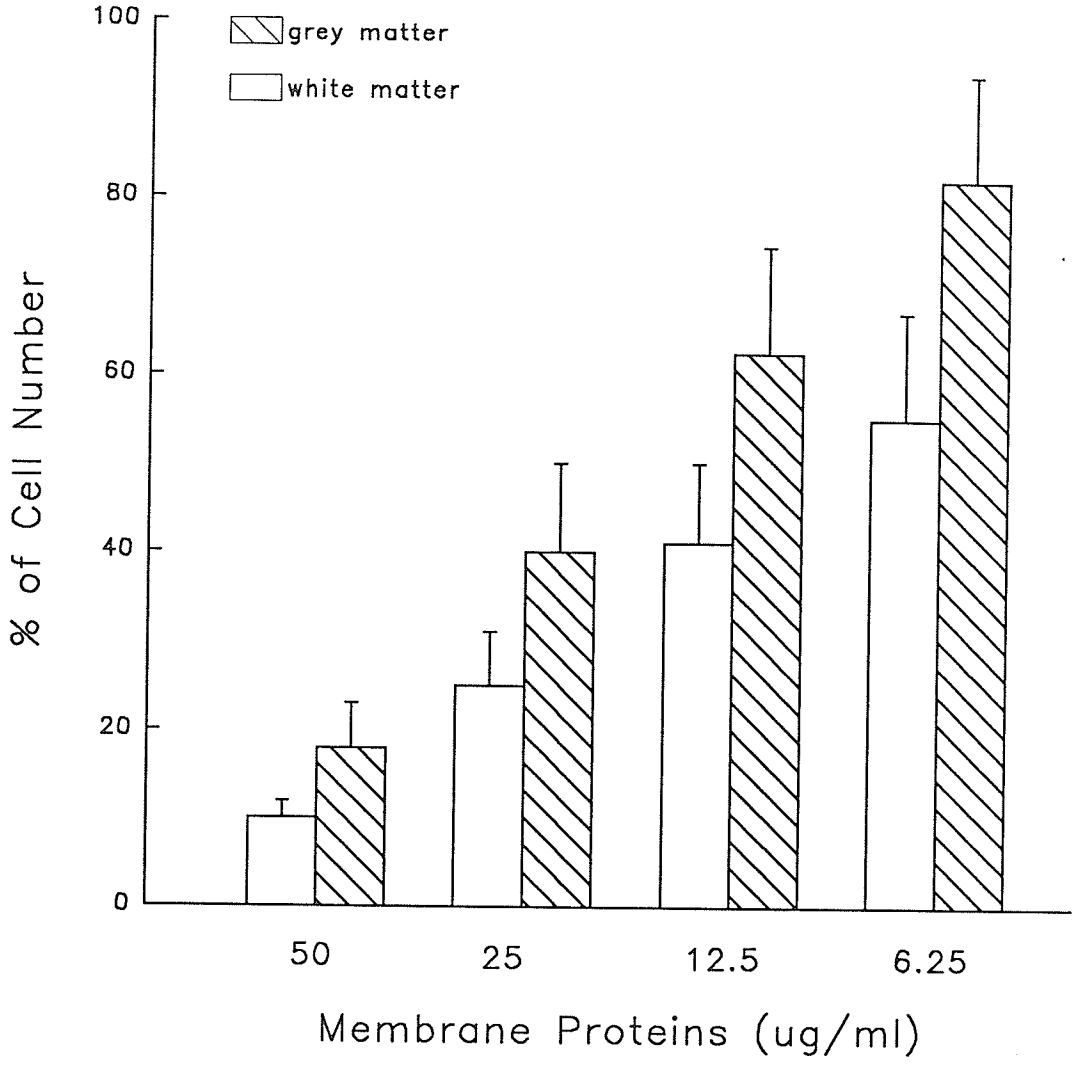

Figure 11. Comparison of nonpermissive activity between plasma membranes isolated from adult rat brain white and grey matters by the cell number assay of NG108-15 cells. The nonpermissive substrate activity was monitored in triplicates at various protein concentrations of 50, 25, 12.5 and 6.25 μg/ml. Values are means ± S.D. of three experiments in triplicates.

activity of the rat CNS plasma membranes. Our findings indicate that the solubilized plasma membrane proteins from rat brain and spinal cord are highly nonpermissive and the activity appears to be different from those neurite outgrowth inhibitory proteins (NI35 and NI250) from the rat CNS myelin reported by Caroni and Schwab (1988a).

5.3 Preliminary characterizations of the plasma membrane-associated nonpermissive substrate activity

In order to isolate the nonpermissive factor, it is imperative that some knowledge on the properties of the activity has to be obtained. These preliminary studies were carried out to characterize the biological and chemical properties of the nonpermissive activity in the alkaline extract of plasma membranes from adult rat brain and spinal cord.

5.3.1 Precipitation of the nonpermissive protein by ammonium sulphate.

Extracts of adult rat CNS plasma membranes were precipitated by ammonium sulphate from 0~30%, 30~50%, and 50~70% saturation at 4°C. The various fractions were redissolved in PBS, dialysed against 0.25% NH_4HCO_3 overnight, and activity was monitored by the cell number assay of NG108-

15 cells. Most of the nonpermissive activity was recovered in the 0~30% and 30~50% fractions, as shown in Figure 12. Therefore, both fractions of 0~30% and 30~50% were pooled and lyophilized for further studies.

5.3.2 Stability of the nonpermissive substrate activity protein at 4°C and -70°C

Stability of the nonpermissive protein was tested at various experimental conditions to establish the optimal conditions for extraction and storage. Aliquots of samples in 0.25% NH_4HCO_3 were sealed in microcentrifuge tubes and stored at 4°C and -70°C for different time intervals and then assayed on NG108-15 cells. Figure 13 shows that the nonpermissive activity at 4°C was deteriorating daily from day 1 to 4, and storage at -70°C was significantly better than that at 4°C for keeping the nonpermissive activity in extracts of solubilized membrane proteins.

5.3.3 Thermal stability of the nonpermissive substrate activity.

For heat stability, aliquots of the nonpermissive protein in 0.25% NH_4HCO_3 were sealed in microcentrifuge tubes and placed in a water bath at various temperatures as indicated. After an incubation of 20 minutes, samples were

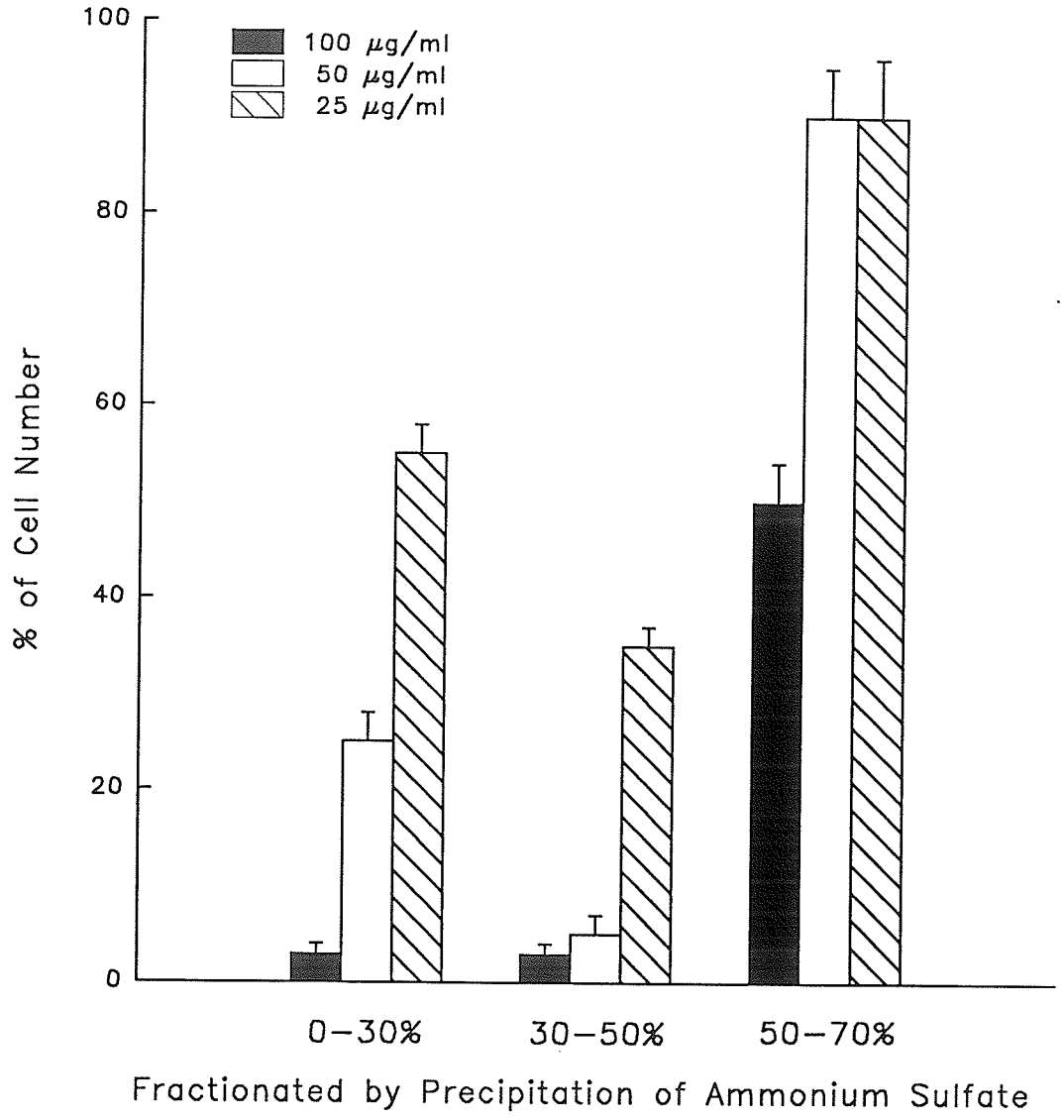

Figure 12. Fractionation of nonpermissive activity in alkaline extracts of rat spinal cord plasma membranes by ammonium sulphate precipitation. The solubilized proteins of nonpermissive substrate activity was precipitated by ammonium sulphate at 0~30, 30~50 and 50~70% saturations. The redissolved precipitates were dialysed against 0.25% NH_4HCO_3 and assayed for nonpermissive activity on NG108-15 cells in triplicates.

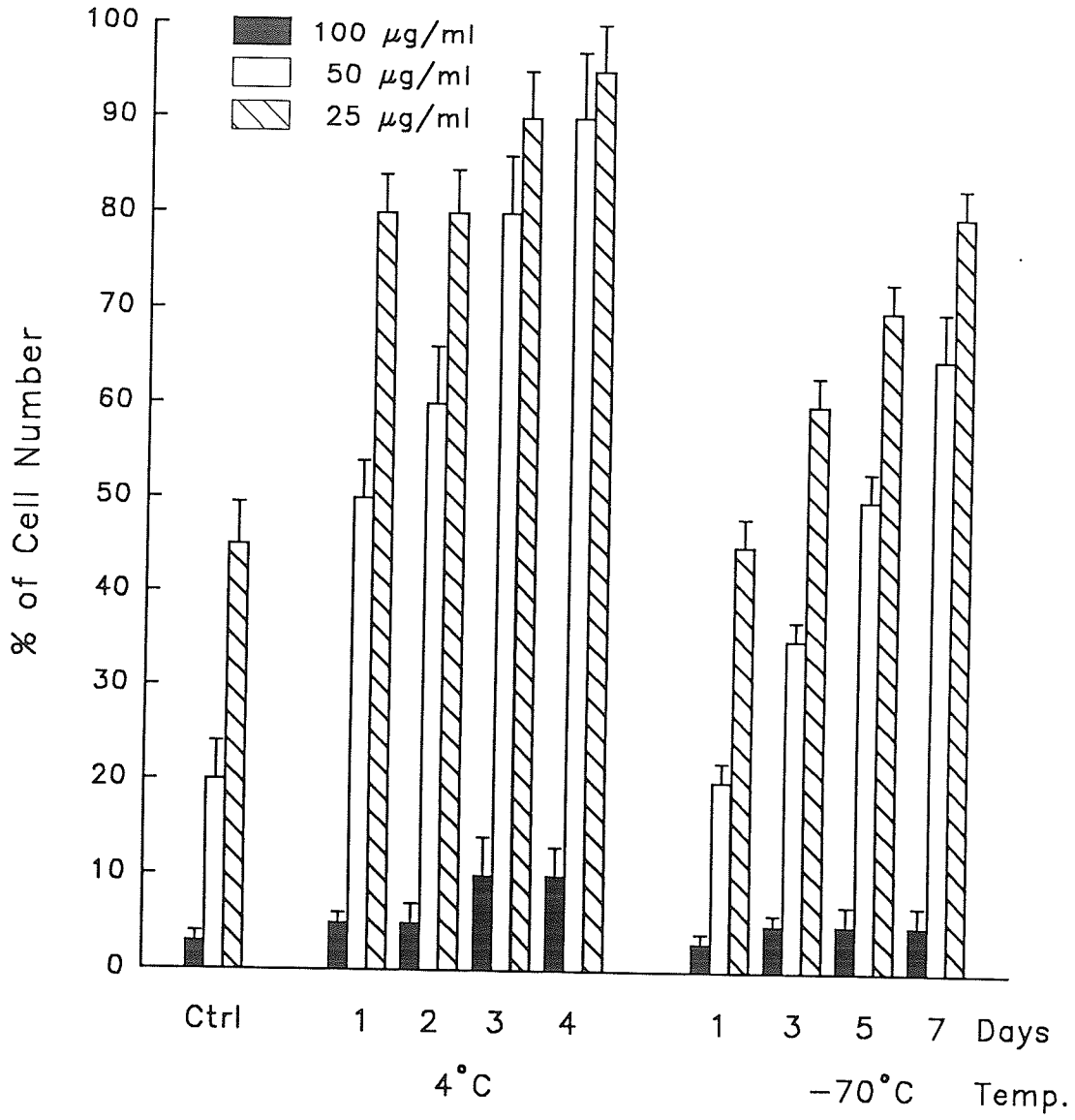

Figure 13. Stability of the nonpermissive activity after various time intervals of storage at 4°C and -70°C. Samples of alkaline extract of nonpermissive proteins from adult rat CNS were stored at 4°C or -70°C after various indicated time intervals, and then monitored by the assay of NG108-15 cells in triplicates.

spun and subjected to the cell number assay of NG108-15 cells by precoating culture dishes at different concentrations. The nonpermissive activity was stable from 0°C to 70°C for 20 minutes, and was partially destroyed at 100°C for 5 minutes as shown in figure 14. These results indicate that the nonpermissive activity in the rat CNS is relatively heat stable.

5.3.4 Effects of pH on the nonpermissive substrate activity.

Aliquots of the alkaline extract of nonpermissive protein solution from adult rat CNS plasma membranes were adjusted to the indicated pH in: 0.1 M sodium acetate buffer at pH 5 or 6, 0.1 M sodium phosphate buffer at pH 7 or 8, Tris buffer at pH 9 or 9.5, and incubated for 24 hours at 4°C. For assays, the pH of the solutions was readjusted to pH 8.2 by dialysing overnight in 0.25% NH_4HCO_3 and then subjected to the NG108-15 cell bioassay at various concentrations.

Figure 15 shows that the nonpermissive substrate activity was destroyed almost completely by pH less than 6, but relatively stable at pH range 7~9, and decreased slightly at pH 9.5.

5.3.5 Effects of enzymic digestion and chemical treatment

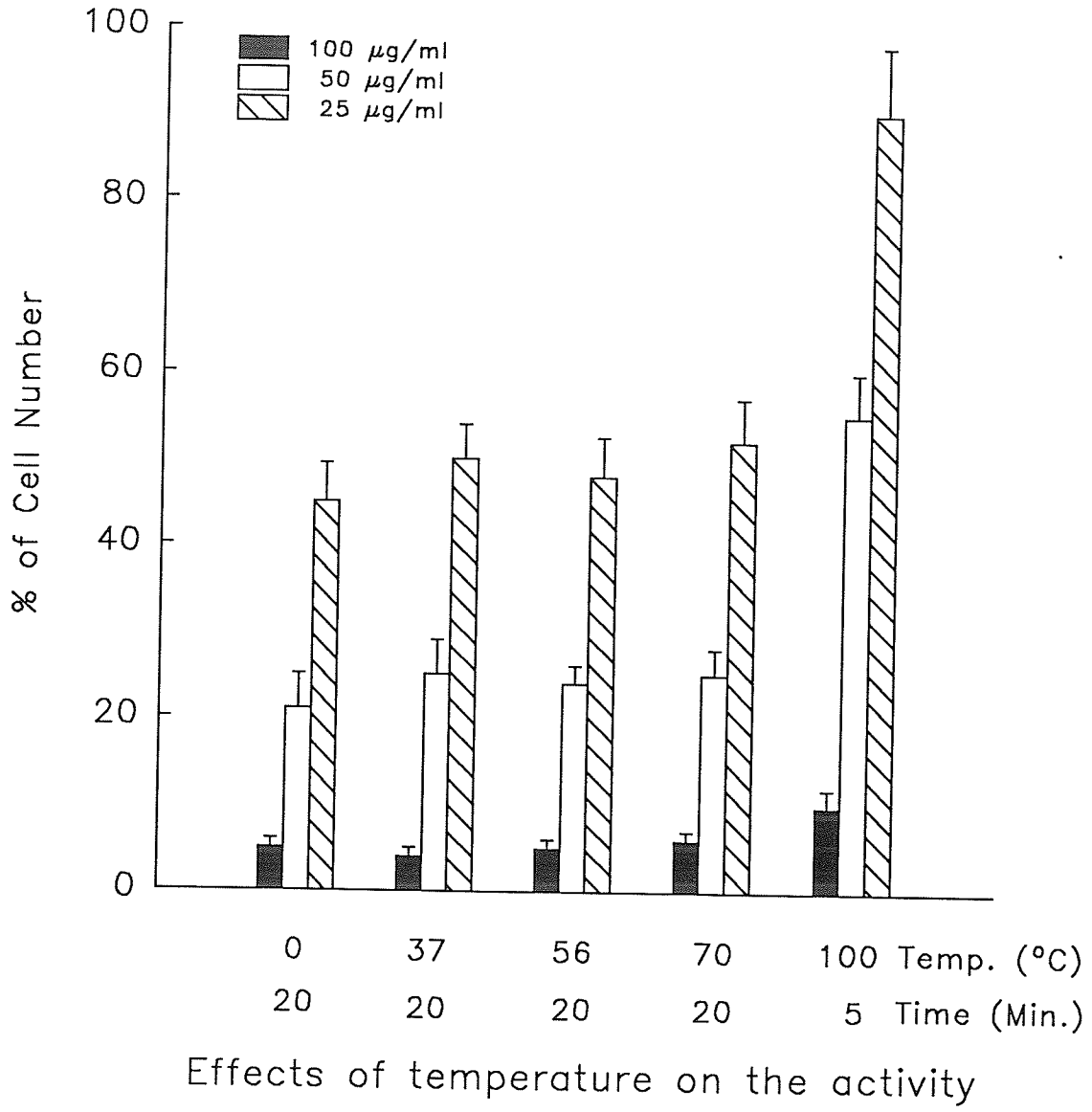

Figure 14. Thermal stability of the nonpermissve substrate activity in alkaline extracts of adult rat CNS plasma membranes. Samples of 200 μl of alkaline extract were sealed in microcentrifuge tubes. Each tube of protein samples was incubated at indicated temperatures and time intervals. After centrifugation, the supernatants were diluted to appropriate concentrations and subjected to the cell number assay of NG108-15 cells in triplicates.

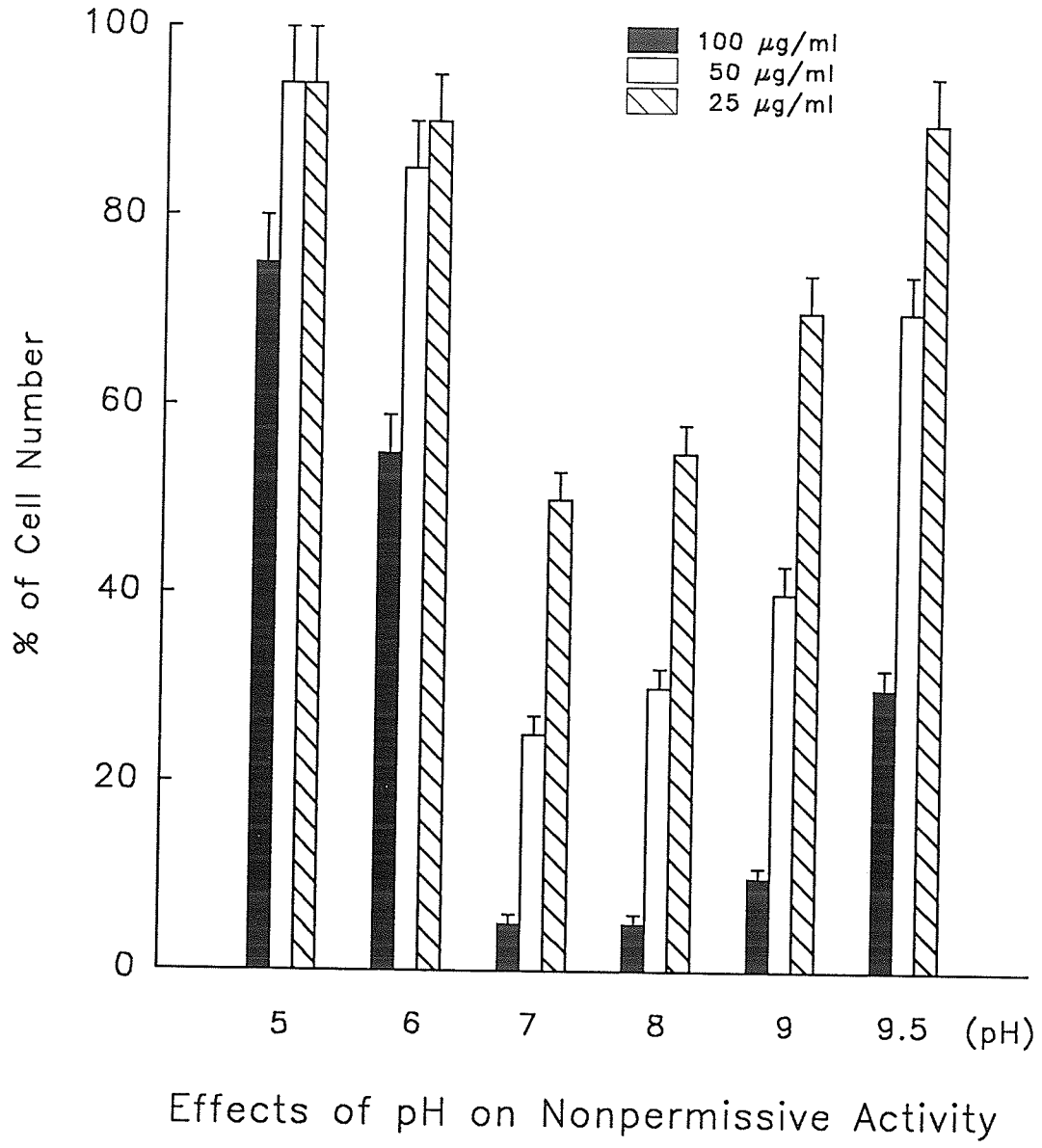

Figure 15. Effects of pH on the nonpermissive substrate activity. Samples of 200 μl were incubated in buffers of different pH at 4°C for 24 hours, and then dialysed against 0.25% NH_4HCO_3, followed by subjecting to the cell number assay of NG108-15 cells in triplicates.

of the nonpermissive activity.

For enzyme digestion, samples of solubilized proteins (100 μl of 500 μg/ml) were digested at 37°C for 1 hour with the following enzymes and conditions. Digestions of trypsin (25 μg/ml), neuraminidase (0.1 units/ml), phospholipase D (0.5 unit/ml), deoxyribonuclease I (5 μg/ml), chondroitinase AC (0.5 units/ml), and keratanase (0.1 unit/ml) were carried out in 20 mM sodium phosphate buffer, pH 7.2. At the end of the digestion, reaction mixtures were boiled at 70°C for 2 minutes, and then subjected to the cell number assay of NG108-15 cells for nonpermissive activity. For chemical treatments, samples were incubated overnight at 4°C in the presence of 8 M urea, 1% β-mercaptoethanol and 1 M NaCl followed by dialysis against 0.25% NH_4HCO_3 overnight before subjecting to the NG108-15 cell bioassay. The nonpermissive substrate activity was not significantly affected by denaturing treatments of 8 M urea or reducing conditions of β-mercaptoethanol, but destroyed by the digestion of trypsin, though not by neuraminidase, phospholipase D, or DNase as shown in Table 1. Similarly, the nonpermissive activity was not significantly affected by digestions of chondroitinase AC or keratanase, indicating that chondroitin sulphate and keratan sulphate are not essential for its activity (Table 1). These findings suggest that the rat CNS plasma membrane-associated nonpermissive factor is of protein nature, but not a

Table 1. Comparison of nonpermissive substrate activity after treatments with denaturing agents and enzymic digestions. Aliquots of 100 μg/ml of solubilized protein samples, after treatments under various experimental conditions, were treated at several dilutions as coating substrates for the cell number assay of NG108-15 cells. Bovine serum albumin (BSA) was used as a positive standard.

Treatment	**Cell Number (%)**
A. Denaturation	
Control (BSA)	100%
8 M urea	<20%
1% β-mercaptoethanol	<20%
1M NaCl	<20%
B. Enzyme Digestion	
Trypsin	80-100%
Neuraminidase	<20%
Phospholipase D	20-50%
DNase I	<20%
Chondroitinase AC	20-50%
Keratanase	<20%

proteoglycan, and its tertiary structure and disulphide bonds, if present, are probably not essential for its nonpermissive substrate activity.

5.4 Preliminary studies on partial purification of the plasma membrane-associated nonpermissive protein

5.4.1 Fractionation by ammonium sulphate

Alkaline extract of nonpermissive activity from adult rat spinal cord was precipitated by ammonium sulphate with 0~50% saturation. The activity containing fractions was redissolved and dialysed overnight against 0.25% NH_4HCO_3, and assayed for nonpermissive activity. Figure 16 shows that ammonium sulphate at saturation 0~50% saturation precipitated approximately 60% of the total solubilized proteins, and contained over 95% of the nonpermissive activity in the crude extract. The 0~50% precipitate fraction was therefore used as the first purification step. Several other separation methods, including gel permeation on Sepharose CL-4B, chromatography on DEAE-cellulose, and preparative polyacrylamide gel electrophoresis were characterized as possible means to obtain partially purified nonpermissive protein from rat CNS plasma membranes.

5.4.2 Gel permeation on Sepharose CL-4B

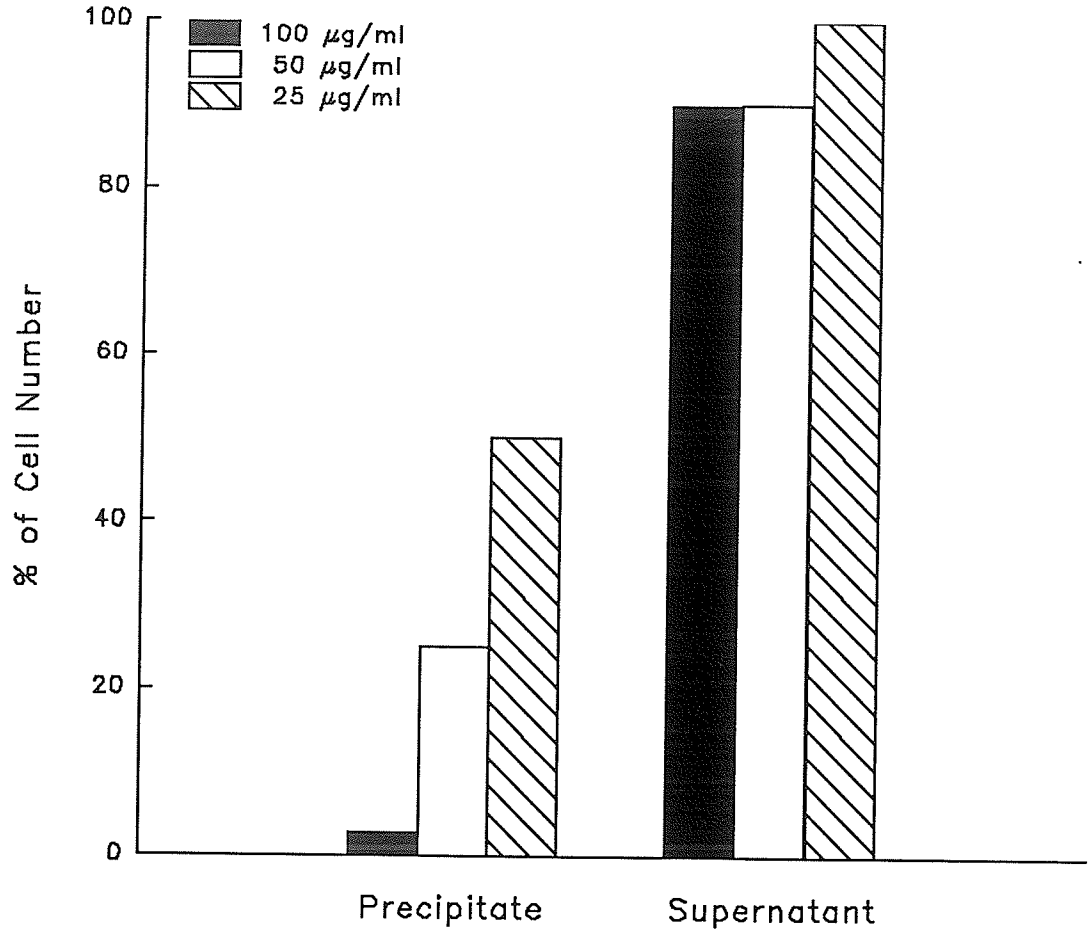

Figure 16. Fractionation of the nonpermissive protein by ammonium sulfate precipitation. The alkaline extract of solubilized proteins from rat spinal cord plasma membranes was precipitated by adding solid ammonium sulfate slowly to a 50% saturation to precipitate proteins with nonpermissive activity. After centrifugation, the precipitate was redissolved, dialysed against 0.25% NH_4HCO_3, and subjected to the cell number assay of NG108-15 cells for nonpermissive activity.

The alkaline extract of solubilized proteins of plasma membranes was applied onto a Sepharose CL-4B column, equilibrated in 20 mM sodium phosphate buffer, pH 7.4, containing 0.05% CHAPS. The solubilized proteins were fractionated by this column into one small peak, corresponding to V_0, followed by one large peak of proteins. Various fractions were monitored by the cell number assay of NG108-15 cells, and the activity was found in the eluents of number 65~85, corresponding to molecular mass of 70~80 KDa, as shown in figure 17. Fractions with nonpermissive activity were pooled, and concentrated for further characterization studies.

5.4.3 Stepwise elution on DEAE-cellulose ion-exchange chromatography

A concentrated sample of the nonpermissive protein precipitated by 0~50% saturation of ammonium sulphate was applied onto a DEAE-cellulose ion-exchange column, equilibrated in 20 mM Tris-HCl buffer at pH 8.2. After washing extensively, the adsorbed proteins were eluted with increasing concentrations of sodium chloride in the same buffer, as shown in Figure 18. The appropriate fractions were pooled, dialysed against 0.25% NH_4HCO_3 overnight at 4°C, and then assayed by the cell number assay of NG108-15 cells for nonpermissive activity. The fractions eluted by 0.2 M sodium chloride was found to contain most of the nonpermissive

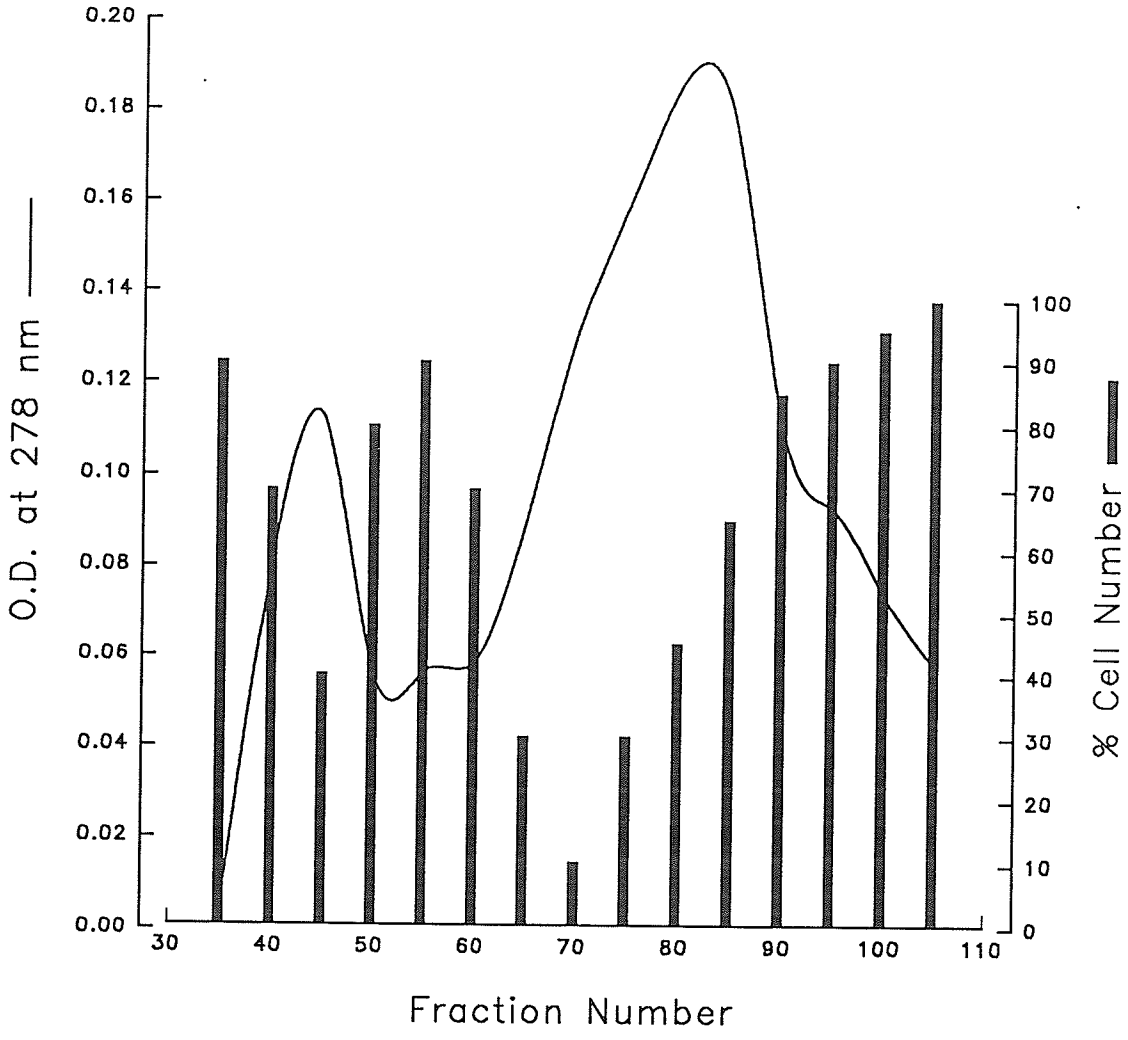

Figure 17. Gel filtration on Sepharose CL-4B. The dialysed alkaline extract of the nonpermissive substrate activity of adult rat spinal cord plasma membranes was applied onto a column of Sepharose CL-4B, equilibrated in 20 mM sodium phosphate buffer, pH 7.4, containing 0.05% CHAPS. Fractions were collected at 2 ml/tube with a flow rate of 15 ml/hour. Various fractions were monitored by the cell number assay of NG108-15 cells for nonpermissive activity.

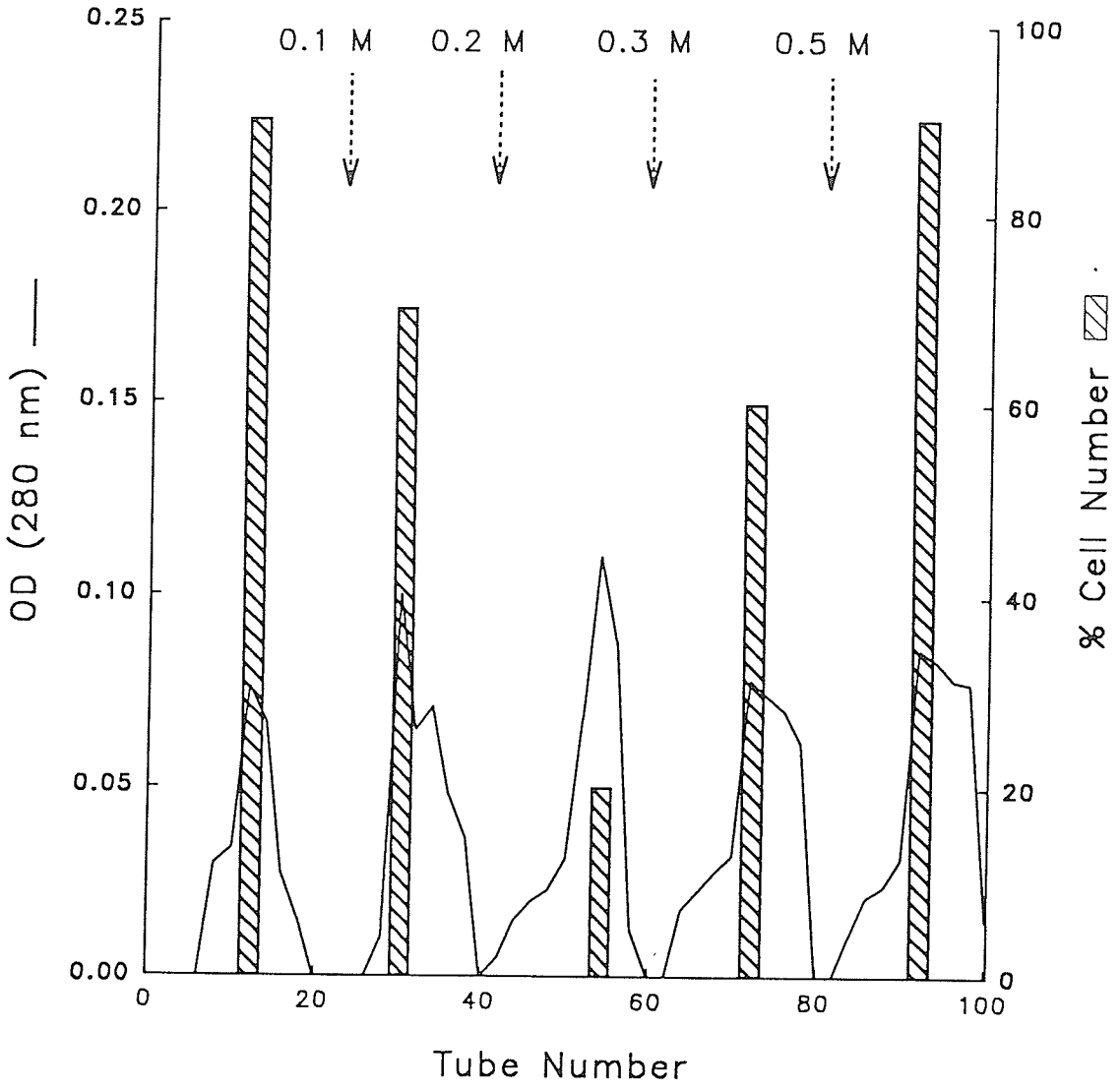

Figure 18. Fractionation of the alkaline extract of plasma membrane proteins on DEAE-cellulose chromatography. Adsorbed proteins were eluted by increasing concentrations of 0.1, 0.2, 0.3 and 0.5 M NaCl, as indicated by readings at 280 nm. The nonpermissive activity of each fraction was monitored by the cell number assay of NG108-15 cell, as indicated by the stripe bars. The nonpermissive activity is expressed as percent of cell number comparing with a BSA control.

activity in the alkaline extract of the plasma membranes.

5.4.4 Fractionation of nonpermissive substrate activity by preparative gel.

A concentrated sample of nonpermissive protein precipitated by 0~50% saturation of ammonium sulphate was subjected to preparative polyacrylamide gel electrophoresis for purification under non-denaturing conditions. One side lane was cut and stained with Commassie Brilliant Blue R-250 for protein bands, and was carefully aligned with the unstained gel as a guide for gel segmentation. The unstained gel part was then cut into segments and each region was homogenized and extracted in 0.25% NH_4HCO_3, pH 8.2, for 2~3 hours at 4°C. The homogenate was centrifuged and the supernatant lyophilized. Salts were removed by passing through a mini-column of G-25. Extracts of the gel segments were monitored by the bioassay of NG108-15 cells for nonpermissive substrate activity. Most of the nonpermissive activity was recovered in gel segment number 7 as shown in Figure 19.

5.4.5 Determination of molecular weight by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate

The molecular weight of the partially purified

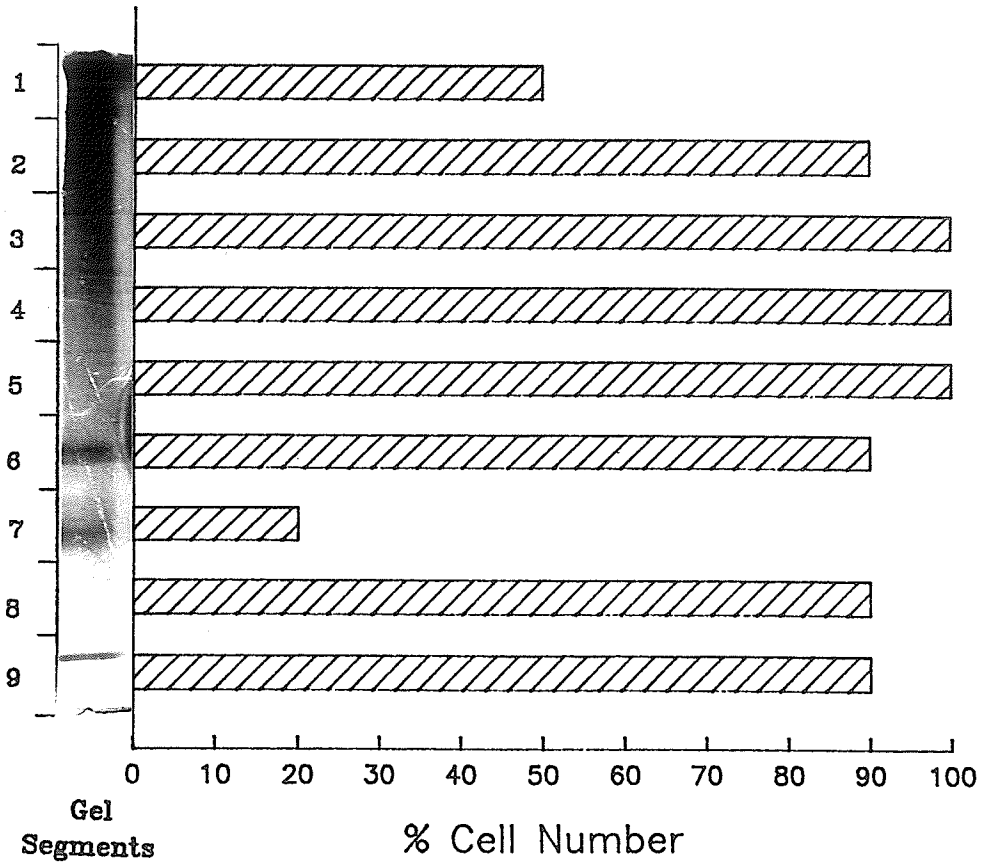

Figure 19. Fractionation of the alkaline extract of plasma membrane proteins by preparative polyacrylamide gel electrophoresis (PAGE). A sample of solubilized membrane proteins was fractionated on a 7.5% separating gel of preparative PAGE at pH 8.9 under non-denaturing conditions. The gel was segmented and then extracted in 0.25% NH_4HCO_3 for 2~3 hours. Eluted proteins from each gel segment were monitored by the cell number assay. Nonpermissive substrate activity was expressed as percent of cell number comparing with a BSA control.

nonpermissive protein fractions was determined by analytical SDS-polyacrylamide gel electrophoresis. Figure 20 shows the electrophoretic protein patterns of crude and partially purified nonpermissive protein fractions. Protein markers used were phosphorylase B (97K), bovine serum albumin (66K), ovalbumin (45K), carbonic anhydrase (29K), soybean trypsin inhibitor (20K), and lysozyme (14K). The active fraction after DEAE-cellulose chromatography consisted of one major band of Mr 50~60 KDa, while that eluted from preparative PAGE contained four bands of Mr 25~60 KDa, including one of Mr 50~60 KDa (figure 20). The estimated molecular weight of the nonpermissive activity protein by SDS-PAGE appeared to be 50~60 KDa.

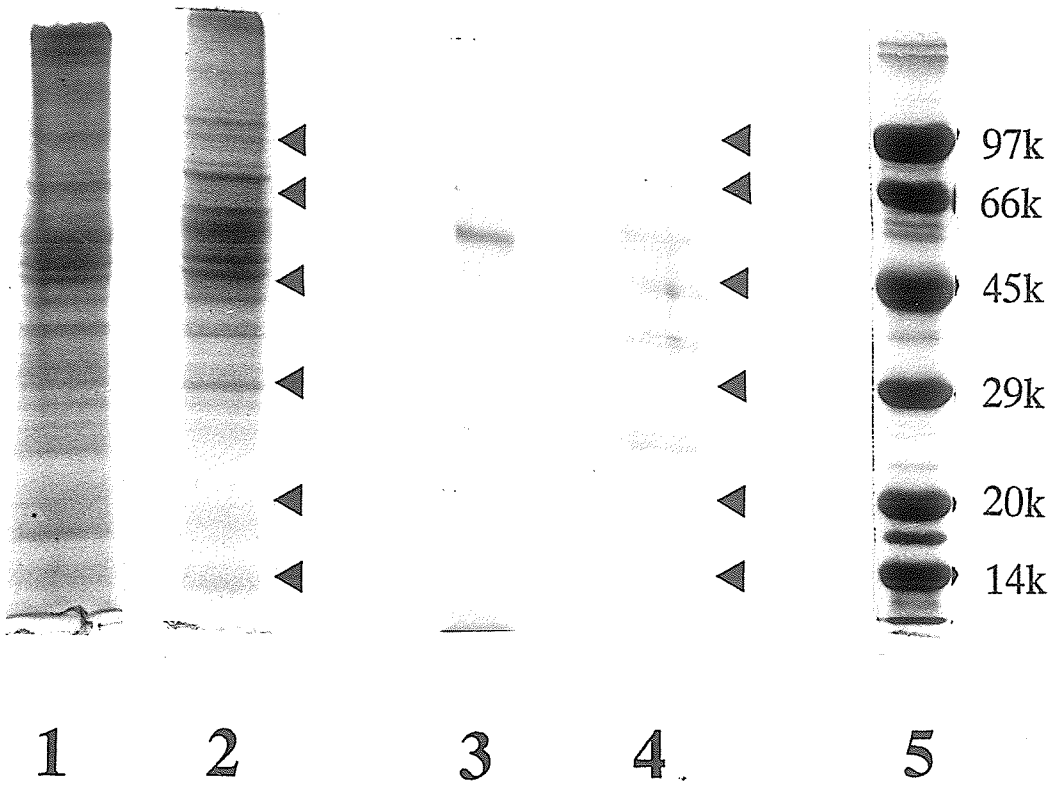

Figure 20. Analytical SDS-polyacrylamide gel electrophoresis of partially purified nonpermissive protein fractions after DEAE-cellulose chromatography and preparative polyacrylamide gel electrophoresis. (1), Total proteins of the adult rat spinal cord plasma membranes; (2), Solubilized proteins in the alkaline extract (0.1 M NH_4OH) of plasma membranes; (3), 0.2 M NaCl eluent of nonpermissive protein fraction after DEAE-cellulose chromatography (Figure 18); (4), Nonpermissve substrate protein fraction extracted from gel segment 7 of preparative PAGE (Figure 19); (5). Molecular weight standards: phosphorylase B (97K), bovine serum albumin (66K), ovalbumin (45K), carbonic anhydrase (29K), soybean trypsin inhibitor (20K), and lysozyme (14K).

6. DISCUSSION

6.1 Characterization of the bioassay of NG108-15 cells for the nonpermissive substrate activity

The bioassay for nonpermissive activity, using the hybrid NG108-15 cell line, has been adapted as a routine assay system for monitoring activity in tissues from various CNS regions of adult and neonatal rats, and in various fractions at different steps of isolation. A simple and reproducible assay system to follow the distribution of activity is essential for successful purirication of the protein factor. Treatment of the NG108-15 cells with 1 mM dBcAMP resulted in dramatic morphological changes of neuronal differentiation. The cells quickly attached to the BSA substrate and neurite outgrowth began almost immediately (within 2 hours). By 48 hours, the cells had both large axon-like processes and short, branched, dendrite-like processes; as well, the shape of the cell soma was characteristic of neurons. In contrast, significantly fewer NG108-15 cells adhered or survived when cultured on a coating of alkaline extract of plasma membrane substrate. Of those cells that eventually adhered to the plasma membrane substrate, very few became highly differentiated. The generally round appearance of their cell bodies was unlike terminally differentiated neurons. Very few of these cells grew any processes and did so only after 24 or even 48 hours

in culture (Figure 2). The differentiation of NG108-15 cell requires a minimal amount of substrate adhesion, which appears to be absent or diminished in the alkaline extract of plasma membranes. At present, the molecular mechanism regulating cell adhesion and differentiation of the NG108-15 cell by the nonpermissive molecule(s) in the CNS plasma membranes remains unclear.

We have further demonstrated that the plasma membrane isolated from adult rat CNS was a highly nonpermissive substrate not only on clonal NG108-15 cells but also on primary cultures of fetal rat cortical neurons (unpublished observation). Most of the nonpermissive activity in the plasma membrane could be extracted under alkaline conditions; and the nonpermissive substrate activity on cultured neuronal cells was time and concentration dependent (Figure 2 & 3).

Some bioassay systems have been developed by other investigators, such as growth cone collapse assay (Raper & Kapfhammer, 1990) and stripe assay (Walter et al., 1987b). When comparing the bioassay using NG108-15 cells with other assay systems, such as growth cone collapse assay (Raper & kapfhammer, 1990) and assays with cultures of primary fetal rat CNS neurons (unpublished observation), for monitoring nonpermissive substrate activity, it is clear that the NG108-15 cell bioassay is simple and significantly less time

consuming and a more convenient assay system for routine measurements. This bioassay system measures primarily substrate activity of cell adhesion on protein-coated surfaces. The number of adhering cells, correlates quantitatively with the nonpermissive activity in the solubilized plasma membrane proteins. However, it has been observed that the adhesive NG108-15 cells on the permissive substrate (e.g. BSA) adjacent to the border of an nonpermissive substrate (e.g. spinal cord plasma membranes) extended neurite processes only in the directions along the border of the nonpermissive coating, whereas cells inside the permissive environment extended neurites at different directions. Only after 2~3 days in culture, some (10~20%) of the already differentiated cells began to extend neurites over the border onto the initially nonpermissive coatings, indicating that neurite outgrowth of these cells has initially been inhibited by the nonpermissive membrane proteins (Ethell et al., 1993). These findings suggest that monitoring nonpermissive activity by this simple and convenient bioassay of NG108-15 cells is likely corresponding to the neurite outgrowth inhibitory activity.

6.2 Isolation of rat CNS myelin and plasma membranes

Procedures to obtain purified plasma membranes, relatively free of myelin, have been reported for rat brain

tissues (Agrawal et al., 1974; Sun et al., 1988) and oligodendroglia (Poduso, 1975; Polak & Szuchet, 1988). Recently, it has been reported that the flotation technique of centrifugation in a discontinuous sucrose gradient, comparing with the sedimentation technique, provides not only higher yields of myelin but also myelin-free proteins in the non-myelin particulate fractions, including the plasma membranes (Royland et al., 1992). In the present study, we therefore used the flotation method to obtain purified plasma membrane and myelin from rat brain, brainstem and spinal cord tissues. The differences between myelin and plasma membranes were analyzed by SDS-PAGE: myelin was rich in low molecular weight proteins (14~23 KDa) of myelin basic protein (MBP) and proteiolipid protein (PLP), whereas plasma membranes consisted of major proteins of molecular weight 30~70 KDa (Figure 6 & 8). These results indicated that our purified plasma membrane preparations were essentially free of myelin contaminations.

6.3 Nonpermissive substrate activity in plasma membranes from grey and white matters

Rat CNS white matter is composed of axons, astrocytes and myelin-producing oligodendrocytes. Several reports have shown that grey matter regions of the mature rat CNS supported neurite regeneration, whereas the major white matter tracts generally did not support neurite growth (Crutcher, 1989;

Savio & Schwab, 1989; Siegal et al., 1990). These results are consistent with the hypothesis of the presence of growth-inhibiting factors associated with CNS white matter. In the present study, the plasma membrane from rat brain grey matter was observed to be a relatively nonpermissive substrate for cell adhesion and neurite outgrowth, even though slightly lower than that of the white matter (Figure 11). The discrepancy between our present observation of nonpermissive substrate and some previous reports on the permissive substrate effects of brain grey matter could be due to differences in the assaying methods of tissue sections as substrate as compared to coating of solubilized membrane proteins. However, the present observation of nonpermissive substrate activity in plasma membranes from adult rat brain grey matter is in excellent agreement with the findings of Cook et al (1992) on the identification of a neurite inhibitory protein in chick grey matter. Whether the nonpermissive activity in the rat grey matter is identical to that of the white matter remains to be answered.

6.4 Myelination and oligodendrocyte during development

In vitro, oligodendrocytes have been shown to be inhibitory to both cell spreading and neurite outgrowth (Schwab & Corani, 1988; Fawcett et al., 1989; Bandtlow et al., 1990), and the active inhibitory principles in rat CNS myelin

have been reported to be two myelin-associated proteins, designated as NI-35 and NI-250 (Caroni & Schwab, 1988). Recently, however, studies have indicated that oligodendrocytes may not be inhibitory for neurite outgrowth because embryonic rat retinal neurite grew well on astrocytes in the presence of mature oligodendrocytes or among oligodendrocytes alone (Ard et al., 1991). Similarly, we have observed that purified myelin from adult rat brain or spinal cord possessed both permissive and nonpermissive properties depending on the method of extraction (unpublished observation). The alkaline extracts of both purified myelin and plasma membrane were nonpermissive substrates for NG108-15 cells (unpublished observation). The nonpermissive activity in purified myelin, however, was substantially less than that of the plasma membrane.

The nonpermissive activity in rat brain, brainstem and spinal cord was observed from postnatal day 1, and the activity increased till postnatal day 26 (Figure 5 & 7). In neonatal rats, the process of myelination appears to occur after day 10 as monitored by the appearance of myelin basic protein (MBP) in the brain and spinal cord of postnatal rats at various developmental stages (Figure 6 & 8). The source of the nonpermissive substrate activity is not known at the present time. Further studies are required to delineate the molecular identity and sites of expression of the plasma

membrane-associated nonpermissive protein.

6.5 Properties of the plasma membrane-associated inhibitory protein

The alkaline extract of adult CNS plasma membranes was a nonpermissive substrate for cell adhesion and neurite outgrowth, and this activity was concentrated by precipitation at 0~50% saturation of ammonium sulphate (Figure 12). The nonpermissive activity was also found to be relatively stable at a pH range of 7~9, but unstable at acidic pH below 6 (Figure 15). The nonpermissive activity was relatively unstable at 4°C but became more stable at -70°C (Figure 13) and was relatively heat-resistant (Figure 14). Furthermore, the rat CNS plasma membrane-associated nonpermissive activity was sensitive to trypsin treatment (Table 1), in contrast to the inhibitory activity reported in membranes of injured brain tissues (Bovolenta et al., 1993). The nonpermissive substrate activity was not destroyed by digestions of neuraminidase, phspholipase D, DNase, or affected by 1% β-mercaptomethanol, 8 M urea and 1 M NaCl (Table 1), suggesting that the nonpermissive activity is protein in nature, but not a proteoglycan, and its tertiary structure and disulphide bonds, if present, are probably not essential for its activity. However, this conclusion should be regarded as tentative since the purity of these commercially available enzymes used were

unknown and the membrane-associated nonpermissive activity studied was a crude preparation of solubilized membrane proteins. Nevertheless, preliminary data on its chemical properties suggest that the rat CNS plasma membrane-associated nonpermissive protein is likely a peripheral membrane-associated protein, possibly similar to the chick collapsin which lacks a transmembrane-spanning region (Luo et al., 1993). However, our rat CNS inhibitor was relatively stable under various denaturing reagents as well as heat-resistant, in contrast to chick collapsin which was heat-labile, but similar to the injured tissue membrane (IM) inhibitory activity which was heat resistant (Bovolenta et al., 1993). However, because these properties of the nonpermissive protein were elucidated in crude preparations of solubilized plasma membrane proteins, they might not reflect the exact nature of the highly purified protein.

6.6 Preliminary studies on partial purification of the nonpermissive protein

Fractionation of CHAPS solubilized injured tissue membrane (IM) was performed using ion-exchange chromatography on DEAE-cellulose under chaotropic conditions of 7 M urea. Under these conditions, sulphated proteoglycans bound to DEAE-cellulose, while the majority of non-GAG-containing proteins did not. The inhibitory activity fraction was eluted with 0.8

M NaCl (Bovolenta et al., 1993). In contrast to the IM-proteoglycan inhibitor, our plasma membrane-associated nonpermissive substrate protein was eluted with 0.2 M NaCl upon DEAE-cellulose ion-exchange chromatography (Figure 18).

The myelin-associated inhibitory proteins of Mr 35 and 250 KDa were separated by SDS-PAGE, and appeared to survive the SDS-denaturing procedure. However, we obtained the rat CNS nonpermissive substrate activity by preparative native-PAGE without denaturing agents (Figure 19), and it appeared to have 4 bands as determined by analytical SDS-PAGE (Figure 20): one of them of 50~60 KDa corresponded to the protein band eluted by 0.2 M NaCl from DEAE-cellulose chromatography. Both were biologically active fractions of highly nonpermissive substrates for NG108-15 cells. Whether the CNS plasma membrane-associated nonpermissive substrate activity is identical, or related, to the myelin-associated NI35 and NI-250 (Caroni & Schwab, 1988) remains to be resolved.

From its estimated molecular weight of 50~60 KDa by polyacrylamide gel electrophoresis in the presence of 1% SDS (Figure 20), the rat CNS plasma membrane-associated nonpermissive protein appears to be smaller than that of the chick brain collapsin with molecular weight of 100 KDa (Luo et al., 1993), and differs from the myelin-associated inhibitory factors with molecular weights of 250 and 35 KDa (Caroni &

Schwab, 1988). Further, the membrane-associated factor is also too small to belong to the tenascin family of J1/160 (Faissner & Kruse, 1990) or proteoglycan (Dou & Levine, 1992), or sulphated proteoglycan inhibitor from kainic acidic-injured rat brain tissue, with molecular weight 160-220 KDa (Bovolenta et al., 1993).

6.7 Possible functional roles of the plasma membrane-associated nonpermissive protein factor

There appear to be more inhibitors in the mammalian CNS both in developing and differentiated conditions. The identification and partial purification of neurite outgrowth inhibitors from various tissues have been reported by several investigators: myelin-associated inhibitors (Caroni & Schwab, 1988), chick brain collapsin (Luo et al., 1993), glycoproteins from posterior tectal membranes (Cox et al., 1990), J1/tenascin (Faissner & Kruse, 1990), NG2 proteoglycan (Dou & Levine, 1992) and IM-associated inhibitor (Bovobenta et al., 1993).

Since the capacity to restore function after an injury would appear advantageous, there must have been a selection pressure to abandon this principle during the evolution of the complex mammalian nervous system. Myelination starts only late in embryogenesis, so myelin proteins are not involved in

early CNS development. Findings of the present study indicate that the nonpermissive substrate activity on CNS plasma membranes may, together with other neurite outgrowth inhibitors and stimulators, play an important role(s) during the development and differentiation of the mammalian CNS. It is possible that the presence of nonpermissive inhibitors serves to separate territories of different fibre tracts from each other, and is essential for the rate and direction of neurite extension during early development of the mammalian CNS; as well, it may exert a stabilizing function for the differentiated CNS. However, whether this speculation contains some truth or not remains to be investigated. It is possible, therefore, that elucidation of the molecular mechanism by which regeneration is inhibited will help to facilitate the repair processes in the mammalian CNS following injury.

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