The Role of Glycoproteins in Neural Plasticity in Domestic Chick

Thesis

THE ROLE OF GLYCOPROTEINS IN NEURAL PLASTICITY IN DOMESTIC CHICK

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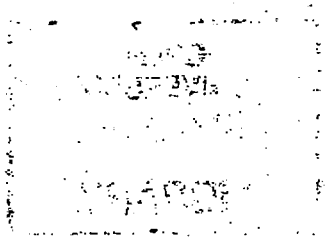
TO MY DEAREST FATHER...



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LIST OF ABBREVIATIONS

BSA	Bovine serum albumin
CNS	Central nervous system
2DGal	2-deoxy-D-galactose
DAB	Diaminobenzidine
DAPI	4,6-diamido-2-phenylindole
ECM	Extra cellular matrix
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
Fuc	Fucose
GalNAc	N-Acetylgalactose amine
GlcNAc	N-Acetylglucose amine
HEPES	4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid
HRP	Horseradish peroxidase
IMC	Nucleus isthmi pars magnocellularis
IMHV	Intermediate medial hyperstriatum ventrale
IgG	Immunoglobulin G
INT	*p*-iodonitrotetrazolium violet
Ic	Intracerebral
Ip	Intraperitoneal
IPC	Nucleus isthmi pars parvocellularis
LPO	Lobus parolfactorius
Iv	Intraventricular
LTP	Long-term potentiation
Man	Mannose

MeA	Methylanthranilate or Methylanthranilate-trained
Mr (kD)	Molecular weight in kilodaltons
NANA	N-Acetylneuraminic Acid
N-CAM	Neural -cell adhesion molecule
NGF	Nerve growth factor
NILE / L1 / Ng-CAM	NGF Inducible Large External glycoprotein
O.G.	*n*-octylglucoside
PA	Paleostriatum
PBS	Phosphate-buffered saline
Pia	Pia mater
PKC	Protein kinase C
PNS	Peripheral nervous system
PIS	Pre-immune serum
PMSF	Phenylmethylsulfonylfluoride
PSD	Postsynaptic membrane
PTM	Post translational modification
RER	Rough endoplasmic reticulum
SAG	Stratum album centrale
SGC	Stratum griseum centrale
SGF	Stratum griseum et fibrosum superficiale
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SER	Smooth Endoplasmic Reticulum
SO	Stratum opticum
SPM	Synaptic plasma membrane
TBS	Tris-buffered saline
TCA	Trichloroacetic acid
TEMED	*N, N, N', N'*-tetramethylethylenediamine
W	Water or water-trained

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ABSTRACT

In this project various neurochemical and immunological approaches were used to identify and analyse the role of glycoproteins in neural plasticity. Where appropriate, the one-trial passive avoidance paradigm was used (Gibbs & Ng 1977).

Initially, the role of a glycosylation inhibitor, 2-deoxy-D-galactose (2DGal), was examined both *in vivo* and *in vitro*. The results of these studies agreed with previous reports that 2DGal inhibits incorporation of fucose and galactose into glycoproteins. However, 2DGal inhibited fucosylation and galactosylation of only a sub-set of these macromolecules. The localization of these glycoproteins in synaptic plasma membranes (SPMs) and postsynaptic densities (PSDs) suggested differential glycoprotein synthesis and distribution across the synaptic cleft.

Subsequent studies attempted to identify the fucosylglycoproteins involved in learning and memory formation and to characterize their structure, sub-cellular distribution and the timecourse of their expression. An *in vitro* increase in the rate of fucosylation was found in the right forebrain base after training. Further, the rate of fucosylation of a protein component of Mr 110-120kD increased significantly within the first four posttraining hours.

A number of glycoproteins showing significant increase in fucosylation either 6hr or 24hr after training, or at both posttraining time-points were identified *in vivo*. Most were concentrated in SPMs and PSDs of LPO as summarised in figure A.1.

Time after training		6hr				24hr	
Structure		LPO		PA		LPO/PA	
Location		SPM	PSD	SPM	PSD	SPM	PSD
No.	Mr (kD)						
1	150-180	⇓L&R	-	-	-	-	⇑L
2	100-120	-	-	-	-	⇓L	⇑L&R
3	62-80	-	⇑R	-	-	-	⇓R
4	55	-	⇓L	-	-	-	⇑L
5	50	⇑L	-	-	-	⇑L	-
6	41	⇑L	⇓R	⇑L	-	-	⇓L
7	33	⇑R	⇓R	-	⇑L&R	-	⇓L, ⇑R
8	27	-	⇓R	-	-	-	⇓R

Figure A.1 : Identified glycoproteins of chick forebrain that show changed fucose incorporation 6hr and 24hr after training. ⇑, increased fucosylation; ⇓ decreased fucosylation; L, left hemisphere; R, right hemisphere; LPO/PA, combined LPO and PA samples.

Polyclonal antibody R15 was raised and it recognized a 60-62kD antigen in SPM and PSD fractions. It rendered chicks amnesic when injected bilaterally 30min before training. Immunocytochemical studies showed medium density labelling of axonal membranes and SPMs and PSDs.

Finally, the sugar contents of SPM and PSD membrane-bound glycoproteins were analysed using specific lectins. Chick brain glycoproteins were separated by two-dimensional gel electrophoresis and identified using the lectin Ulex Europaeus type I (UEA.I). Some 55 fucosylated polypeptides were found in SPMs and PSDs. Specific

antibodies whose antigens have been implicated in learning and memory formation were used to examine whether their immunoreactivity changed as a result of passive avoidance training. Both anti-N-CAM antibodies and anti-ependymin antibodies cross-reacted with chick antigens from SPM and PSD fractions, but immunoblotting did not show training-induced changes.

The results reported in this thesis are discussed in the light of current understanding of neural mechanisms of learning and memory. Several hypotheses are considered which collectively recognize glycoproteins as major contributors to neural network formation/re-formation during learning and formation and consolidation of memory.

CHAPTER ONE

Glycoconjugates And Their Role In Neural Plasticity

CHAPTER ONE

GLYCOCONJUGATES AND THEIR ROLE IN NEURAL PLASTICITY

PART ONE : AN OVERVIEW

Over the last few decades research into biological properties of glycoconjugates [glycoproteins, glycosphingolipids and proteoglycans] has grown into new ground. Work on glycoconjugates ranges from fundamental biological and developmental aspects (e.g., Berger et al., 1982) through pathological manifestations (e.g. Volk & Aronson, 1972; Dowson and Hancock, 1989) to behavioural effects (e.g., Rahman 1984; Rose et al., 1987).

Research areas such as serology, virology, immunology, toxicology, oncology, and neuroembryology (encompassing growth, differentiation, cell-cell interaction, and development) have benefitted from this extensive work on glycoconjugates.

In neurology, the important role of these macromolecules is increasingly recognized. The sheer quantity and structural diversity of glycoconjugates, and particularly recognition of the fact that almost all neural cell membrane proteins are glycosylated, indicates their evolutionary and hence functional importance. Glycoproteins are constituents of most membrane systems and most secreted macromolecules (Margolis & Margolis, 1983; Hughes, 1983). Their structural diversity seems to reside more in the number or type of polysaccharide chains attached to the macromolecules than in their polypeptide backbones (Brunngraber, 1972).

Glycoconjugates and neural development

Glycoconjugates research has concentrated mostly on embryonic neurogenesis, neural differentiation and migration as well as synaptogenesis and neural regeneration and degeneration. The expression and alteration of cell surface glycoconjugates, for example, have been monitored in many differentiating and developing systems. Researchers use specific carbohydrate binding lectins (see Chapter 6) to identify the structure, tissue distribution and developmental role of glycoconjugates. For example, the increase in concanavalin A binding glycoproteins on the membranes of newly formed parallel fibres in the developing cerebellum and simultaneous appearance of a mannose-specific lectin on the surface of Purkinje cells are said to serve important functions during the period of active synaptogenesis (Zanetta et al., 1978 and 1985). This is believed to be a highly dynamic process, since in order to generate functional neural circuits, selective connections between distinct subsets of neurons must be formed (Margolis & Margolis 1989).

What is common to all synaptic glycoconjugates, however, is that their oligosaccharides are located within the synaptic cleft and therefore changes in their carbohydrate composition will alter the general molecular environment of the cleft (Margolis & Margolis 1989). The size of some of these large, complex, N-linked oligosaccharides (see below) is such that it can cover an area of 20-25nm^2 (Montreuil, 1984). This enables opposing membranes to make contact through their carbohydrate chains.

During embryogenesis, several distinct steps are necessary before precise coordinated intercellular recognition can take place. First, there is a process of selective adhesion to segregate neural cells from non-neural epithelial cells. This results in the formation of neural tissue. Then follows the migration of neuroblasts and their differentiation into distinct subsets of neurons, the axons of which project to their specific cellular targets

under the influence of the extracellular matrix (ECM) and its associated proteins. The innervation of the tectum by axons of retinal ganglion cells has provided a classical model system.Glycoconjugates are likely candidates as mediators of retinotectal recognition (Margolis & Margolis 1989). The growth cones then appear to recognize and select appropriate cellular targets with which to form stable contacts. The mechanism of recognition involves homophilic interactions between molecules present on the surface of participating neurons (Edelman, 1986; Edelman et al., 1987; Rutishauser & Jessell, 1988). Calcium independent and dependent adhesion molecules like N-CAM and N-cadherin are involved in this process. These molecules are, therefore, implicated in the process of synapse formation (Jørgensen et al., 1987). The presence of the terminal saccharide (polysialic acid) on N-CAM, for example, seems to affect the binding of opposing membranes during embryonic stages (Hoffman & Edelman, 1983).

Growth cones are motile organelles, situated at the tip of growing nerve fibers, and have fine projections called filopodia. They contain microfilaments which are responsible for testing the substratum for its adhesiveness (Carbonetto, 1984; Landis, 1983; Letouruean, 1983). Time-lapse studies have shown that adhesion of filopodia to their substrata appears to initiate contractile forces that help extend the neurite (Carbonetto, 1984). To do this, filopodia containing integrins make contact with the ECM at one end and through an integrin linkage to the cytoskeleton (For more detailed account of the role of ECM see below). This sequence of coupling helps accomplish contractile activity of the growth cone and extend the axons (Margolis & Margolis 1989). Decoupling of integrins (through, for example, phosphorylation or protease activity) and their recoupling with the next ECM-embedded glycoconjugates along the line is essential for further axonal growth.

Several lines of evidence point to the necessity of integrins for in neural growth.

Antibodies against integrins inhibit avian neural crest cell migration, resulting in malformed neural tubes *in vivo* (Duband et al., 1986). Anti-integrin antibodies also inhibit neurite growth from PNS and CNS neurons on laminin or fibronectin (Cohen et al., 1986 and 1987).

It is clear, therefore, that the development of a synapse is an ongoing process which involves initial contact and recognition between the opposing membranes, the formation of an adhesive membrane junction, the stabilization of the synaptic contact and subsequent morphological, biochemical, and functional maturation of the synapse. Glycoproteins are considered to be particularly involved in the initial recognition and adhesion between putative pre- and postsynaptic cell membranes (Margolis & Margolis 1989). Indeed, changes in cell surface glycoconjugates have been observed in many differentiating and developing systems, including cerebral tissue (Ziesk & Bernstein, 1982; Raedler et al. 1981).

During neural development, the protein and glycoprotein contents of the synapse change and the oligosaccharides associated with glycoproteins of the synaptic junction undergo structural modification. For example, the number of chains containing eight mannose, equal in quantity with those containing five mannose ($mannose_5$), at day 10, diminishes by day 28. This leads to $mannose_5$ chain predominance (Fu & Gurd, 1983). During the same period, there is a small increase in the amount of sialylation of fucosylated glycoproteins (Stanojev, 1987).

Synaptic plasticity

The role of the ECM in neural growth and development was briefly mentioned above but further discussion of the role of this complicated network of macromolecules is warranted. The extracellular space occupies about 40% of the immature brain

(Bondareff & Pysh, 1968), and then decreases to 20% in the adult brain (Nevis & Collins, 1967). This space is filled with ECM and consists of insoluble glycoconjugates like laminin and fibronectin and other glycoproteins and proteoglycans through which neural cell precursors migrate to their final positions. Through this process, adhesion of cells to glycoconjugates of the matrix or other cells is essential, for example, the adhesion between ECM glycoproteins, laminin and fibronectin, and their receptors on the cell surface (Margolis & Margolis 1989). Laminin is a large glycoprotein (900kD) that consists of 3 sub-units (400, 215 and 205kD), each expressed by a different gene, and disulphide-bonded to form a molecule shaped like a crucifix. Laminin has multifunctional properties as known by its various binding sites for ECM and cell surface molecules. For example, it contains a heparin binding site which can potentially bind to N-CAM (Ott et al., 1982).

Laminin can induce neurite outgrowth from CNS as well as PNS neurons (Kleitman et al., 1988). It has been localized immunocytochemically in the embryonic CNS, but is thought to be absent from the adult mammalian CNS (Manthorpe et al., 1988). Therefore, the process of synapse formation in adult mammalian brain would have to involve matrix glycoconjugates other than laminin. Indeed, the ECM contains adherons that are associated with the cell surface and as such play a major role in cell-cell adhesion (Margolis & Margolis, 1989). It also contains a number of matrix-bound factors such as proteases, protease inhibitors, soluble forms of membrane protease and growth factors. Consequently, the ECM of the CNS does not only play a structural role in maintaining the integrity of the tissue, it also acts as a guiding medium. For example, in chick CNS, in both retina and tectum, the ECM forms oriented channels along which optic axons grow (Krayanek, 1980; Krayanek & Goldberg, 1981).

The past decade has seen a growing role for cell surface oligosaccharides as mediators of cell-cell recognition and adhesion in several diverse systems. Studies of sperm-egg

interactions in mammals, for example, have established that the oligosaccharide structures associated with glycoproteins serve as functional receptors in cell adhesion and recognition, independent of their protein backbone (Margolis & Margolis, 1989).

Glycosylation is not only confined to the phenomena discussed above. Certain proteins seem to contain oligosaccharide chains for a number of other reasons. Thus the glycan moiety of glycoproteins may directly interact with, and influence the conformation of, its associated polypeptide as, for example, in the Fc fragment of IgG (Sutton & Phillips, 1983) whose degalactosylation may lead to the autoimmune disease exhibited as rheumatoid arthritis.

Furthermore, the addition or removal of certain key sugars may change the oligosaccharide conformation and alter molecular interactions of the glycoprotein, as with N-CAM and its polysialic acid chain (Hoffman & Edelman, 1983). The presence of the terminal polysaccharide on this molecule during embryonic stages seems to affect the binding of opposing membranes.

Glycoproteins are also important in stabilizing membrane structure. Thus, a PNS myelin-specific glycoprotein called PO crosses lipid bilayer and, through the cytoplasmic medium, reaches the lipid bilayer on the other side, and by doing so, is believed to play an important role in stabilizing both the intraperiod and major dense lines (Morell et al., 1989).

The carbohydrate chains on a number of glycoproteins are known to confer important physical properties such as conformational stability, protease resistance (through blocking access to the peptide core that would otherwise be susceptible to protease activity (Kozarsky et al., 1988)), charge- and water-binding capacity, host-pathogen interactions and protection from freezing (Snider, 1984; Sadler, 1984).

PART TWO : BIOCHEMISTRY OF GLYCOCONJUGATES

1. GLYCOPROTEINS

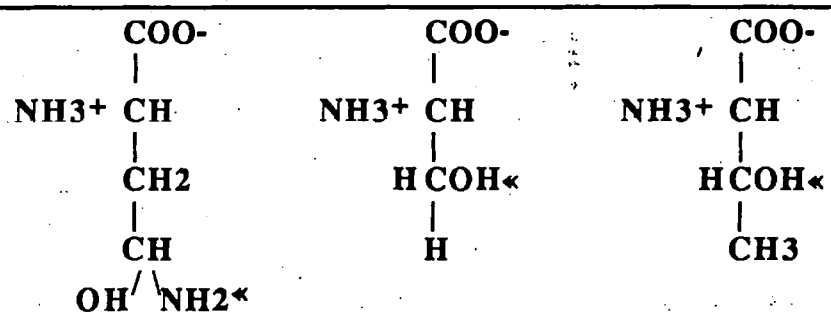
1.1. Classification and structures

Glycoproteins are complex molecules that consist of various lengths of polypeptide chain and one or more covalently bound carbohydrate chains of varied lengths, structures, and sub-structures. As a rule, the predominant sugars in glycoproteins are: N-acetylneuraminic acid (NANA), mannose (Man), galactose (Gal), fucose (Fuc), N-acetylgalactosamine (GalNAc), and N-acetylglucosamine (GlcNAc) (Brunngraber, 1972). With the exception of collagen type proteins (Ginsburg, 1969), glucose (Glc) is rarely present in these molecules, but is the major precursor for the formation of monosaccharide components of mammalian glycoproteins. However, galactose, mannose, galactosamine and glucosamine from the diet or from glycoprotein catabolism can also enter the metabolic cycle and undergo conversion to other sugars (Schachter, 1978; see Fig.1.1).

The relative amount of these sugars may vary a great deal depending on a variety of factors. For example, chick brain neural-cell adhesion molecule (N-CAM) contains about 30% sialic acid (Kornfeld & Kornfeld, 1985).

Glc <-> Glc-6-p <-> Fru-6-p <-> Man-6-p <-> GDP-Man*-> GDP-Fuc <-> Fuc-1-p -> Fuc

Figure 1.1 : The synthetic pathway of fucose from glucose. GDP-fucose acts as a nucleotide derivative donor of this sugar in the assembly of oligosaccharide chain (Hughes, 1983). * Irreversible reaction (Coffey et al., 1964).



Asparagine Serine Threonine

Figure 1.2 : The molecular structure of asparagine, serine, and threonine; « the site of glycosidic linkage

There is another group of glycoproteins called proteoglycans that consist of glycosaminoglycans covalently attached to serine residues of the protein backbone. Glycosaminoglycans are high molecular weight linear carbohydrate polymers (as opposed to branched carbohydrate chains which are the usual conformation in glycoproteins) that consist of repeating disaccharide units of a uronic acid (glucuronic or iduronic acids) and a hexosamine (GlcNAc or GalNAc). For example, chondroitin sulphate proteoglycans contain an average of 56% protein, 24% glycosaminoglycans and 20% N-type and O-type linked oligosaccharide (Margolis & Margolis, 1989). In all tissues studied, including brain, chondroitin sulphate and heparan sulphate are O-glycosidically linked to the serine residues of proteins and account for less than 1% of the soluble brain protein (Kiang et al., 1981).

There are, in general, two types of carbohydrate linkages to the protein backbone, namely N-glycosidic and O-glycosidic linkages (see Berger et al., 1982). In the former case the carbohydrate chain forms a linkage with the asparagine (Asn) residue of the nascent glycoprotein, and in the latter with either serine (Ser) or threonine (Thr) (see Fig. 1.2).

Ten to fifteen percent of the oligosaccharide chains in the brain are O-glycosidically linked (O-type glycoprotein). They consist of the core disaccharide galactose(β1-3)GalNAc, which may occur either as such or substituted with sialic acid residues at C-3 of galactose and/or C-6 of GalNAc (Margolis & Margolis, 1989). Approximately 85-90% of the carbohydrate chains in the brain are linked via GlcNAc to the amide nitrogen of asparagine residues in the protein moiety (N-type glycoprotein) (Margolis & Margolis, 1989). Some N-glycosidic oligosaccharides are also reported to contain sulphated residues and are identified as galactose 6-sulphate and GlcNAc 6-sulphate (Margolis & Margolis, 1970). Several well characterised sulphated glycoproteins have

been identified, such as myelin-associated glycoprotein, the ependymins (a family of soluble glycoproteins found in goldfish and mammalian brain), and several neural cell adhesion molecules like N-CAM and NILE/L1/Ng-CAM (Margolis & Margolis, 1989).

Attachment to hydroxyproline has also been found in both vertebrates and invertebrates (Hughes, 1983). The first sugar attached to asparagine is invariably GlcNAc, whereas GalNAc attaches to serine or threonine residues. It should be noted that the division of glycoproteins into N- and O-types is arbitrary, since there are reports on both types of oligosaccharide linkages on a given protein such as the glycoprotein galactosyltransferase (Sadler, 1984).

The oligosaccharides are not distributed uniformly along the peptide chain. Typically, they are clustered in heavily glycosylated domains in which glycosylated serine and threonine residues comprise 25-40% of the sequence (Jentoft, 1990). The glycosylated regions in glycoproteins often consist of 20-70 amino acids and, since the carbohydrate contents in these regions ranges from 65-85%, it is believed to dominate their chemical and physical properties (Jentoft, 1990). O-type glycoproteins may contain fucose, sialic acid, galactose, GlcNAc in addition to GalNAc, but not mannose (see below). N-type glycoproteins, on the other hand, have a common core containing mainly mannose and GlcNAc. In general, the carbohydrate portion of both N- and O-type glycoproteins may represent between 1% to 80% of the weight of the molecule and may be made up of varying proportions of sugars (Spiro, 1963a&b).

In addition to "conventional" N- and O-type glycoproteins, novel oligosaccharide linkages to the polypeptide backbone have recently been reported. For example, Hart and associates (1990) identified a type of glycosylation in which GlcNAc formed O-glycosidic linkages. These proteins have been almost exclusively found in the cytoplasmic and neucleoplasmic compartments (Hart et al. 1989, also see Finne et al.,

1979). In addition, there are unusual types of O- linked mannose-containing proteins that are exclusively found in the cytoplasm of adult rat brain (Hart et al., 1989).

Carbohydrates can form glycosyl linkages via different carbon atoms of the molecule and can form branched carbohydrate chains. These properties can potentially result in stupendous diversity of complex oligosaccharide structures (Margolis & Margolis, 1989). Indeed, three molecules of the same hexose may form many different glycosidic linkages between themselves and hence, given the presence of appropriate processing enzymes, produce up to 176 different configurations. Thus, many thousands of different oligosaccharide structures could be assembled in the presence of the combined and/or sequential action of glycosidic enzymes. In fact, the synthesis of only a limited number of structures is seen in any one organism. This is due to the rigid substrate specificity of the enzymes combined with their activity and relative concentration and availability any given cell. Each cell also determines which glycoproteins/glycolipids are synthesized. Pollack and Atkinson (1983) noted that the site of glycosylation in the polypeptide chain correlates with the extents at which the carbohydrate chains are processed. For example, glycosylation sites in the first 100 amino acid residues were found to be enriched in complex units of N-type glycoproteins, whereas high mannose forms predominated further along the chain (i.e., from the 200th amino acid onwards). However, the actual mechanism for this arrangement is not clear, since by the time that the polypeptide chain reaches the Golgi it has already folded into a 3-D structure. This may indicate that more emphasis should be directed towards configurational rather than sequential factors. Further, the control of oligosaccharide structure must be determined genetically; that is, in terms of the type and the relative concentration of transferases. For example, different blood groups arise from different oligosaccharide chains of glycoproteins and glycolipids of red blood cells (Berger et al., 1982).

Structurally, O-type glycoproteins exhibit all the different forms of carbohydrate

configuration, and contain from one to more than twenty saccharides per chain (Jentoft, 1990). But in most systems there seem to be only five different core structures defined by the sugars directly attached to the initial GalNAc residue (Paulson, 1989. See Fig. 1.3). Three major sub-types of N-type glycoproteins have been identified. They are called high mannose, complex, and hybrid. They have a common core structure consisting of five sugar molecules (see Fig. 1.3). The high mannose sub-type contains mainly mannose in the outer core, the complex sub-type has a highly varied and branched carbohydrate chain and beside mannose contains other carbohydrate molecules. The hybrid, as the name implies, shares features of both high mannose and complex sub-types features (For more details see Berger et al., 1982; Paulson, 1989).

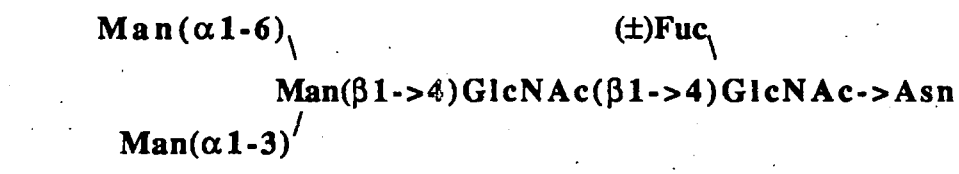


Figure 1.3 : The common core structure of the N-type glycoproteins.

1.2. Distribution

Since 1967 there have been a number of reports concerning the presence of carbohydrate rich proteins in the region of the synaptic cleft (Pfenninger, 1973; Ramboueg & Leblond, 1967). The use of carbohydrate binding proteins (lectins) revealed the presence of glycoproteins on postsynaptic membranes and in particular postsynaptic densities (PSDs). Analysis of the composition of PSDs, synaptic plasma membranes (SPMs), and synaptic junctions (SJs) has confirmed their high glycoprotein contents.

Brunngraber (1972) provided a detailed set of data on the subcellular and anatomical distribution of glycoproteins in the rat brain. Briefly, 20% of the total glycoproteins were found in the crude nuclear fraction with the same percentage occurring in the soluble fraction (for a review of nuclear and cytoplasmic glycosylation and the presence of their processing enzymes, glycosidases and glycosyltransferases, see Hart et al., 1989). However, Margolis & Margolis (1972, 1979a&b and 1989) found approximately 90% of the brain glycoproteins to be integral components of cell surface and internal membranes (see also Schachter, 1978; Zatz and Barondes, 1970). Finally, through partial purification of soluble (Saraswatti & Bucchawat, 1970) and insoluble glycoproteins (Brunngraber, 1969a; Dutton & Barondes, 1970), about 80% of brain glycoproteins were reported to be insoluble and membrane bound.

The high degree of heterogeneity of glycoproteins raises the possibility that the amount of any one glycoprotein in a given organism will be rather limited (Quarles & Brady, 1971). As for the type of oligosaccharide linkage, most of the glycoprotein sugar chains in SPM, PSD and SJ have N-type linkages; O-linked sugar chains account for only 1-2% of the total glycoprotein carbohydrate (Krusius et al., 1978).

Relatively little information is available regarding the oligosaccharide structures of

synaptic glycoproteins. Krusius et al. (1978) reported the identification of five O-linked sugar chains in rat's SPMs. Analyses of N-linked oligosaccharides have identified triantennary complex chains (between 60-80% of the total sugars), biantennary structures (21%), and high mannose chains (13-16%) (e.g., Krusius et al., 1978). Almost no hybrid oligosaccharides are associated with synaptic glycoproteins (Gurd, 1989).

Given the large amount of membrane area in the axon and terminal fields of most neurons, one would expect a considerable percentage of the glycoprotein labelled in neuronal cell bodies to be transferred via the axonal transport system to the nerve terminal and also to dendrites. In Aplysia only 45% of the incorporated labelled fucose was reported to have been exported into the axon within 10 hr after labelling (Ambron et al. 1974). In the mammalian optic system, however, 70-95% of the labelled glycoprotein may be transported to the nerve terminal (Gurd, 1989). Most of the axonally transported glycoproteins are integrated into either axonal or terminal membranes (e.g., Bennett et al., 1973; Gammon et al., 1985). There is also evidence suggesting that some transported glycoproteins, perhaps confined to the lumen of transported vesicles, are secreted from axons and their terminals (Caroni et al., 1985; Gurd, 1989).

Postsynaptic cells also become labelled by transported glycoconjugates. Some of this label is subsequently axonally transported to the terminals of these postsynaptic cells (Bennett et al., 1973; Casagrande & Harting, 1975; Matthews et al., 1982; Specht & Grafstein, 1977). This may be due to either the uptake and reincorporation of labelled sugar following metabolic turnover of transported glycoconjugates, or to the transfer of intact glycoconjugates, or a combination of both (Gurd, 1989).

1.3. Metabolism

During the last decade much has been learned about the synthesis of glycoconjugates. It is known that, with the exception of N-linked oligosaccharide synthesis which occurs in the rough endoplasmic reticulum (RER), all the processing steps take place in the Golgi apparatus (Golgi).

1.3.1. Biosynthesis : There is no template, as in the case of polypeptide synthesis, for the structure and elongation of the carbohydrate chains of N- and O-type glycoproteins. Their final structure depends on the specificity of the glycosyl transferases present and their relative concentrations at the site of synthesis/assembly (Berger et al., 1982). The enzymes transfer the sugars from their activated nucleotide derivatives and link them to specific glycose acceptors. There are also structural and functional influences in the biosynthesis of these sugar chains; the amino acid sequences around the site of glycosidic linkage as well as the polypeptide structure and the total number of amino acid residues play important roles in the final structure of the oligosaccharide chain (Pollack & Atkinson, 1983; also see Berger et al., 1982).

Nearly all glycoproteins are synthesized by ribosomes bound to the endoplasmic reticulum and contain covalently linked carbohydrate chains (Palade, 1975; Stryer, 1981; Hubbard & Ivatt, 1981). Glycosylation takes place either as polypeptides are being synthesized, or shortly after (Berger et al., 1982). The carbohydrate chain may both orient the nascent glycoprotein in the membrane and also determine its destination; that is, whether it will be secreted or become bound to the plasma membrane or transported to the lysosomes (Stryer, 1981). The "signal" sequences of these secretory and membrane proteins which are excised in the RER under normal circumstances can be seen when the proteins are synthesized *in vitro* in the presence of free ribosomes (Stryer, 1981). However, ecto-galactosyl-, fucosyl- and sialyltransferases have been

found on the extracellular surface in neuronal cultures and adult CNS, suggesting that they have a role in cell-cell interaction (Matsui et al., 1983; Rostas et al., 1981).

N-type glycoproteins contain a common inner core structure (see Fig. 1.3). These core units are transferred from a lipid carrier, called dolichol phosphate (PDol), to the growing polypeptide chain on the luminal side of the RER membrane (for a more detailed study of the role of dolichol see Hughes, 1983; Schwarz & Datema, 1982). The activated donor carbohydrates are usually nucleotide sugar derivatives, but dolichol phosphoryl mannose (Man-Pdol) and dolichol phosphoryl glucose (glucose-Pdol) also act as potential donors in the synthesis of N-type glycoproteins (Berger et al., 1982). Lipid-linked oligosaccharides are synthesised first and then transferred to the protein moiety thereby releasing the lipid. The oligosaccharide chain is synthesised by the sequential addition of sugars to the lipid moiety, dolichol phosphate (PDol). Here PDol is anchored to the RER membrane and sugars are then transferred by membrane-bound transferases (Fig. 1.4).

GlcNAc + P* -> GlcNAcP*

GlcNAcP* + Dol-P -> GlcNAcP*P*-Dol -> ◊◊GlcNAcP*P*-Dol ->

P*P*-Dol + ◊◊GlcNAc-Asn (protein)

Figure 1.4 : A summary of the sequential steps from GlcNAc to its linkage with asparagine. P*, activated phosphate group; ◊◊, oligosaccharide chain; GlcNAcP*, activated oligosaccharide.

1.3.2. Sub-cellular sites of glycosylation : It is generally accepted that the majority of glycoproteins found at the nerve terminal are synthesized in the cell soma, processed in the membrane systems of RER and Golgi, and transported in particulate form, by fast axoplasmic transport to the region of the synapse at a rate of approximately 70-80mm/day (as compared to 1mm/day for proteins) (Bondy, 1971). However, within the same neuron, different glycoproteins can be transported at different rates. Levin (1977) reported two waves of transported glycoprotein in rat moving through the hypothalamus at 96 and 48 mm/day, respectively. Glycoproteins are not transported faster than non-glycosylated proteins.

The presence of certain glycosylating enzymes in nerve endings has also been reported by several investigators (eg., Forman et al., 1972; Zatz & Barondes, 1971). Margolis and Margolis (1975), for example, provide evidence for sialic acid incorporation at nerve terminals. A fucosyltransferase in the region of the synaptic junction has also been reported (Rostas et al., 1981). Fucose is rapidly incorporated at nerve endings (Autilio et al., 1968; Morgan & Austin, 1968) and acetoxycycloheximide can inhibit this phenomenon (Zatz & Barondes, 1970).

It is well documented that the transfer of oligosaccharide to protein takes place in the lumen of RER (Morre et al., 1979; Bretscher & Raff, 1975; also see Ralph et al., 1982). During their synthesis, those glycoproteins destined to become integrated in the membrane remain bound to the RER membrane by hydrophobic polypeptide chains. Glycoproteins are then separated from the binding proteins which, during translation, bind them to ribosomes, and transferred to the smooth endoplasmic reticulum (SER). Glycoproteins are transferred to the Golgi for the final stages of posttranslational modification. Glycoproteins destined to be soluble are released in the cisterna after the completion of the translation process. In addition, the Golgi apparatus is recognised as a site for further glycosylation (Haddad et al., 1971). The enzymes N-

acetylgalactosaminyltransferase and a glucuronosyltransferase, involved in the synthesis of the repeating polymer of chondroitin sulphate have been identified in the Golgi of developing chick embryo brain (Jourdian, 1979).

The biosynthesis of glycoproteins and, in particular, their mode of glycosylation is highly ordered and shows specific temporo-spatial characteristics (Hughes, 1983). Some sugars like fucose, galactose, and ManNAc are added to the growing carbohydrate chain in the Golgi. Here, the terminal sugars of the common carbohydrate cores of N-type glycoproteins are trimmed and remodeled (Stryer, 1981). Glycoproteins are then sorted and packaged for different destinations.

The synthesis and translocation of gangliosides and glycoproteins in chick CNS after an intraocular injection of N-acetylneuraminic acid (NANA) has been reported by Donowicz et al. (1988). Four hours after injection, the highest concentration was found in the vesicles of the retinal ganglion cell layer. Under the influence of anaesthesia, shown by Richards (1983) to reduce the release of neurotransmitters, more gangliosides and glycoproteins were found in the vesicles than in synaptic plasma membranes (see also Donowicz et al., 1988).

Membrane-bound glycoprotein processing enzymes have also been identified. For example, cell surface N-acetylglucosamine(β1-4)galactosyltransferase has been found in chick embryonic retina (Roth et al., 1971). Most if not all synaptic glycoproteins are oriented with their oligosaccharide moieties facing the synaptic cleft (Gurd et al., 1983). The structural modification of oligosaccharides would therefore require that the necessary enzymes and substrates be present on the outer surface of the membrane and there is already evidence supporting the presence of these processing enzymes in neural tissues (e.g., Matsui et al., 1986).

1.4. Biological effects of glycoproteins

One concept of glycoprotein function is based on the fact that certain carbohydrates may represent highly specific compounds acting as carriers of biological information. A few well documented functions of glycoproteins, attributed to their carbohydrate chains, may be summarized as follows:

A. Cell-cell recognition : A specific sequence of these chains can act as a complementary structure for other molecules such as, proteins, glycoproteins, or even glycolipids. This phenomenon is known as cell-cell or cell-molecule recognition. Thus, certain glycoproteins function as membrane receptors with a high degree of specificity for endogenous ligands (Palade, 1975; Rothman & Fine, 1980; Merlie et al., 1982). Glycoproteins can act as highly specific cell adhesion molecules. This is important in the formation of tissues and organs during embryogenesis (Hughes, 1983). Processes such as cellular proliferation, differentiation, sorting, translocation, and terminal development depend on specific intercellular reorganisation and selective adhesion between cells.

B. The type of glycosylation may determine the function of glycoproteins. For example, sulphated glycoproteins tend to interact with pharmacological agents containing basic amines, making them good candidates to bind certain neurotransmitters such as monoamines (Simpson et al., 1976). A number of neural receptors such as cholinergic, opiate, and GABAergic receptors are known to be glycosylated (eg., Merlie et al., 1982). The oligosaccharides present in the acetylcholine receptor (an N-type glycoprotein) play a role in determining the assembly and folding of the newly synthesized polypeptides (Merlie et al., 1982). Similar results have been reported with respect to opiate receptors and the role of terminal fucose, as shown by 2-deoxy-D-galactose (Richter et al., 1991).

C. The structure of the oligosaccharide chain itself can influence the configuration of the protein backbone to which it is bound (Berger et al., 1982). Co-translational transfer of oligosaccharides to the growing polypeptide chain in the RER may be a determining factor in the final molecular configuration of the protein (Berger et al., 1982).

D. Both glycoprotein and glycolipid oligosaccharide chains can stabilize the cell membrane, and influence its fluidity and permeability (see Rahman, 1982). Cell surface glycoproteins may be attached to cytosolic microfilaments and microtubulin to give shape and mobility to the cell (Berger et al., 1982).

E. The role of glycoproteins in the formation of new neuronal circuitry and in morphological changes during memory formation and consolidation, has been extensively investigated in several laboratories. The biological functions of glycoproteins such as adhesion, recognition and so forth, are considered to be important in developing a theory of memory and learning (for detailed discussions see Edelman, 1987; Matthies, 1989; Rose, 1981a, 1983, 1989; Schmidt et al., in press).

1.5. Fucose containing glycoproteins

Fucosylglycoproteins are major constituents of synaptic plasma membranes. Indeed, 26% of the total membrane proteins and 86% of the total glycoproteins of SPM are known to contain fucose (Zanetta et al., 1977). As such fucosylglycoproteins would be expected to play an important role in cellular events that involve the plasma membrane, including cell-cell interaction. During epidermal differentiation in rat there is a threefold increase in the synthesis of fucosylglycoproteins (Zieske & Berstein, 1982). By using sequential lectin-affinity chromatography and [^{3}H]fucose labelling of on SPM glycoproteins, Gurd and Mahler (1974) were able to identify up to 25 fucosylglycoproteins in rat. Similarly, Zanetta et al. (1977) reported the presence of

some 40 glycoproteins from their rat SPM preparation, using mannose (Concanavalin A) and fucose (Ulex europaeus type I) specific lectins. The significance of this diversity of glycoproteins present in SPMs or of the heterogeneity of their oligosaccharides is not known, but may reflect the occurrence of related glycoproteins with distinct sugar compositions in functionally different synaptic subtypes or the presence at individual synapses of structurally diverse glycoproteins, or a combination of these factors (Gurd, 1989).

Fucose in glycoproteins is present either at the terminal position or the non-reducing position in the carbohydrate chain (Berger et al., 1982). It is usually, but not always, linked to galactose, (see Fig. 1.3). The most common linkage between galactose and fucose is fucose(α1->2)galactose. The enzyme β-galactoside(α1-2)fucosyltransferase has been identified in many animal and human tissues (Beyer et al., 1981).

A number of fucosylglycoproteins have been identified in the nervous system. For example, Thy-1, the smallest member of immunoglobulin family is heavily fucosylated (Willams & Barclay, 1988). It has been implicated in neural growth during development, showing a 100 fold increase in the brain during early postnatal development (Morris, 1985).

In general, about 60% of identified neural glycoproteins contain galactose or galactose and fucose (Goodrum et al., 1989). The heavily glycosylated myelin associated (N-type) glycoprotein (MAG) is another example of neural fucosylglycoproteins (Quarles et al., 1973) (Table 6.2 in Chapter 6 contains a list of glycoproteins that have been identified in the nervous system).

The metabolic pathways of fucose and fucoglycoproteins have been investigated in a

number of laboratories. Following an intraperitoneal injection of [^{14}C]fucose a large proportion was found to be excreted, leaving the rest almost exclusively incorporated into fucosylglycoproteins (Brunngraber, 1972). It was, therefore, suggested that either fucose was not converted to other sugars, or it underwent a very slow conversion (Brunngraber, 1972). Fucose catabolism was also reported to be very slow (Coffey et al., 1964). In his study Coffey et al. found no [^{14}C]fucose breakdown into [^{14}C]CO_2. Bekesi and Winzler (1967), however, refuted Coffey's claim when they observed about 1.6% conversion of [^{14}C]fucose into [^{14}C]CO_2 six hours after injection. Durand and associates (1969) reported on the presence of fucose-containing glycolipids, gangliosides, and mucopolysaccharides in the nervous system, amounting to less than 0.1% of the total fucose content. Two types of enzymes act on fucose before it is linked to the oligosaccharide chain. Fucokinase links fucose to its nucleotide GDP, and fucosyltransferase transfers GDP-fucose to galactose.

Fucosylglycoproteins may undergo deglycosylation before they reach their final destination. Indeed, several glycosidases have been identified in synaptosomal preparations (e.g., Gustavsson et al., 1982, Ohlson & Karlsson, 1983; Towsend et al., 1979). Fucosylglycoproteins are reported to exhibit two different turnover rates, one fast with a half-time of about one day, the other with a half-time of 7-10 days (Goodrum et al., 1989).

2. GLYCOLIPIDS

2.1. Classification and structures

Glycosphingolipids are one of the major constituents of the neural lipids; the others being phospholipids and, to a less extent, cholesterols. In general, sphingolipids are divided in to four groups: cerebrosides, sulphatides, ceramidepolyhexosides, and gangliosides (Rouser et al., 1978, Routtenberg, 1982). They all contain a basic building block known as sphingosine (Suzuki, 1981, Järnefelt et al., 1978), which consists of a long aminodiol chain with one unsaturated bond (Fig.1.5). The structure of the oligosaccharide moiety of glycolipids is determined by the availability and concentration of specific processing enzymes. The sugar molecules in glycolipids can vary in number, type, and proportions. Gangliosides, for example, contain as many as seven carbohydrates. Cerebrosides, the simplest glycolipids with only one sugar, either contain glucose or galactose.

2.2. Metabolism

2.2.1. Biosynthesis and degradation of brain glycosphingolipids : The biosynthetic mechanism of glycolipids is essentially the same as that of glycoproteins (Ginsberg and Neufeld, 1969). Some of the transferring enzymes have already been identified (Robinson et al., 1966), and the Golgi apparatus is suggested as the site of glycosylation and sulphation. The major site of the synthesis has been found to be the perikarya, with a small amount of synthesis taking place in synaptosomes (Caputto et al., 1976; Maccioni et al., 1974b; Arce et al., 1971). During glycosylation the addition of carbohydrate molecules to ceramide is carried out in a stepwise manner. Carbohydrate chains are similarly degraded through sequential hydrolysis of glycosyl groups in lysosomes by exoglycosidases only (Järnefelt et al., 1978; Schachter, 1978). The glycosidases involved act in association with certain protein factors possessing detergent-like properties. For reviews of the biosynthesis and degradation of glycolipids see Ledeen (1983), Radin (1983), and Kishimoto (1983).

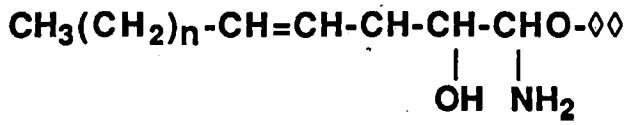


Figure 1.5 : The Molecular Structure of Sphingosine.

The amino group of sphingosine is usually acylated with a long chain fatty acid ranging from C14 to C26. This hydrophobic part of the molecule is incorporated in the membrane bilayer with the hydrophilic part, ie. carbohydrate chain (◊◊ in above figure), emerging into the intercellular medium (Suzuki, 1981) (n=10-16, but commonly n=12).

2.3. Distribution

All neural cell membrane lipid bilayers consist mainly of glycosphingolipids (including gangliosides), cholesterol, a number of glycerophospholipids, and a trace amount of sphingomyelin (Suzuki, 1981). Most of the glycolipids and glycoproteins of the animal cell are associated with the surface membrane (Schachter, 1978). In the CNS, gangliosides comprise up to 5-10% of the total lipid of cell membrane, with the synaptosomal fractions containing up to 12% of total brain gangliosides (Ledeen, 1978). In fact, the highest concentration of glycolipids are to be found at the nerve endings (Volk & Aronson, 1972).

2.4. Fucose containing glycolipids

In the last twenty years the role of fucose in glycolipids has been increasingly recognised, especially in the area of hæmatology and neurology. For example, certain

glycosphingolipids related to blood groups have been observed to contain fucose (see e.g., Stellner & Hakomori 1973; Presper et al., 1978; Yang and Hakomori, 1971). Sonnino et al. (1978) identified a fucosylglycolipid in pig cerebellum. Other fucosylgangliosides have been isolated from human brain (Ando & Yu, 1979) and pig nervous tissue, in dorsal root ganglia, spinal cord, and forebrain (Morrison, 1986). Fucosylglycolipids have a slow turnover. Thus, 24hr after intracerebral injection of [^{14}C]fucose no detectable fucosylglycolipid fractions can be found in the mouse brain (Dutton & Barondes, 1970; Zatz & Barondes, 1970).

The biochemical functions of fucosylglycolipids are not well understood (Mc Kibbin, 1978). They do share some of the functions of glycoproteins (for a summary of glycolipid functions see the section entitled "Glycolipids and Information processing"). Fucosylglycolipids are often present in rapidly proliferating tissues, such as gastrointestinal epithelium, salivary gland, bone marrow, testis, and so on. However, they are generally absent, or present in lower amounts, in tissues that do not undergo cell division such as the brain and kidney (McKibbin, 1978).

3. FUCOSE AND ITS METABOLISM

Coffey et al. (1964) observed that fucose became incorporated into glycoproteins without prior conversion to other sugars (see also, Sturgess et al., 1973; Kaufman & Ginsberg, 1968). However, certain enzymes capable of converting fucose to other hexoses and lactose (a disaccharide of glucose and galactose) have been identified (Wilkinson, 1957).

Fucose has been used in many studies to assess the turnover rate of glycoconjugates. The turnover of fucose-containing glycoproteins has two very different rates, one fast (around 1 day), and the other slow (around 30 days); the latter representing partial reutilization of labelled fucose (Margolis & Margolis, 1989). Longer turnover rates

have also been seen, in different system as shown in Fig. 1.6.

When incorporated in macromolecules, fucose is either linked to galactose via a 1->2 linkage, or to GlcNAc via 1->3, 1->4, or 1->6 linkages. In glycoproteins, fucose, having a half-life of one month, occurs at non-reducing terminals of carbohydrate chains and thus acts as a "chain stopper" controlling the extent of chain elongation (Zatz & Barondes, 1970; Flowers, 1981).

Tissue	Fast $t_{1/2}$ (days)	Slow $t_{1/2}$ (days)
Chick cilliary ganglion	–	7
Pigeon optic lobe	3	38
Pigeon optic tract	7	69
Goldfish optic tectum	–	20
Rabbit OT, LGB, SC	–	10
Mouse LGB and SC	2-4	7-10
Rat sciatic nerve	–	7

Figure 1.6 : Turnover rate of fucosylglycoproteins in the nervous system using [^{3}H]fucose as precursor. OT, Optic tract; LGB, lateral geniculate body; SC, superior colliculus; –, no data is available. Adapted from Goodrum et al. (1989).

PART THREE : NEURAL PLASTICITY IN THE CHICK

"That there are metabolic changes consequent upon training has made life relatively easy for those working in this field. It would have been more bothersome if there had been no such metabolic events...." (Rose 1981b).

Learning and memory in birds

During the past few decades a large body of research in avian learning has developed with the aim of acquiring an understanding of neural plasticity which would be applicable to all species possessing an organized nervous system. It includes the neurobiology of song learning, food storage and retrieval, imprinting and learning and memory.

A case for the chick : Research into neural mechanisms of memory and learning in birds is merited because they possess certain highly developed behavioural repertoire such as flying, intercontinental migration, singing, and food storing. The young domestic chick is a valuable model in which neural mechanisms of plasticity can be investigated. Chick is precocial (exhibiting spontaneous environmental exploration and food searching shortly after hatching), cheap to keep, easy to handle, and has a partial blood brain barrier which facilitates certain pharmacological experiments. On the other hand, for behavioural studies, there are a number of set-backs when using chicks; the CNS of the young chick is rapidly developing which may confound the study of neural processes related to learning and memory.

Imprinting

Imprinting, found in many vertebrate species, and especially common in precocial birds, is an adaptive process in which animals, during a sensitive period, learn to restrict their social preferences to a specific class of objects (Hess, 1959; Bateson,

1966). During this time the young bird makes use of as many cues as it can perceive [e.g., visual, olfactory, and auditory (Scott, 1962)] in order to imprint on its mother (or both parents, or in the case of birds, any moving object which may or may not resemble parents). They then follow them and actively avoid other objects (for a review see Dudai, 1989). In birds, the sensitive period usually starts from hours after hatching to a few days later. According to Lorenz (1981) imprinting serves two purposes, first; it ensures survival (filial imprinting, during dark days of feebleness!), and second; it serves to minimize filial genetic pooling during mating, sexual imprinting).

A convenient way to demonstrate filial imprinting is to place dark-hatched chicks individually in a free rotating, but *stationary*, cylindrical cage with a vertical opaque base and top and horizontal transparent wire-meshed sides (Horn, 1985). They are then exposed to imprinting objects (a rotating cube, flashing light, a stuffed-jungle fowl etc.) during which time they may try to get close to the object by walking on the wire mesh. After initial imprinting, the degree of preference to the imprinted, as opposed to a neutral, object is assessed by placing the chick in a similar but moving cage, which is designed such that, when chicks try to get near to the preferred object, it moves away from it. This provides a measure of the degree of preference (Bateson & Wainwright, 1972).

The incorporation of labelled lysine into protein and uracil into RNA during imprinting have been investigated, and shown to be experience- and brain region-specific. In split-brain chicks (with an ablated dorsal supraoptic commissure leaving the contralateral pathways intact), birds that imprinted with one eye open showed increased RNA expression and protein synthesis in the contralateral anterior forebrain roof (Horn et al., 1971; 1973, Bateson et al., 1972). Furthermore, the degree of experience (measured by the amount of time that chicks were exposed to imprinting objects) was positively correlated with lysine and uracil incorporation. In these experiments control birds were

not imprinted on any object but kept them in the dark during the course of the experiment. It was therefore, possible that light deprivation contributed to low incorporation of precursors. Based on the assumption that the degree of learning correlated with increased metabolic rates, an improved version of the above experiment was devised. Here, when two groups of birds (A and B) were imprinted on an object for different periods in day one (i.e. group A, shorter, and group B, longer), but exposed to the object for equal amount of time on day two, group A showed more incorporation of precursors into the anterior forebrain roof, measured on day two, than group B (Bateson et al., 1973). Moreover, imprinted chicks that showed more intensity of preference towards the object exhibited more uracil and lysine incorporation (Bateson et al., 1975). Autoradiography of brain sections obtained from chicks in group A resulted in more uracil incorporation into intermediate medial hyperstriatum ventrale (IMHV) than those obtained from group B (Horn et al., 1979).

Radiolabelled uracil and 2-deoxy-glucose incorporation was used to investigate which structures responded to visual and acoustic imprinting (Koshka et al., 1979, and Maier & Scheich, 1983). The length of time that chicks followed a floating red balloon during retrieval was taken as the criterion for successful imprinting; those that followed the balloon for over 20min were considered imprinted, and those with a pursuit a time of less than 5min non-imprinted. Another study exploited the criterion of preference between two distinct tones (1.8 and 2.5kHz, with chicks imprinted on the lower frequency tone). Here, besides IMHV (as identified by Horn, 1985), hyperstriatum accessorium and the lateral and medial neostriatum and hyperstriatum ventrale were also found to incorporate this metabolic precursor (Maier & Scheich, 1983).

In order to assess the involvement of these anatomically identified structures in imprinting, lesion studies were carried out. Bilateral lesions to the IMHV before

imprinting abolished the ability of chicks to imprint and post-imprinting lesions made them less able to remember the imprinted object (McCabe et al., 1981; 1982; Horn et al., 1983). The IMHV is therefore, necessary for both acquisition and retrieval. However, the involvement of IMHV in imprinting has proved to be more complicated than it first seemed. For example, chicks imprinting on a rotating coloured box were more severely affected by IMHV lesions than those imprinting on a stuffed fowl (Horn & McCabe, 1984). Thus, it seems that chicks have a genetically acquired predisposition towards their mother hen.

However, chicks exhibit equal preferences for a disarticulated stuffed fowl, intact stuffed fowl, stuffed duck and even stuffed polecat when compared with other stimulants such as the rotating red box (Johnson et al., 1985, and Horn, 1985). When IMHV- and Wulst-lesioned chicks were compared for their ability to distinguish between familiar and unfamiliar stuffed fowls (slightly differing in shape), the former failed the task, indicating that they had lost their 'fine-tune' capability (Johnson & Horn, 1987). The IMHV, therefore, enables chicks to distinguish the fine qualities of a particular type of object (Johnson & Horn, 1987), and its ablation abolishes the ability to acquire and remember, say, a rotating red box and the individuality of chickens, but does not abolish the (adaptive) innate preference for a "platonic chick" [see Dudai (1989) for a more detailed review].

Learning

Passive avoidance training paradigm : One trial passive avoidance training in the chick was first introduced by Cherkin & Lee-Teng (1965), and later modified by Gibbs & Ng (1977). It is argued that for an animal to learn a task it must contain some important elements of survival such as fear, danger, pain, or solution to pressing problems like hunger (Rose, 1981a). Passive avoidance training employs some of these survival aspects, such as environmental exploration and the search for food and water.

Unpleasant-tasting objects, once tried, would be avoided thereafter (Rose, 1985). In passive avoidance training the bitter tasting liquid methylanthranilate (MeA) is used. It is presented to birds by dipping a chrome bead attached to a metal rod in the solution and holding the rod in front of the bird; the control birds are presented with a bead dipped in water. The actual training is preceded by what are known as equilibration and pretraining periods. During the equilibration period, chicks are placed in pairs in individual pens for some time so that they become familiarized with the new environment. By the time of training any psychological stress is assumed to have disappeared. The pre-training procedure involves presentation of dry white beads to the birds. The actual training then follows shortly after by presenting the birds with beads coated with water or MeA. Birds are then tested after a fixed interval, during which time they may undergo specific treatments, like brain lesions, electroconvulsive shock, injection, and so forth. At the time of testing, the birds that remember the taste of the MeA avoid peaking at the bead which they associate with the bitter taste. [For a detailed account of passive avoidance training and its justification as a tool for investigating the neural mechanisms of learning and memory see Rose (1985)].

Passive avoidance is perhaps the simplest form of training; it involves a single discrete and effective presentation and as such it is a useful tool in studies that depend on accurate timing, like investigation of immediate (or early) posttraining neural activities, and the role of pharmacologically active agents with a specific mode of action (e.g., the use of agonists or antagonists of membrane channels, or inhibitors of posttranslational modification).

Background : It is generally accepted that the formation of a long lasting memory trace involves a chain of neuronal events as regards their temporal, physiological and molecular properties (e.g., Matthies, 1989; Rose, 1981b and 1991a). Early neuronal activities, like organized nerve impulses and ion channels activation, would lead to

more or less permanent (in some cases life-long) structural changes. The immediate post experience neural events, in the form of K^+ channel activation, lasting only a few minutes would necessarily have to lead to some intermediate changes, in the form of Na^+/K^+ pump activities, and perhaps receptor down- or up-regulation. These events would then result in sequential molecular changes in the form of *de novo* macromolecular synthesis (e.g., Gibbs & Barnet, 1976; Gibbs et al., 1978; Gibbs & Ng, 1976; Patterson et al., 1986 and 1987). In parallel with, or as part of, immediate and intermediary changes, a sequence of events involving posttranslational modification may also occur. Routtenberg (1982) would argue that, given; 'a', the slow pace of protein synthesis machinery; 'b', the complications involved in transport of specifically synthesized macromolecules from the cell soma to *specific* dendritic branches for the formation of neural contacts (considering a value of 10^4 postsynaptic sites per neuron); and 'c', the picosecond timecourse of each posttranslational modification, allowing several such modifications per excitatory postsynaptic potential (EPSP), it is conceivable that *de novo* synthesis of macromolecules may not take place during long-term memory formation. Synaptic connectivity can, therefore, take place *in situ* without a need for selective "differential transport-tagging mechanism". This argument, despite its strength, runs counter to some reports that show activation of immediate early genes and elevation of RNA and protein precursor incorporation after training and the amnesic effect of protein synthesis inhibitors (Anokhin & Rose, 1991; Anokhin et al., 1991; McCabe & Rose, 1987; Patterson et al., 1986 and 1988). It is possible that both *de novo* protein synthesis and posttranslational modification take place. There may be two phases of posttranslational modification, one taking place immediately and the other some time (perhaps hours) after training. The former phase would act to satisfy earlier neuronal requirements such as increases in synaptic efficacy and connectivity, while the second phase acted to provide a more permanent enhancement of earlier established mnemonic traces.

Neurochemical and molecular changes associated with passive avoidance training

Some of the changes associated with passive avoidance training are short lived. For example, the observed rise in muscarinic receptor binding from 10 min to about 40min after training and significant decrease in [^{3}H]α-bungarotoxin (nicotinic receptor ligand) binding in chick forebrain roof which reverts to control level after 3h, suggesting the involvement of acetylcholine (Ach) in the process of short-term memory formation (Aleksidze et al., 1981; Rose, 1981a). Other changes may last for days. Increased protein and glycoprotein incorporation have been shown to last at least 24hr (Schliebs et al., 1985; Mileusnic et al., 1980).

In general, MeA chicks show various neurochemical, morphological and physiological changes which are mainly localized to the IMHV in the dorsal forebrain, the paleostriatum augmentatum (PA), and lobus parolfactorius (LPO) in the ventral forebrain (Rose & Csillag, 1985). In 2-deoxy-glucose uptake experiments, Kossut & Rose (1984) found that 30min after training the autoradiographically measured uptake of 2-deoxy-glucose-6-phosphate (the end-product of 2DGlc metabolism) in posterior hyperstriatum ventrale (forebrain roof), PA, & LPO (forebrain base) was significantly greater in MeA chicks than in W birds. Thirty minutes after training the increased 2-deoxy-glucose metabolism persisted in the left IMHV, thus showing lateralisation (Rose & Csillag, 1985).

Protein synthesis is argued by Davis and Squire (1984) to be an essential step in the formation of long term memory (LTM), and is believed to be initiated during, or shortly after, training. Thus, one hour after passive avoidance training in a day-old chick Schliebs et al (1985) observed a 23% increase in [^{14}C]leucine incorporation into forebrain slices. Previously, Mileusnic et al (1980) reported anatomical differences in incorporation of [^{14}C]leucine into brain proteins 30min to 24hr after passive avoidance

training. [^{3}H]fucose incorporation into glycoproteins, labelled leucine uptake into tubulin were mainly confined to the anterior forebrain roof, but muscarinic changes occurred throughout the forebrain roof.

If protein synthesis is required for long-term memory formation, inhibiting this neural response should interfere with learning. Intracerebral injection of cycloheximide (CX), and other inhibitors of protein synthesis, has been reported by several investigators to impair formation and retention of memory, or cause retardation in learning [e.g., Hambley & Rogers, 1979, Rogers et al., 1974, and for a review see Davis & Squire (1984)]. Several acidic amino acids like GABA, glutamate (Glu), aspartate, and taurine injected intracerebrally can also impair learning. CX is claimed to increase intracellular glutamate and aspartate levels but not GABA or taurine. This suggests that, besides its "primary" function as a protein synthesis inhibitor, CX may induce a "secondary" effect by increasing certain neurotransmitters (see also Howard et al., 1980). Furthermore, CX (1mM) *in vitro* could only inhibit 60% of glycoprotein fucosylation (McCabe & Rose, 1985). Three hours after passive avoidance learning fucose uptake persisted with a 16-19% increase in the right forebrain base even after CX treatment *in vitro* (McCabe & Rose, 1985), but not *in vivo* (McCabe & Rose, 1987). There may already exist an incompletely modified pool of glycoproteins "waiting" for fucosylation, and all CX does is to prevent *de novo* synthesis of glycoproteins. This suggests that a system of posttranslational modification (both early and late) is involved in memory formation. The activity of fucokinase, one of the enzymes involved in fucose processing, was reported to increase 1hr after passive avoidance training in the chick (Lössner & Rose, 1983).

Another system of posttranslational modification is protein phosphorylation, a process by which extracellular signals elicit various intracellular responses in the form of gene expression, protein synthesis, receptor activation, or posttranslational modification.

Thus, 30min after passive avoidance training a significant decrease in the rate of B-50 phosphorylation was found in SPMs of the IMHV of MeA birds (Ali et al., 1988a&b; Bullock et al., 1990a). B-50 (F1/GAP43) is an important neural phosphoprotein located mainly at the presynaptic membrane and is a substrate of Ca^{2+} dependent protein kinase (PKC). It is believed that, during memory formation, the catalytic subunit of this enzyme translocates to the membrane and phosphorylates its substrates (Burchuladze et al., 1991). No change in B-50 modification was seen 10min or 1hr after training indicating a time-window for the earlier phase of memory processing. Although inhibitors of PKC cause amnesia when injected immediately before or after training, the amnesic effect is not detectable for the first 90min of the posttraining period, indicating that posttranslational modification of B-50 is perhaps involved in intermediate term memory rather than the short term phase. Interestingly, a unilateral injection of PKC inhibitors (like melettin) into the left IMHV is sufficient to cause amnesia (Burchuladze et al., 1991).

Several attempts have been made to explain how the process of posttranslational modification may help to consolidate memory despite the short half-life of the substrate molecules (Crick, 1984, Lismen, 1985, Burgoyne, 1989). Another question that presents itself is; if for each memory "quantum" a given number of substrates are permanently phosphorylated, would this entail the chick being constantly haunted by the bitter taste of MeA!? Clearly, there must be a system of selection that decides which memory quantum, already consolidated, is to be re-activated at the appropriate time. It, therefore, seems more likely that newly synthesized proteins (perhaps permanently modified by fucosylation) ; located at the synaptic terminals form new trans-synaptic connections, or increase the efficacy of existing synapses. The newly formed network would then reflect the memory trace for, for example, the *Gestalt* of MeA-related experience. However, there must exist a cellular mechanism, against the background of

permanent turnover of macromolecules, to "immortalize" the newly established network; that is, if chicks are to avoid the bead for days, this network must remain functional but only active on appropriate times (perhaps through encountering the *same* or *similar* events).

According to Dudai (1989), a mechanism for generating LTM may be based on alteration in gene expression which would result in long lasting modification of protein synthesis (or posttranslational modification of proteins) and hence, in changes in neuronal properties such as synaptic efficacy. The immediate-early oncogenes such as *c-fos* and *c-jun* are believed to act as nuclear switches. Upon stimulation of cells they quickly activate a chain of cellular responses and are presumed to promote long-lasting cellular changes (Halazonetis et al., 1988; Nakabeppu et al., 1988; Vogt & Bos, 1989; Weinberg, 1985). Interestingly, the activation of *c-fos* is dependent on the elevated influx of intracellular calcium through NMDA channels (Fitzgerald, 1990); application of the channel blocker MK801 results in a substantial reduction in *c-fos* induction following noxious stimulation (Fitzgerald, 1990). Increased intracellular Ca^{2+} concentrations activates the phosphatidyl inositol (PI) cycle, part of a chain of intracellular messenger systems, resulting in a lasting cellular response (Berridge, 1986; Burgoyne, 1989; Vogt & Bos, 1989).

In MeA chicks the level of *c-fos* mRNA increased significantly (2-4 fold) 30min after training. However, *c-fos* is sensitive to external stimuli such as heating, electrical stimulation or even non-noxious stimulation, like repeated brushing of the hindlimb, and showed an increased expression in W-trained chicks (Anokhin et al., 1991; Fitzgerald, 1990). Therefore, a two-day food-discrimination paradigm (pebble floor ± food particles) was used in which three groups of chicks underwent either, no training (no food), training (food on day two only), or overtraining/habituation (food on both days) (Anokhin & Rose, 1991). The only group that exhibited increased *c-jun*

expression was found to be the trained group in which birds *naturally* learned to only peck at the food particles. Similar results have recently been reported in rats undergoing brightness discrimination training (Tischmeyer et al., 1990).

The role of brain structures

Rose (1981a) has argued that the mechanisms involved in memory formation should take place in anatomically defined regions whose ablation would interfere with the process. Earlier reports suggested a strong case for the involvement of the IMHV. Ablation of the posteriolateral portion of the telencephalon (the major part of the forebrain) resulted in complete loss of retention and relearning of passive avoidance training (Benowitz, 1972). Dorsomedial ablations that include parts of the hyperstriatal complex caused a deficit in new learning but not in the retention of a presurgically acquired experience (Benowitz, 1972). Similar results have been obtained when IMHV was lesioned bilaterally before training, indicating a role for this structure in memory processing (Davies et al., 1988). It was later found, however, that lesions to the left IMHV would suffice (Patterson et al., 1990). Furthermore, 1 to 6hr posttraining bilateral lesions to the same structure were found to be ineffective (Patterson et al., 1990). This suggested that left IMHV is involved in acquisition rather than just recall and memory.

Other structures that were labelled by 2-deoxy-glucose (Kossut & Rose, 1984; Rose & Csillag, 1985) were LPO and PA. It seemed likely that these areas play a role in some aspects of information processing complementary or synergistically to that of IMHV. There do not seem to be direct neural connections between LPO and IMHV, except perhaps through the archistriatum (Benowitz, 1980), but direct ipsilateral and reciprocal connections between IMHV and PA have been identified (Bradley et al., 1985). In contrast to IMHV lesion studies, pre-training bilateral lesions to the LPO proved to be ineffective; but when LPO was bilaterally, but not unilaterally, ablated 1hr after

training, chicks became amnesic (Gilbert et al., 1991). It seems, therefore, that chicks need their left IMHV to acquire and their LPO to retain and recall memory. Similar results were also reported earlier by Benowitz (1972) where the frontal section of the forebrain, when ablated, impaired retention of a presurgically learned response but did not affect acquisition after surgery. However, chicks with pre-training bilateral LPO and post-training left IMHV ablation did still remember, but if the right IMHV instead of the left IMHV is lesioned, chicks became amnesic (Gilbert et al., 1991). Therefore, after acquisition and processing of the memory in the left IMHV, the right IMHV takes over, probably for further processing. However, when the right IMHV was lesioned before training and the LPO lesioned after training, birds retained their memory. This suggested that, under these circumstances, the left IMHV should have retained the memory trace, and that, in normal circumstances, LPO would receive the memory trace via the right IMHV. Therefore pretraining lesion to the right IMHV and posttraining lesion to the left IMHV should be amnestic. In fact, this is not so. Thus, it seems that, in the absence of the right IMHV at the time of training, other structures become involved in processing of the memory trace (Fig. 1.7).

The results reviewed here should be viewed with caution since lesion to the brain is bound to trigger a number of compensatory reactions that may not be otherwise active, particularly in a fast developing organism like the young chick. It may be possible that memory is first processed in a localized "executive centre", (say, the left IMHV) and then dissipated to other regions such as the right IMHV and LPO, as well as other nuclei capable of storage and recall of different *aspects* of the experience. However, each one of these nuclei may only become active during a specific time-window.

1	Lesion (left IMHV)	Train	No lesions	Test ->	Amnesia
2	No lesions	Train	Lesion (Both IMHV)	Test ->	Avoidance
3	No lesions	Train	Lesion (Both LPO)	Test ->	Amnesia
4	Lesion (Both LPO)	Train	Lesion (left IMHV)	Test ->	Avoidance
5	Lesion (Both LPO)	Train	Lesion (right IMHV)	Test ->	Amnesia
6	Lesion (Right IMHV)	Train	Lesion (Both LPO)	Test ->	Avoidance
7	Lesion (Right IMHV)	Train	Lesion (Left IMHV)	Test ->	Avoidance

Figure 1.7 : A summary of lesion studies discussed in the text.

Physiological changes

Anatomically localized physiological changes have been observed in the chick brain after passive avoidance training. A significant increase in high frequency neuronal bursting of IMHV was found by Mason and Rose (1987) when recording between 1 to 12hr after training in anaesthetized chicks. This effect was found to be specific to memory formation since subjecting the trained chicks to sub-convulsive electric shock immediately after training not only rendered the birds amnesic but also abolished the physiological changes. Chicks that, despite receiving the shock, still remembered to avoid the bead also still exhibited the typical high frequency activity (Mason & Rose, 1988).

Recent studies have demonstrated that there is a more specific mode of bursting during the first 12hr post-training. Thus, spike activities are shown to be high within two

specific time-windows, one about 1hr and the other between 7-9hr after training (Gigg, 1991). However, in MeA-trained birds, the right IMHV shows a significantly higher bursting activity during the second time-window than the left. As for the LPO, it too shows high spike activity during the second posttraining phase but with no apparent lateralisation. Again, these physiological activities were shown to be specific for memory processing since application of electroconvulsive shock within the first few minutes of training abolished these activities in birds that became amnesic. It is difficult to explain what kind of relations may exist between the IMHV and the LPO, particularly during the second time-window, since no direct neural connection between them has yet been reported.

Morphological changes

There is ample evidence that associates morphological changes with long term memory formation. Morphological changes are classified by Dudai (1989) into two categories: first, those that are confined to existing connections and cause no anatomical alteration to neural connectivity but alter the function, and, second, changes that lead to the growth of new (or elimination of some) anatomical connections.

The translation of biochemical into structural changes during memory formation is thought to take place mainly at the trans-synaptic level. Figure 1.8 summarizes the results of a large number of studies in this area. Analysis was carried out 24hr after training. Morphometric measurements indicated that, beside training-related changes, certain phenomena such as mean bouton density did not show changes after training. The application of transcranial subconvulsive shocks a few moments after training resulted in the absence of training-related morphological changes only in those birds that became amnesic after the shock treatment. If the shock is delayed by a few minutes, however, no amnesia is seen in chicks and similar morphological changes are found again.

Glycoprotein fucosylation

Approaches to the study of glycoprotein involvement in cognitive processes include carbohydrate analysis of glycoproteins and monitoring of their influence on acquisition, storage and retrieval of memory, structural and subcellular distribution of these molecules during different phases of memory, analysis of signal transduction pathways necessary for the expression of fucosylglycoproteins, and the application of neuroimmunological tools for characterisation and identification of specific glycosylated antigens. The results so far indicate that glycoprotein fucosylation is a specific neural response to training and memory formation; it does not occur as a result of, for example, merely exposing the chicks to the taste of MeA (without the accompanying training procedure) (Rose, 1981a).

The biochemical and biological properties of glycoproteins have been reviewed in Parts One and Two of this chapter. However, since Chapter 4 is specifically devoted to detailed analysis of fucoglycoproteins that are involved in memory formation, further review as to the role of these molecules in cognitive processes are accounted for in that chapter.

Neuroimmunological changes

One of the most effective ways to identify and assess the role of neural glycoconjugates during memory formation and consolidation is the use of specific antibodies. Antibodies can be used to show whether the presence or the expression of an antigen is *concomitant* to or *necessary* in learning, to examine the *time course* of such expression, and to map the *anatomical* and *morphological distribution* of the antigens. Antigens like Thy-1 (Bernard et al., 1983; Lappuke et al., 1987), ependymin (Schmidt, 1987; Schmidt & Piront, 1985; Schmidt et al., 1991; Shashoua & Moore, 1978), N-CAM (Doyle et al., 1990; Nolan et al., 1987b; Persohn & Schachner, 1990), 411B (Bullock et al., 1987, and 1988), F-3-87-8 (Nolan et al., 1987a), as well as

vasopressin (Croiset et al., 1990), S-100 (Cicero et al., 1970; Shtark et al., 1987), 14-3-2 (Cicero et al., 1970), B6E11 (Stanton et al., 1987), L1 (Persohn & Schachner, 1990), GAP43/B50 (DiFiglia et al., 1990; Campagne et al., 1990), and many others have been identified and their role in learning and memory formation and neural growth, development, and organization studied. For a brief review of these investigations see Chapter 6.

Structure	Measurement	W L *vs* R	M L *vs* R	Reference
IMHV	Mean length of postsynaptic thickening	>	= (⇑)	Stewart et al., 1984
	Synaptic vesicle number per unit neuropil	<	>	
	Number of vesicles per synapse	-	> (61%)	
	Presynaptic bouton density	-	> (22%)	
	Number of spines	-	> (60%)	
	Size of spine head	-	>	Patel and Stewart (1988)
	Spine stalk length	-	⇓	
LPO	Postsynaptic thickening	<	>	Stewart, 1991
	Number of vesicles per unit neuropil	-	>	
	Number of vesicles per bouton	-	>	
	Mean bouton density	=	=	
		M = W		
	Average bouton volume	-	>	
	Numerical density of synaptic vesicles	<	=	Stewart et al., 1987
	Number of vesicles per synaptic bouton	<	=	

Figure 1.8 : A summary of major morphological changes in the forebrain of day-old chick upon passive avoidance training. >, increase in the left structure; <, increase in the right structure; ⇓, decrease after MeA training; =, absence of lateralisation; ⇑, increase after training; -, no changes found.

2-Deoxy-D-galactose, a specific memory inhibitor

For several years a number of laboratories including the Brain and Behaviour Research Group (BBRG), the Institute of Brain Research in Magdeburg, have been engaged in exploring the function and the role of 2DGal in chick, rat and goldfish brain. Because of its relevance to some of the work carried out in this project, a brief review of these 2DGal studies is included in the introductory section of Chapter 3. Briefly, the results indicate that fucosylation of glycoconjugates is a concomitant and necessary process in the formation of long lasting memory traces, and that this phenomenon can be specifically blocked by 2DGal without significantly interfering with all other hitherto examined neuronal and physiological processes.

Glycolipids and information processing

Glycosphingolipids are assumed to play a specific role in the transmission of information and memory formation. Their amphiphilic property allows them to form complexes with Ca^{2+}. The resulting complex is capable of "modulating the very local zone of synaptic contacts following ionic induction and thus facilitating the transmission of information from one cell to the other" (Rahman, 1984; see also Wherrett & Hakomori, 1973). This hypothesis stems from a number of reports that are summarized below:

The sialic acid residue of gangliosides together with those of membrane-bound glycoproteins are the main sources of the negative charge of the cell surface (Nagai & Iwamori, 1980), making them good candidates for ligand binding. There is indeed evidence to suggest that some gangliosides act as receptors for hormones and neurotransmitters (Dette & Wasemann, 1978; Dowson & Stoolmiller 1976; Hakamori, 1981; Flexner & Flexner, 1967; Kanfer & Richards, 1967; Ledeen, 1978; Lee et al., 1976; Nagai & Iwamori, 1980; Ochoa & Bangham, 1976). Ganglioside GM1, for example, plays a role in hippocampal glutaminergic transmission, while striatal

cholinergic transmission is mediated by polysialogangliosides (Seifert et al., 1986). The binding of glutamate to glutamate receptors in the rat brain SPM is mediated by GM1 in a Ca^{2+} dependent fashion.

The functional involvement of gangliosides is also supported by the finding that activation of protein kinases in SPM preparations occurs only in the presence of specific Ca^{2+}-ganglioside complexes (Rahman, 1984). It is assumed that the arrival of an impulse at the nerve ending will cause the release of Ca^{2+} from Ca^{2+}-ganglioside complexes and consequently render SPMs permeable to Ca^{2+}. The highly negatively charged sialoglycoconjugates in SPMs are shown to participate in the displacement of Ca^{2+} during neural transmission (Seifert, 1986; Tobias, 1964).

Anti-neural ganglioside antibodies have been used in a number of studies and have resulted in interesting findings emphasising the important role that these antigens play in neural development and plasticity. Savaki (1977) has shown that passive avoidance learning can be inhibited by applying antiserum raised against rat brain gangliosides. Furthermore, the GM1-antibodies used by Karpiak and associates (1976 and 1978) caused epileptiform spike activity and inhibited a learned avoidance response. They also reported that their antibody specifically inhibited the consolidation phase of memory but not the acquisition phase (Karpiak & Rapport, 1979). Antibody to the same ganglioside, administered intraventricularly immediately after training, was also reported by Rapport et al. (1979) to impair learning processes (for further discussions of neurobiological function of gangliosides and their role in the long term storage of information consequent to synaptic transmission see Järnefelt et al., 1978; Maccioni et al., 1974a&b; Rahman, 1984; Rapport, 1981).

CHAPTER TWO

General Methodology

CHAPTER TWO

GENERAL METHODOLOGY

In order to carry out this project, that is, characterisation and identification of glycoproteins that are involved in memory formation and consolidation, a variety of laboratory techniques were employed. A few methods are commonly used in most of the succeeding chapters and, in order to prevent repetition, they are described below. However, where mentioned later, some modifications to these methods were introduced.

MATERIALS

p-Iodonitrotetrazolium violet (INT), n-octyl-β-D-glucopyranoside (OG), glucose, galactose, and 2-deoxy-D-galactose (2DGal) were from Sigma Chemical, U.K. [^{3}H]fucose and [^{3}H]galactose (specific activity 16.7 Ci/mmol), and [^{14}C]fucose (specific activity 58.7 mCi/mmol) were from Amersham International plc, U.K. Lectins Concanavalin A and Ulex Europaeus type I (UEA I) were bought from Sigma Chemicals Co. USA, and Sambucus nigra bark (SNB) and Maackia Amurensis (MAA) from Boehringer Mannheim Biochemica. Antibodies anti-UEA I conjugated to horse radish peroxidase was bought from Dakopatts Denmark, anti-biotin conjugated to horse radish peroxidase from Sigma Chemical Co. USA, and anti-rabbit IgG conjugated to horse radish peroxidase from Dakopatts Denmark. Normal goat serum was obtained from Vector Laboratories USA. Prestained molecular weight markers were from Sigma Chemical Co. USA. All other chemicals used were reagent grade.

METHODS

1. Passive avoidance training paradigm

The general paradigm of passive avoidance training was described in chapter one. Chicks were hatched in our communal brooders and, after approximately 24hr, were transferred to pens designed to house two chicks at a time. Each pen was illuminated with a dim red light (25W), and the room was kept at a constant warm temperature (30°C). Training was preceded by equilibration and pretraining stages. Equilibration involved pairs of chicks being placed in individual pens, containing a small amount of chick crumb, for a minimum of 45min. This familiarized them with their new environment. Psychological stress was assumed to have been overcome by the time of pre-training.

The pre-training procedure involved presentation of a dry white bead (3mm diameter) to the birds. The bead was slowly introduced to the pen away from the chicks. It was then moved towards the chicks to enabled them to see it. The chicks would spontaneously peck the bead. No more than 20sec was allowed for each chick to peck. During this time the behaviour of the chicks was recorded; they either pecked (P), actively avoided (A), or did not show any response (0) towards the bead. Pretraining consisted of three such bead presentations, and each time the chick's reactions were recorded. Those birds that pecked at the bead at least two out of three times were used for the next step.

Two groups of chicks, control and experimental, were chosen and trained. Training followed shortly after and involved presenting the birds with a chrome bead (5mm diameter) which was coated with either water (for the control group) or methyl anthranilate (MeA) (for the experimental group). After pecking at MeA-beads, chicks exhibited a characteristic "disgust" reaction by vigorously shaking their head and rubbing their beak against the floor. Care was taken to ensure that chicks would form

a strong association between the taste of MeA and the appearance of the bead. This was done by holding the bead in front of the chicks for a few seconds after they had pecked at it. If chicks were to remain in the pen for several hours before being tested, they were given more food and some water in a small bowl.

For testing, chicks were shown an identical but dry chrome bead for not more than 20sec. In the absence of any interventions 80% of MeA-trained birds avoided the test bead. With W-trained birds a success rate of 90% was achieved (a schematic diagram of passive avoidance training and protocol is shown in Fig. 2.1).

2. *In vitro* incubation of tissue slices

The *in vitro* technique employed in this project was as follows. After killing the chicks and removing their brain, the forebrains were placed on a few layers of dry filter paper on a pre-cooled McIlwain chopper platform. The brain tissue was wetted with ice cold preincubation buffer (see below) and sliced with the McIlwain chopper set at 400µm thickness (McCabe & Rose, 1985 & 1987). The sliced brain tissue together with the filter paper were then transferred *en masse* to a petri dish containing a few milliliters of preincubation buffer and slices were gently separated with the aid of a pair of long and narrow-headed paint brushes. Brain slices were transferred in pairs to separate clear plastic vials containing preincubation buffer and left at room temperature for 15min. At the end of this period 50-100µl of radiolabelled sugars were added and the mixtures were transferred to a water bath and incubated at 42°C.

During the two-hour incubation period samples were agitated at about 100 shakes per minute to insure adequate O_2/CO_2 exchange. Both preincubation and incubation media were *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES)-Ringers (pH 7.4) adopted from Dunlop et al. (1975 & 1977) pregassed for 15min. They consisted of the following in mM concentration; HEPES (25), K_2HPO_4 (1.5), NaCl (118), KCl

(4.4), $MgCl_2$ (1.3), glucose (12); $CaCl_2$ (2.6), and the non-radioactive sugar isotope (0.020). Both preincubation and incubation buffers were gassed with a N_2/O_2 (80:20 ratio) gas mixture for at least 15min to ensure saturation. At the end of incubation, vials were quickly transferred to an ice-water mixture, the incubation solution was drained off and 3ml of fresh ice-cold preincubation solution containing unlabelled sugar (1mM) was added. The mixture was shaken for a few seconds and the solution drained off. Remaining droplets of buffers were adsorbed with a piece of filter paper. This washing step was repeated twice more. The unlabelled sugar was added in order to displace [^{3}H]- or [^{14}C]-labelled sugar from nonspecific sites.

After transferring the samples to Eppendorf tubes, 1ml of ice-cold double-distilled water was added and samples were homogenised by mild sonication. Small aliquots of the homogenates were set aside for protein estimation and radioactivity measurements and the remainder quickly acid hydrolysed with ice-cold trichloroacetic acid (TCA) (20% final concentration). Samples were left on ice for at least 30min and then spun at 10.6kg_{max} in a Microcentaure bench centrifuge for 10min. Pellets were washed twice with water and prepared for gel electrophoresis. The corresponding supernatants were pooled and kept for radioactivity measurements.

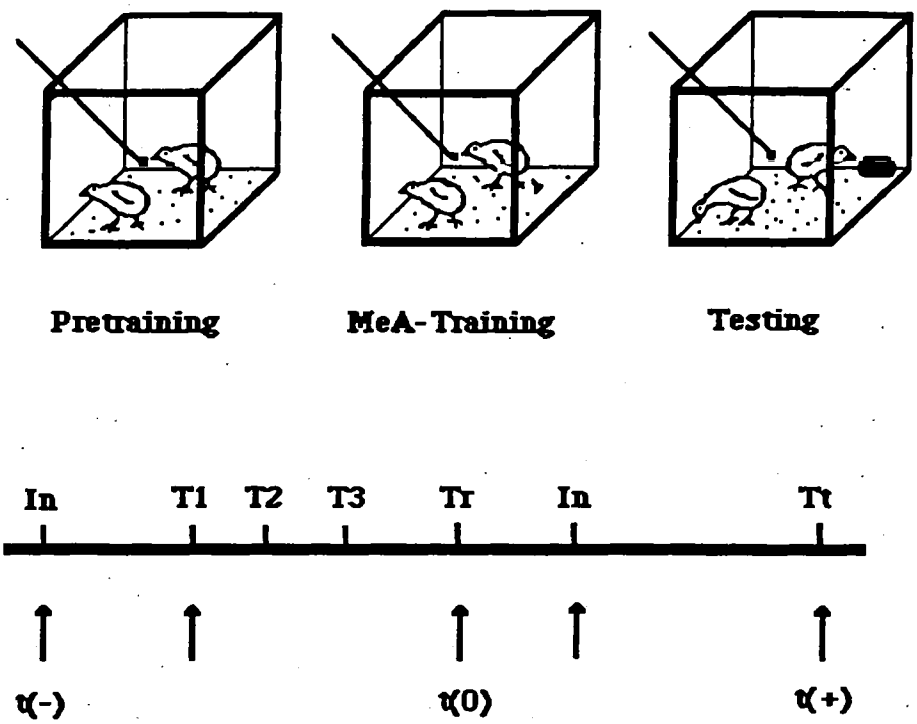


Fig. 2.1 : A schematic diagram of passive avoidance training paradigm and protocol are shown above. In, intervention (eg. injection, lesion); T1, first pretraining; T2, second pretraining; T3, Third pretraining; Tt, Test; t, time.

3. Protein estimation

Protein estimations were carried out to calculate the specific activity of samples and hence the turn-over rate of a radiolabelled agent (e.g., [^{3}H]Fuc), and to calculate the recovery rate of a preparative method (e.g., subcellular fractionation). The procedure for estimating protein content used Coomassie blue dye (Bradford, 1976) and was briefly as follows;

A calibration curve was constructed by using known amounts of protein. For this purpose bovine serum albumin standard dissolved in double distilled water was used. A range of solutions, in triplicate, contained between 0 to 2mg/ml of protein. First to each Eppendorf tube 30µl of a 75mM solution of NaOH was added to solubilize the protein. Equal volumes of solutions containing different amounts of protein were then added to the Eppendorf tubes. To these mixtures 500µl of Bradford dye was added, and after a few minutes, 3x100µl of each solution were transferred to ELISA plates for absorption measurement which was carried out at 595nm. The first vertical column in each ELISA plate was filled with distilled water (DDW) to standardize inter-plate reading.

Bradford dye was made by adding 100ml of H_3PO_4 to 50ml of ethanol solution that contained 5ml of DDW and 100mg of Coomassie brilliant blue G250. The volume of the mixture was adjusted to one litre and the resulting solution filtered and stored at 4°C. A calibration graph was constructed and used to estimate the protein content of test samples. A similar procedure to that described above was used to prepare sample solutions. In general, a protein content of 9-18µg/ml gave satisfactory results since a protein content above 20µg/µl took the absorption:protein concentration relation beyond the range of linearity.

4. Delipidation

Lipid extraction was carried out after homogenisation of tissue samples. This was done by a simple ethanol, methanol:chloroform, ethanol procedure.

I. About 1mg of protein was dissolved in 1ml of ethanol, and spun at $12.8k_{gmax}$ 10krpm for 10min in Beckman J2-21 centrifuge. The supernatant was set aside and the pellet carried to next step.

II. The pellet was mixed with 1ml of methanol:chloroform (2:1, v/v), and spun at $28k_{gmax}$ 15krpm for 20min. The supernatant was again retained and the pellet was washed once more. The supernatant was added to previous pool.

III. The pellet was finally mixed with 1ml of ethanol and spun as before. The supernatant was added to the pool and the pellet retained for analysis.

IV. The pools of supernatants were transferred into scintillation vials and air-dried in a fume cupboard overnight. One ml of water and 8ml of scintillant (Emulsifier-safe™, Packard) were added to the pellets and the radioactivity was measured in a Beckman LS.1710 scintillation counter at an efficiency rate of 80%.

5. SDS-Polyacrylamide gel electrophoresis

One-dimensional and two-dimensional reducing gel electrophoresis were carried out using the techniques described by Laemmli (1970), O'Farrell (1975), O'Farrell et al. (1977), and Rodnight et al. (1988).

5.1. One dimensional gel electrophoresis : Two different sets of apparatus were used throughout this project, a Mighty Small™ apparatus (Bio-Tech, Slough) and an LKB polyacrylamide gel running apparatus. Some modifications of the latter set-up were introduced. For example, an outlet tap was fitted to the upper tank to

facilitate the exchange of the upper tank buffer during the running of the second dimension (see below). Further more, in order to economize on the use of buffers, a smaller upper tank was designed that held not more than 450ml of solution without affecting the quality of gel running.(see Fig. 2.2a). Also, a large gel caster was made in which four slab gels were made at a time (see Fig. 2.2b). A large gradient gel maker purchased from Biorad was used for making linear gradient gels.

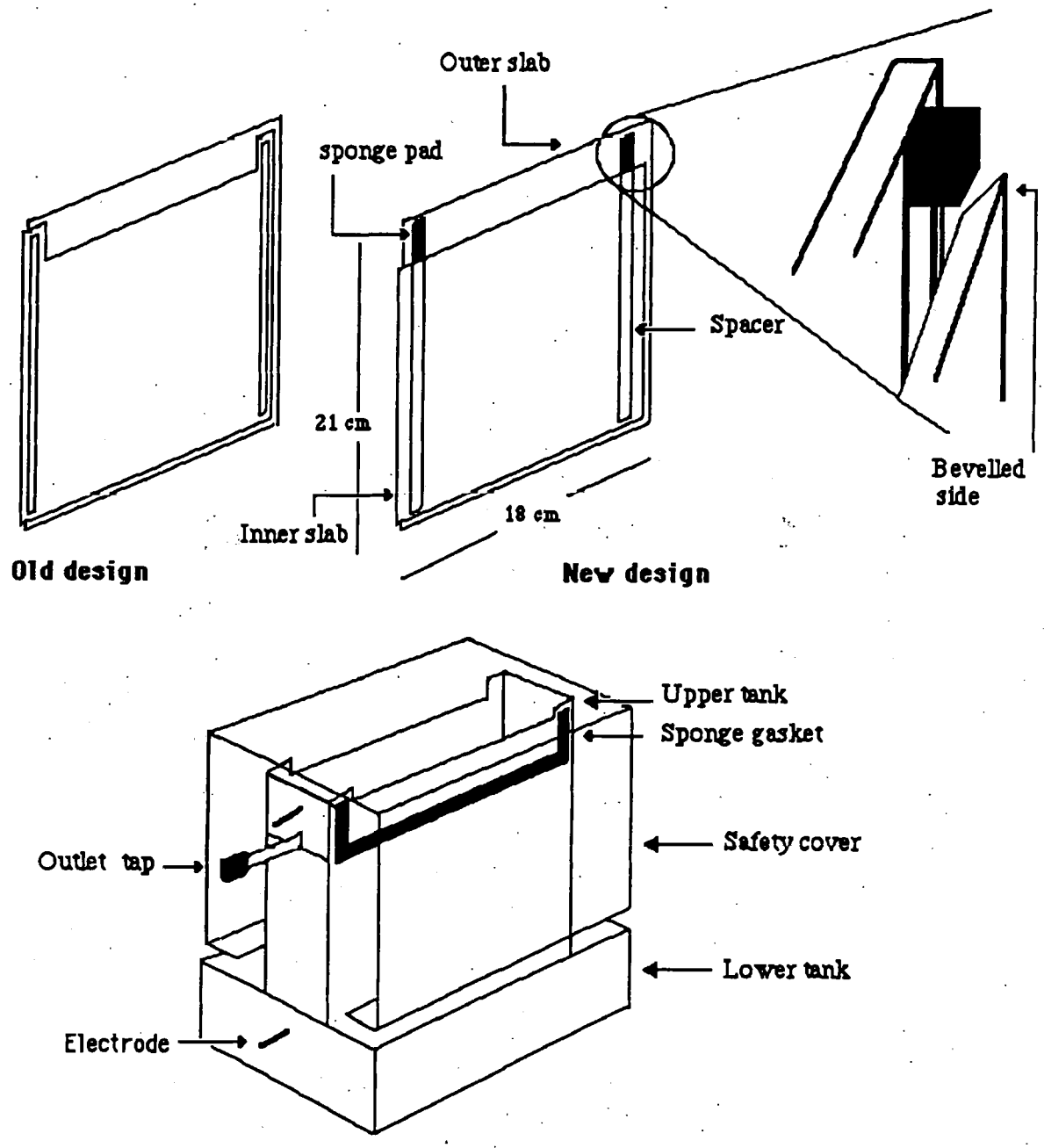


Fig. 2.2a : A schematic diagram of the electrophoresis equipment..

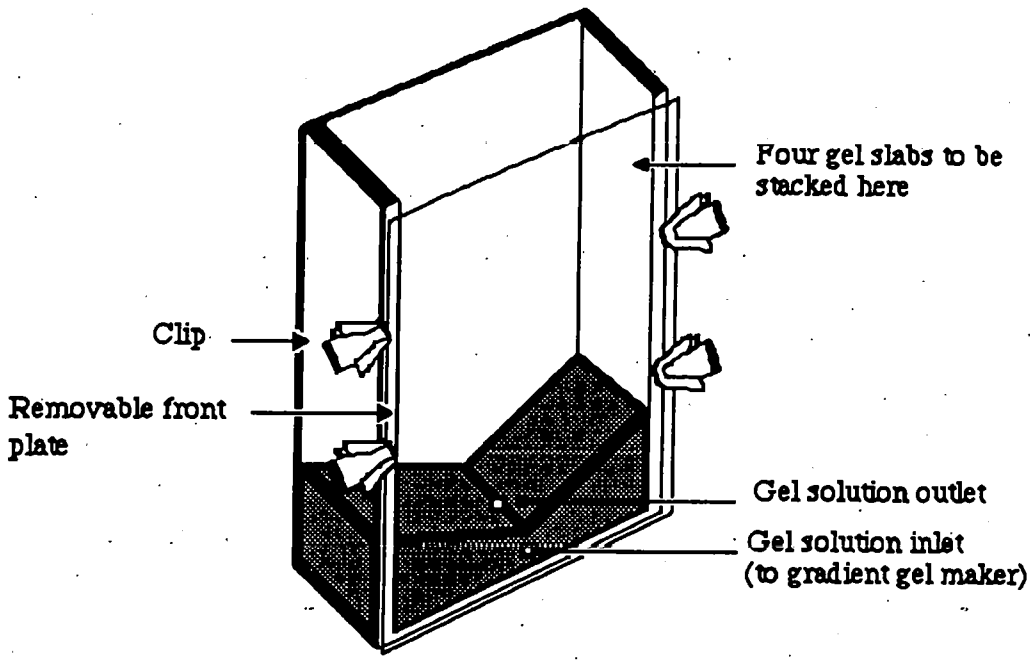


Figure 2.2b : A schematic diagram of the gel caster making four gradient gels at a time.

To run the protein samples on a gel, they were first TCA-precipitated and washed twice in double distilled water (DDW) to remove excess acid (see above). Laemmli buffer was added the pellets to give a solution containing 1mg/ml of denatured protein. The solution was loaded onto the gel slab using a Hamilton syringe, and the gel run at 20mA constant current overnight. At the end of the electrophoresis, gels were stained with methanol:acetic acid:water (40:10:50 v/v) containing 0.1% Coomassie blue R-250. Gels were then destained, first in stain solution without the dye, and then in 10% acetic acid solution. This would destain and rehydrate the gels.

To make 100ml of Laemmli buffer the following reagents were used; glycerol (20ml), 2-β-mercaptoethanol (10ml), SDS (30ml of 20% solution made in DDW), bromophenol blue (BPB) (5ml of 0.1% solution made in DDW), stacking gel buffer (25ml), and water (10ml). The stacking gel buffer consisted of Tris buffer (0.5M, pH 6.8) containing 0.4% SDS. The resolving gel buffer consisted of Tris buffer (1.5M, pH 8.8) containing 0.4% SDS. The electrophoresis running buffer consisted of a tris buffer (25mM, pH8.3) containing 192mM glycine and 0.1% SDS. 10-Fold concentrated stock solutions of these buffers were made.

To make 100ml of stacking gel solution following reagents were used; 13.3ml of 30% acrylamide, 27.8ml of stacking gel buffer, 1ml of 10% SDS, 0.15ml of *N, N'*-methylenebisacrylamide and *N, N, N', N'*-tetramethylethylenediamine (TEMED), 56.1ml of DDW, and 2ml of 10% ammonium persulfate (APS). To make a linear gradient gel 5% and 15% resolving buffers were made. The former solution consisted of, in 100ml, 16.5ml of 30% acrylamide, 32ml of resolving buffer, 2.6ml of 10% SDS, 0.15ml of TEMED, 48.ml of DDW, and 0.15ml of 10% APS. The latter solution consisted of, in 100ml, 50ml of 30% acrylamide, 32ml of resolving buffer, 2.6ml of 10% SDS, 0.3ml of TEMED, 15ml of DDW, and 0.15ml of 10% APS.

5.2. Two-dimensional gel electrophoresis : 2-D SDS-PAGE was exclusively carried out in Chapter 6 and is, therefore, described there.

6. Subcellular fractionation: Preparation of synaptic plasma membranes and postsynaptic densities

The methods for the preparation of synaptic plasma membranes (SPMs) and postsynaptic densities (PSDs) were based on the procedures described by Murakami et al. (1986) and Gurd et al. (1982, 1983).

SPM isolation : Chick forebrains were removed rapidly and placed in ice, and either processed individually or combined, depending on the design of the experiment. Unless mentioned otherwise, the entire procedure was carried out at 4°C. The wet weight of each sample was recorded before homogenization in a teflon-glass homogenizer. This was done by placing the samples in glass homogenization tubes containing 0.32M sucrose in 2mM HEPES (10% wet weight/v), and homogenising at 700rpm, for seven up-and-down strokes. An aliquot of the homogenate was set aside.

The homogenates were spun at $1kg_{max}$ 3krpm for 5min, and the pellet (P1) (tissue debris and nuclear fraction) washed once in the same manner. The resulting supernatants (cell fragments, synaptosomes and mitochondria) were pooled and spun at $15kg_{max}$ 11krpm for 20min. The pellet (P2) (crude mitochondria) was washed twice in the sucrose buffer to remove microsomal contamination (Gurd et al. 1974). It was then resuspended in lysis buffer (50mM Ca^{2+} in 5mM Tris, pH8.1) (10ml/g wet weight tissue) by glass-teflon homogenization, and left on ice for 30min. The mixture was centrifuged at $120kg_{max}$ 25krpm for 20min and to the pellet 3ml/g tissue of freshly made *p*-iodonitrotetrazolium violet (INT) and 1.1ml/g tissue of 0.2M sodium phosphate buffer (pH7.4) were added.

The mixture was homogenised using a teflon-glass homogeniser and incubated for 30min at 25°C. The amount of INT used to precipitate the mitochondria is critical since excess INT can precipitate membrane fragments as well as mitochondria and hence reduce the final yield; too little INT, on the other hand, causes an increase in mitochondrial contamination of membrane fractions (Nieto-Sampedro et al., 1981).

At the end of incubation, the lysate was centrifuged at $70kg_{max}$ 20krpm for 15min, using a Beckman high speed centrifuge machine with an SW28 swing-out rotor. The pellet was washed once in 0.16M sucrose buffer containing 2mM HEPES (pH7.4). The resulting pellet was resuspended in 0.25ml lysis buffer/g tissue using a glass homogeniser. To the homogenate, 2.3ml of 48% sucrose, containing 2mM HEPES (pH7.4)/g tissue was added and mixed well. The homogenate was transferred to polyallomer tubes and overlaid with a solution of 28.5% sucrose containing 2mM HEPES (pH7.4). The gradient was centrifuged at $120kg_{max}$ 25krpm for 75min.

The SPM layer was removed from the interface between the two sucrose solutions and washed in 50μM Ca^{2+} ($120kg_{max}$ 25krpm, 20min), and resulting pellet was resuspended in 50μM Ca^{2+} (1.2ml/g wet weight of tissue). The pelleted mitochondria formed during gradient centrifugation were retained for analysis.

PSD isolation : PSDs were prepared from SPM fractions. A mild non-ionic detergent, n-octyl-β-D-glucopyranoside (O.G.), at 0.7% (w/v) final concentration was used in a two-phase partitioning procedure. The immiscible polyethylene glycan 6000/dextran T500 gradient was made up as follows; In 5g total weight of gradient, 1ml of 20% polyethylene glycan, 1.2ml of 25% dextran T500, 1.32ml Ca^{2+} (50μM, made in DDW) 0.4ml $NaHCO_3$ (0.5M), and 80μl O.G. were added to 480μl of SPM solution (having a concentration of 1.5-3mg/ml). The mixture was vigorously mixed on a vortex mixer and centrifuged at $2.3kg_{max}$ 3.5krpm for 7.5min. The PSD ring

was removed from the interface, transferred into Eppendorf tubes, and washed in 50mM TBS (pH7.6), 50μM Ca^{2+} (10.6kg_{max} in a Microcentaure bench centrifuge for 10min). The PSD pellet was resuspended in the same buffer (0.24ml/g wet weight of tissue).

CHAPTER THREE

2-deoxy-D-galactose And Chick Brain Protein Glycosyiation

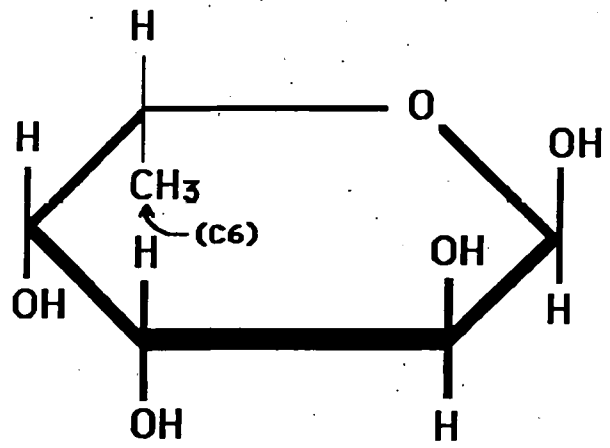
CHAPTER THREE

2-DEOXY-D-GALACTOSE AND CHICK BRAIN FUCOSYLGLYCOPROTEINS

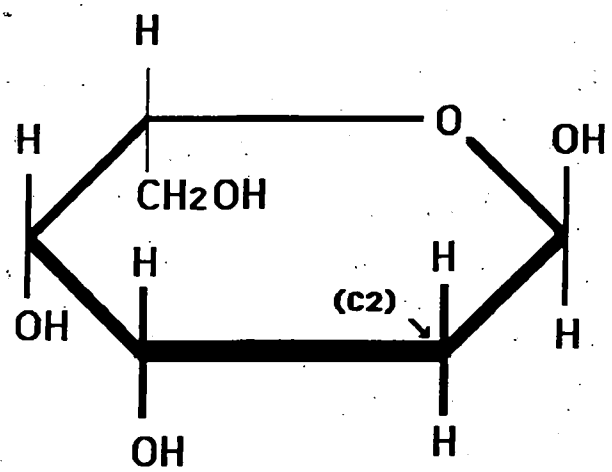
INTRODUCTION

2-deoxy-D-galactose (2DGal) when converted into its nucleotide derivative UDP-deoxy-D-Gal inhibits fucosylation (Schwarz and Datema, 1982 and Büchsel et al., 1980). The absence of a hydroxyl group at the C-2 position of 2DGal specifically blocks the formation of (α1->2) L-fucosyl linkage by β-galactoside(α1-2)fucosyltransferase (Bretscher and Raff, 1975 and Morre et al., 1979) (see Diagram 3.1 for 2DGal and fucose structures). The metabolic route of 2DGal conversion is known as the Leloir pathway. In their study on rat liver Büchsel et al. (1980) found that *in vivo* 2DGal inhibited galactose incorporation into glycoproteins without affecting protein synthesis, and that 1hr after the injection of [^{14}C]2DGal the level of UDP-glucose and UDP-galactose decreased by 50%. Smith and Keppler (1977) investigated the metabolism of 2DGal in a hepatoma cell line and found 99.3% conversion of [^{14}C]2DGal to [^{14}C]2DGal-1-phosphate, and only 0.4% to [^{14}C]UDP-2DGal and UDP-2-D-glucose.

As will be pointed out below, 2DGal when administered at the time of training induces amnesia both in rat and chick. It may be thought that 2DGal could induce it effect via uridylate trapping rather than directly competing with Gal. However, in a report by Schmidt et al. (1989), 2DGal, at amnesia-inducing doses, was found not to trap uridine phosphates in the rat brain. These results were interpreted by the authors to indicate a more specific metabolic effect of this sugar derivative being responsible for its amnestic action. 2DGal does not affect the activity of fucosyltransferase or other



L-FUCOSE (6-DEOXY-L-GALACTOSE)



2-DEOXY-D-GALACTOSE

Diagram 3.1 : The ring structures of fucose and 2-deoxy-D-galactose.

transferases activity for the incorporation of their sugar substrates into carbohydrate chains of glycoconjugates (Büchsel et al., 1980).

2DGal is thought to require the enzyme galactosyltransferase for the formation of the pre-terminal glycosidic linkage. Several transferases have already been identified in different systems including the CNS, and localized at the synaptic junction (Rostas et al., 1981; Sadler, 1984). The presence of galactosyltransferase at the synaptic junction suggests that *in situ* posttranslational modification (PTM) may take place almost immediately after the administration of 2DGal into the brain, thereby inhibiting fucosylation. However, the limited amount of epimerisation of 2DGal-1-phosphate to 2DGlc-1-phosphate (Starling and Keppler, 1977) would ensure that the remaining 2DGal in the brain will become incorporated into the nascent glycoproteins destined for membrane integration (mainly SPM and PSD) sometime later.

The inhibition of fucosylation by 2DGal in rat hippocampus was investigated by Jork et al. (1986). they found that intraventricular injection of 2DGal showed both dose- and time-dependent effects in the reduction of the rate of fucosylation. Retention performance of animals in a foot shock-motivated brightness discrimination task was considerably impaired following 2DGal injection either before or immediately after training (Jork et al., 1986). It was concluded that "an effective metabolic inhibition of glycoprotein completion by 2DGal at the time of training is crucial to interfere with morphological alterations in the neural network underlying the formation of a memory trace" (Jork et al., 1986). A direct link between inhibition of fucosylation and the amnestic effect of 2DGal was later reported by Jork and associates (1989). They found that the training induced increased [^{3}H]fucose incorporation in rats was blocked by amnestic dose of intrahippocampal 2DGal injection. Similar results have been reported in studies involving passive avoidance training in chicks. An intracerebral injection of 2DGal (20μmole) just before or after training produced long-lasting

amnesia (up to 24hr), whereas 2DGal injections 4hr before or 3hr after training had no effect (Rose and Jork, 1987). There was a significant (26%) drop in glycoprotein fucosylation in the forebrain within 3hr after passive avoidance training. Simultaneous injection of galactose abolished the amnestic effect of 2DGal. This was expected since they are thought to compete for the same transferase for their incorporation into oligosaccharide chains. Furthermore, simultaneous injection of either fucose, 2-deoxy-D-glucose (2DGlc), or glucose with 2DGal did not stop it from causing amnesia. This is because these sugars use different processing enzymes and, consequently, have no effect on 2DGal incorporation (see also; Büchsel et al., 1980).

The effect of 2DGal on long term potentiation (LTP) has also been investigated. LTP is a widespread neuronal phenomenon regarded as a mnemonic device in the mammalian brain. Results from a study of the effect of LTP induced in rat hippocampus through tetanization of perforant path on fucose incorporation showed no significant difference between control stimulated tissues and LTP induced preparations (Pohle et al., 1986). However, a consistent and significant elevation of fucosylation in hippocampal areas CA1 and dentate of LTP induced brains were seen. Recently, the effect of inhibition of protein fucosylation on LTP was investigated (Krug et al., 1991). Two models of LTP, one in freely moving rats, the other in hippocampal sections, were used and in both 2DGal was found to inhibit the second phase of LTP, known as maintenance, without interfering with the first phase, induction, and synaptic transmission, or spike generation. Therefore glycoproteins are thought to be crucial to the maintenance of LTP as well as in long term memory consolidation.

The mechanism of action of 2DGal has been studied in a Garcia-type training paradigm (Barber et al., 1989). Here, too, protein fucosylation was found to be necessary for the non-Pavlovian formation of long term memory trace (that is,

temporal discontiguity between conditional and unconditional stimuli). Chicks made sick by intraperitoneal administration of lithium chloride (LiCl) sometime (about 30min) after peaking at a dry bead avoided it in future encounters. Their avoidance was specific to the dry bead since they did not avoid an LED lit bead presented to them at the time of testing. However, when they were given an intracerebral dose of 2DGal about 10min before training chicks did not avoid the test bead when they encountered it for the second time. It was therefore argued that chicks must form a memory trace for every novel stimulus for at least 30min; this then became associated with the experience of LiCl-induced sickness, resulting in their avoidance of the test bead subsequently. This memory trace is said to be dependent on fucosylation of neural cell membrane glycoproteins.

The specificity of the mechanism of action of 2DGal was tested for state-dependency. A number of drugs that have been used to induce behavioural alteration are state dependent. For example, amnesia induced by intraperitoneal injection of anisomycin in trained chicks is abolished when it is injected before testing (Patterson et al., 1989). In their study, Barber and associates (1990) tested 2DGal by administering the sugar analogue, once to induce amnesia -10min before training- and again 10min before testing the birds on the sickness-induced learning task. Four groups of birds were chosen; group (a) received, chronologically, 2DGal and saline, group (b), 2DGal on both occasions (test group), group (c), saline on both occasions ("pure control" group), and group (d) saline and 2DGal. No state dependency effect was recorded since groups (a) and (b) both became amnesic whereas the remaining two groups avoided the test bead. It can, therefore, be concluded that 2DGal exerts its amnestic effect via inhibition of fucosylation, and that once mnemonic relevant fucosylglycoproteins are converted into 2-deoxy-D-galactosylglycoproteins the amnesia is immortalized.

Thus, results from a large many behavioural paradigms and from LTP experiments indicate that fucosylation of glycoconjugates is a necessary process for the formation of long term memory, and that this process can be specifically inhibited by 2DGal. It was the aim of the work described in this chapter to gain more insight into the mechanisms through which this inhibitor exerts its role and to identify the proteins of chick CNS whose fucosylation is inhibited by 2DGal. The first sections investigate *in vitro* and *in vivo* characteristics of 2DGal action, and the last section concentrates on fucosylglycoprotein identification using subcellular fractionation and electrophoretic analysis.

MATERIALS

See chapter two for details of materials used in this study.

METHODS

The role of 2DGal in inhibiting the incorporation of its analogue Fuc and Gal with which it shares the processing enzymes for its incorporation, was studied both *in vitro* and *in vivo*

The *in vivo* study

Day-old chicks were placed in pairs in individual pens and after 1hr they received either, bilateral intracerebral (ic) or, intraperitoneal (ip) injections. For the ic injections 20µl of sugar solutions of various concentrations were made in saline and administered into each hemisphere. The head-holder used in our lab was modified to accurately deliver solutions into IMHV. The part containing the "syringe tunnel" was made of one inch thick perspex (see Fig. 3.12). For the ip injections 200µl of saline-diluted sugar solutions of various concentrations were used.

The *in vitro* incubation of tissue slices

The *in vitro* technique used here was essentially as described in the Chapter 2.

Delipidation

In order to obtain an accurate measure of substrate incorporation into proteins lipid extraction was carried out after homogenisation of tissue samples. This was done by a simple ethanol, methanol:chloroform, ethanol process as described in Chapter 2.

Subcellular fractionation and gel electrophoresis

After the preparation of subcellular fractions their protein contents were measured and the rest of samples solubilised in Laemmli Buffer and run on a 5-15% gradient gels. These were carried out essentially as described in Chapter 2.

About 35 Coomassie blue R-250-bands stained, were cut out from each gel lane, and dissolved in 2ml of Protosol (NEN Research Products). After 24hr, 1ml of water and 8ml of scintillant (Emulsifier-safe™, Packard) were added and their activity counted in a Beckman LS.1710 scintillation counter at an efficiency rate of 80%. Water was added to reduce chemiluminescence.

RESULTS

Leucine and fucose incorporation into proteins *in vitro*

The incorporation of [^{14}C]leucine (1μCi, 2.87 nmoles) in 2ml incubation medium was assessed using various concentrations of unlabelled amino acid (0.01-10mM). Each concentration was incubated in duplicate. Tissue slices were preincubated for 15min and incubated for 1hr. The results are shown in Fig. 3.1a&b. As was expected the uptake of labelled leucine was inhibited by its unlabelled analogue (more than 90% competition was achieved in this system at 10mM concentration).

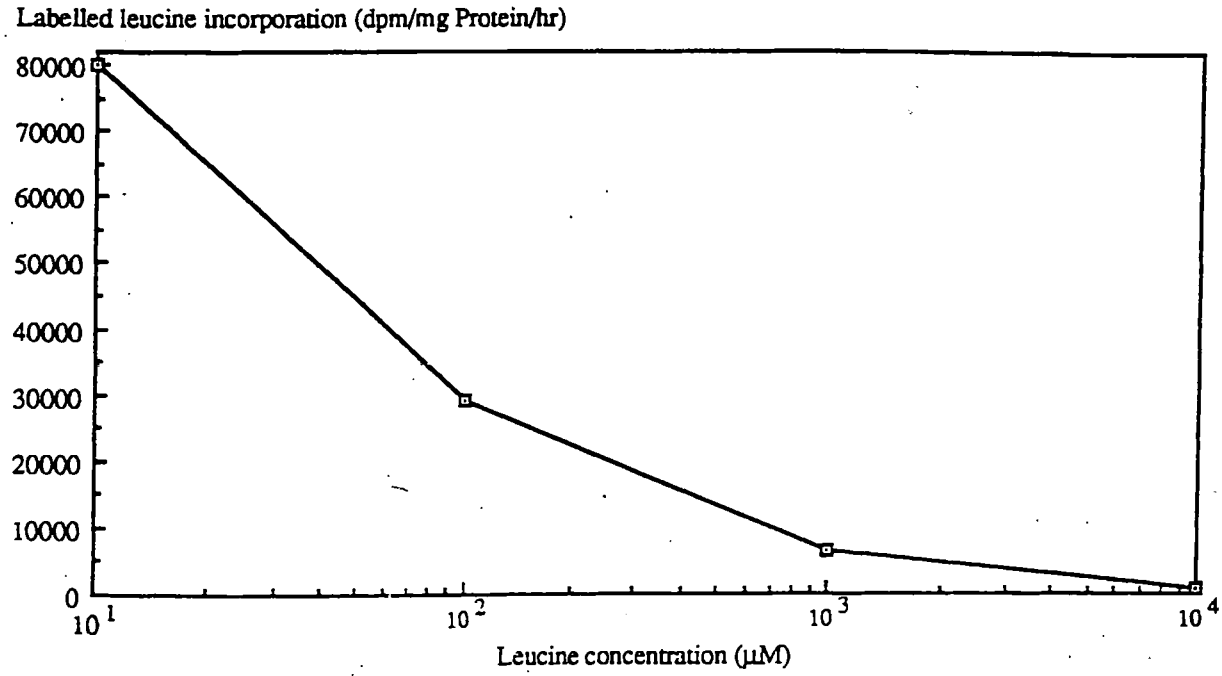


Fig. 3.1

Figure 3.1 : The incorporation of radiolabelled [^{14}C]leucine into TCA insoluble material in the presence of a range of unlabelled leucine *in vitro*.

Two samples were used under each condition, and each point on the curve is the average of the two. Corresponding values differed from each other by not more than 5%.

The incorporation of [^{3}H]fucose (5μCi, 0.3μmole) and [^{14}C]fucose (1μCi, 0.017μmole) were assessed in the presence of a range of unlabelled fucose concentrations (0-100mM). Up to 95% competition was observed under these conditions (see Fig. 3.2a-d). When different amounts of [^{3}H]fucose (0.5-10μCi/2ml) were used in the presence of either 0mM or 1mM unlabelled fucose, the incorporation of the labelled isotope dropped by some 75-80% in the latter condition (see Fig. 3.3).

2-Deoxy-D-galactose inhibition of galactose and fucose incorporation

The *in vitro* study : Having established the *in vitro* system described above, the effect of 2DGal on the incorporation of fucose and galactose was examined.

A range of 2DGal concentrations (0-100mM) were used to inhibit the uptake of [^{3}H]galactose (10μCi/2ml, 1.45μmoles) and [^{3}H]fucose (10μCi/2ml, 0.6μmoles) *in vitro*. Four groups each consisting of 5 chicks were used in this study. At 1mM, 2DGal significantly inhibited galactose incorporation. As shown in Figs 3.4a&b, 2DGal inhibited fucose and galactose uptake in a dose-dependent manner. The incorporation of fucose dropped significantly (by about 17%) at a 2DGal concentration of 10mM (Fig. 3.4a).

The *in vivo* study : A range of 2DGal concentrations was used to examine inhibition of galactose and fucose incorporation *in vivo*. Such data is more useful when designing behavioural experiments. In a galactose/2DGal study, 20μCi of [^{3}H]galactose [2.9nmoles/40μl of saline containing either unlabelled galactose (1mM) or 2DGal (1mM)] were injected bilaterally into the IMHV, using the head holder. Four hours later the incorporation of labelled galactose into chicks' forebrains was analysed. As shown in Fig. 3.5 2DGal at 1mM concentration inhibited the incorporation of [^{3}H]galactose by 73%.

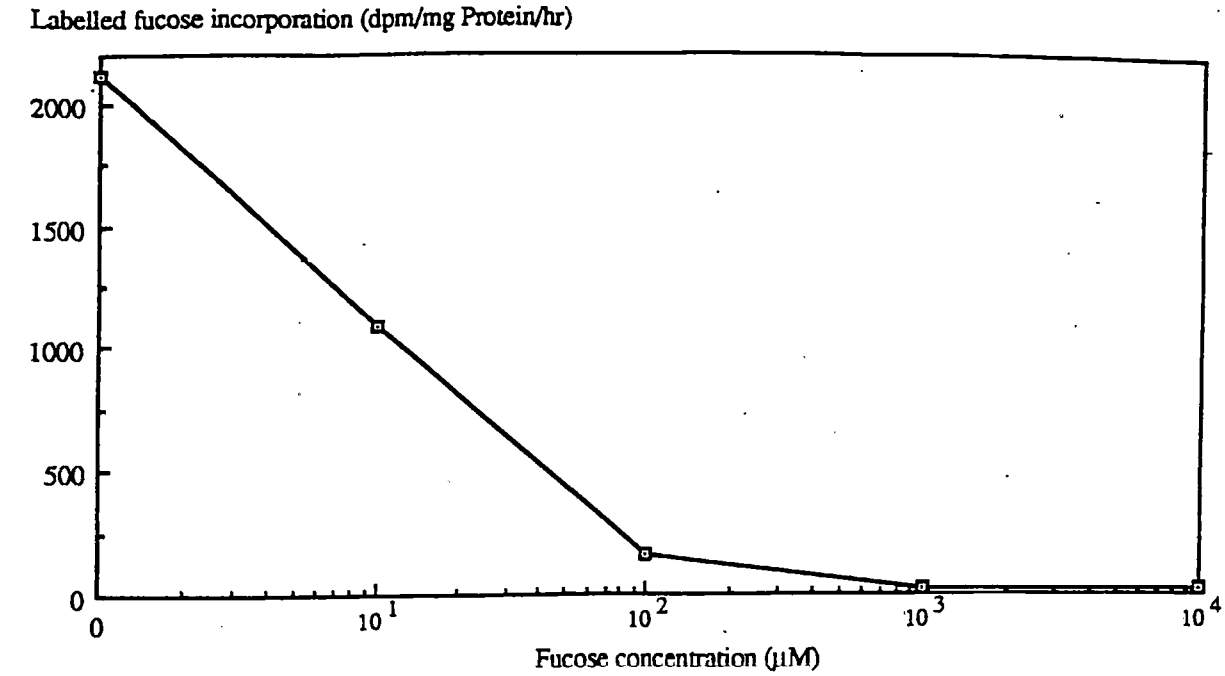


Fig. 3.2a

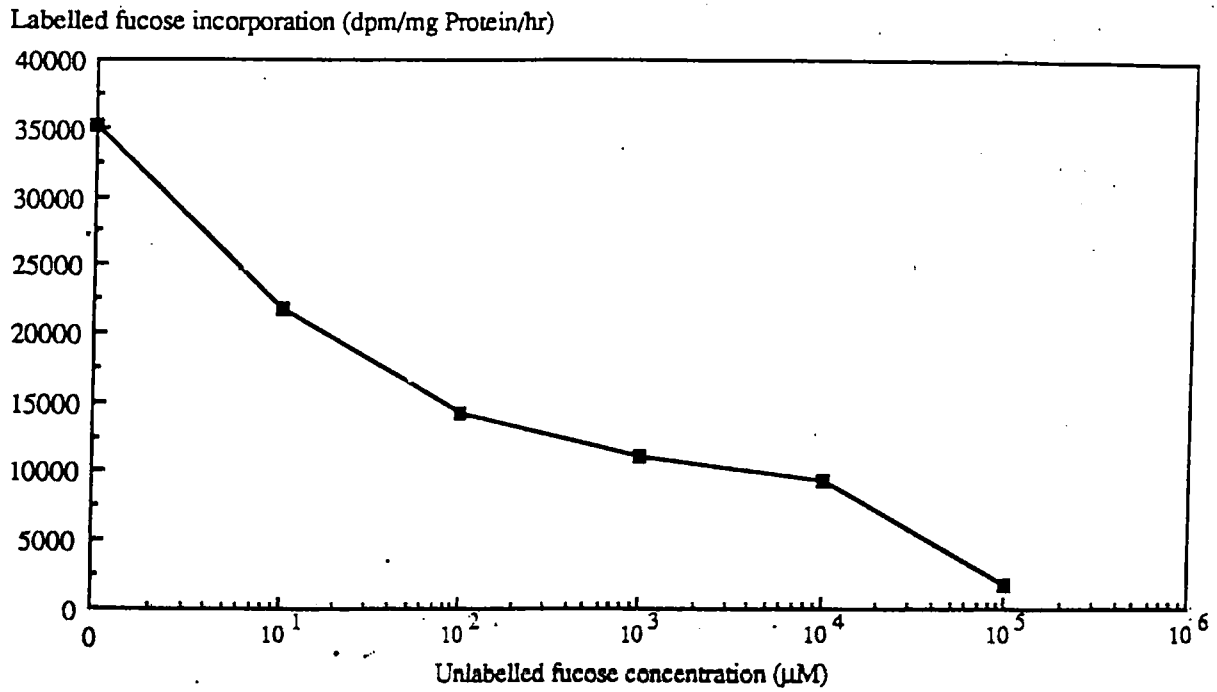


Fig. 3.2b

TCA insoluble material in the presence of a range of unlabelled sugar *in vitro*. Two samples were used under each condition, and each point on the curve is the average of the two. Corresponding values differed from each other by not more than 5%.

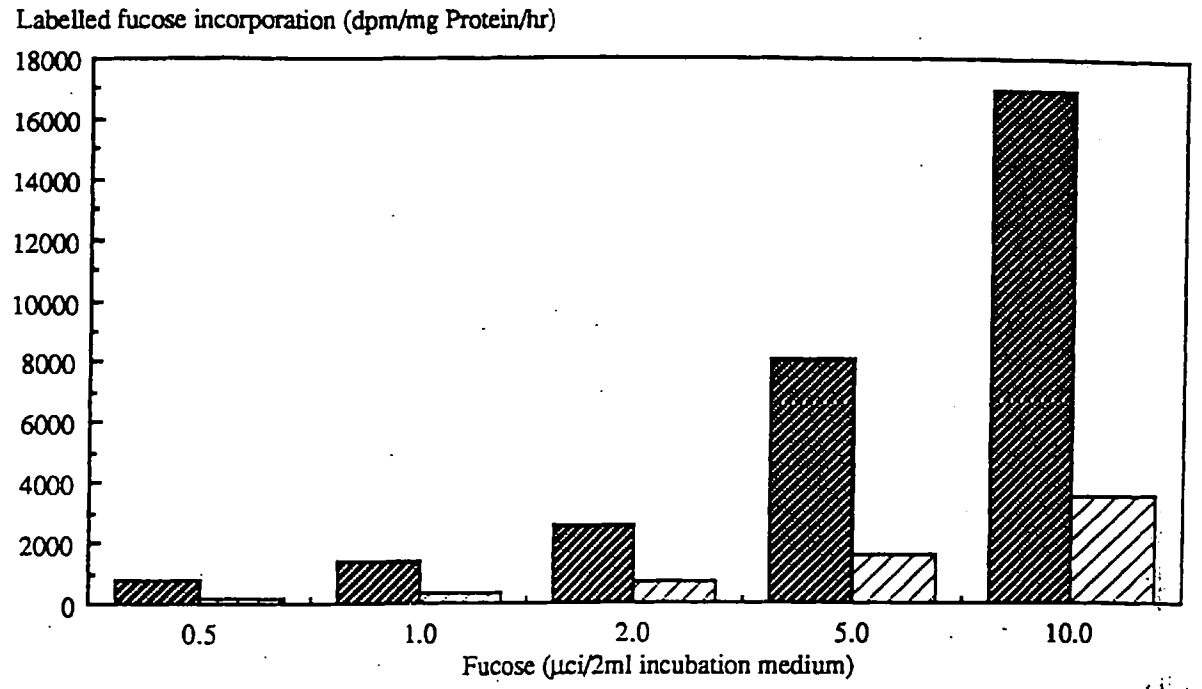


Fig. 3.3

Figure 3.3 : The incorporation rate of [^{3}H]fucose into TCA insoluble material *in vitro*.

Each incubation medium contained different amount of [^{3}H]fucose. Two samples were used under each condition, and each point on the hostogram is the mean of the two. Corresponding values differed from each other by not more than 5%. Half the samples were incubated in the presence of 1mM unlabelled fucose (light shaded bars).

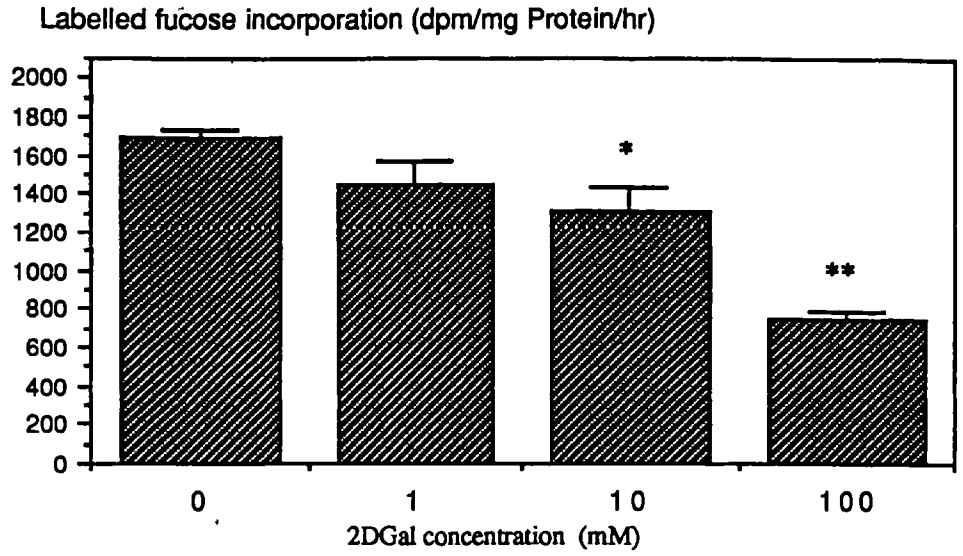


Figure 3.4a : The incorporation of [^{3}H]fucose into TCA insoluble material in the presence of a range of 2DGal concentrations *in vitro*.

Four samples were used under each condition, and each point on the histogram is the mean of the four with s.e.m. bars. * $p<0.002$; ** $p<0.001$ (Students t. test)

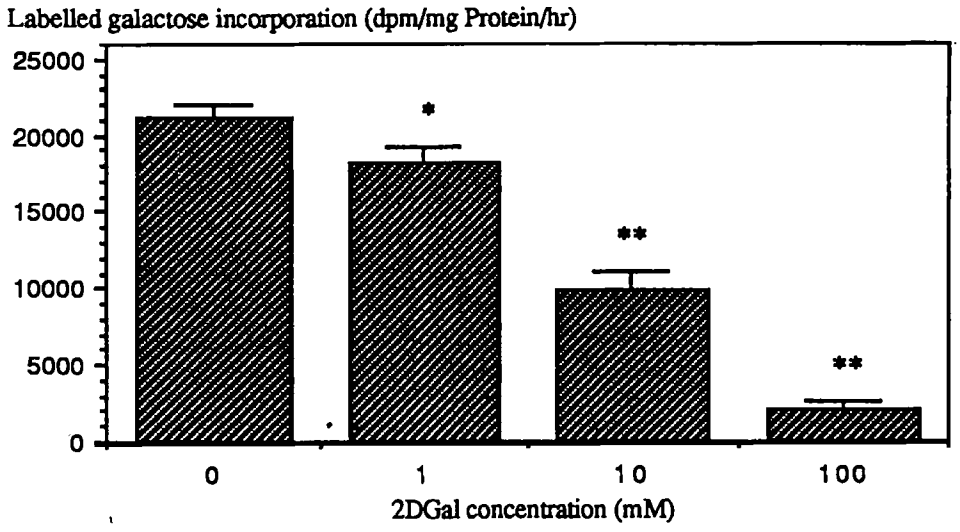


Figure 3.4b : The incorporation of [^{3}H]galactose into TCA insoluble material in the presence of a range of 2DGal concentrations *in vitro*.

Four samples were used under each condition, and each point on the histogram is the mean of the four with s.e.m. bars. * $p<0.010$; ** $p<0.001$ (Students t. test).

The inhibition of fucose uptake by 2DGal was also studied using both intracerebral and intraperitoneal injection. Three chicks were used in each group and various concentrations of 2DGal used to assess the its characteristics of inhibition. Contrary to the *in vitro* experiments, when the substrates were administered simultaneously 2DGal failed to show detectable inhibition of fucose. If, however, fucose injection was delayed by 1hr after the injection of 2DGal, detectable inhibitions occurred. When administered intraperitoneally, the incorporation of fucose dropped by 22.5% and 84.2% in the presence of 48μmoles/40g body weight (240mM injection solution) and 750μmoles/40g body weight (200μl of 3.75M injection solution) of 2DGal respectively. Less inhibition was observed when the substrates were administered intracerebrally. 2DGal inhibited [^{14}C]fucose incorporation (2.5μCi, 42.5nmole/ 5μl/hemisphere) by 23%, 33%, and 40.7% at 20μmoles, 50μmoles, and 100μmoles respectively (see Fig. 3.6 and Fig. 3.7).

Analysis of glycoproteins by gel electrophoresis

Results from the above experiment suggested that detailed analyses of chick brain fucosylated and galactosylated proteins were feasible since 2DGal *in vivo* and *in vitro* was found to compete with fucose and galactose in a dose-dependent manner, and the degree of competition was relatively large. In a number of experiments, therefore, forebrain slices were incubated for 3hr in the presence of labelled sugars. The duration of incubation was based on results from earlier studies in which labelled leucine was used to measure the rate of protein synthesis *in vitro*. Incorporation of leucine increased in a linear manner for the first 3hr and beyond this time-point it started to drop.

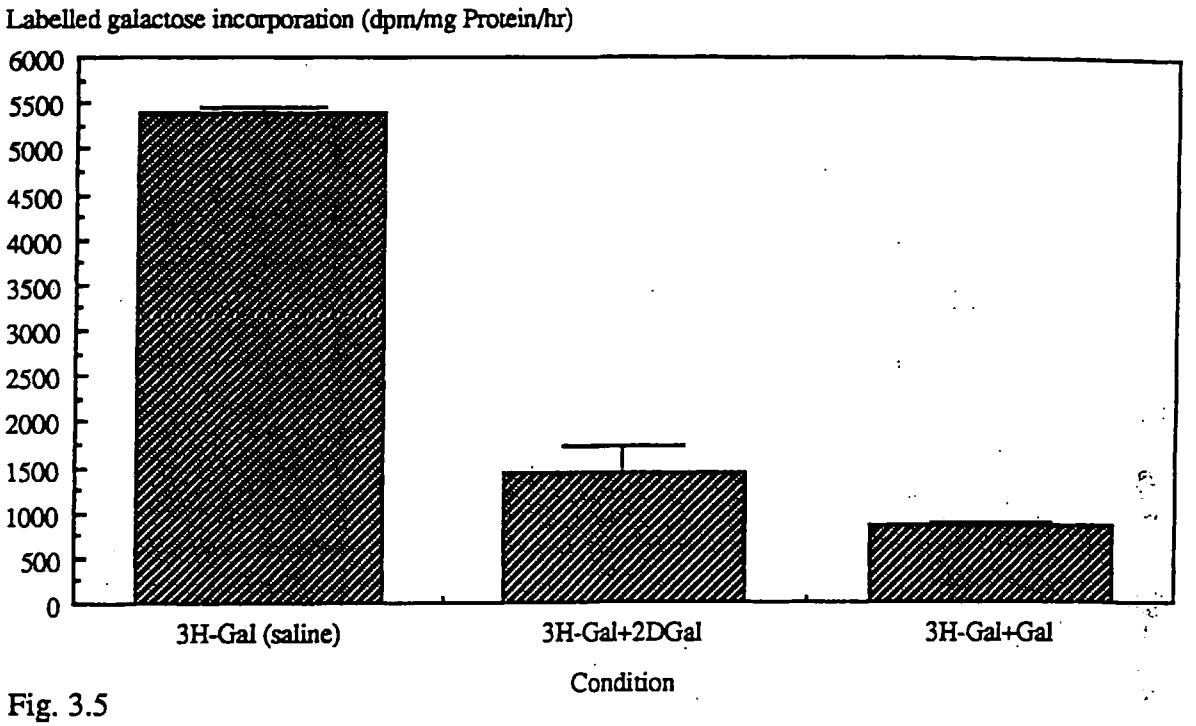


Fig. 3.5

Figure 3.5 : The incorporation of [^{3}H]galactose into TCA insoluble material in the presence either 20µmoles of 2DGal or galactose *in vivo* (with intracerebral injections).

Three birds were used under each condition, and each point on the histogram is the mean with s.e.m. bars.

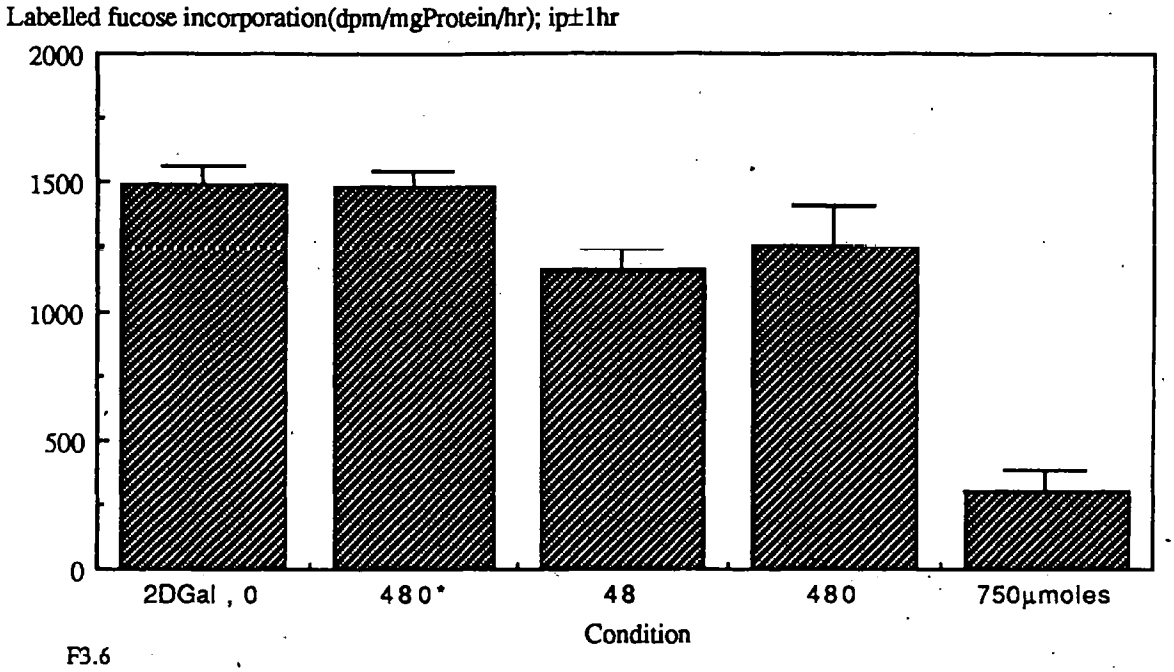


Figure 3.6 : The incorporation of labelled [^{3}H]fucose into TCA insoluble material in the presence of a range of 2DGal *in vivo* (with intraperitoneal injections either given simultaneously (480*µmoles), or with an hour lapse between 2DGal and fucose injections (48, 480, and 750µmoles)).

Three birds were used under each condition, and each point on the histogram is the mean with s.e.m. bars.

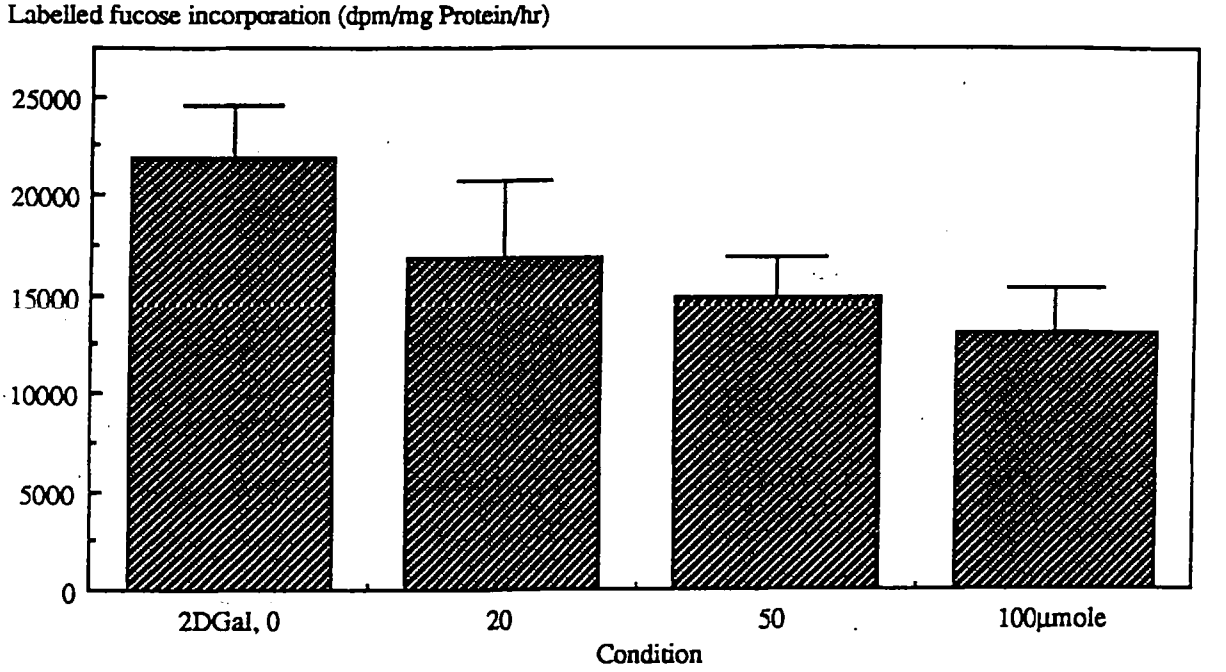


Fig. 3.7

Figure 3.7 : The incorporation of [^{14}C]Fuc into TCA insoluble material in the presence of a range of 2DGal *in vivo* (with intracerebral injections).

The injection of 2DGal preceded that of fucose by 1hr. Three samples were used under each condition, and each point on the histogram is the mean with s.e.m. bars.

In each study two groups of samples were used, one incubated in the presence of 100mM 2DGal (test) and one in the absence of the inhibitor (control). The rate of sugar incorporation was measured by incubating samples in the presence of labelled fucose and galactose. After incubation a small aliquot of each homogenate was taken for total brain glycoprotein analysis and the rest of the homogenates were fractionated into SPMs and PSDs. In order to increase the yield, one untreated carrier forebrain was added to each sample before subcellular fractionation. Figs 3.11a&b show representative electron micrographs of SPMs and PSDs obtained from these studies. Between 5-10% cross-contamination was visually observable in this type of preparation.

Representative electrophoretic patterns of proteins and glycoproteins of homogenate, SPM and PSD samples are shown in Fig. 4.3c. Figs. 3.8a&b show representative activity traces of gel tracks containing SPM (Fig. 3.8a) and PSD (Fig. 3.8b) samples. Various bands were then grouped together and relative molecular weight of each resulting peak measured. In order to make incorporation rates comparable, activity counts from each sample were standardized per unit weight of corresponding material before solubilisation in laemmli buffer. A histogram of activity (incorporation rate) against relative molecular weight was plotted for each sample.

DISCUSSION

Results from leucine and fucose incorporation studies indicated that the *in vitro* system was sensitive to small changes in concentration of unlabelled substrate. Incorporation of labelled leucine fell by more than 60% in the presence of only 100μM concentration of unlabelled amino acid, and that of [^{14}C]fucose fell by 50% at 10μM and 90% at 100μM concentrations of unlabelled sugar. A similar range of drop in the incorporation of fucose was observed when [^{3}H]fucose was used (some 60% at

100μM concentration of unlabelled sugar).

2DGal inhibition of fucose and galactose incorporation was concentration-dependent both *in vivo* and *in vitro*. The greater fall in incorporation rate of galactose *in vitro* when compared to that of fucose (91.3% & 44.4%), was as expected since 2DGal directly competes with galactose for the same site in the carbohydrate chain but only competes indirectly with fucose for glycosidic linkage processing. *In vivo,* at 1mM (20nmoles/20μl/ hemisphere) 2DGal inhibited galactose incorporation by over 75%, when injected intracerebrally (Fig. 3.5). However, inhibition of fucose proved to be more problematic, in that 2DGal did not show detectable inhibition when chicks received fucose and 2DGal simultaneously, a phenomenon seen regardless of the mode of injection (ie. intraperitoneal or intracerebral injections). However, when administration of fucose was delayed by 1hr, 2DGal showed a concentration-dependent inhibition (Figs. 3.6 and 3.7). At 10μmoles/20μl/hemisphere, the amnestic dose, 2DGal showed 25% inhibition of labelled fucose [85nmole of [^{14}C]fucose (5μCi)/10μl/hemisphere]. Inhibition of this degree of fucosylation leading to amnesia suggests that only a subset of these glycoproteins are directly involved in processes involved in memory formation and consolidation.

Figs. 3.9a&b show results of an experiment in which *in vitro* fucosylation and galactosylation of forebrain proteins were studied in the presence and absence of 2DGal. Seven major glycoprotein components were recognised in samples obtained from TCA precipitable material before subcellular fractionation. They were, in descending order of molecular weights, 160-180, 110-120, 65-84, 55-60, 48-50, 36-40 and 26-30kD. Consistent with results from the previous section, 2DGal showed a larger degree of inhibition to galactose incorporation than to fucose.

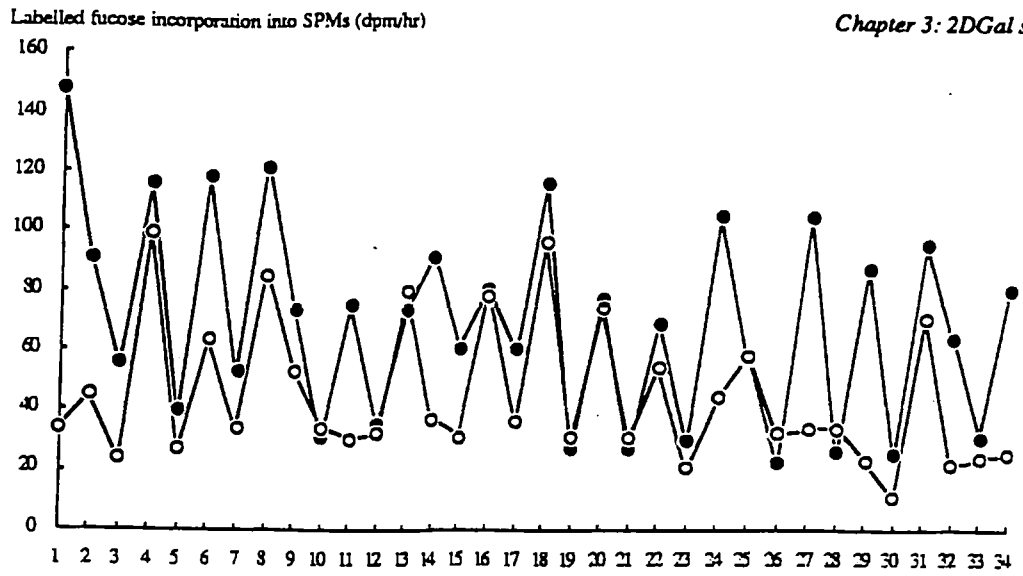


Fig. 3.8a

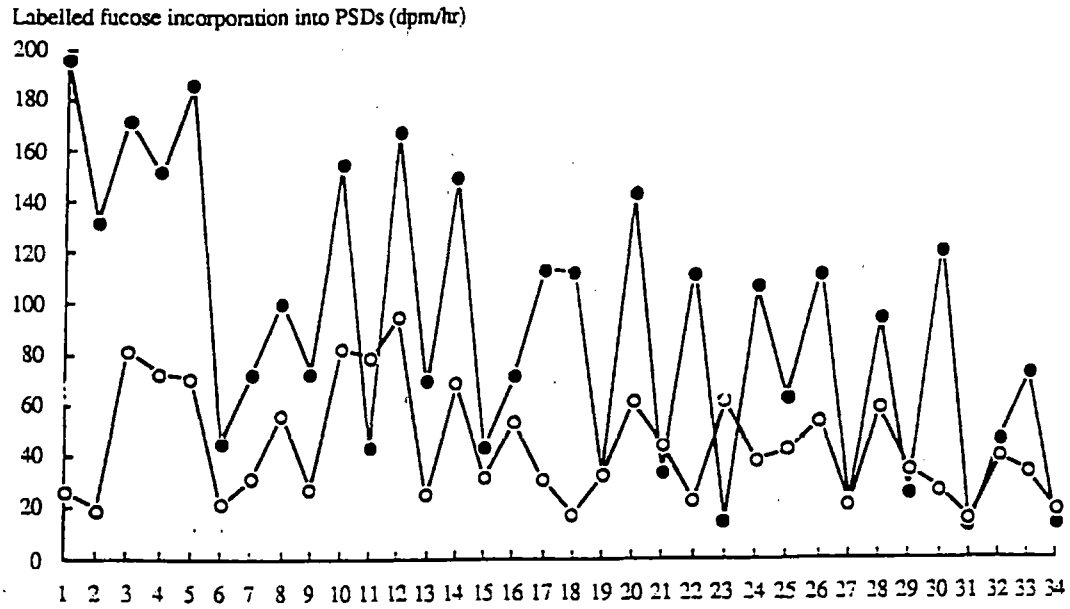


Fig. 3.8b

Figure 3.8 : Representative patterns of the specific activity of protein bands obtained from SPMs (a) and PSDs (b).

Samples were run on 5-15% gels and stained as described in Chapter 2. Stained components were sliced from the gel lanes and their activity counted. The activities of bands falling under each peak in the above graph were combined to provide a more simplified pattern of these proteins for analysis (see Figs. 3.9 and 3.10). Filled circles, [^{3}H]fucose incorporation in the absence of 2DGal; empty circles, [^{3}H]fucose incorporation in the presence of 2DGal.

When analysing fucosylglycoproteins of subcellular fractions *in vitro*, 7 major protein components were recognized in SPM and 10 in PSD samples. Fucosylation of these glycoproteins was found to be inhibited by 2DGal (Figs. 3.10a&b). Major fucosylglycoprotein components of SPMs were, 160-180, 110-120, 100, 65-84, 48-55, 36-40 and 26-30kD. And major fucosylglycoproteins of PSDs were, 160-180, 130, 110-120, 100, 65-84, 55-60, 48-50, 40, 36 and 26-30kD.

Results from the gel electrophoresis study are only a first step towards a detailed identification and characterisation of fucosylglycoproteins involved in memory formation. Analysing of the protein patterns of total TCA precipitable material recognised seven components (see results). 2DGal mainly inhibited both fucosylation and galactosylation of high and medium range glycoproteins *in vitro*. Further more, there was a larger degree of glycosylation inhibition between galactose and 2DGal than fucose and 2DGal (Figs. 3.9a&b). However, compared with *in vivo*, in most *in vitro* systems there is less low molecular weight protein synthesis (and possibly posttranslational modification). Thus in her study, Bullock et al. (1990) investigated chick brain glycoproteins *in vivo*. Eight major protein components were recognised in total TCA precipitable material. They were, 150-180, 100-120, 62-84, 55, 50, 41, 33 and 28kD. These components are, however, comparable with those reported here especially, with those of high molecular weight.

The subcellular distribution of fucosylglycoproteins showed that although the pattern in SPMs was similar to that in total TCA insoluble material, a different pattern, containing more protein components, was recognised in PSDs (see results). This may be due to the existence of glycosylation processing enzymes at both sides of synaptic junction which makes independent protein glycosylation at pre- and post synaptic sites possible. Therefore, there may be different sources of glycoproteins in SPM and PSD fractions whose fucosylation is inhibited by 2DGal.

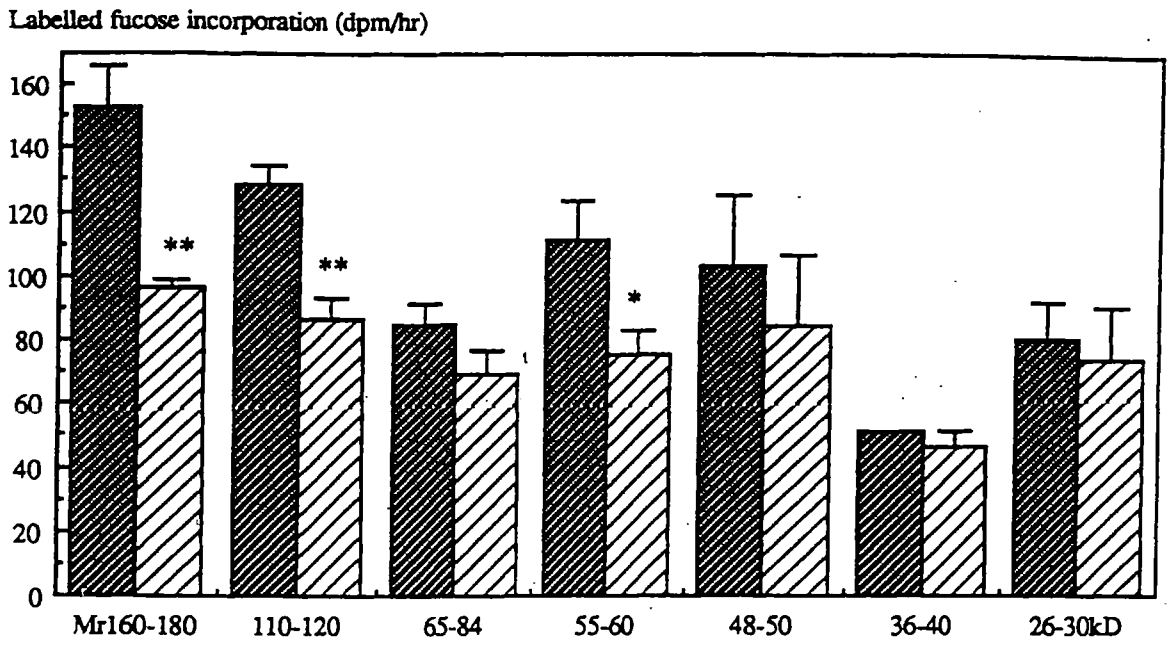


Fig. 3.9a

Figure 3.9a : A representative pattern of fucosylated glycoproteins in the absence (dark shaded bars) and presence (light shaded bars) of 2DGal.

Each result is the mean of 5 measurements with sem bars. As can be seen 2DGal exerts its influence mainly on the medium and higher molecular weight components *in vitro*. * $p<0.01$; ** $p<0.001$

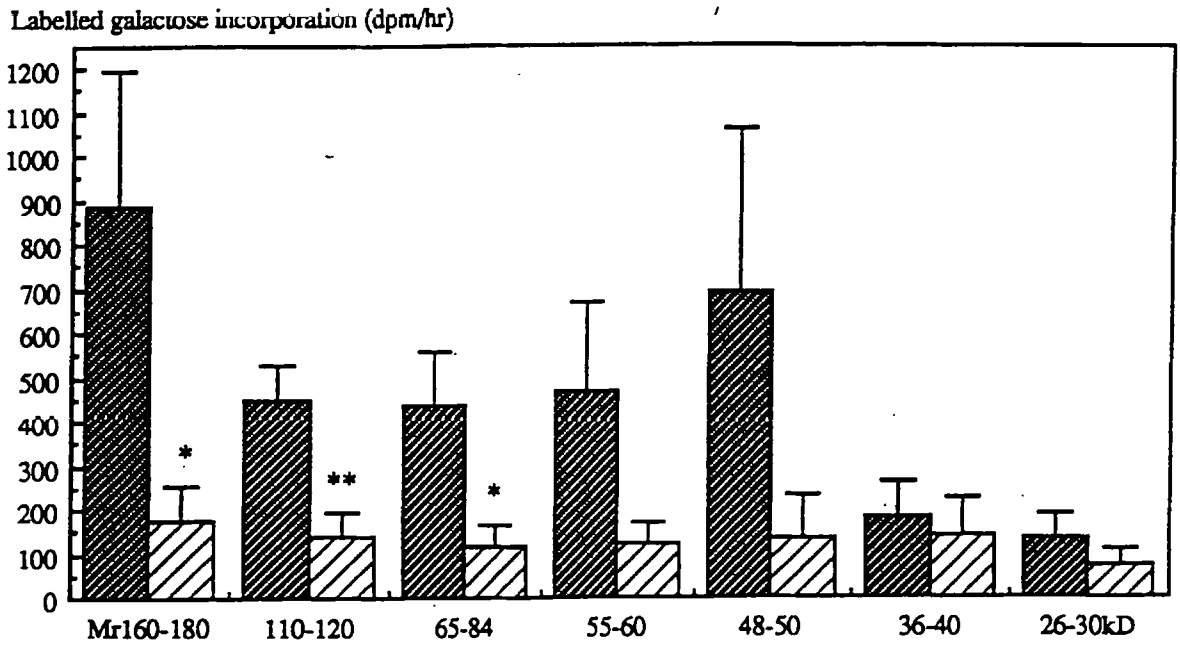


Fig. 3.9b

Figure 3.9b : A representative pattern of galactosylated glycoproteins in the absence (dark shaded bars) and presence (light shaded bars) of 2DGal.

Each result is the mean of 4 samples with s.e.m bars. * $p<0.05$; ** $p<0.01$

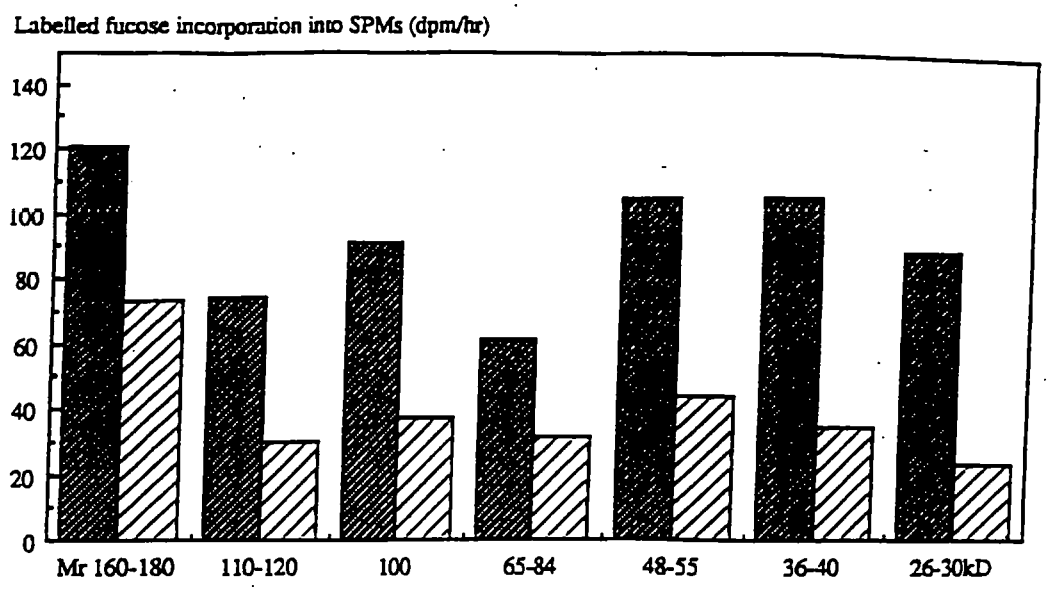


Fig. 3.10a

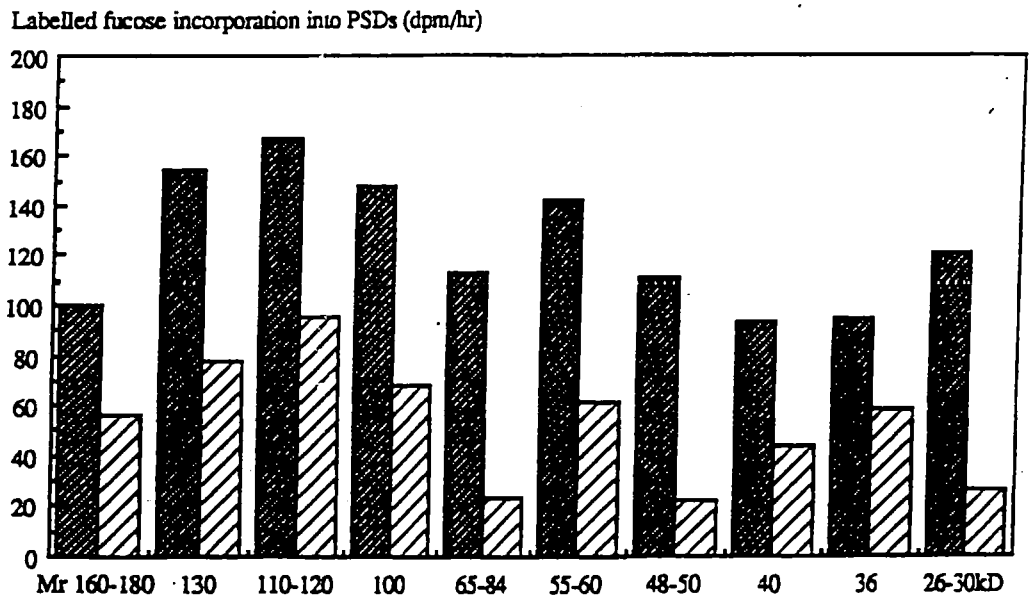


Fig. 3.10b

Figure 3.10a&b : Patterns of fucosylated glycoproteins in the absence (dark shaded bars) and presence (light shaded bars)of 2DGal obtained from SPM (a) and PSD (b) fractions of day-old chick forebrain.

Each result is the mean of 2 samples. The corresponding values differ from each other by not more than 5%.

The relative degree of inhibition of galactose and fucose incorporation by 2DGal for corresponding protein components was different (Figs. 3.9a&b). This is a reflection of the different distribution of galactose and fucose in oligosaccharide chains of glycoproteins. It is known that a large proportion of fucose molecules are linked with N-acetylgalactosamine, a situation unaffected by 2DGal. Further, not all galactose molecules in oligosaccharide chains are linked with fucose through α1-2 glycosidic linkages.

These results indicate that the mechanism of action of 2DGal is similar in both *in vitro* and *in vivo*, and that it specifically competes with galactose for incorporation into oligosaccharide chains of glycoconjugates, thereby inhibiting fucose linkage formation to oligosaccharide chains of glycoproteins. These results are in agreement with those of Bullock et al. (1990) showing that the electrophoresis patterns obtained from 2DGal labelling are similar to those obtained after fucose labelling of MeA-trained birds. Those glycoproteins that show training related increased fucosylation now need to be identified and characterised.

Figure 3.11 : Electron micrographs of synaptic plasma membranes (panel a) and postsynaptic densities (panel b). Arrows point to the membrane fractions.

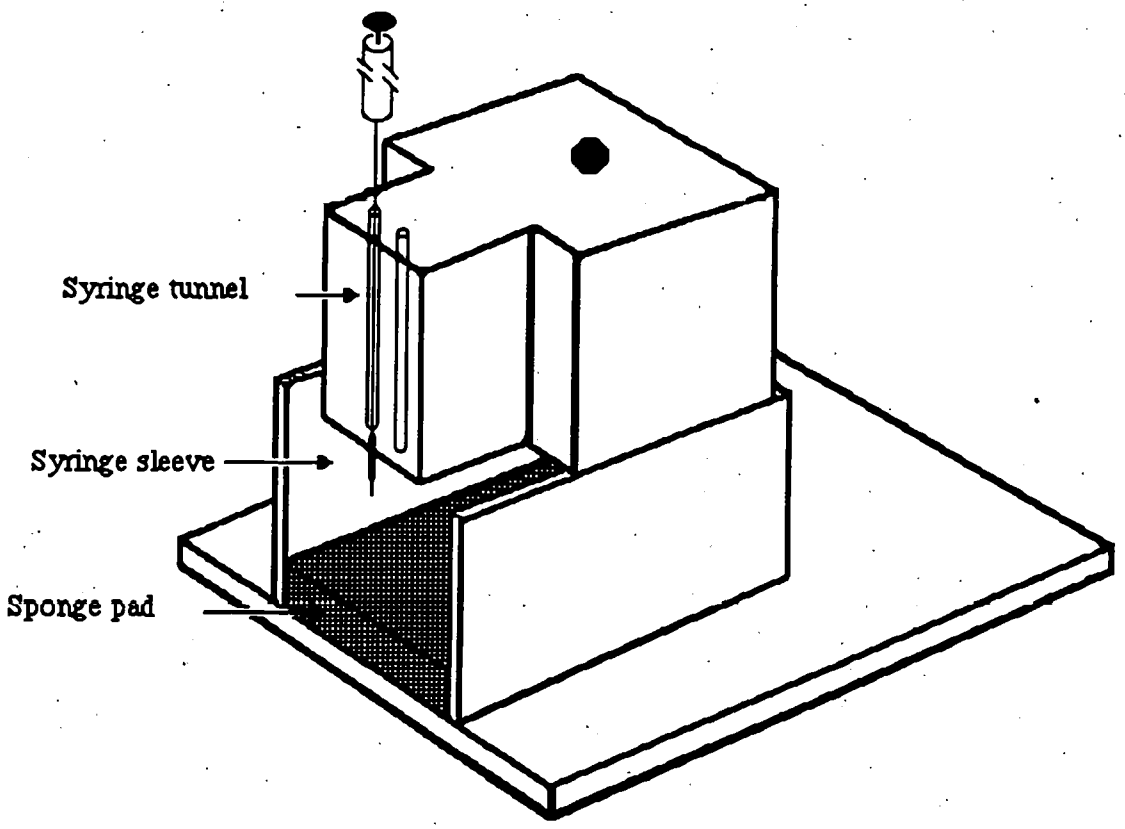


Figure 3.12 : A schematic diagram of the modified head-holder used for intracerebral injections.

CHAPTER FOUR

Investigation Of Chick Forebrain Proteins That Show Changed Fucosylation During Memory Formation

CHAPTER FOUR

INVESTIGATION OF CHICK FOREBRAIN PROTEINS THAT SHOW CHANGED FUCOSYLATION DURING MEMORY FORMATION

INTRODUCTION

General Findings

The biochemical properties of glycoproteins and their role in cognitive processes were briefly described in chapter 1. In this chapter the specific role of fucose and fucosylated glycoproteins in memory processes is investigated.

The functional role of the carbohydrate moieties of cell surface glycoproteins has been studied in many laboratories and it has become clear that they are important in cell-cell recognition processes during differentiation, development, axonal guidance, dendritic growth and synaptogenesis. The oligosaccharide groups of synaptic glycoproteins are orientated towards the synaptic cleft, so that changes in sugar composition cause alterations in the molecular structure of this microenvironment. The physical dimensions of complex, N-linked oligosaccharides are such that they presumably cover a large part of the membrane surface area, thus theoretically enabling them to interact with carbohydrate chains of opposing membranes (Gurd, 1989). These changes to oligosaccharide chain structures would require the presence of glycosylating and/or deglycosylating enzymes. A number of membrane-bound processing enzymes have actually been identified at these sites. Changes in oligosaccharide composition can occur both pre- and postsynaptically, and independently from each other (see Chapter 3). Protein synthesis taking place at either sides of the synaptic cleft has already been documented (Bullock et al., 1990).

Polyribosomes have been identified in dendritic spines and their activity has been measured. It increases during the period of synaptogenesis (Steward and Falk, 1985 and 1986) and also during stimulation, for example, in rat visual cortex as a result of exposure to complex and enriched environments (Greenough et al., 1985).

There is a large body of literature concerning the role that membrane-bound fucosylated glycoproteins play in memory processes. The rise in fucose incorporation during memory formation and consolidation, for example, has been observed in rat and chick brain [eg., Burgoyne & Rose, (1980); McCabe & Rose, (1985); Jork et al., (1989).

Whilst chicks that have undergone passive avoidance training avoid pecking at the bead for several days, the increase in [^{3}H]fucose incorporation is reported to last only for about 48hr (Burgoyne & Rose, 1980). This may be due to several factors such as rapid turnover of the relevant glycoproteins. Margolis and Margolis (1975) observed a biphasic turnover rate modality for the polypeptide backbone of glycoproteins. The rapid phase could last up to 6 days, and then the slow rate would generally start from the second week and last about two weeks.

To establish whether protein fucosylation is necessary for memory formation, chicks were given a subconvulsive transcranial electric shock minutes after training (Rose, 1984; Rose & Harding, 1984). The treatment resulted in both behavioural and neurochemical changes; it rendered chicks amnesic and prevented the increase in glycoprotein fucosylation that otherwise occurred. However, in those birds that despite the treatment remembered the task, protein fucosylation was similar to the levels seen in unshocked MeA-trained birds. The results, therefore, suggest that increased fucosylation was "directly associated with memory formation rather than with non-specific concomitants of the training experience" (Rose & Jork, 1987).

Increased fucosylation has also been demonstrated in other studies that do not involve passive avoidance training or brightness discrimination tasks. For example, exposing dark-reared rats to 3hr of light, induces a 23% increase in the rate of fucosylation (Burgoyne & Rose, 1978), and after 24hr of light exposure the level of fucosylation returns to pre-exposure levels. This is an indication of the universality of these neurochemical changes that reflect behavioural experiences across species.

Wetzel et al. (1982) studied protein fucosylation in the central nervous system and found that injection of fucose 30min before training rats on both shuttle box avoidance and brightness discrimination tasks improved retention of learned behaviour tested 24hr after training. It was suggested that the availability of free fucose influences the rate of glycosylation. However, Wetzel and associates claimed that fucose injection caused increased protein synthesis as measured by [^{3}H]leucine incorporation. It is also possible that fucose may partially inhibit lysosomal degradation of the polypeptide backbone of glycoproteins rather than stimulate *de novo* protein synthesis. Moreover, intracellular fucose has been shown to inhibit fucosidase, the enzyme that cleaves fucose from the carbohydrate chain (Zatz and Barondes, 1970).

The effect of other sugars like galactose and mannose commonly found in glycoproteins has also been tested and none seem to exert a strong positive influence on the acquisition performance of rats. However, the intracerebral administration of fucose, galactose, and N-acetylneuraminic acid (NANA) to rats before training on a brightness discrimination paradigm, did improve retention of the acquired task. Mannose did not show any effects even at the highest dose used, while galactosamine produced some improvement (Popov and Matthies, 1986). It is noteworthy that, in the training paradigm described here, at least 24hr had elapsed before brain tissues were examined for their carbohydrate content; during this time labelled sugars could have

undergone limited conversion to fucose (with the exception of fucose). This would confound only specific attribution of the role of these precursors. Training paradigms such as one-trial passive avoidance learning, due its short time-course, may offer a better alternative.

Anatomical and subcellular specificity of fucosylation

In their investigations on brain slices, McCabe and Rose (1985a) found increased fucosylation both in W-trained and MeA-trained chicks, compared with untrained chicks, but MeA-trained birds showed a grater and significant increase (16%) in the right forebrain base, a region containing LPO, 30min after training. A similar trend was also observed *in vivo* (McCabe & Rose, 1987).

Popov and colleagues (1976 and 1980), in their *in vivo* studies involving intraventricular injection of [^{3}H]fucose, reported an increased glycoprotein fucosylation in areas CA1 and CA3 of the hippocampus and dentate gyrus after training rats on a brightness discrimination task. There was no increased fucosylation in the visual cortex. Furthermore, the increased fucosylation in the dentate area was seen during the first hour of post-training period, whereas areas CA1 and CA3 'lagged behind' by about 7-9hr. Popov et al. (1980) suggested that this phenomenon is an index of "stepwise signal processing from dentate area to CA1 and CA3".

Burgoyne and Rose (1980) carried out a number of *in vivo* subcellular distribution studies involving passive avoidance learning in day-old chicks. Three hours after training, birds injected intraperitoneally showed a significant increase in fucose incorporation into glycoproteins of SPMs in the anterior forebrain roof and of mitochondrial fractions, whereas those injected intracerebrally revealed a 40% rise in SPM fractions only. No data on PSD fucosylation were reported. However, the

electrophoretic patterns of glycoproteins obtained by both procedures were comparable. The significant increase (26%) in the level of incorporation of [^{3}H]fucose into the particulate fraction of the anterior forebrain roof of MeA-trained birds could be observed for as long as 24hr after training (Sukumar et al., 1980)

Second messenger systems and protein glycosylation

Jork and associates (1982a) examined the *in vivo* effects of several neurotransmitters and their pharmacological ligands on protein synthesis and fucosylation. Amongst those reagents tested only dopamine increased the fucosylation of glycoproteins. This effect was accompanied by a decrease in leucine incorporation. The injection of apomorphine, a dopamine agonist, into the hippocampus significantly improved the retention of footshock-motivated brightness discrimination learning. Jork and associates (1982b) also carried out a series of *in vitro* brain slice studies on rat hippocampus and found an increase dose response correlation between dopamine concentration in the incubation medium and fucose incorporation. The administration of non-hydrolysable cAMP (dibutyryl cAMP) gave a similar effect, suggesting that dopamine acts through a second messenger system. Since the activity of the enzyme fucosyltransferase remained constant during the length of incubation (2hr), it was suggested that the dopamine 'system' may act to increase the activity of fucokinase, and hence, increase fucose-1-phosphate concentration. The activity of the kinase increases immediately after training but that of the transferase decreases during this time-window and does not increase until some 7-9hr later (Popov et al., 1983). These findings, together with reports that intrahippocampal apomorphine administration leads to a significant improvement in the retention of footshock-motivated brightness discrimination through glycoprotein fucosylation, and that the same drug is capable of inducing theta rhythm in the hippocampus (Matthies, 1978), may explain the mechanism of the rapid rise of glycoprotein fucosylation observed shortly after training, an effect which is compatible with the post translational modification

hypothesis advocated by Routtenberg (1982) (See chapter 3).

The relation between the dopaminergic neurotransmitter system and the second messenger system involving PKC (a diacylglycerol stimulating protein kinase) has been studied because both mechanisms have been implicated in memory processing. The activity of fucokinase is influenced by the intracellular calcium concentration; chelating the calcium with EGTA leads to a 2-fold increase in kinase activity (Jork et al., 1984a). It has been reported that dopamine decreases the phosphorylation of B-50, a PKC substrate, in SPM fractions by some 30% (Jork et al., 1984b). This is suggested to be due to a decrease in mobilizing Ca^{2+} and hence a drop in PKC activity. There is a contrast between these results and those indicating a posttraining rise in PKC translocation and activity which is predominantly presynaptic. However, since protein glycosylation is assumed to be postsynaptically triggered (Matthies, 1989), it may be that initial dopaminergic activation can lead, through a network of interneurons, to a drop in the presynaptic Ca^{2+}concentration, thus influencing PKC activity. By such time the activity of PKC would have already increased and initiated a chain of cellular response. As suggested by Matthies (1989), "the timing of distinct metabolic events induced by different converging transmitter-mediated inputs during learning and the interference of their second messenger systems surely induces more complex conditions that are still far from being understood".

Detailed analysis of training-related fucosylglycoproteins revealed that there were two waves of fucosylation (Popov et al., 1980 and 1983). The electrophoretic analysis of hippocampal tissues from rats trained on a brightness discrimination task showed a significant increase (25-30%) in medium and high molecular weight membrane-bound fucosylglycoproteins, 7-9hr after training (Popov et al., 1976, and 1980). In 1983, this group, examined the time-course of the activity of two enzymes involved in fucosylation, namely fucokinase and fucosyltransferase. As predicted, the activity of

these enzymes varied during the first 9hr posttraining. Immediately after a brightness discrimination task, fucokinase activity increased significantly. The activity of the transferase, however, decreased significantly during this period but then showed a significant increase 7-9 hr later, by which time the activity of the kinase had returned to control levels. The authors postulated that "during acquisition of such a behaviour distinct systems operating in the hippocampus can influence metabolic processes by controlling enzymic reactions through a second messenger system" (Popov et al., 1983). The cellular events that take place during the period between the two fucosylation phases are still unknown. Increased fucokinase activity is also seen in chicks 1 and 6hr after passive avoidance training (Lössner and Rose, 1983; McCabe, 1985b). There is also lateralisation. The activity of the kinase increases significantly in the right forebrain base (containing PA and LPO) and persistently in the left base (Lössner and Rose, 1983). Six hour after training, however, there is shift in the region of significantly increased activity from the base to the right forebrain roof, containing IMHV (McCabe, 1985b). The effect of passive avoidance training on chick fucosyltransferase has not been reported.

The results discussed above suggest that glycoprotein fucosylation is a specific neural response to training and memory formation (Rose, 1981a). Until now there has been no specific attempt to identify fucosylglycoproteins in chick brain that are involved in processes leading to memory formation. In their study, Bullock and associates (1990b) used 2DGal to help identify 8 major fucosylated protein peaks that may be involved in memory formation. The following experiments aimed to identify and localize in details some of the molecules that show elevated fucose incorporation following training.

METHODS

Two major techniques one *in vitro* and one *in vivo*, both followed by 1-D PAGE, were used. The *in vitro* technique, by virtue of its controllability and inexpensiveness, was a logical first step in a general analysis of learning-related chick forebrain fucosylglycoproteins. It was therefore decided that a detailed analysis of these molecules using *in vivo* techniques was warranted. Furthermore, when a 24hr incorporation period for the labelled sugar was required the *in vivo* technique was the only option.

The *in vivo* analysis was carried out at two different times 6hr and 24hr after training. The decision to use the former time was based on work by Zamani and Rose (1990) in which 2-deoxy-D-galactose (2DGal) was found to cause amnesia in two distinct time-windows. It rendered chicks amnesic if injected during the first hour posttraining and again when chicks received the inhibitor 6hr after training they became amnesic. 2DGal did not have amnestic effect when injected at other times up to 12hr after training. These results suggested that, in chicks, there must exist two waves of protein fucosylation, one around the time of training, the other 6hr after training. It was therefore decided to examine fucosylglycoproteins during these two time-windows, and assess whether different molecules are fucosylated during the two phases.

Passive avoidance training

Chicks were trained essentially as described before (see Chapter 2). They were tested for recall 30min later and only those that responded correctly (i.e., W-trained birds pecking, and MeA-trained chicks avoiding the dry chrome bead) were used for analysis of fucosylation.

Passive avoidance learning and *in vitro* fucosylation

Fifteen minutes after testing chicks for recall, their forebrain bases were removed, cooled on ice and prepared for *in vitro* incubation. This was carried out as described in Chapter 2. Chicks were coded and decoded only after analysis of the results was complete. The forebrain base region was chosen since in their earlier study McCabe and Rose (1987) found a significant elevation in fucose incorporation into the right forebrain base 3hr after training.

After incubating tissue slices for 3hr with 4μCi [^{14}C]fucose/2ml HEPES containing 10μM fucose (pH7.4), they were homogenised in 1ml of 2M HEPES containing 0.32M sucrose (pH7.4) and crude synaptosomal/mitochondrial fractions were prepared. All steps were done at 4°C. Small aliquots were also taken, where appropriate, for protein estimation and activity measurements. Each homogenate was divided into two parts. A small part was taken for TCA precipitation, as described before, and the larger part for crude membrane preparation. The homogenates were spun at 1000g_{max} (3000rpm) for 10min. The supernatant was separated from the crude nuclear pellet (P1) and spun at 15kg_{max} (11000rpm) for 1hr. The pellet (P2) was taken as the crude membrane fraction. The membrane fractions were solubilised in Laemmli buffer and run on 5-15% linear gradient one-dimensional gels as described before. The individual glycoprotein bands were cut from gels and their activity measured.

Calculation of data (*in vitro study*)

Graphs of glycoprotein activities (dpm of [^{14}C]fucose) against gel band numbers were plotted for each gel separation. The total activity under each major peak on the was calculated, and the percentage activity of each peak against the total activity of that sample was calculated. This step eliminated any variations due to different amounts of sample being loaded onto gels and different recovery rates of radioactivity during

electrophoresis.

Passive avoidance learning and *in vivo* fucosylation

Chicks were trained, as described above and 30min later they were tested for recall. Those that responded correctly were injected with fucose. The injection protocol was as follows; MeA-trained birds received 40μCi[^{14}C]fucose and W-trained chicks 10μCi [^{3}H]fucose. The isotopes were freeze-dried and dissolved in 20μl 0.9% saline. The injection was done using the head-holder described in Chapter 2 (Davis et al., 1982). In order to control for differences in the metabolism of the two isotopes, two groups of W-trained chicks were injected, one with [^{3}H]fucose, and the other with [^{14}C]fucose. In all six chicks were used in each condition.

Chicks were killed either 6hr or 24hr after training and the left and the right IMHV, PA and LPO were quickly dissected using a resin mould developed by Rose and Csillag (1985), and described by Bullock et al. (1987). Corresponding structures, one from a W-trained and one from a MeA-trained chick were combined and added to the whole forebrain of an untreated bird which acted as carrier material. The same procedure was carried out on naïve groups that received the isotopes. Subcellular fractions were prepared and TCA precipitated and delipidated as described before. [^{3}H]fucose and [^{14}C]fucose were counted simultaneously after dissolution of pellets in Protosol for 24hr, addition of 1ml of water to reduce chemiluminescence, and 8ml of scintilant (Emulsifier-safe™, Packard). Counting was carried out in a Beckman LS.1710 scintillation counter at an efficiency rate of about 80% for both isotopes.

Calculation of data (*in vivo* study)

The ratio of [^{14}C]fucose/[^{3}H]fucose was calculated for each subcellular fraction and was expressed as the percentage of the corresponding homogenate from which they

were derived [^{14}C]fucose/[^{3}H]fucose. This was called the raw ratio. This ratio was independent of any variations in the subcellular fraction recovery, the relative amount of tissue taken for analysis, and relative amount of isotope injected. A raw ratio of 100% would be achieved if all subcellular fractions in both W- and MeA-trained chicks incorporated the same amount of each isotope. The raw ratios obtained from MeA/W samples were then divided by the raw ratios of W/W samples prepared at the same time. This standardized the sample ratios against any basal metabolic variations of the different isotopes of the same sugar. Any deviation from 100% in standardized ratios, therefore, indicated relative enrichment or impoverishment of incorporation into that fraction in samples from MeA-trained chicks. These deviations were statistically compared to the null hypothesis (i.e., no enrichment or impoverishment) using the Student's t. test. The relative incorporation ratios between the left and the right hemisphere were carried out by calculating the left/right ratios of each pair of samples and applying the same statistical technique. The distribution of incorporation between PSDs and SPMs of corresponding samples were calculated similarly.

Of the labelled glycoproteins separated by gel electrophoresis, 8 major peaks, similar to those reported by Bullock et al. (1990), were recognised, and the activity of individual bands calculated as described above. The [^{14}C]fucose/[^{3}H]fucose ratio of each band was expressed as a percentage of the corresponding ratio for the whole gel. This eliminated variations due to differences in the amounts of isotopes incorporated into each sample. This raw ratio was obtained for all samples and the mean of raw ratios from W/W samples calculated. The raw ratio of each MeA/W sample was then expressed as a percentage of the mean W/W ratio. Deviations from the null hypothesis for these standardized samples were calculated using the Student's t. test. In the 24hr-posttraining study the LPO and PA samples were combined and analysed together but for the 6hr-posttraining phase samples from the IMHV, LPO, and PA were analysed separately.

RESULTS

The *in vitro* study

Measurements of the specific activity of TCA insoluble pellets are shown in Table 4.1. There was a significant elevation of fucose incorporation into the right forebrain base of MeA-trained chicks as compared to controls ($p<0.05$). No other significant differences were observed either between groups trained and control or between hemispheres. Figs. 4.2a&b confirm that there was no difference in [^{14}C]fucose contents of incubation media and tissue homogenates between samples.

Gel electrophoresis and glycoprotein analysis : The activity of protein components obtained from sets of 4 samples, that is, left and right forebrain base regions of W-and MeA-trained birds (n=5 per group) revealed 6 major glycoprotein peaks. They had molecular weights of 180, 116-120, 55-60, 45-50, 35-40, and 26-30kD. The only glycoprotein component to show a significant increase in fucosylation in the right forebrain base was 116-120kD molecular weight (Fig. 4.1a). The 55-60, 45-50, and 35-40kD peaks also showed a slightly elevated fucosylation. Interestingly, however, the peak at 35-40kD from the left forebrain base of MeA birds showed a significant increase in fucosylation compared to W birds (Fig. 4.1b). This was in contrast to the absence of a significant increase in fucosylation in this region after training.

Fucose incorporation rate (pmoles/mg Protein/hr)				
	W-Trained		MeA-Trained	
Sample	Left	Right	Left	Right
1	11.5	11.7	13.6	14.1
2	8.8	14.8	9.4	21.5
3	17.1	18.1	20.8	18.8
4	8.3	10.1	15.8	25.2
5	12.7	15.5	16.9	19.8
Mean	11.7	13.9	15.3	19.7
sem	1.6	1.5	1.9	1.7

Statistics (Student t.-test) : Right W-trained *vs* Right MeA-trained, $p<0.04$ ($t=2.5$); left W-trained *vs* left MeA-trained, $p<0.18$ ($t=1.5$)

Table 4.1 : The effect of passive avoidance training on *in vitro* fucose incorporation into TCA precipitated protein of chick forebrain base after a 3hr labelling pulse.

Fig. 4.1a

Fucose incorporation into right forebrain base (%, peak dpm/sum total dpm)

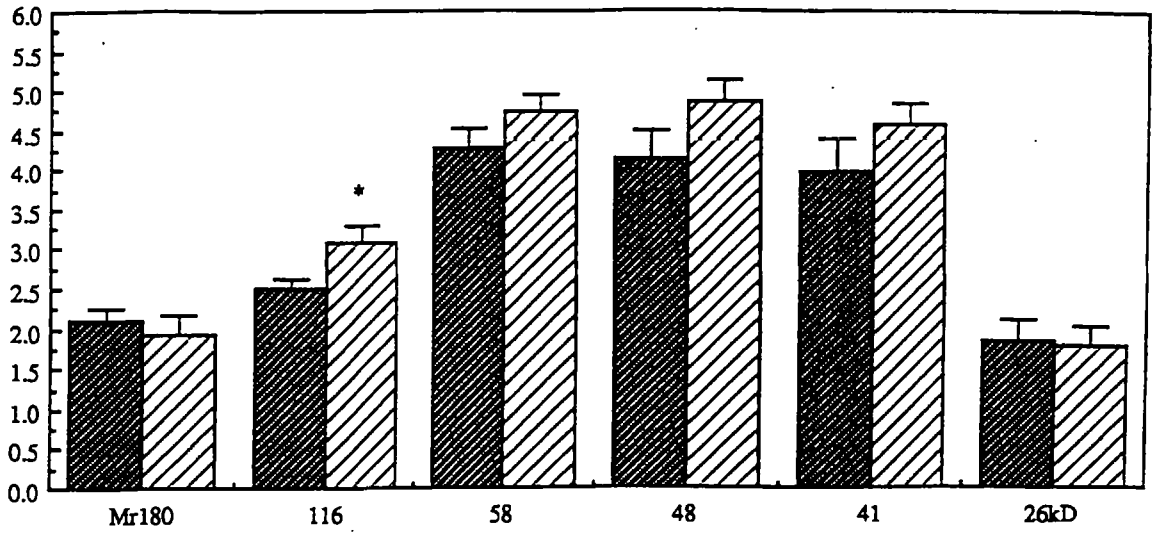


Fig. 4.1b

Fucose incorporation into left forebrain base (%, peak dpm/sum total dpm)

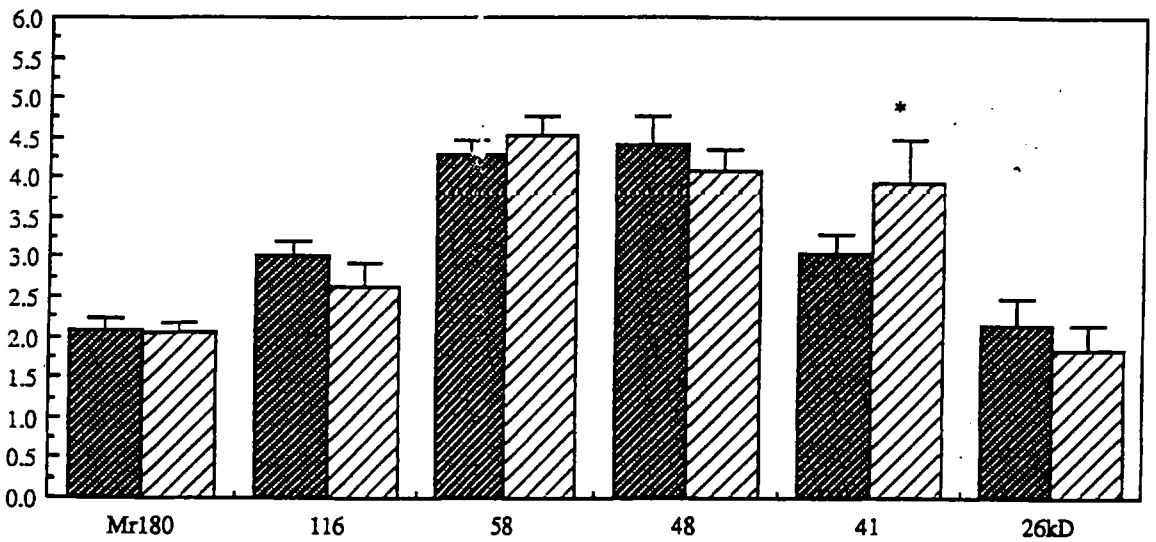


Figure 4.1 : SDS-PAGE analysis of fucose incorporation into TCA precipitable materials obtained from the right (a) and the left (b) chick forebrain base. Dark hatched columns are values for W-trained birds and light hatched columns are values for MeA-trained chicks. Values are expressed in terms of percentage of the activity of each peak against the total activity of the entire sample loaded on to the gel. Each value represents the mean of 5 replicates. *=$p<0.05$.

Fig. 4.2a

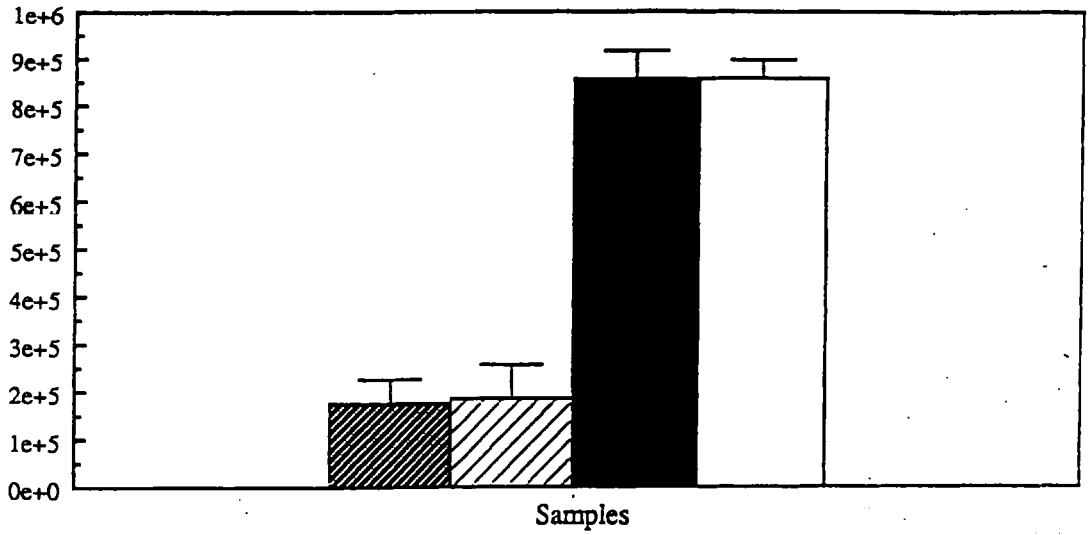


Figure 4.2a : The activity of [^{14}C]fucose in tissue homogenates and incubation medium 3hr after *in vitro* labelling.

The value of each column represents the mean of 12 samples ± standard deviation. These values indicate that enhanced fucosylation seen after passive avoidance learning was not due to the use of more labelled isotopes in incubation media of right forebrain base samples. Dark hatched column, left hemisphere homogenate; light hatched column, right hemisphere homogenate; black column, left hemisphere incubation medium; white column, right hemisphere incubation medium.

Fig. 4.2b
[14C]fucose in incubation media (dpm/ml)

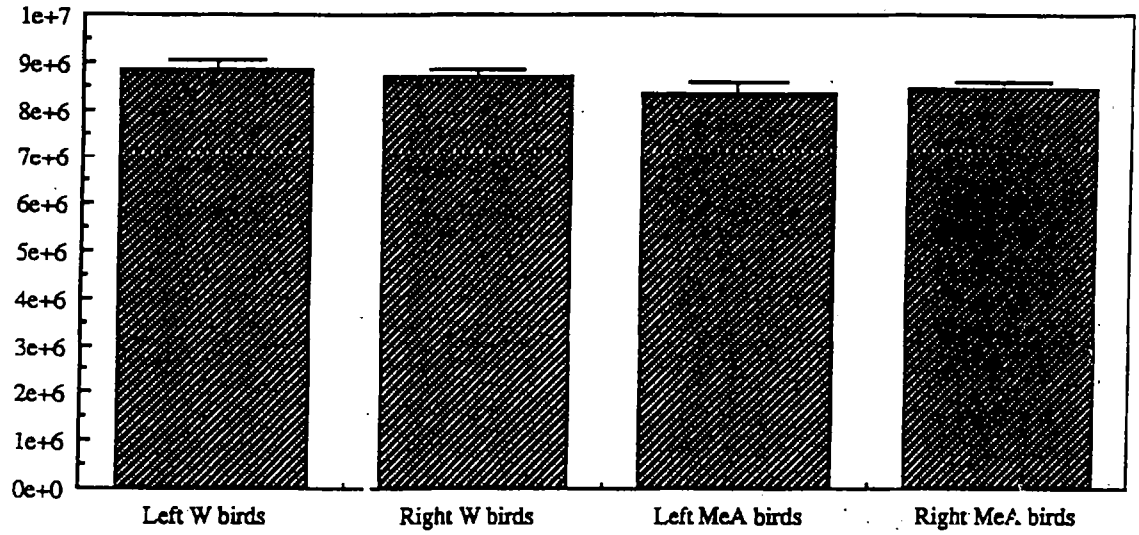


Figure 4.2b : The activity of [^{14}C]fucose in incubation medium 3hr after *in vitro* labelling (Panel b) .

The value of each column represents the mean of 6 samples. Error bars represent sems. These values indicate that enhanced fucosylation seen after passive avoidance learning was not due to the use of more labelled isotopes in incubation media of right forebrain base samples of MeA-trained birds, or the use of less labelled isotope in incubation media of right forebrain base samples of W-trained chicks.

The *in vivo* experiments

Subcellular localisation of fucosylation 24hr after training

The subcellular incorporation of fucose is shown in Table 4.2. In subcellular fractions of the PA, there was a significant 63% increase in fucose uptake in the right SPM, and a significant 115% increase in the right PSD; the left PSD also showed a significant fucose elevation (98%).

A similar set of results were obtained from LPO. Both left SPM and PSD fractions showed significant fucose incorporation elevations (64% and 24% respectively. The 19% rise in fucose uptake in the right SPM was not significant. However, the uptake of fucose in the right PSD dropped by 7%. In PA, passive avoidance learning resulted in a strong postsynaptic effect which was evident in 116% and 32% relative increases in fucose incorporation in left and right hemispheres respectively. LPO, on the other hand, showed a relatively strong presynaptic effect (32% and 29% increase in left and right hemispheres respectively).

As can be seen in Table 4.2, there was no significant fucose enrichment in IMHV, rather the SPM fraction of IMHV from the right hemisphere showed a significant reduction in fucose uptake. The increase of 12% seen in the left SPM was not significant. The relatively strong postsynaptic effect seen in the right hemisphere was at the expense of a 24% fall in fucose incorporation in the SPM fraction.

The interhemispheric differences in fucose uptake are also shown in Table 4.2. In the PA there is a right hemispheric dominance in SPM and PSD fractions, whereas LPO shows a strong left hemispheric dominance. In the IMHV, however, it is the SPM that shows interhemispheric lateralisation in the form of left IMHV dominance.

Sample	IMHV			PA			LPO		
	L	R	L/R	L	R	L/R	L	R	L/R
SPM	111.7 ±2.3	75.6* ±6.0	149.0 ±10.1	91.5 ±8.3	163.3# ±12.1	57.0 ±5.6	164.3* ±2.5	119.3 ±6.4	136.3 ±7.6
PSD	109.5 ±3.5	106.0 ±11.2	101.3 ±7.1	198.0# ±9.3	215.7# ±22.0	91.3 ±12.7	124.0* ±3.5	92.7 ±9.5	136.0 ±24.9
PSD/ SPM	98.0 ±8.6	140.2 ±9.9	68	216.4# ±21.9	132.1* ±11.8	160	75.5* ±7.3	77.7* ±3.5	100

Table 4.2 : The effect of passive avoidance learning on fucose incorporation into subcellular fractions of chick forebrain 24hr after training. Results are expressed as [^{14}C]fucose (MeA)/[^{3}H]fucose (W) percentage ratios standardized against [^{14}C]fucose/ [^{3}H]fucose of untrained controls. Data are means ±sem of 10 samples for each value. *= $p<0.05$; #=$p<0.01$. L, left hemisphere; R, right hemisphere.

Identification of fucosylglycoproteins isolated 24hr after training

The processing of samples at this stage was confined to LPO and PA fractions since the preliminary results, described above, indicated greater changes in fucose uptake in SPMs and PSDs of these structures than in IMHV fractions. Gel electrophoresis was therefore carried out on combined LPO/PA samples, the activity of individual protein bands being measured as described previously. On plotting graphs of band number against activity, 8 major groups of glycoproteins were recognised and ranked from high to low molecular weights, designated peaks 1-8 (see Bullock et al., 1990). They were, respectively, 150-180, 100-120, 62-80, 55, 50, 41, 33, and 28kD. A typical plot of [^{3}H]fucose and [^{14}C]fucose against the band number is shown in Figs. 4.3a&b. The patterns of fucosylglycoproteins in SPMs and PSDs of MeA-trained chicks are shown in Fig. 4.4a&b. Passive avoidance training resulted in a relative drop of fucose incorporation into peak 2 of the left hemisphere SPM fraction. However, peak 5 of the left hemisphere SPMs, representing a Mr 50kD component, showed a significant increase in protein fucosylation. Furthermore, the comparison in fucose uptake between SPMs and PSDs of this component indicated a presynaptic effect (Table 4.3).

In the PSD fraction, peaks 1, 2 and 4 of the left LPO/PA and peaks 2 and 7 of the right LPO/PA showed increases in fucose uptake. However, peaks 3 and 8 of the right forebrain base and peaks 6 and 7 of the left showed significant decreases in their fucose incorporation. Overall, the biggest changes were seen in peak 2 which represented a 150-180kD component indicating a postsynaptic effect. This is evident in Fig. 4.5; peak 2 showed a strong positive PSD-to-SPM ratio, whereas peak 5 of the left LPO/PA showed a strong negative ratio (see also Table 4.3). In addition to peak 5, peaks 6 of the left and peak 3 of the right LPO/PA showed negative ratios, indicating a relative presynaptic enrichment. Peaks 1 of both hemispheres showed a

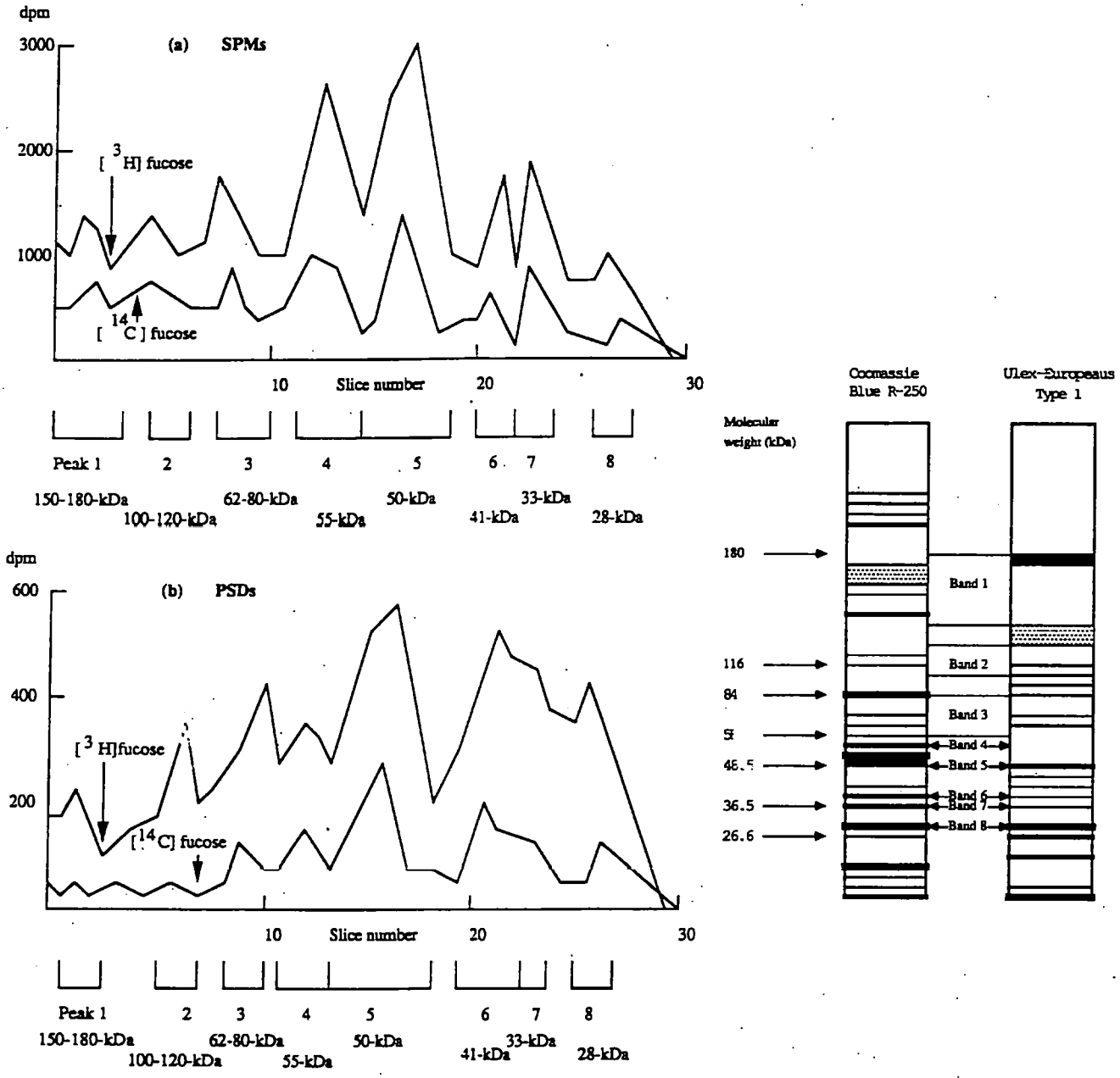


Figure 4.3 : The representative pattern of incorporation of the two fucose isotopes into glycoproteins of SPMs (a) and PSDs (b) resolved by 1-D SDS-PAGE using 5-15% gels.

The chart on the right shows the protein components cut from the gels and used for analysis.

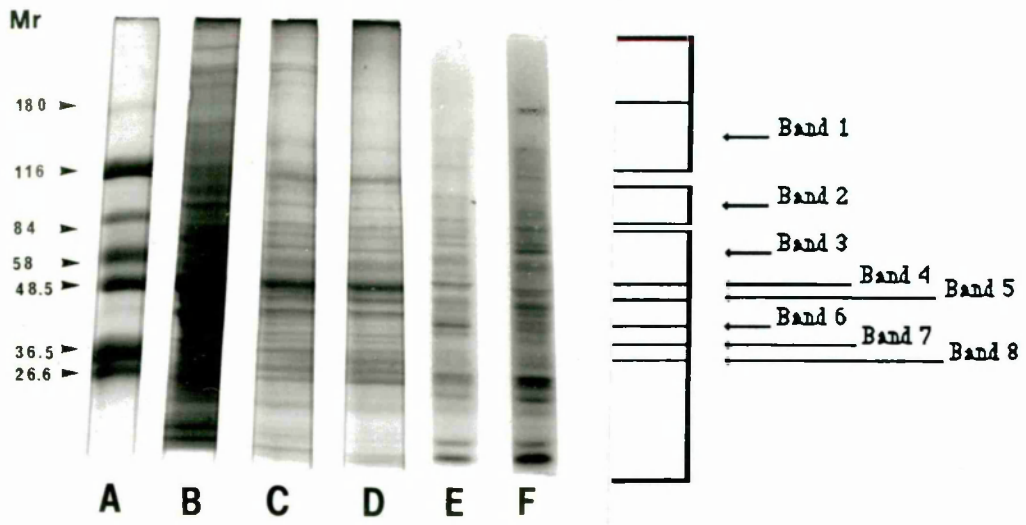


Figure 4.3c : Representative electrophoretic patterns of homogenate, SPMs and PSDs prepared from day-old chick forebrain.

Track A contains molecular weight markers, track B contains whole forebrain homogenate, track C contains SPMs, and track D contains PSDs, all stained with Coomassie Blue R-250. Track E contains SPMs and track F contains PSDs stained with Ulex Europaeus Type I after Western blotting (see Chapter 6 for the technique of Western blotting). Molecular weight values are indicated in kilodaltons.

Fig. 4.4b
MeA/W ratio of fucose incorporation into L&R LPO/PA PSDs (24hr)

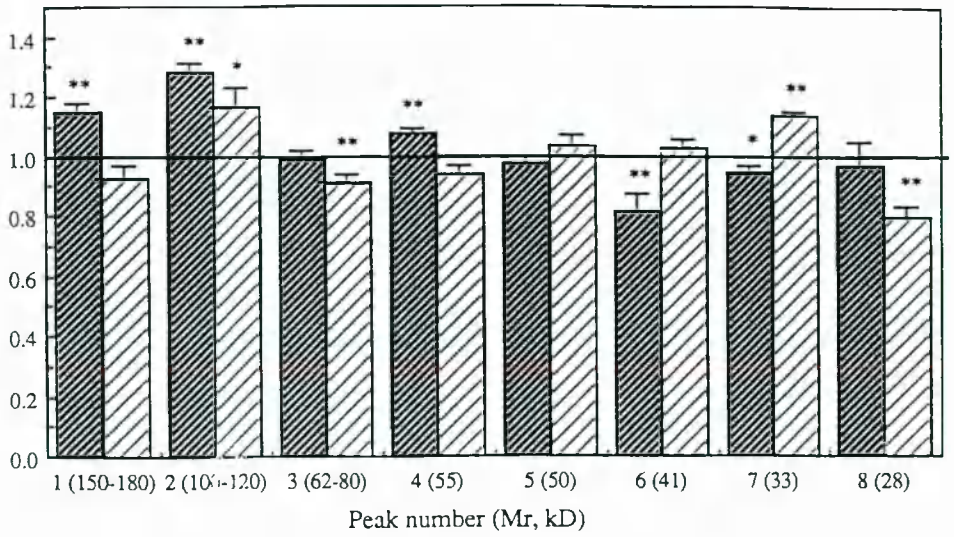


Fig. 4.4a
MeA/W ratio of fucose incorporation into L&R LPO/PA SPMs (24hr)

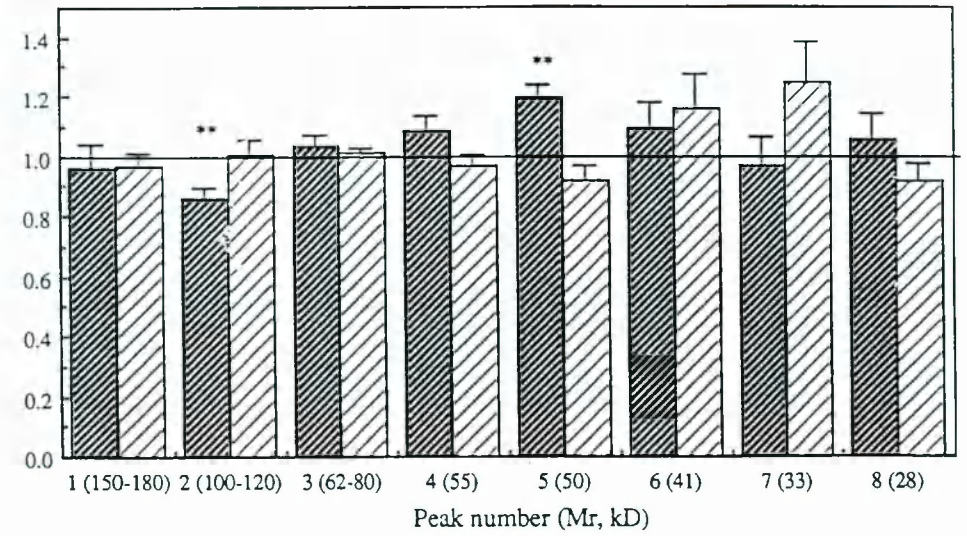


Figure 4. 4 : SDS-PAGE analysis of fucose incorporation into SPMs (a) and PSDs (b) from LPO/PA 24hr after passive avoidance learning.

Dark hatched columns are the values of SPMs or PSDs prepared from left hemisphere LPO/PA, and light hatched columns those for the same fractions prepared from right hemisphere. Data represent the means of 5 standardized values (i.e., [^{14}C]fucose of MeA/[^{3}H]fucose of W divided by [^{14}C]fucose of W/[^{3}H]fucose of W-birds). Error bars are sems. Ratios above 1.0 indicate training induced fucose enrichment, and those below 1.0 indicate fucose impoverishment. *=$p<0.05$; **=$p<0.02$.

similar effect as those of peaks 2. When comparing the relative uptake of fucose between the right and the left hemispheres, the postsynaptic effect of the 150-180kD (peak 2) component was seen to be dominant in the left LPO/PA. So was the the presynaptic effect of the 50kD component. However, the postsynaptic effect of peak 7 was dominant in the right LPO/PA (see Table 4.4).

Subcellular localisation of fucosylation 6hr after training

The distribution of the standardized ratios of protein-bound fucose isotopes is shown in Table 4.5. The only significant training-induced changes seen in the IMHV were in the right SPM and PSD fractions that showed a drop in fucose incorporation by about 22% and 20% respectively.

In the PA, no significant changes were seen in the SPM fractions, but the right PSD showed a significant increase in fucose uptake (17%). This led to a significant postsynaptic effect, reflected in the ratio of PSD/SPM (112%). The postsynaptic effect was significantly lateralised to the right PA.

In the LPO fractions, a significant drop in fucosylation was observed in the right SPM, leading to a left LPO lateralisation (L/R ratio of 135%). The level of fucose uptake remained almost the same in the PSDs of left and right LPO which resulted in a significant presynaptic effect in the left LPO.

PA & LPO		PSD/SPM			
Peak	Mr (kD)	Left	±sem	Right	±sem
1	180-150	1.32*	0.13	1.09*	0.04
2	120-100	1.58**	0.07	1.25#	0.12
3	62-80	0.93	0.02	0.89**	0.02
4	55	1.00	0.06	1.03	0.05
5	50	0.78**	0.03	1.17	0.08
6	41	0.73**	0.08	0.86	0.08
7	33	1.22	0.18	0.83	0.08
8	28	0.97	0.09	0.91	0.03

Table 4.3 : PSD/SPM ratios of fucose incorporation into the major glycoprotein peaks in LPO/PA 24hr after passive avoidance learning. Values are the ratios of [^{14}C]fucose MeA/[^{3}H]fucose W data standardized against [^{14}C]fucose W/[^{3}H]fucose W controls (n=5). *=p<0.05; **=p<0.01; #=p<0.06

Fig. 4.5

PSD/SPM ratio of fucose incorporation into LPO/PA (24hr)

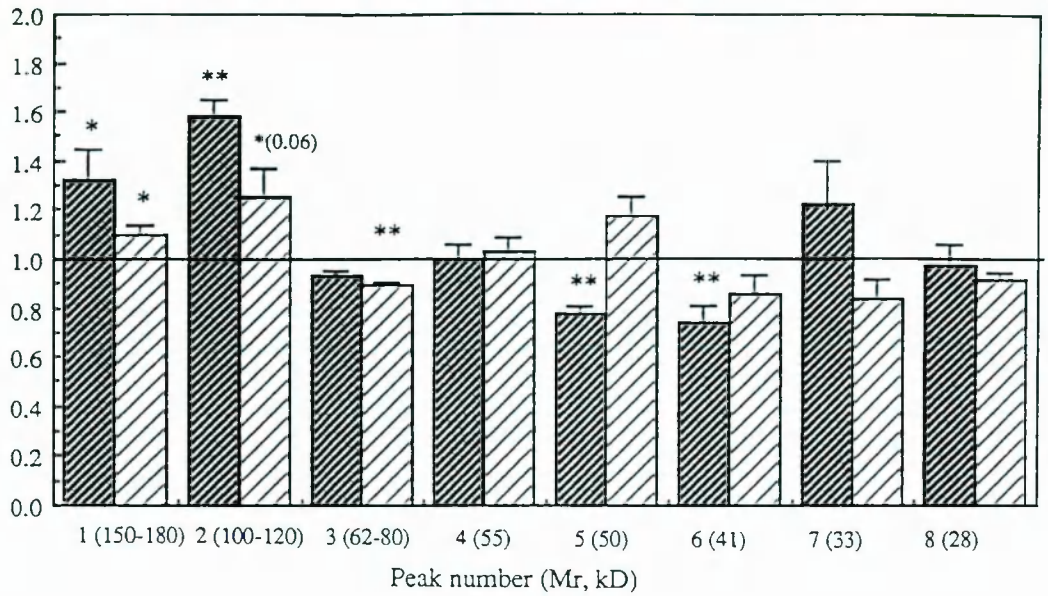


Figure 4.5 : The PSD/SPM ratios of electrophoretically resolved LPO/PA 24hr after training.

Dark hatched columns represent values of the left LPO/PA, and light hatched columns those of the right structures. Error bars are sems.*=$p<0.05$; **=$p<0.02$.

LPO/PA		Left/Right			
Peak	Mr (kD)	SPM	±sem	PSD	±sem
1	150-180	1.00	0.09	1.26**	0.06
2	100-120	0.86**	0.03	1.12	0.08
3	62-80	1.02	0.04	1.09	0.06
4	55	1.14	0.08	1.16**	0.05
5	50	1.37	0.15	0.95	0.04
6	41	0.99	0.09	0.79**	0.05
7	33	1.01	0.02	0.83**	0.02
8	28	1.02	0.05	1.24	0.12

Table 4.4 : Left/Right ratios of fucose incorporation into the major glycoprotein peaks of SPMs and PSDs of the LPO/PA 24hr after passive avoidance learning. Values are the ratios of [^{14}C]fucose MeA/[^{3}H]fucose W data standardized against [^{14}C]fucose W/[^{3}H]fucose W controls for each hemisphere (n=5). *=$p<0.05$; **=$p<0.02$.

Sample	IMHV			PA			LPO		
	L	R	L/R	L	R	L/R	L	R	L/R
SPM	95.9 ±13.3	78.3† ±6.7	124.7 ±18.0	122.8 ±18.0	105.9 ±5.4	114.2 ±13.4	119.6 ±16.0	84.3* ±6.9	134.6† ±19.5
PSD	97.4 ±13.2	80.2* ±8.0	112.4 ±14.8	123.4 ±16.9	117.1# ±6.0	107.2 ±16.1	91.4 ±12.6	98.5 ±10.8	98.3 ±15.1
PSD/ SPM	101.8 ±1.2	113.4 ±10.4	92.7 ±6.2	101.2 ±1.6	112.1† ±7.8	93.1† ±8.1	76.7† ±3.1	105.0 ±4.7	74.3† ±5.4

Table 4.5 : The effect of passive avoidance learning on fucose incorporation into subcellular fractions of chick forebrain 6hr after training. Results are expressed as [^{14}C]fucose (MeA)/[^{3}H]fucose (W) percentage ratios standardized against [^{14}C]fucose (W)/ [^{3}H]fucose (W) of controls. Data are means ±sem of 6 samples for each value. *= $p<0.05$; #=$p<0.01$; †=$p<0.001$. L, left hemisphere; R, right hemisphere.

Identification of fucosylglycoproteins isolated 6hr after training

Figures 4.5a&b, 4.6a&b and 4.7a&b show the patterns of protein fucosylation in SPMs and PSDs obtained from IMHVs, LPOs, and PAs of trained birds. No significant increases in the amount of fucose uptake was observed in either SPMs or PSDs of IMHV fractions. However, peak 5 showed a significant drop in fucose incorporation into SPMs of the right IMHV (Fig. 4.6a). Peaks 3 and 4 of PSD fractions obtained from the right IMHV showed a similar trend. It is assumed that the these decreases in fucose uptake were the major contributory factors in a general decrease in fucosylation in the right IMHV observed when examining subcellular localisation of protein fucosylation (see Table 4.5).

Compared to the IMHV, the patterns of fucosylation in PA were different; in the left SPM, peak 6 (41kD) exhibited a sharp increase in fucose uptake, whereas peak 7 (33kD) of both left and right PSD fractions showed elevated fucosylation (Fig. 4.7a&b). The PSD/SPM ratios of PA are shown in Table 4.6. As can be seen, the effect of fucosylation in peak 7 of both left and right PA is significant postsynaptic enrichment. The postsynaptic effect of the 'right' peak 4 was, however, at the expense of a drop in fucose uptake of the same fraction in the SPM. The incorporation of fucose into left PA SPM of the 33kD component (peak 7) was significantly less than that in the right PA (see Table 4.7). These patterns were also consistent with observations made at the subcellular level (see Table 4.5, the PA column).

The training induced fucosylation patterns, obtained from LPO fractions, proved to be the most varied of the six subcellular fractions (Fig. 4.8a&b). In the SPMs 150-180kD components of both left and right LPOs (peak 1) showed a sharp decrease, whereas peaks 5 and 6 (50 and 41kD) of the left LPO, and peak 7 of the right showed elevated fucosylation. The presynaptic effect of 50kD (peak 5) component was mainly confined in the left hemisphere (see Table 4.9 and Fig. 4.8a).

PA		PSD/SPM			
Peak	Mr (kD)	Left	±sem	Right	±sem
1	180-150	1.08	0.06	0.89**	0.03
2	120-100	1.15	0.07	0.92	0.02
3	62-80	1.03	0.05	0.99	0.03
4	55	0.99	0.02	1.14**	0.03
5	50	1.08	0.06	1.05	0.03
6	41	0.92	0.08	1.06	0.04
7	33	1.19**	0.02	1.12*	0.04
8	28	0.97	0.06	0.88	0.13

Table 4.6 : PSD/SPM ratios of fucose incorporation into the major glycoprotein peaks in PA 6hr after passive avoidance training. Values are the ratios of [^{14}C]fucose MeA/[^{3}H]fucose W data standardized against [^{14}C]fucose W/[^{3}H]fucose W controls (n=5). *=$p<0.05$; **=$p<0.01$.

Fig. 4.6a

MeA/W ratio fucose incorporation into L&R IMHV SPMs

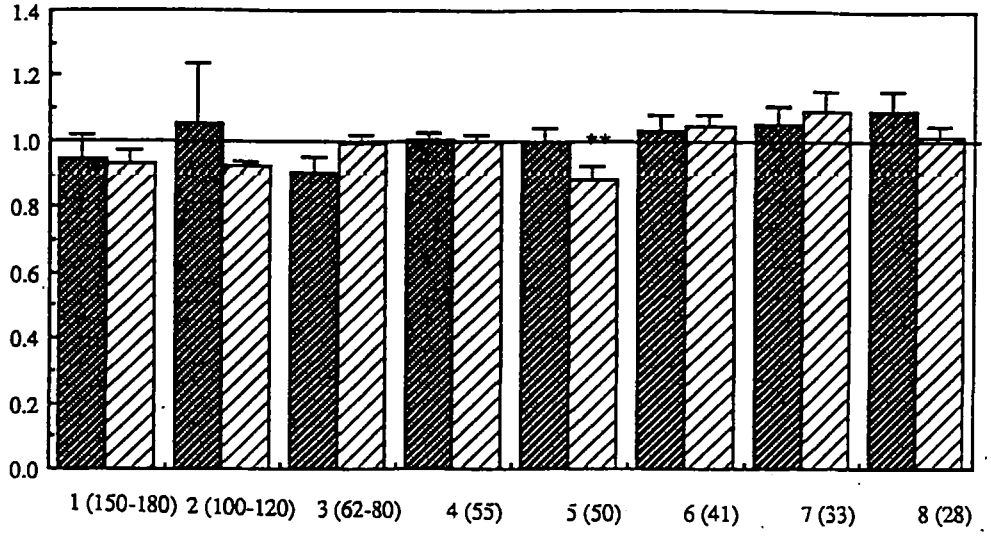


Fig. 4.6b

MeA/W Ratio fucose incorporation into L&R IMHV PSDs

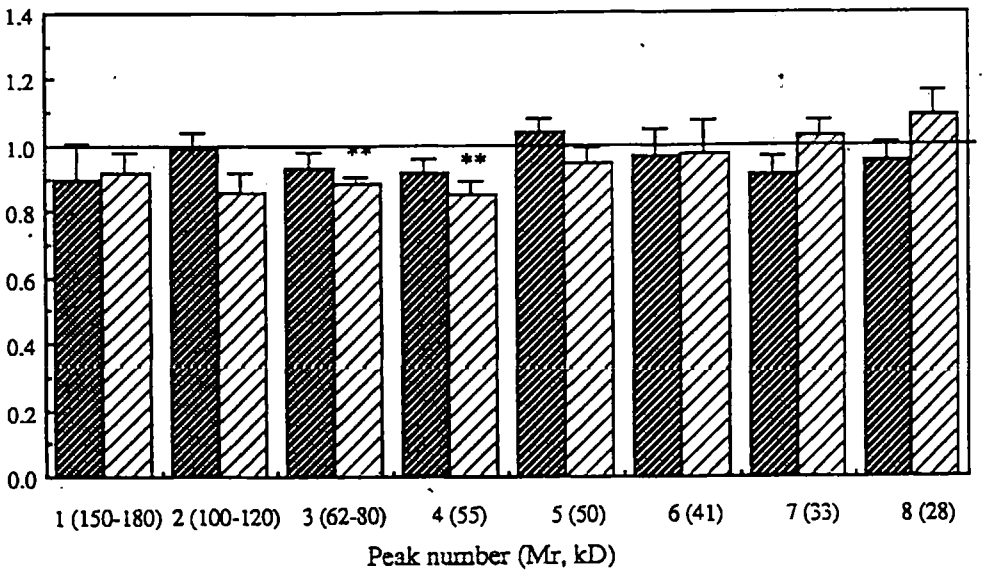


Figure 4.6 : SDS-PAGE analysis of fucose incorporation into SPMs (a) and PSDs (b) from IMHV 6hr after passive avoidance learning.

Dark hatched columns are the values of SPMs or PSDs prepared from left hemisphere IMHV, and light hatched columns those for the same fractions prepared from right hemisphere. Data represent the means of 5 standardized values [i.e., [^{14}C]fucose of MeA/[^{3}H]fucose of W divided by [^{14}C]fucose of W/[^{3}H]fucose of W-birds]. Error bars are sems. Ratios above 1.0 indicate learning induced fucose enrichment, and those below 1.0 indicate fucose impoverishment. **=$p<0.02$.

Fig. 4.7a

MeA/W fucose incorporation into L&R PA SPMs

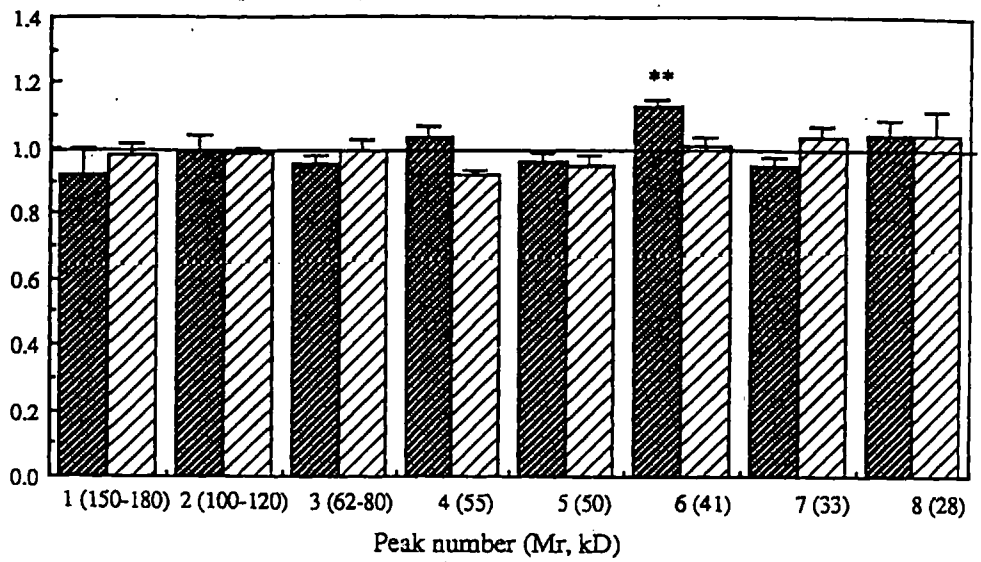


Fig. 4.7b

MeA/W ratio of fucose incorporation into L&R PA PSDs

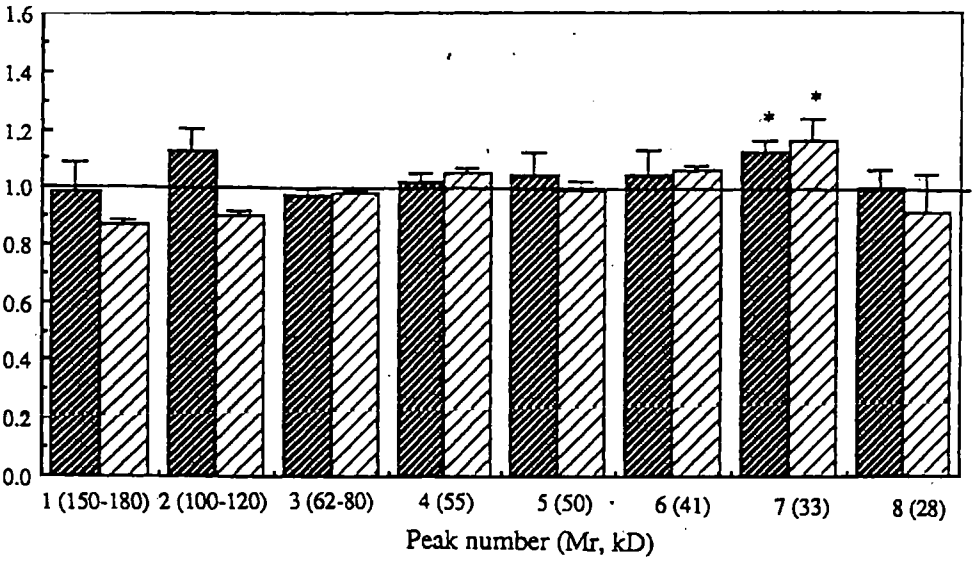


Figure 4.7 : SDS-PAGE analysis of fucose incorporation into SPMs (a) and PSDs (b) form PA 6hr after passive avoidance learning.

Dark hatched columns are the values of SPMs or PSDs prepared from left hemisphere PA, and light hatched columns those for the same fractions prepared from right hemisphere. Data represent the means of 5 standardized values [i.e., [^{14}C]fucose of MeA/[^{3}H]fucose of W divided by [^{14}C]fucose of W/[^{3}H]fucose of W-birds]. Error bars are sems. Ratios above 1.0 indicate learning induced fucose enrichment, and those below 1.0 indicate fucose impoverishment. *=$p<0.05$; **=$p<0.02$.

PA		Left/Right			
Peak	Mr (kD)	SPM	±sem	PSD±sem	
1	150-180	0.93	0.07	1.13	0.09
2	100-120	1.01	0.05	1.25**	0.08
3	62-80	0.96	0.01	0.99	0.03
4	55	1.13**	0.03	0.98	0.04
5	50	1.01	0.01	1.05	0.05
6	41	1.13**	0.04	0.98	0.07
7	33	0.92	0.04	0.98	0.08
8	28	1.01	0.05	1.23	0.24

Table 4.7 : Left/Right ratios of fucose incorporation into the major glycoprotein peaks of SPMs and PSDs of the PA 6hr after passive avoidance learning. Values are the ratios of [^{14}C]fucose MeA/[^{3}H]fucose W data standardized against [^{14}C]fucose W/[^{3}H]fucose W controls for each hemisphere (n=5). *=$p<0.05$; **=$p<0.02$.

LPO		PSD/SPM			
Peak	Mr (kD)	Left	±sem	Right	±sem
1	180-150	1.57**	0.11	1.21*	0.08
2	120-100	1.01	0.08	1.11	0.14
3	62-80	1.24*	0.08	1.26*	0.09
4	55	0.94	0.06	1.13	0.14
5	50	0.92	0.06	1.15	0.07
6	41	0.87*	0.04	0.75*	0.09
7	33	1.12	0.18	0.80**	0.04
8	28	0.95	0.03	0.72**	0.05

Table 4.8 : PSD/SPM ratios of fucose incorporation into the major glycoprotein peaks in LPO 6hr after passive avoidance learning.Values are the ratios of [^{14}C]fucose MeA/[^{3}H]fucose W data standardized against [^{14}C]fucose W/[^{3}H]fucose W controls (n=5). *=p<0.05; **=p<0.01.

Fig. 4.8a

MeA/W Ratio fucose incorporation into L&R LPO SPMs

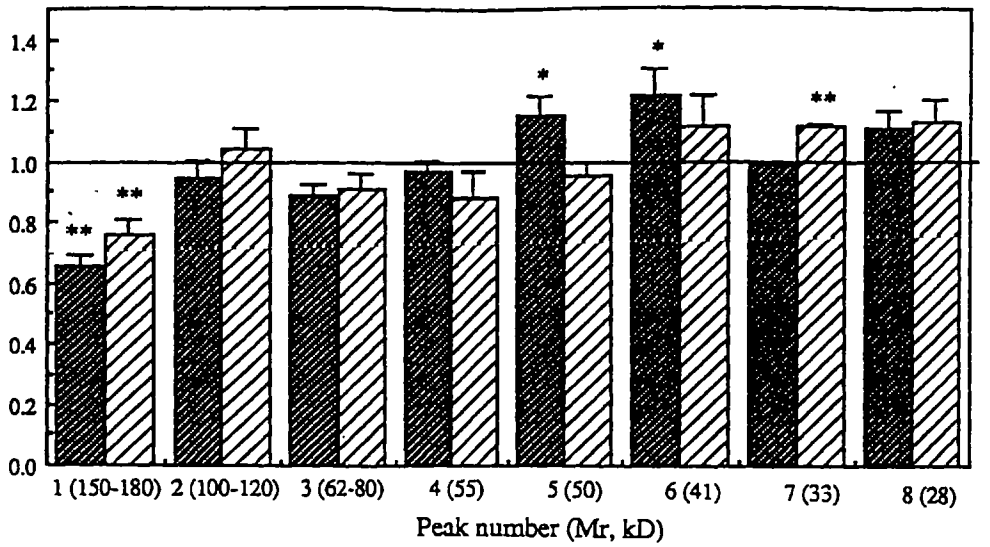


Fig. 4.8b

MeA/W ratio of fucose incorporation into L&R LPO PSDs

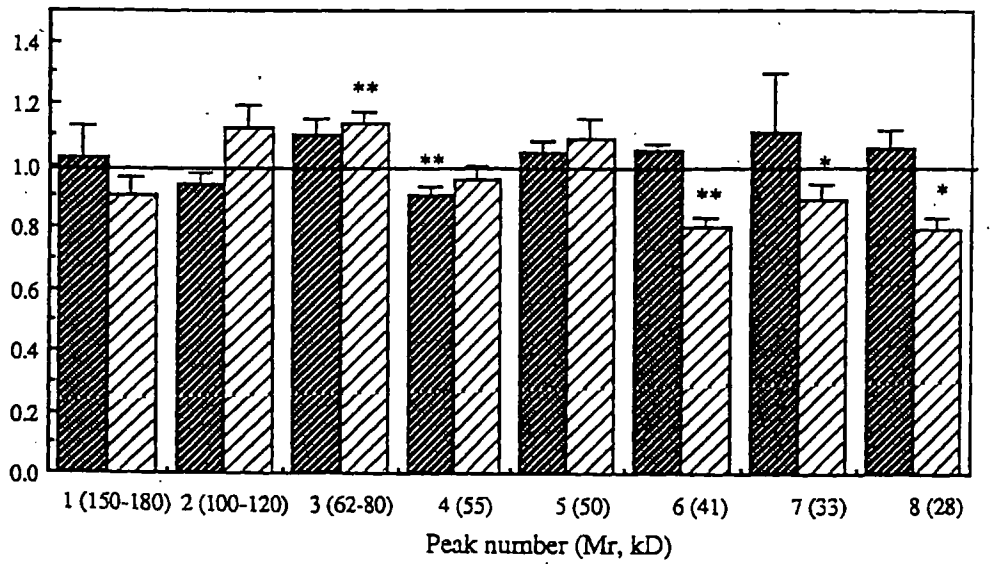


Figure 4.8 : SDS-PAGE analysis of fucose incorporation into SPMs (a) and PSDs (b) form LPO 6hr after passive avoidance learning.

Dark hatched columns are the values of SPMs or PSDs prepared from left hemisphere LPO, and light hatched columns those for the same fractions prepared from right hemisphere. Data represent the means of 5 standardized values [i.e., [^{14}C]fucose of MeA/[^{3}H]fucose of W divided by [^{14}C]fucose of W/[^{3}H]fucose of W-birds]. Error bars are sems. Ratios above 1.0 indicate learning induced fucose enrichment, and those below 1.0 indicate fucose impoverishment. *=$p<0.05$; **=$p<0.02$.

In the PSD fractions, the 62-80kD band (peak 3) showed significantly increased fucosylation. Peak 4 of the left, and 6, 7, and 8 of the right LPO exhibited significant decreases in their fucose uptake after training. When comparing protein fucosylation between SPM and PSD components (Table 4.8 and Fig. 4.9), the 33kD band (peak 7) of the right hemisphere was found to exhibit a significant presynaptic effect. No lateralisation of this component was observed (see Table 4.9). Peak 1 also showed a postsynaptic effect. However, this was at the expense of a sharp drop in fucosylation of this component in SPM fractions.

Fig. 4.9

PSD/SPM ratio of fucose incorporation into LPO

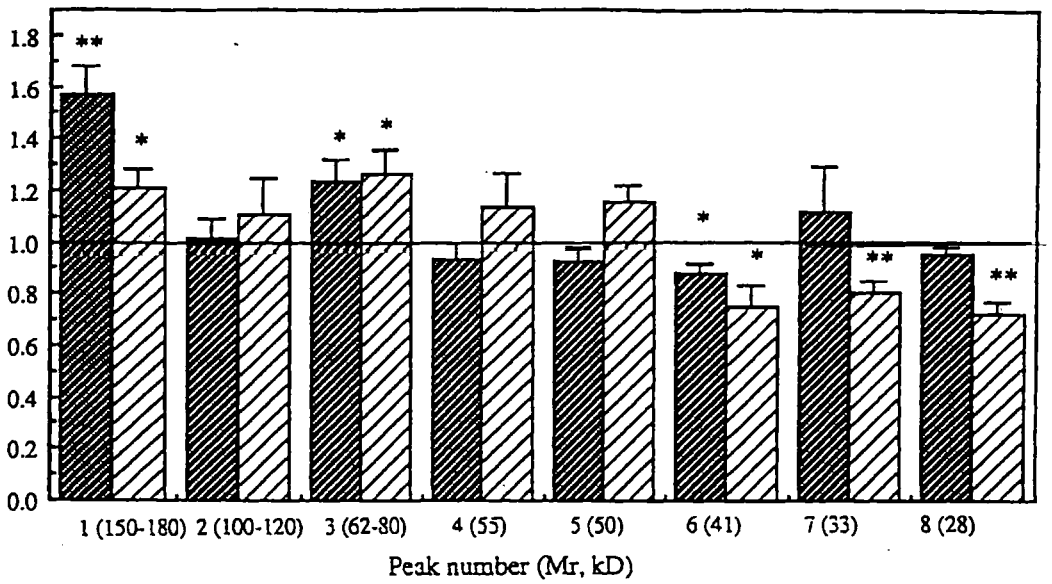


Figure 4.9 : The PSD/SPM ratios of electrophoretically resolved LPO 6hr after training.

Dark hatched columns represent values of the left LPO, and light hatched columns those of the right hemisphere. Error bars are sems.*=$p<0.05$; **=$p<0.02$.

LPO		Left/Right			
Peak	Mr (kD)	SPM	±sem	PSD	±sem
1	150-180	0.88*	0.05	1.14	0.09
2	100-120	0.91	0.05	0.85*	0.06
3	62-80	0.98	0.04	0.97	0.06
4	55	1.16	0.16	0.95	0.03
5	50	1.23*	0.09	0.98	0.07
6	41	1.13	0.13	1.31**	0.04
7	33	0.89	0.01	1.26	0.23
8	28	1.00	0.09	1.33**	0.06

Table 4.9 : Left/Right ratios of fucose incorporation into the major glycoprotein peaks of SPMs and PSDs of the LPO 6hr after passive avoidance learning. Values are the ratios of [^{14}C]fucose MeA/[^{3}H]fucose W data standardized against [^{14}C]fucose W/[^{3}H]fucose W controls for each hemisphere (n=5). *=$p<0.05$; **=$p<0.02$.

DISCUSSION

The earlier *in vitro* study was based on the reports by McCabe and Rose (1985a) and Lössner and Rose (1983) who found the right forebrain base as the site showing maximum fucosylation and significant fucokinase activities a few hours after training on the passive avoidance task. Results of the *in vivo* studies reported here have confirmed and extended McCabe and Rose's findings. In other words, the IMHV did not show significant fucosylation increases 6 & 24hr after passive avoidance learning.

Results of a number of other studies, however, point to the importance of IMHV function during and after learning. Indeed, the use of labelled 2DGlc showed an increased metabolic activity in the IMHV during the first hour posttraining period (Kossut and Rose, 1984; Rose and Csillag, 1985).

Lesion studies have shown that the left IMHV is necessary for the acquisition of learning (Patterson et al., 1990). Morphological studies, on the other hand, showed that 24hr after training the mean length of postsynaptic thickening in the left IMHV increased to the extent that the greater length seen in the right IMHV of W-trained birds was no longer apparent (Stewart et al., 1984). Interestingly results reported here (MeA-trained standardized against W-trained values) showed a decrease in the right IMHV fucosylation, both in SPMs and PSDs -at 6hr after training (Table 4.5)- and in the right SPMs -at 24hr after training. This may indicate that some training specific protein fucosylation does indeed take place in the left IMHV, but only to "close the gap" between W and MeA-trained fucosylation. On the other hand, it is possible that some training-specific reduced glycosylation takes place in order to reprocess previous memories and reorganize neural connectivity for future experience. During training redundant connections face regression and make way for incoming processing. After all, continual addition of new glycoproteins and/or increased synapse formation in confined volume of the brain does not seem feasible. Evidence for synapse loss or

regression during neural development and learning (imprinting) is presented by a number of researchers (e.g., Cowan et al., 1984; Scheich, 1987). However, there are hitherto no reports of specific glycoproteins that are deglycosylated during neural plasticity.

The other two brain structures studied were PA and LPO. No significant changes were seen in SPMs 6hr after training. But by 24hr, there was 63% increase in the amount of protein fucosylation in the right SPMs. The right PA seemed to be metabolically active as early as 6hr posttraining with increased activity still after 24hr. At this time point the left PA almost doubled its fucose incorporation into PSD fractions, which compared to the corresponding SPMs, resulted in an overall postsynaptic fucosylation dominance. This effect, however, changed laterality; that is, from the right PA 6hr after passive avoidance training to the left PA after 24hr.

Changes in fucose incorporation into LPO were more prominent 24hr after training than after 6hr. The non-significant 20% increase in fucose labelling in the left SPM at 6hr developed into a significant 64% increase after 24hr. At this latter time the PSD fractions of left LPO showed a 24% increased fucosylation, an effect which was absent 6hr after passive avoidance training. No change in protein fucosylation took place in PSD fractions of the right LPO at either time point.

Results obtained from subcellular analysis of fucosylation indicate that different brain structures are active at different periods (and perhaps for different lengths of time) after passive avoidance training. Some of these changes are present at the earlier time point, but by 24hr they are no longer detectable. Some changes present at 6hr persist for at least 24hr, and yet others only appear during later stages of memory formation. The changes seen in the left and right LPO, for example, are consistent with

morphological and neurochemical changes that suggest (new) synapse formation and incorporation of glycoproteins into synaptic membranes.

Gel electrophoresis results from the *in vitro* study were encouraging. When TCA precipitable materials of total right forebrain base tissue homogenates were resolved, a fucosylated glycoprotein component of Mr 100-120kD showed a significant elevation after MeA-training (Fig. 4.1a). A protein component of Mr 40-45kD in the left forebrain base also showed an increased rate of fucosylation 3hr after training (Fig. 4.1b). Based on these results, gel electrophoresis was carried out on subcellular fractions of IMHVs, PAs, and LPOs 6 and 24hr after training.

Chronologically, the 24hr study was done before the earlier time point. This was justified for several reasons, such as maximization of labelled fucose incorporation. That the morphological results were obtained 24hr after training was another reason. However, since LPO and PA exhibited the highest amounts of fucosylation, it was decided, in order to maximize radioactivity counts, to combine the two structures and then resolve out fucosylglycoproteins by gel electrophoresis. In retrospect, having obtained interesting results from processing LPO and PA separately 6hr after training, it might have been advisable to analyse these structures separately at 24hr also.

IMHV electrophoresis at 6hr after training showed no significant changes in any of the 8 characterised protein peaks were observed. It is, therefore, assumed that, given the similar rates of fucosylation in subcellular fractions 6 and 24hr after training (see Tables 4.2 & 4.5), the electrophoretic resolution of the IMHV at the longer time-point would be similar to that obtained at 6hr posttraining time.

In order to make a comparative analysis of these glycoproteins, the separate data obtained, 6hr after training, from LPO and PA were combined (Fig. 4.10a&b). The

only protein component in the SPM fractions that showed increased fucose uptake was the 41kD (peak 6) component of the left LPO/PA. Interestingly, this component was also identified in the *in vitro* study (see Fig. 4.1b). By 24hr the degree of fucose uptake in this component reverted to control levels. Instead, the 50kD component of the left LPO/PA structure exhibited significant elevation in fucosylation after training.

In the PSDs of LPO/PA, a 62-80kD (peak 3) component was identified that showed increased fucose uptake, mainly in the right hemisphere. However, 24hr after training, the 62-80kD component showed a significant decrease in its fucose uptake. Instead, peak 2 (100-120kD) of both right and left LPO/PA and the 180kD component (peak 1) of the left LPO/PA showed significant fucose incorporation.

A closer examination of these protein components revealed that the elevated fucose uptake of peak 3, at 6hr after training, was confined to the postsynaptic LPO, and that it occurred in both left and right hemispheres (Fig. 4.7b). The 100-120kD (peak 2) component of the right LPO also showed an increased fucose uptake, though this was not significant. This peak did show significant fucose incorporation in the *in vitro* study. The elevation in 41kD (peak 6) fucosylation, on the other hand, was contributed to by presynaptic fractions of both LPO and PA (Fig's. 4.6a & 4.7a).

Using the electrophoresis technique fine but significant variations in neurochemical events were elicited that would otherwise have remained un-noticed in more crude techniques.

Fig. 4.10a
MeA/W ratio of fucose incorporation into L&R of LPO/PA SPM (6hr)

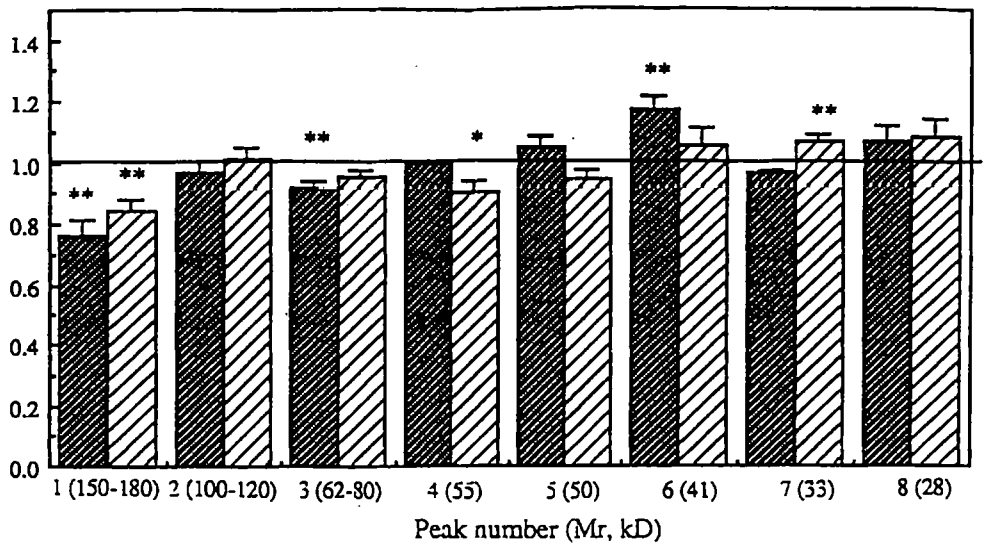


Fig. 4.10b
MeA/W ratio of Fuc incorporation into L&R of LPO/PA PSD (6hr)

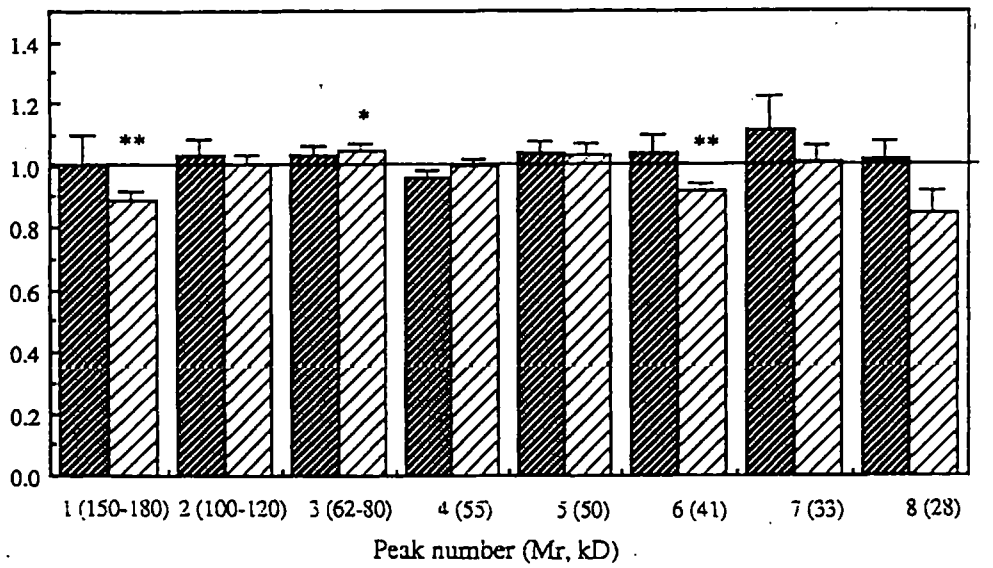


Figure 4.10 : SDS-PAGE analysis of fucose incorporation into SPMs (a) and PSDs (b) from combined LPO & PA 6hr after passive avoidance learning.

Dark hatched columns are the values of SPMs or PSDs prepared from left hemisphere LPO/PA, and light hatched columns those for the same fractions prepared from right hemisphere. Data represent the means of 5 standardized values [i.e., [^{14}C]fucose of MeA/[^{3}H]fucose of W divided by [^{14}C]fucose of W/[^{3}H]fucose of W-birds]. Error bars are sems. Ratios above 1.0 indicate learning induced fucose enrichment, and those below 1.0 indicate fucose impoverishment. *=$p<0.05$; **=$p<0.02$.

In summary, the results reported here are consistent with findings reported from other neurochemical and morphological studies. They suggest that there are different mechanisms governing memory formation and storage at different times after training and in different structures. The induction of amnesia by 2DGal, at around the training time and again 6-8hr after training, also supports this notion (Zamani and Rose, 1990). A further analysis of these fucosylglycoproteins should, therefore, be able to show whether their increased turnover and fucosylation are necessary if memory is to be formed and stored. One effective way to examine this criterion is the employment of neuroimmunological techniques. Antibodies raised against these proteins could be used to demonstrate whether their injection into the chick brain renders them amnesic, and if so, whether their antigens/epitopes show alterations in turnover after passive avoidance training. Such investigations are discussed in the next chapter.

CHAPTER FIVE

The Use Of Polyclonal Antibodies In Identification And Characterization Of Glycoproteins Involved In Passive Avoidance Learning : A Case For R15

CHAPTER FIVE

THE USE OF POLYCLONAL ANTIBODIES IN IDENTIFICATION AND CHARACTERIZATION OF GLYCOPROTEINS INVOLVED IN PASSIVE AVOIDANCE LEARNING: A CASE FOR R15.

INTRODUCTION

Since 1964 when the term immunoneurology was introduced by Schmidt, the application of neuroimmunological techniques as sensitive tools for understanding both the brain structure-function relationship and the brain and behaviour dichotomy has been increasingly appreciated.

The majority of research concentrates on addressing several fundamental questions, including identification and characterization of neuron-specific antigens such as receptors and gated channels, the morphological distribution of these antigens, their roles in neural growth and development such as synaptogenesis, the study of pathological conditions resulting from abnormal expression of the antigens, and their role in various behavioural and cognitive changes such as learning and memory (see Jankovic, 1985 for a review).

Considering the fact that thousands of proteins exist in the nervous system, some of which are specific to the system, it seems likely that it has an immense immunogenic potential.

The early study by Reichner and Witebsky (1934) in which they used polyclonal

antibodies against neural alcohol soluble fractions and caudate nucleus and hippocampus provided a method for distinguishing between white and grey matter. Further cytochemical differentiation was achieved when Herschman and co-workers (1972) reported that the plasma membrane of cortical synaptosomes differed immunologically from the plasma membranes of cells in other organs, and from other subcellular fractions of the CNS.

One of the first antigens that was shown to possess immunogenicity was S-100. When first discovered, it was thought to be neuron specific, but later studies revealed that in fact other tissues such as skin respond to anti-S-100 antibodies (Cocchia et al., 1981).

A number of antibodies have been raised to brain proteins in order to study animal models of neuropathological disorders such as experimental allergic encephalomyelitis, multiple sclerosis, post infectious and post vaccinal encephalitis, and epilepsy (for a review see Jankovic, 1985).

Neural glycolipids and, in particular, gangliosides have been studied by neuroimmunological techniques (e.g., see Karpiak et al., 1978). Antiganglioside antibodies are reported to have a selective mode of action despite being widespread in the CNS. For example, they are claimed to induce epileptiform spiking activity (Karpiak et al., 1976a), and to suppress synapse, dendrite, and myelin formation (Jankovic, 1985). Certain anti-brain protein antibodies have also been found to disrupt these processes. An antibody to a 50kD protein secreted at the site of rat denervated muscles was found to prevent neuronal sprouting when introduced at the site of denervation (Gurney, 1984).

Neuroimmunological techniques have been increasingly employed in assessing the role of specific neural antigens in alteration of behaviour and in cognitive processes. In one

such study reported by Jankovic et al.(1968), cats were trained to discriminate between two tones of different frequencies, one of which was associated with an aversive stimulus. After receiving a single intraventricular injection of antiribonucleoprotein antibody, they showed a marked and long-lasting deficit in this response. Further, Jankovic (1985) also reported that anti-hippocampal antibodies and anti-caudate antibodies severely affected monkeys' performance in delayed visual discrimination task. In rats, injection of anti-SPM antibodies was shown to impair recall mechanisms (Kobiler et al., 1976).

Antibodies raised against isolated brain specific proteins have been used in behavioural investigations. The role of S-100 has been reported in various paradigms. For example, the prior intraventricular injection of anti-S-100 antibodies was shown to prevent rats from maze learning or, if injected during the course of learning, it prevented them from further learning (Hyden and Lange, 1971; see also Karpiak et al., 1976b).

Production of monoclonal antibodies to neural specific antigens has proved to be difficult. The reason for this is a matter of conjecture. However, it is possible that these antigens are evolutionarily conserved and, as a result, have little immunogenicity. It may also be possible that specific immunogenic epitopes of some of these molecules become masked by the process of posttranslational modifications. Nevertheless, the production of a number of monoclonal antibodies has been reported and their role in development and behavioural alteration investigated.

One such antibody 411B (Bullock et al., 1987 & 1988) was found to recognize a 170kD chick brain postsynaptic protein and was implicated in post-hatch neural development. It showed an increased titre 4 days and, even larger increase, 14 days after hatch. Further, an increased titre of 411B was detected in the rat brain after haloperidol treatment, indicating an involvement in the mechanism of dopamine

supersensitivity (Lössner et al. 1988). Recently, Scholey (1991) reported an increased binding of 411B in dentate gyrus sections of rat brain 8hr after induction of long-term potentiation. No evidence as regards 411B glycosylation has been reported.

Other monoclonal antibodies that have been raised to subcellular fractions include Mab-gp50 and Mab-gp65 (Beesley et al., 1987). The former recognizes two immunologically related antigens of Mr 45 and 49kD, and the latter, two related synaptic glycoproteins of Mr 55 and 65kD. Although they were found to be brain specific, they were not exclusively localized at the synapse. Mab-gp50, for example, was found to label cell bodies as well as dendrites of both fore- and hind brain. The Mab-gp65 antigen was, however, relatively enriched at PSDs.

Long term studies on the development of behavioural alteration in progeny from mothers injected with anti-brain antibodies have also been carried out. In one such study, rats born to mothers that received anti-hippocampal antibodies exhibited behavioural deficits in a number of conditioning paradigms such as, passive avoidance and T-maze learning (Jankovic, 1985; Karpiak & Rapport, 1975). It is, however, quite likely that the effect of these antibodies during embryonic neural development was non-specific.

Another way of determining whether a particular antigen plays a role in neural plasticity is by intervention studies. Results from several relevant investigations were cited above. In those studies, the antigens, with the exception of GM1 and S-100, were not identified. However, a number of antibodies have been raised to purified antigens and their role in neural plasticity determined. The function of some of these antigens in other organs is already known. For example, Croiset et al. (1990) demonstrated that anti-vasopressin antibody when injected intraventricularly immediately after training

caused inhibition of retention in rats trained on a passive avoidance paradigm. The response deficit was not, however, evident earlier than 6hr posttraining. Also, anti-GM1 antibody injected intraventricularly immediately after training rats impaired maze learning (see, Jankovic, 1985). Antibodies to the same ganglioside were found to affect learned avoidance response (Karpiak et al., 1978), or, if injection is delayed, the antiganglioside antibody inhibited memory consolidation, but did not affect learning (acquisition) (Karpiak et al., 1978).

As noted before, glycoproteins play an important role in neural development and plasticity. The use of antibodies raised against membrane-bound glycoproteins could shed more light on their mode of activity. One such molecule is the extracellular matrix glycoprotein ependymin. According to Shashoua (1985), polymerization of ependymin in the synaptic junction milieu resulting from a local drop in Ca^{2+} concentration increases synaptic efficacy (see also, Shashoua et al., 1990). Thus, injection of anti-ependymin antibody 30min to 24hr after training fish to learn to swim in straight line when they have a float attached to them caused behavioural deficit. ELISA showed that the concentration of the antigen in extracellular fluid dropped by 20% in fish during the acquisition phase of a shock avoidance paradigm (Shashoua & Hesse, 1989). This is accompanied by an increased synthesis of the antigen (Schmidt, 1987). The interference of anti-ependymin in processes leading to long-term memory consolidation has also been demonstrated in mice trained on a T-maze (Shashoua, 1983).

Thy-1 glycoprotein, the smallest member of the immunoglobulin family, which is located almost exclusively at the postsynaptic site, is also implicated in processes involved in memory formation. Thus, when chicks received intracranial injections of either polyclonal or monoclonal anti-Thy-1 antibodies 10min before or after passive avoidance learning, they became amnesic (Bernard et al., 1983; Lappuke et al., 1987).

Finally, antibody raised to the neuron specific glycoprotein N-CAM is reported by Nolan and associates (1987a; Doyle et al., 1990) to render rats amnesic when administered 6-8hr after training on a passive avoidance paradigm. However, the amnesia was only evident 48hr after training. In addition, the injection of the antibody earlier than 6hr posttraining did not cause memory deficit. In contrast to N-CAM, the authors showed that the brain specific anti-protein 2 antibody rendered rats amnesic when it was administered 5min before training, and no amnesia was observed when injection was carried out 6-10hr after training. Similar results were reported when a monoclonal antibody to the neural glycoprotein F-3-87-8 was administered to rats undergoing passive avoidance learning (Nolan et al., 1987b).

In summary, one of the most effective ways to identify and assess the role of neural glycoconjugates during memory formation and consolidation is by the use of specific antibodies. In general, antibodies serve several functions; first, they show whether the expression of an antigen is *concomitant* to or *necessary* in learning and memory formation; second, they can be used to examine the *time course* of such expression; and third, they can be used to map the *anatomical* and *morphological distribution* of the antigens. Thus, we decided to raise a number of antibodies to chick forebrain antigens, to characterize their antigens. Those that recognized specific antigens of interest, that is those with molecular weights similar to synaptic glycoproteins which showed increased fucosylation after training (see Chapter 4), were selected for further analysis.

MATERIALS

See chapter two for details of materials used in this study.

METHODS

Preparation of antibodies

In order to raise polyclonal antibodies, TCA precipitable material from chick forebrain homogenate was resolved by 1-D PAGE as described in Chapter 2. A small strip was cut out from the middle of the gel along the direction of electrophoresis and stained with Coomassie blue. This was done to ensure that a suitable electrophoretic separation was achieved. Eight horizontal strips were cut out of the gels corresponding to the already established protein peaks that showed increased fucosylation after training (Chapter 4) that is; Mr 150-180, 100-120, 62-80, 55, 50, 41, 33, and 28kD and homogenized in saline using a teflon-glass homogenizer. Rabbits were injected with 5ml of homogenates and given a booster injection after two weeks. Regular injections and bleeding were followed alternately at weekly intervals. The polymerized acrylamide was used as a substitute for Freund's adjuvant.

To test their immune response, rabbits were bled and the blood was kept at 4°C for 48hr to coagulate. It was then spun at $1.5k_{gmax}$ for 5min and the serum removed from the pellet using a Pasture pipette. The serum was filtered through a sterile Schleicher & Schnell filter (0.2μm pore size) using a 5ml sterile syringe. Forebrain homogenate, SPMs and PSDs were then resolved electrophoretically and transferred to nitrocellulose paper by Western-blotting. Immunoblotting, according to the method described in Chapter 6, was used to test the activity of each serum.

Antibody purification

Purification of total serum IgG was by affinity chromatography using protein G-sepharose columns. Serum was buffered to pH7.0 using 100mM phosphate buffer. The protein-G-sepharose beads were transferred to a sterile syringe and connected to a

peristaltic pump which was adjusted to allow a flow of 1ml per min. The column was washed 5 times bed-volume with water and equilibrated with 5 times bed volume of 20mM phosphate buffer (pH7.0). The serum (about 10ml/ml of protein-G column) was run through the column and 1ml aliquots were collected in ice-cold plastic tubes. The column was then washed with 20mM phosphate buffer (20 times bed-volume). Aliquots of 1M tris/HCl buffer (55µl, pH9.0) were added to the remaining collection tubes. Serum IgG was then eluted from the column at a slightly higher flow rate using 100mM glycine/HCl (pH2.7). One milliliter aliquots were used to measure the optical density of each fraction. The column was washed with 20 times bed-volume of 20mM phosphate buffer (pH7.0) and 20mM phosphate buffer containing 20% ethanol (pH7.0). It was then sealed and stored at 4°C for reuse.

A plot of fraction number against absorption was constructed and those fractions containing IgG were pooled and dialyzed overnight against saline at 4°C. The protein content of the resulting solution was measured (as described in Chapter 2), and the IgG solution was aliquoted into Eppendorf tubes and stored at -20°C. For intervention studies, the protein concentration of the IgG solution was adjusted to 1mg/ml using Minicon™ concentrators (Amicon corporation, USA). To ensure the purity of IgG preparations, aliquots of eluants were electrophoresed on 1-D PAGE and the protein bands stained with Coomassie blue. Two heavily stained protein bands corresponding to the heavy (Mr 50kD) and light chains (Mr 25kD) of IgG, were seen.

Passive avoidance training

Day-old chicks were transferred from the communal brooder and placed in pairs in pens that were lit with dimmed red light. They were trained and tested 24hr later as described in Chapter 2. The effect of antibodies on avoidance learning was examined by bilateral intracerebral injections (10µg/10µl/hemisphere) at specified time-points, before or after training. Retention scores were compared with those of saline injected birds. The

statistical method of two-way X^2 was used to measure the level of significant difference between the two groups. In some experiments pre-immune serum obtained from the same rabbits was used in place of saline to assess the specificity of raised IgG.

Enzyme linked immunosorbent assay

The principles of solid phase protein coupling and antibody-antigen interactions were exploited in enzyme linked immunosorbent assays (ELISA). By calibrating the antibody-antigen reactivity, nanogram variations in antigen contents of samples were detectable. The procedure for calibration and antigen measurement was as follows.

The strength of each antibody was measured by calibration using a range of antigen and antibody concentrations. To prime microtitre plates, 100µl of the coating solution containing 50mM carbonate/bicarbonate buffer (pH9.6) were added to each microwell and left for 2hr at room temperature. Plates were then emptied by shaking and striking them against clean tissue papers. To store microtiter plates, they were covered with parafilm sheets and kept a room temperature.

Pre-coated plates were loaded with 50µl aliquots of solutions containing different amounts of antigen (e.g., 1mg/ml to 1µg/ml) and kept in a moist box overnight at 4°C. Each assay was in duplicate. Plates were washed 5 times with phosphate buffered saline (PBS) (pH7.5) containing 0.05% Tween 20. Microwells were blocked with 100µl PBS, containing 0.05% Tween 20 and 3% BSA (w/v), for 1-2hr at room temperature. Plates were then washed twice with PBS and loaded with 50µl aliquots of wash buffer containing varying amounts of antibody (e.g., 1:100 - 1:1000 dilution). Plates were then covered and kept at room temperature for 4hr in a moist box.

Plates were washed 5 times and loaded with 50µl aliquots of horseradish peroxidase (HRP)-conjugated anti-rabbit antibody (1:500 v/v, in wash buffer) and left covered for

3hr at room temperature in a moist box. They were then washed 5 more times and loaded with 50µl of HRP substrate, O-phenylene diamine dihydrochloride, in 100mM citrate buffer (pH5.0) containing 0.02% H_2O_2. The time taken for optimum colour development was recorded and the reaction was stopped by adding 2M sulphuric acid. The optical density was measured at 496nm.

The first track of each plate was loaded with wash buffer instead of antigen solutions to provide a blank reading. The second track was loaded with the highest concentration of antigen and no primary antibody solution was added. This controlled for interaction between the antigen and secondary antibody. A calibration plot of absorption against antigen concentration at different antibody concentrations was drawn. From the graph an optimum concentration of antibody for a given range of antigen expression in the linear portion of the curve was selected and used in behavioural experiments in which the variations in antigen immunogenicity between MeA-trained and W-trained birds were examined.

Chick brains were freshly prepared and dissected on ice as described in Chapter 2. Four different regions, left and right forebrain roof, and left, and right forebrain base were obtained and their R15 antigen titers tested 6hr and 24hr after training. After dissection, brain tissues were rapidly homogenized in Tris buffered saline containing 0.01% phenylmethylsulfonylfluoride (PMSF), 1µg/ml leupeptin and 5mM ethylenediaminetetraacetic acid (EDTA) (pH7.6). The protein concentration of samples was adjusted to 1mg/ml and they were then stored at -70°C. By standardizing the protein contents of samples before ELISA smaller variances between duplicate readings were obtained.

Immunocytochemistry

Immunocytochemistry was carried out at both light and electron microscopic levels (LM and EM respectively). An indirect method of immunolabelling involving HRP-conjugated anti-rabbit antibody was used.

I. LM immunocytochemistry : Freshly dissected forebrains were dipped into dry ice-cooled isopentane for 5sec and removed with a pair of forceps. They were then either kept at -70°C or mounted on a Cryostat platform and brain slices of 20μm thickness cut at -20°C. Slices were laid on poly-L-lysine coated microscope slides and kept at -70°C pending further processing. Tissue sections were fixed with acetone for a total of 10min and washed 3 times for 15min in 100mM PBS (pH7.4). Endogenous peroxidase activity was blocked by treating the sections with 15% H_2O_2 for 15min. Sections were washed as before and placed in block solution for 1hr. The block solution was PBS (pH7.4) containing 20% normal goat serum. Sections were then washed and overlaid with either primary antibody (R15, 1:250) or pre-immune serum (1:250) or block solution. Dilutions of R15 were made in the block solution. The latter two conditions were set up to examine the specificity of R15 labelling. Sections were then kept in a moist box overnight at room temperature.

On the following day, sections were washed 3 times and secondary antibody, HRP-conjugated goat anti-rabbit (1:250), was added to the sections for 3hr. Sections were washed 3 more times and overlaid with substrate solution (diaminobenzidine (DAB)). DAB (0.05% w/v) was freshly made in TBS containing 0.03% H_2O_2 (pH7.4). Preparations were fully developed within 10min. The reaction was quenched by several washes with PBS. DAPI (4,6-diamido-2-phenylindole) (1% solution) a fluorescent reagent specific for the cell nucleus, was then used on some sections as a counter stain. The appearance of different areas of forebrain and cerebellum were recorded using an Axiophot™ microscope (Zeiss, Germany) fitted with fluorescent filters. Sections were

then dehydrated gradually by passing through 30%, 50%, 70%, 80%, 90% and 100% ethanol and preserved using DPX Mountant ™ (BDH).

II. EM Immunocytochemistry : In order to establish the optimum conditions for tissue preparation and antibody-antigen interactions several procedural variations were examined. The IMHV and cerebellum from day-old chick brains were dissected out using a brain mould. Small tissue cubes ($1mm^3$) from these structures were rapidly prepared and immersed in a freshly prepared fixative solution consisting of 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1M phosphate buffer (pH7.4) for 1hr at room temperature. The following steps were then taken:

A. Osmication : Samples were osmicated by placing them in a phosphate buffer (0.1M) containing 1% osmium tetroxide for 1hr at room temperature. This was done to preserve membrane integrity and maintain a high level of ultrastructural detail. However, since the effect of osmium on antigen presentation was unknown in our system, samples were divided in two portions with one portion not being osmicated.

B. Dehydration : Samples from both portions were dehydrated through a graded series of 25% to 100% ethanol.

C. Embedding : Various resins were used to embed the samples. This was done to assess which type best preserved antigen immunogenicity. Samples were first immersed in a 50% resin solution for 4hr, and then transferred into neat resin solution with two to three changes. Resins used were: Polarbed (polymerization at 60°C for 24hr), LR White (polymerization at 50°C for 24hr), and Araldite (polymerisation at 60°C for 24hr).

D. Ultrathin sections (approximately 100nm thickness) were cut on a Reichert™ OMU4

ultramicrotome and collected on 200 mesh copper grids coated with carbon/formvar (polyvinyl formal) film (BioRad).

E. Etching : The surface resin was etched off from the surface of Polarbed and Araldite resin embedded sections using 10% H_2O_2 treatment for 10min. This and the following steps were performed in duplicate by placing the section side of grids onto 20µl drops of appropriate reagents held on parafilm. Before placing grids onto the next reagent, they were blotted on a piece of filter paper without touching the sections themselves. The subsequent immunocytochemical procedure was essentially the same as that described under LM immunocytochemistry.

Etched sections were washed 3 times with double distilled water (DDW) for 15min before immunoblotting. They were then blocked with 1-5% solutions of bovine serum albumin (BSA) in PBS (pH7.4) for 30min at room temperature. In a number of studies 1% Tween 20 was included in the block solution. Sections were overlaid with R15 (1:2, 1:5, 1:10, 1:50, 1:100, and 1:1000 dilutions) or pre-immune serum (1:5, 1:10, and 1:50 dilutions) made in the block solution and incubated overnight at 4°C, or at room temperature for 2hr, in a moist atmosphere. Samples were then washed 3 times in block solution for 15min, and transferred to 20µl drops of protein A/gold solution (15nm diameter gold particles) at 1:50 dilution made in block solution for 1hr at room temperature. Samples were washed as before and then stream-washed with DDW for 30sec and air-dried. Sections were stained with aqueous uranyl acetate and lead citrate in an LKB Ultrastainer™ and examined under a JEOL 100S transmission electron microscope.

RESULTS

In this study 19 rabbits (R) were injected with different putative glycoprotein components resolved by SDS-PAGE. Results from the serum activity of R0, R2, R4, R5, R6, R10, R11, R13, and R15 on chick forebrain TCA precipitable material (homogenate), SPMs, and PSDs are presented in Fig. 5.1. Each serum contained a different combination of antibodies recognizing different epitopes on Western blots. R2, R13, and R15 showed a high degree of specificity by labelling a small number of epitopes. R2 strongly recognized a protein component of Mr 90kD and several other protein components. R13 and R15 were, however, more specific; R13 recognized a doublet of Mr 49 and 55kD, and R15 recognized a single protein band of Mr 60-62kD.

It was, therefore, decided to purify the R15 IgG by affinity chromatography and examine its affinity. Fig. 5.2 shows the result of purified R15 immunolabelling on homogenate, SPMs and PSDs. In order to control for the specificity of R15 labelling, parallel immunoblots were run in the presence and absence of pre-immune serum as primary antibody. R15 was found to have retained its affinity for the 60-62kD protein component.

In order to establish whether R15 antigen is fucosylated, homogenate, SPMs and PSD component were resolved on 1-D gels and transferred onto nitrocellulose paper as described in Chapter 6. The lectin Ulex Europaeus Type 1 (UEA.I) was used on one blot to label fucosylglycoproteins while, R15 was used on the other blot to label the 60-62kD component. Fig. 5.3 shows that the protein component recognized by R15 (5.3a) also showed a high affinity for UEA.I (5.3b). It was, therefore, concluded that the R15 antigen was a fucose-containing glycoprotein.

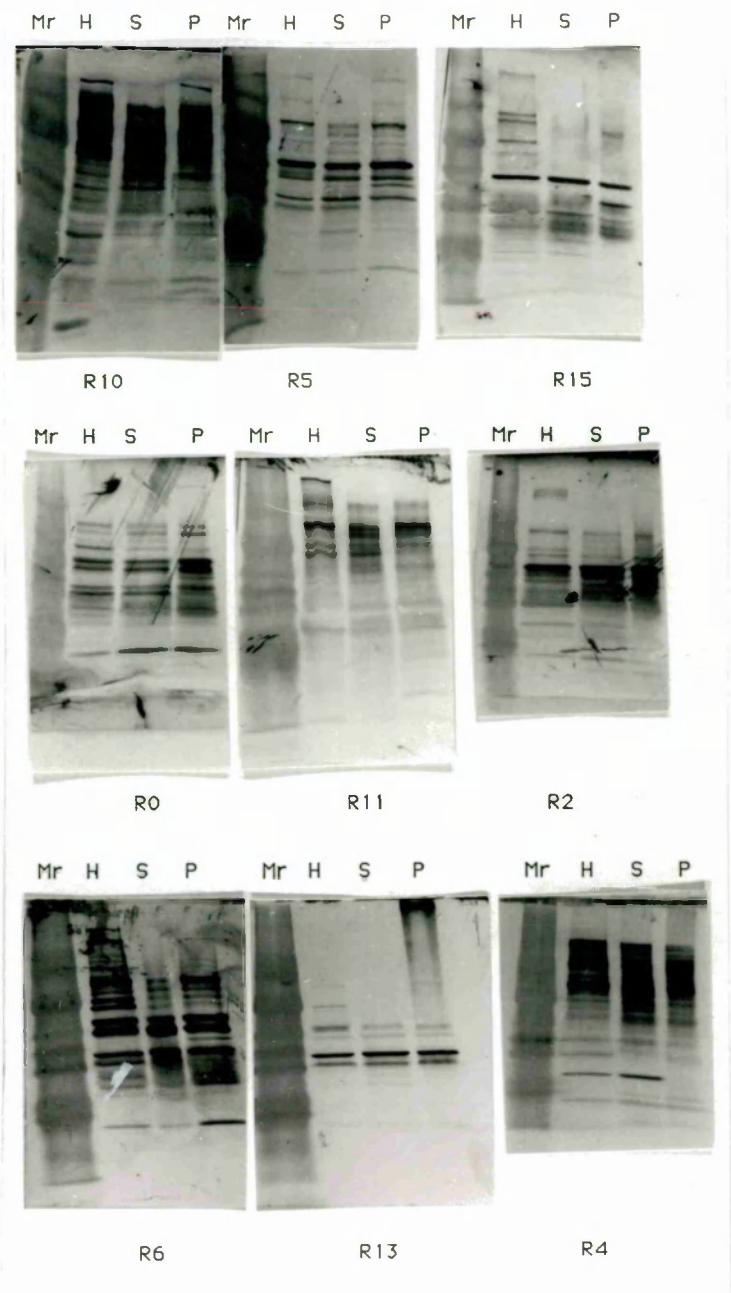


Figure 5.1 : The patterns on Western blots of chick forebrain antigens recognized by various rabbit sera containing different polyclonal antibodies.

TCA precipitable material (homogenate) resolved by 1-D PAGE was divided into 8 major molecular weight bands, homogenized and administered to rabbits as described under Methods. Each blot contains, from left to right, standard molecular weight markers, homogenate, SPM and PSD samples.

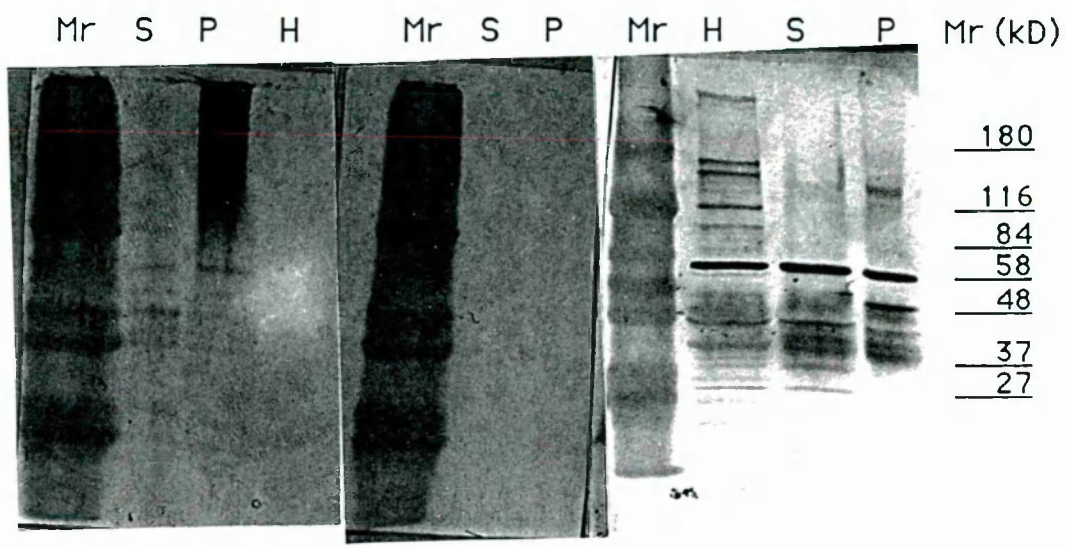


Figure 5.2 : Immunoblotting patterns of homogenate (H), SPM (S) and PSD (P) samples.

Part (a) shows an immunoblot using pre-immune serum; part (b) shows the same blot in the absence of primary antibody; (c) shows an immunoblot using R15. The antibody recognized a Mr140kD protein in the homogenate and a 60-62kD protein in SPMs and PSDs.

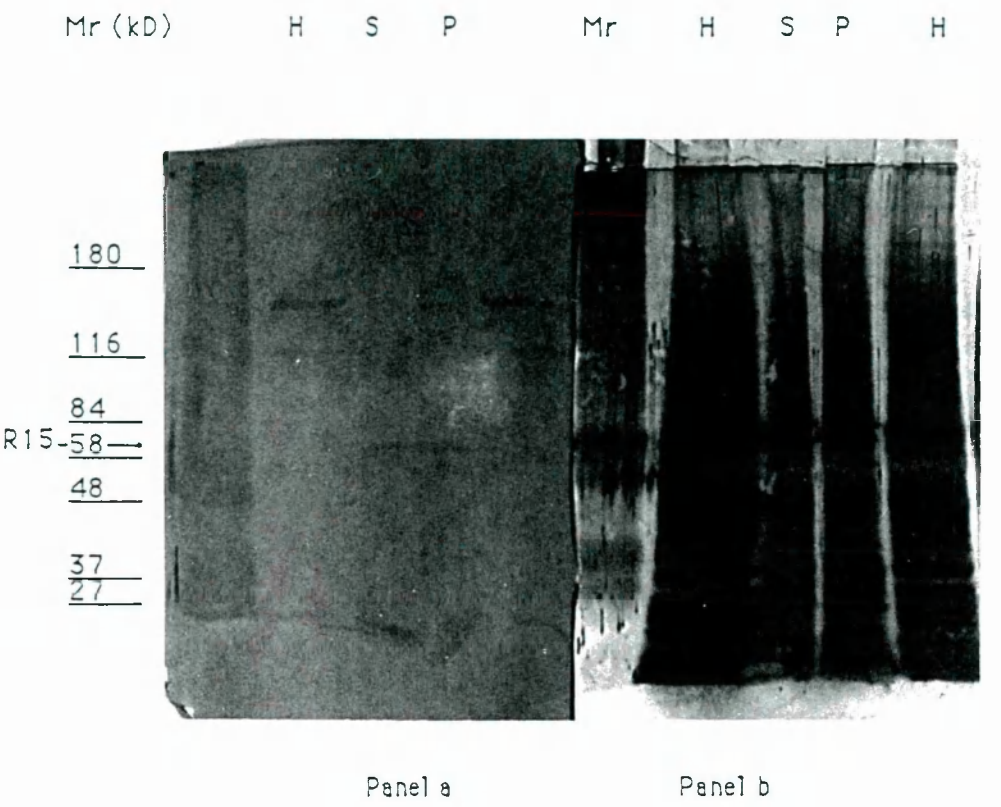


Figure 5.3 : Pattern of R15 antigen recognition (a) alongside that of fucosylglycoprotein occurrence (b) in chick forebrain detected using UEA.I a fucose-specific lectin.

The R15 antigen was shown to co-migrate with a 60-62kD fucosylglycoprotein in SPMs (S) and PSDs (P). This was apparently absent in the homogenate (H). Mr, standard molecular weight marker.

Effect of R15 on passive avoidance learning

Two groups of chicks were trained on MeA beads and were given bilateral intracerebral injections of either R15 (10μg IgG/10μl/hemisphere) or saline (10μl/hemisphere). In a parallel study, two groups of MeA birds were given bilateral intracerebral injections of either pre-immune serum (10μg protein/10μl/hemisphere) or saline (10μl/hemisphere). The latter study was carried out to examine whether pre-immune serum interfered with memory processing. Four injection time-points were chosen, 1.5hr before, 30min before, 30min after, and 5.5hr after training. All chicks were tested blind 24hr after training.

Fig. 5.4 shows that 44% of chicks injected with R15 30min before training showed retention 24hr after training. This contrasted with the 81% retention seen in saline-injected birds. A similar result was obtained from chicks that received R15 30min after training (44% retention). However, no results were obtained from the corresponding saline injected birds since, 24hr after training, saline injected birds showed signs of illness, and consequently were not tested. No clear explanation for this finding was apparent although the possibility of saline contamination was not ruled out. No statistical tests were carried out for this time-point. The retention values of R15 injected birds for other time-points were similar to those that received saline. Chicks injected with R15 were inspected several times after injection and before test; no visible behavioural side-effects were seen throughout the entire period. Also, 24hr after training the avoidance scores of saline injected birds were similar to those that received pre-immune serum injections.

In order to assess whether R15 injection influenced the pecking tendency in chicks the percentage of pecks during pretraining was calculated in both the saline- and R15-injected groups. Results are shown in Table 5.1. R15 injection did not seem to increase chicks' tendency to peck when compared, either with saline-treated groups, or those that received the antibody after training.

Number	Agent injected	Injection Time-point	Percent pecking
1	R15	-1hr	88
2	Saline	-1hr	90
3	R15	-30min	90
4	Saline	-30min	91
5	R15	+5hr	81
6	Saline	+5hr	90

Table 5.1 : The percentage of pecks during pretraining. Chicks were pretrained and trained on passive avoidance task and given either saline or R15 injections before or after training. Each group consisted of 16-70 birds. -, before training; +, after training.

Enzyme linked immunosorbent assay

ELISA was used to assess whether the immunogenicity of R15 antigen was affected during learning and memory formation. Two time-points, 6hr and 24hr after training, and four different brain regions, forebrain roof (left and right) and forebrain base (left and right) chosen to test possible regional variations were used. ELISA was carried out on tissue homogenates. The number of birds used in each W and MeA group ranged between 18 to 21.

Fig. 5.5 shows results of an R15 calibration experiment. The antibody was found to be sensitive and, therefore, a 1:500 dilution that gave relatively a wide range of linearity was chosen for the next experiment. For this dilution, 1.25μg of antigen was used in each microwell.

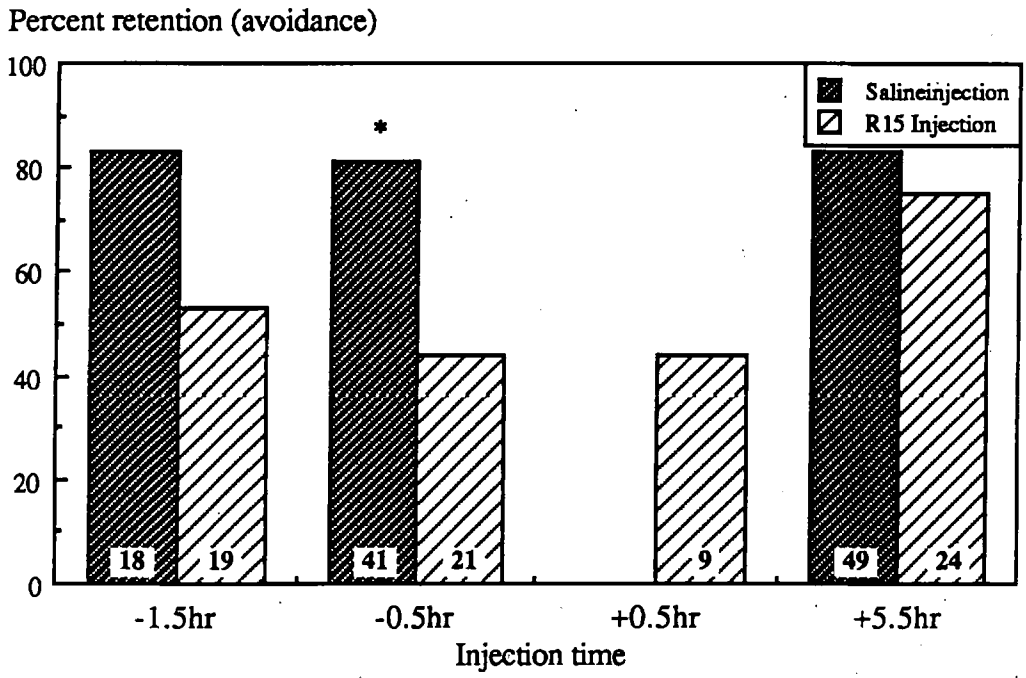
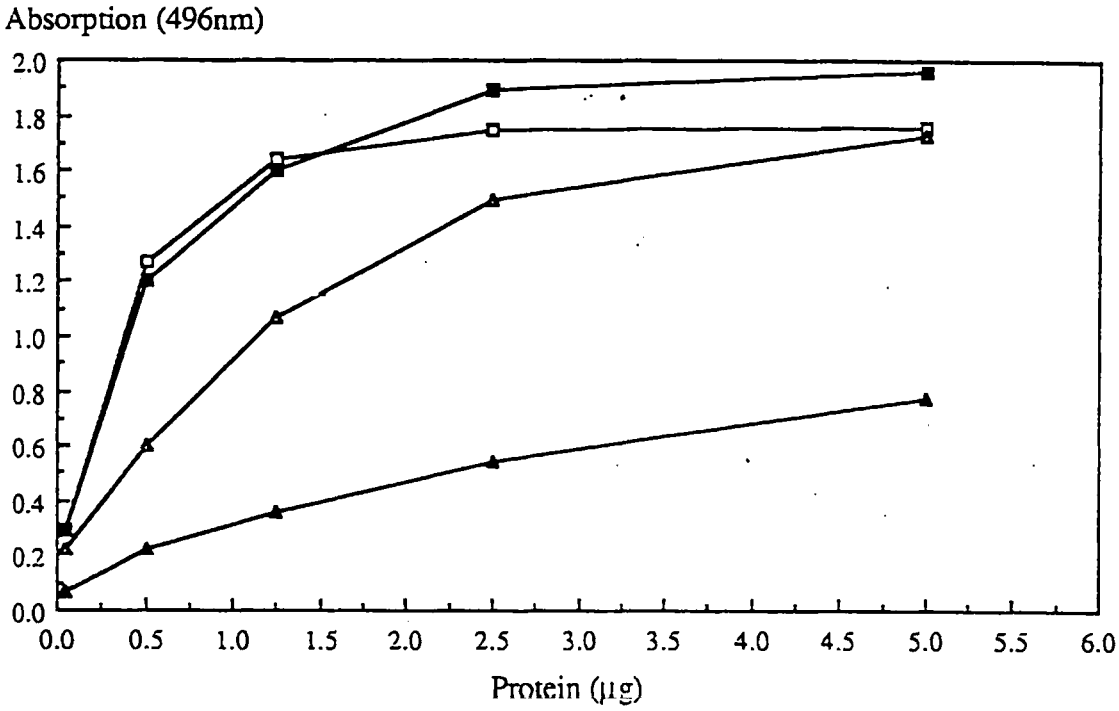


Figure 5.4 : The effect of R15 on passive avoidance learning.

Day-old chicks were trained and given bilateral intracerebral injections of saline (control) and R15 at different times before or after training. All chicks were MeA-trained and tested 24hr after training. The number of birds used in each condition is given in each bar. *$p<0.001$.



5.5 : The ELISA calibration graph of R15 using four different antibody

1:100; solid squares, 1:250; empty triangles, 1:500; solid

0. The longest linear region was given by the R15 dilution of

Figs. 5.6a&b and Table 5.2 show the results of R15 antigen immunogenicity 6hr and 24hr after passive avoidance learning. As can be seen, 6hr after training, the R15 absorption in MeA birds was significantly higher than in corresponding control groups in all four brain regions studied. By 24hr, however, this relationship was reversed; all MeA birds exhibited a large drop in R15 absorption as compared W birds. When studying R15 absorption of control groups at the two time-points (Fig. 5.7b), and also the experimental groups at the two time-points (Fig. 5.7a), the relative changes in absorption were seen to be much larger in the latter rather than former the groups.

Cellular and sub-cellular localization of R15 antigen

Results for both forebrain and cerebellum areas at different magnifications are shown in Figs 5.8-5.10. In the cerebellum, Purkinje cells and the molecular and Granular cell layers were strongly labelled by R15 (Figs. 5.3.10a & b). In the forebrain, structures like hippocampus and hyperstriatum ventrale, neostriatum and LPO were also strongly and uniformly labelled by the antibody (Fig. 5.8a-e). The staining patterns in the optic lobe was very regular with major identified layers (Karten & Hodos, 1967) like stratum album centrale (SAC), stratum griseum centrale (SGC), stratum griseum et fibrosum superficiale (SGF) and stratum opticum (SO) showing strong affinity for R15 (Fig. 5.9a). Nucleated structures, such as nucleus isthmi, pars magnocellularis (IMC) and nucleus isthmi pars parvocellularis (IPC) in this region, were characteristically labelled by R15 (Fig. 5.9b). To test the specificity of R15, a negative control (pre-immune serum) was used in parallel with the antibody and showed no labelling (Fig. 5.8c). Also, the affinity of secondary antibody (horseradish peroxidase-conjugated goat anti-rabbit IgG) was tested by excluding primary antibody during immunocytochemistry, and again no labelling was observed.

Fig. 5.6a

Absorption Values at 496nm, 6hr posttraining (x10exp-3)

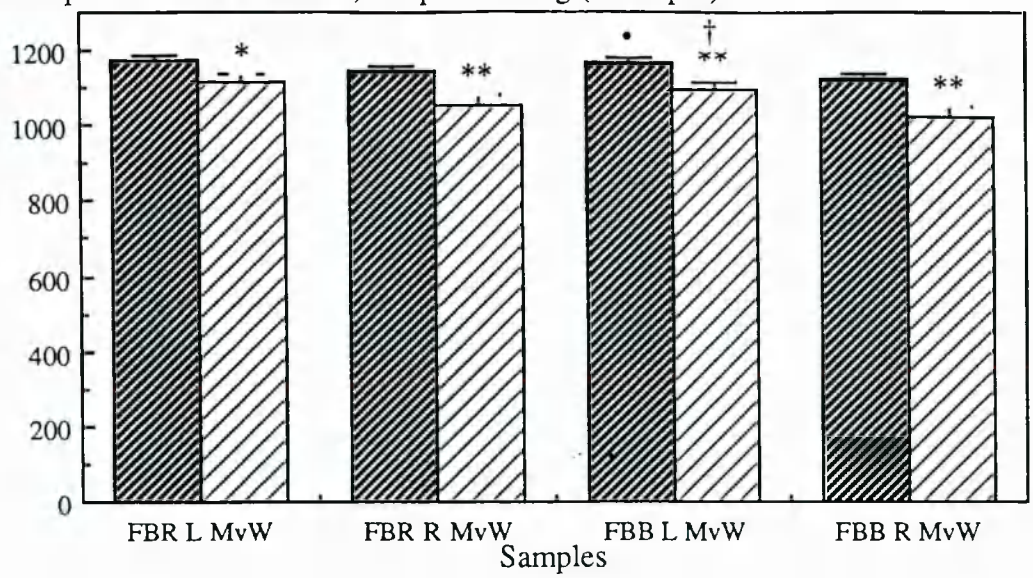


Fig. 5.6b

Absorption Values at 496nm, 24hr posttraining (x10exp-3)

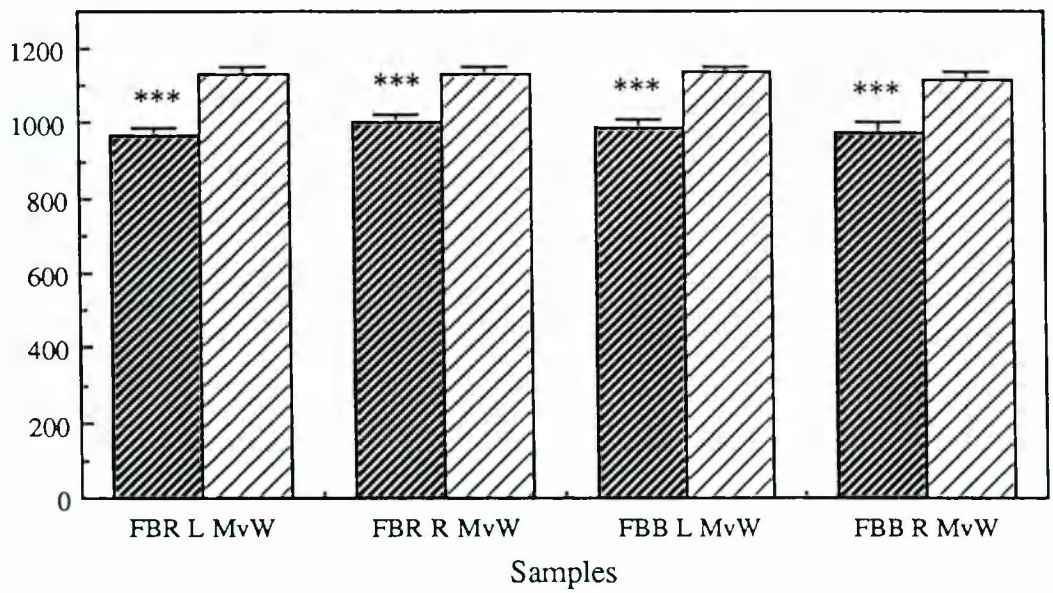


Figure 5.6 : The effect of passive avoidance learning on R15 immunoreactivity absorption 6hr (panel a) and 24hr (panel b) after training.

Dark-shaded bars, MeA birds; light-shaded bars, W birds. FBR, forebrain roof; FBB, forebrain base; L, left; R, right; *p< 0.03; **p<0.005; ***p<0.0005 between W and MeA groups; •p<0.03 between FBBLM and FBBRM; †p<0.03 between FBBLW and FBBRW.

Fig. 5.7a
Absorption Values at 496nm, 6&24hr posttraining, MeA chicks (x10exp-3)

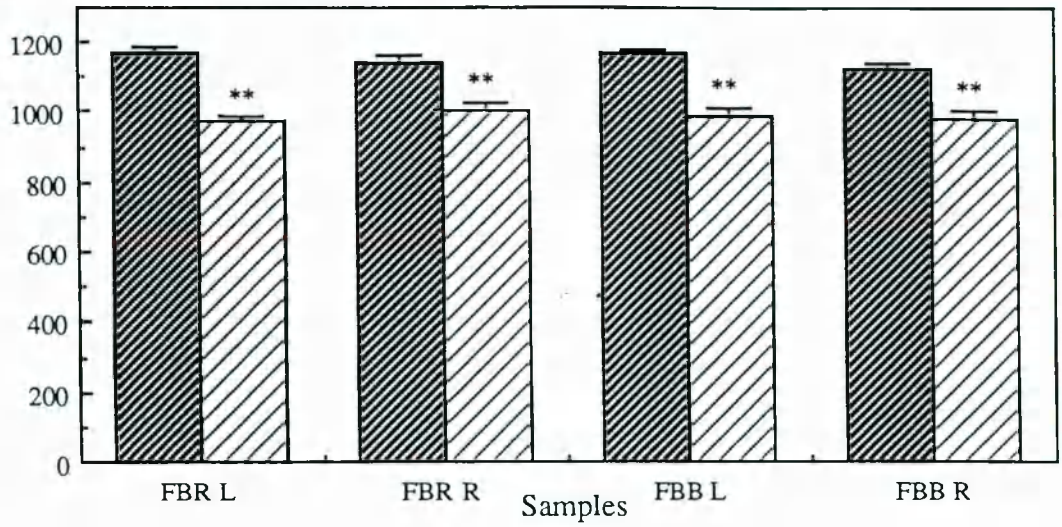


Fig. 5.7b
Absorption Values at 496nm, 6&24hr posttraining, W chicks (x10exp-3)

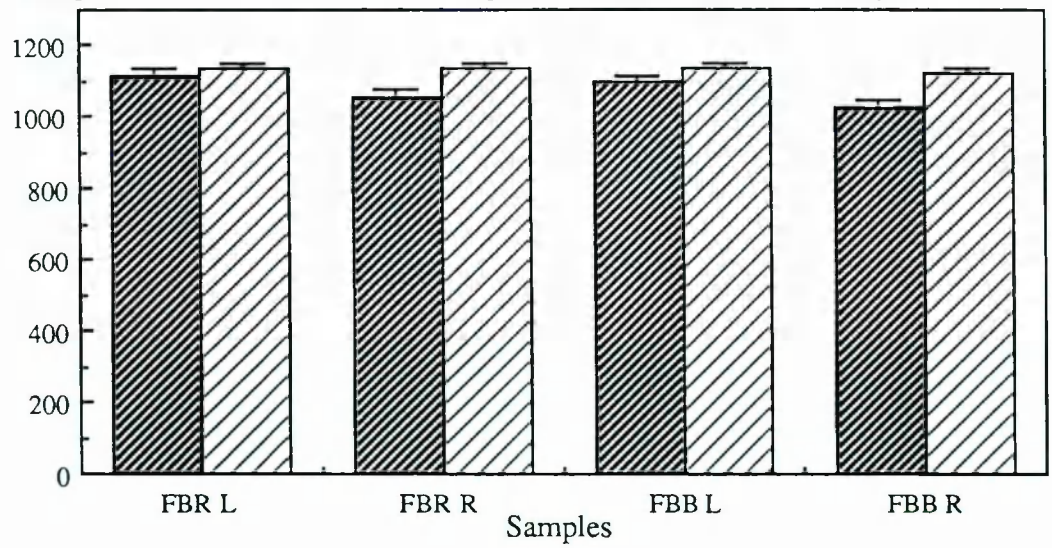
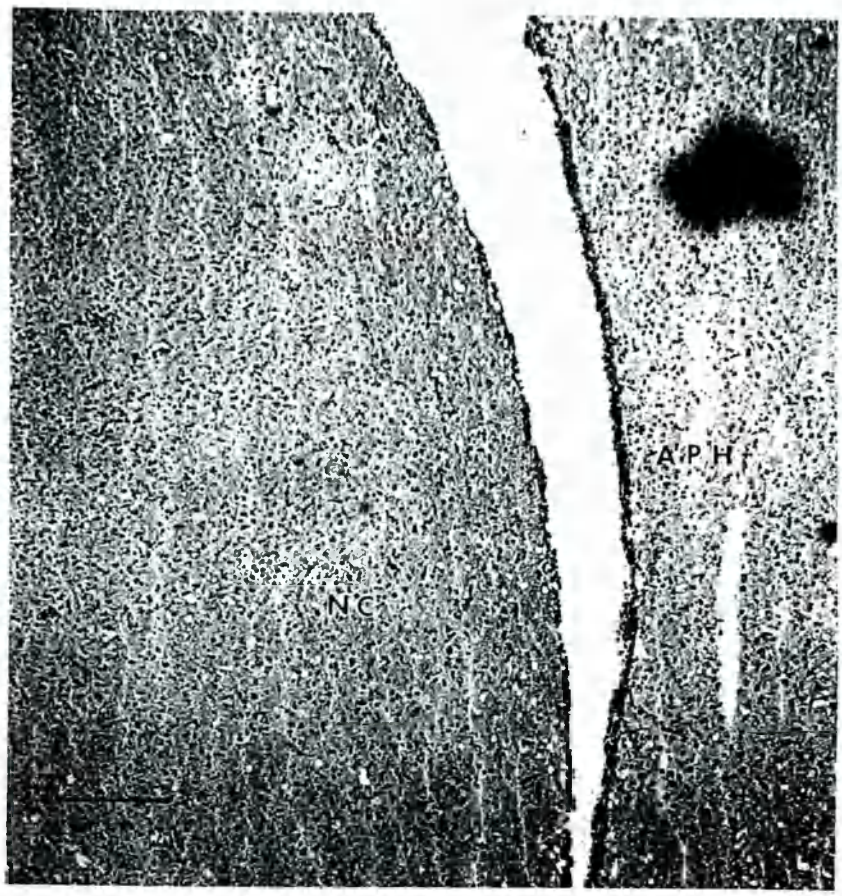


Figure 5.7 : Comparison of immunoreactivity absorption of MeA birds (panel a) and W birds (panel b) at 6hr and 24hr post-training times.

Dark-shaded bars, 6hr posttraining, light-shaded bars, 24hr posttraining. FBR, forebrain roof; FBB, forebrain base; L, left; R, right. **p<0.0005.



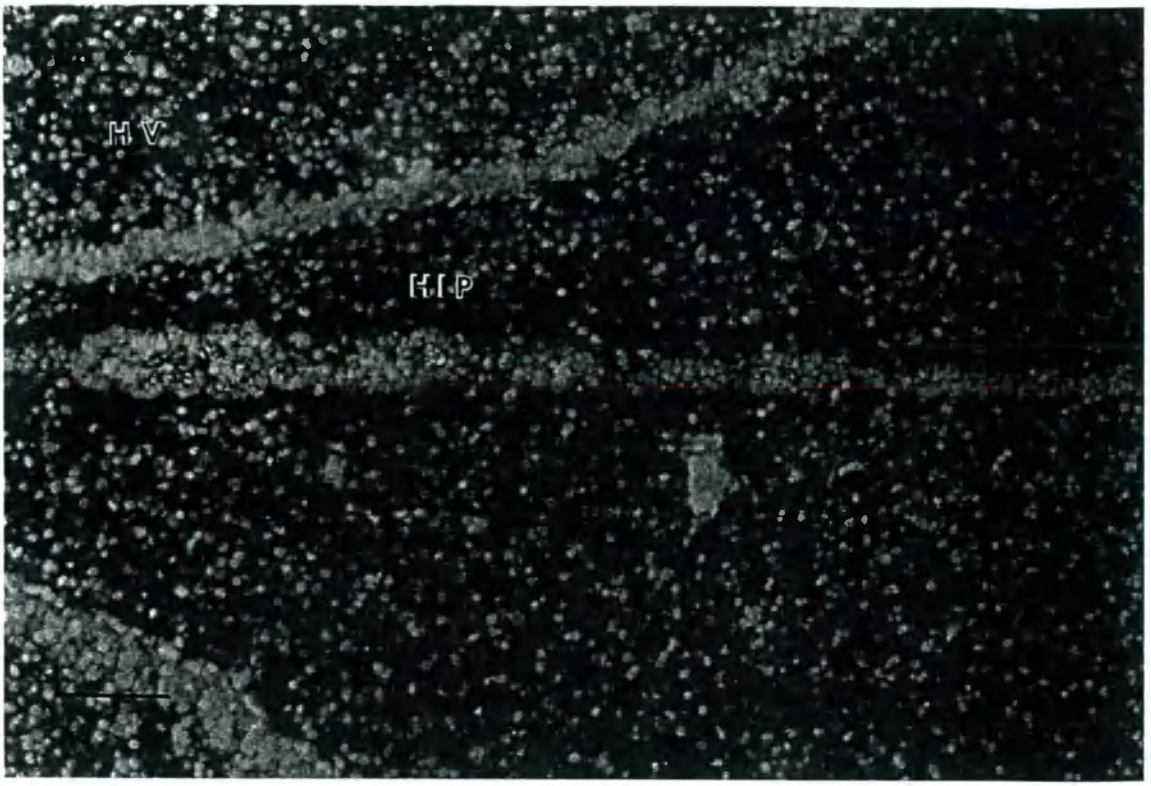
Panel a

Figure 5.8 : Structural localization of R15 antigen in day-old chicks brain (panels a, b and c).

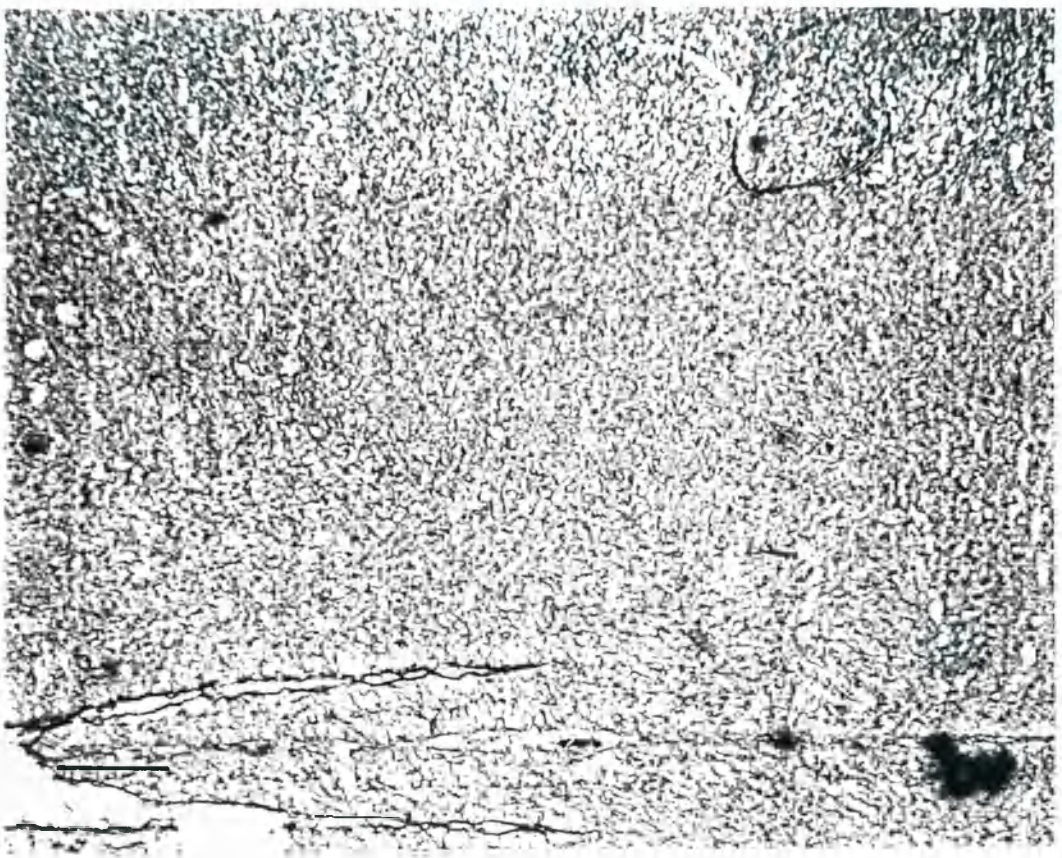
The pattern of nuclear labelling by DAPI counter-stain is shown in panel d. Results of pre-immune serum labelling suggested that the labelling pattern obtained when using R15 was exclusively due to the antibody (panel e). APH, Area parahippocampalis; Hip, hippocampus; HV, hyperstriatum ventrale; N, neostriatum; NC, neostriatum caudale. Scale bar 500μm.

Panel b

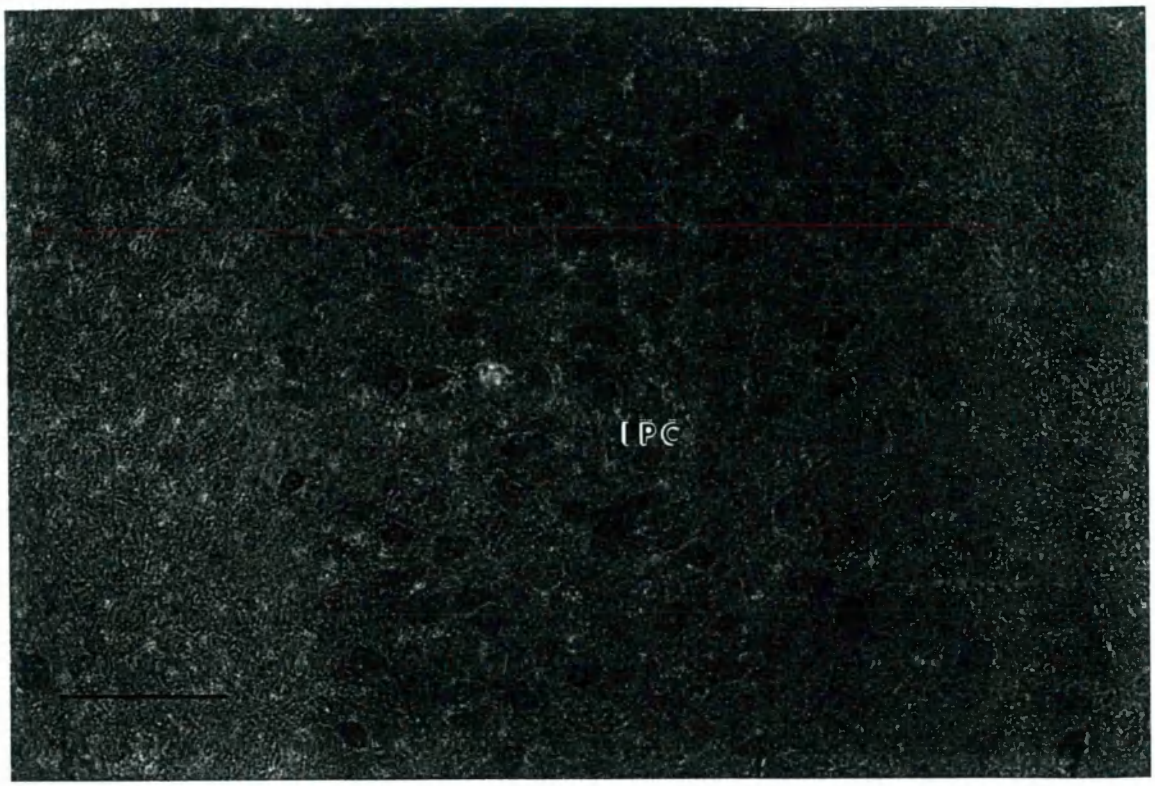
Panel c



Panel d



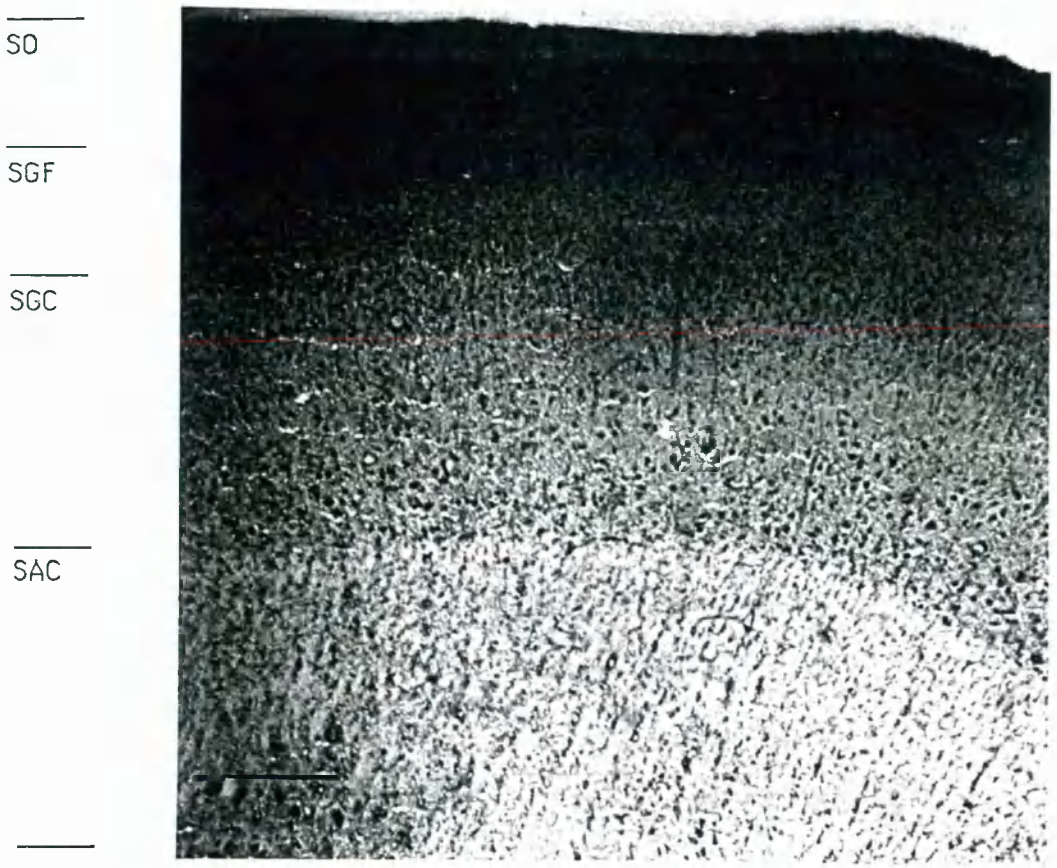
Panel e



Panel c

Figure 5.9 : The pattern of R15 immunolabelling in the optic lobe showing at least 13 major layers in this structure (panels a & b).

Immunocytochemical localization of R15 antigen in area IMC at higher magnification and nuclear counter-staining by DAPI is shown in panel c. Cell body, nuclear and occasional neural processes labelled by R15 are evident. IMC, nucleus isthmi, pars magnocellularis; IPC, nucleus isthmi pars parvocellularis; SAC, stratum album centrale; SGC, stratum griseum; SGF, stratum griseum et fibrosum superficiale; SO stratum opticum. Scale bar 500µm.

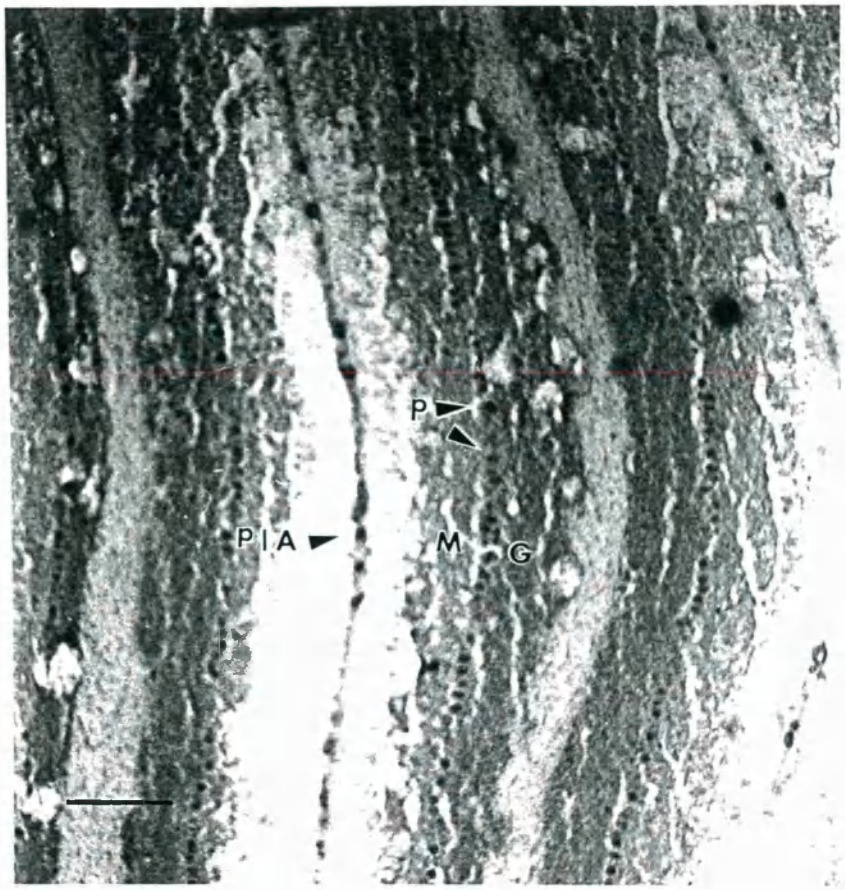


Panel a

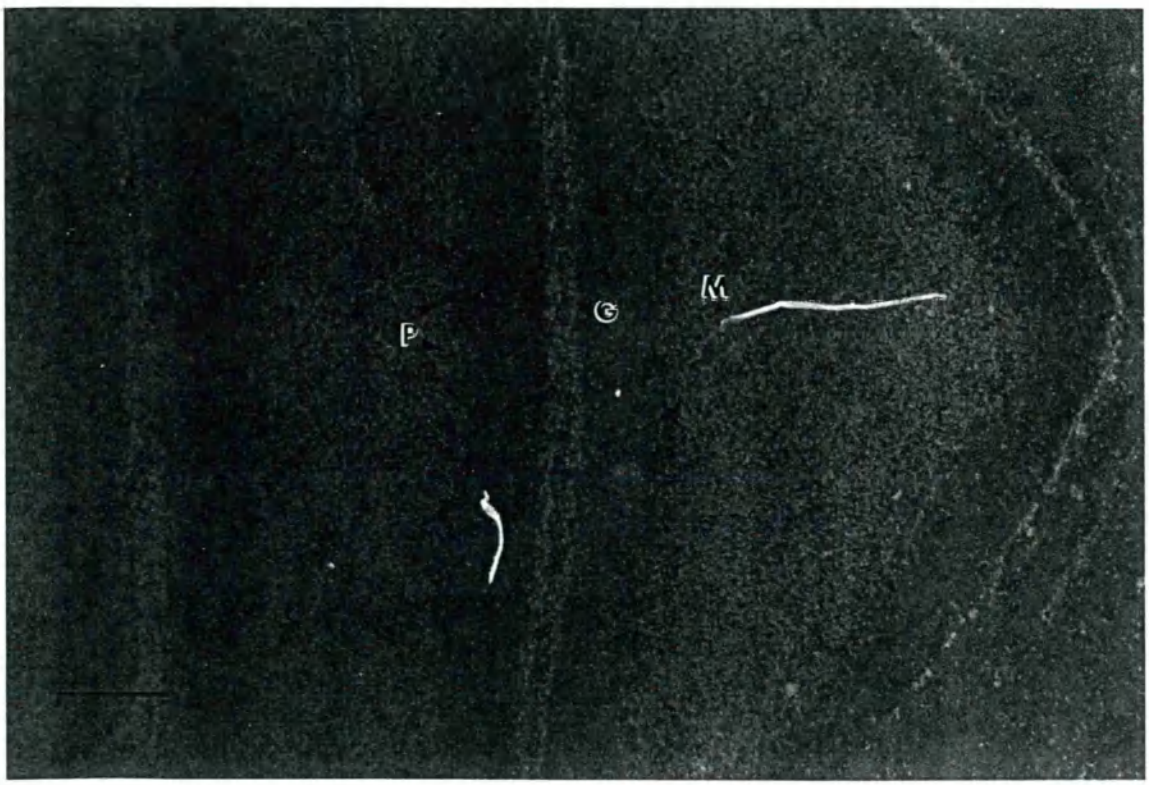
Panel b

Figure 5.10 : The pattern of R15 immunolabelling in cerebellum.

Purkinje cell layer (P), molecular layer (M) and granular layer (G) are positively labelled. Purkinji layer cells gave a relatively high degree of staining (panel a). Nuclear counter-staining is also shown in this structure (panel b). Pia, pia mater. Scale bar 500μm.



Panel a



Panel b

Sample		Absorption	sem	MeA/W (%)
FBRL MeA	(6hr)	1170*	±14.5	105.1
FBRL W		1113	±20.1	
FBRR MeA		1141**	±18.8	108.5
FBRR W		1052	±27.2	
FBBL MeA		1168**	±12.1	106.5
FBBL W		1097	±18.8	
FBBR MeA		1125**	±13.8	109.9
FBBR W		1023	±25.3	
FBRL MeA	(24hr)	971***	±16.1	85.7
FBRL W		1133	±19.3	
FBRR MeA		1003***	±20.3	88.5
FBRR W		1133	±15.8	
FBBL MeA		991***	±16.4	87.3
FBBL W		1136	±13.1	
FBBR MeA		977***	±24.3	87.3
FBBR W		1119	±18.0	

Table 5.2 : R15 absorption obtained by ELISA 6hr and 24hr after training chicks on passive avoidance paradigm.

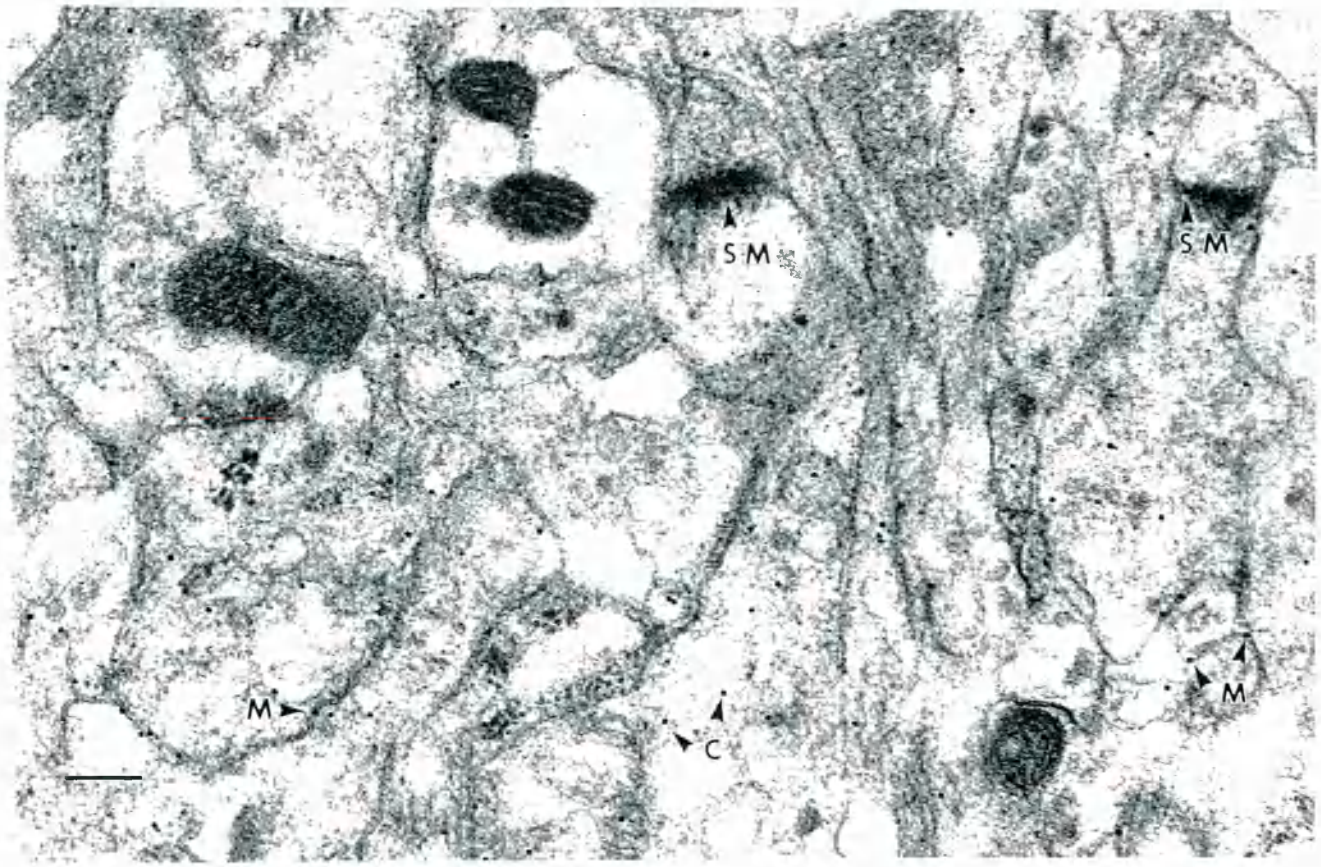
1.25µg/50µl antigen was used per microwell. This was overlaid with R15 at a dilution factor 1:500. Statistical comparisons (Student t test) were carried out on test and control groups from corresponding forebrain regions for each time-point. FBR, forebrain roof; FBB, forebrain base; R, right hemisphere; L, left hemisphere; . *=$p<0.03$; **=$p<0.01$; ***=$p<0.0005$.

Under the light microscope the antibody recognized cell nuclei/nuclear envelope. In fact when DAPI, a specific fluorescent nuclear stain, was used on R15-treated slides, the labelling patterns were found to differ from those of R15. Under close inspection (magnification 400X), the nuclei stained by R15 were also seen to have been labelled by DAPI. However, as seen in Fig. 5.9c, R15 labelled only a specific subset of nuclei, implying that its antigen is distributed rather specifically, depending on the cell type.

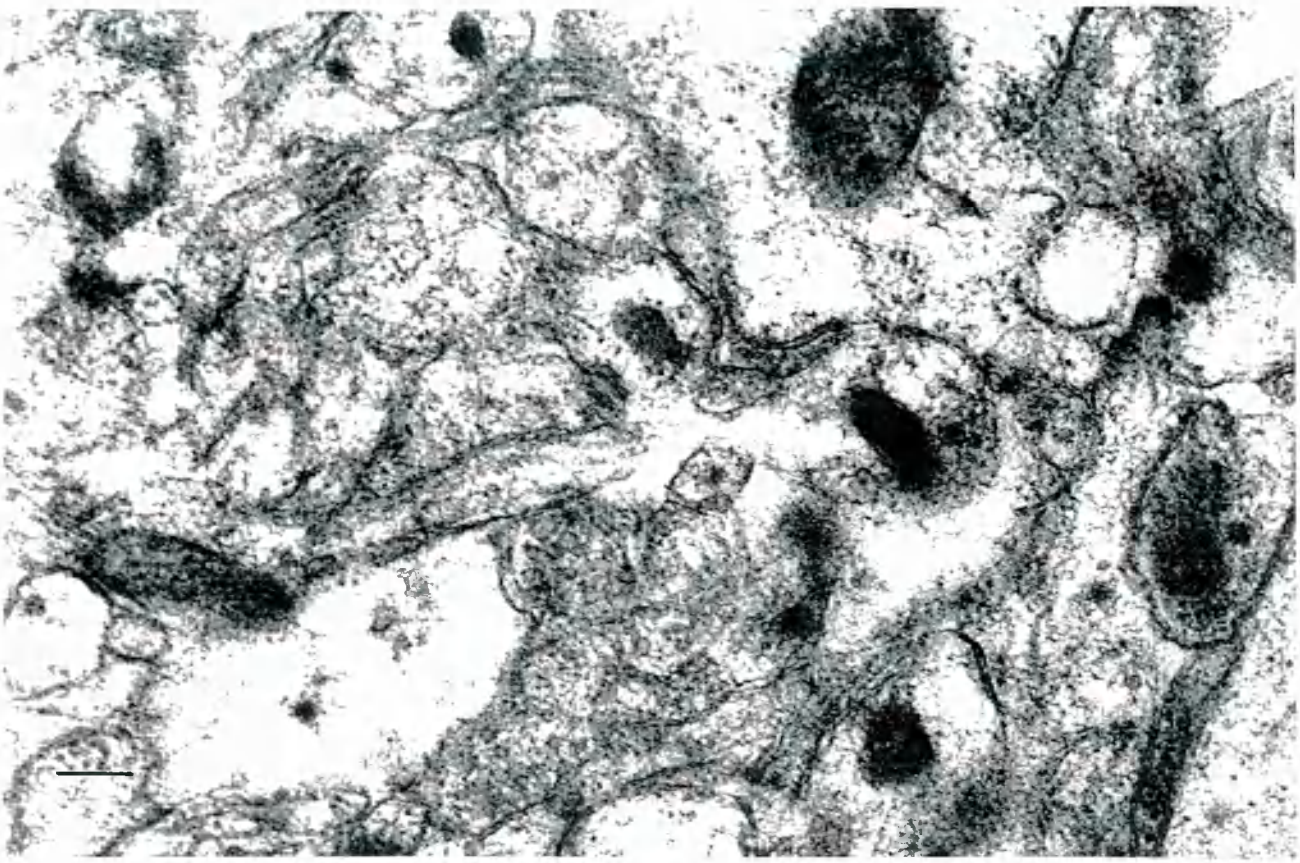
The best EM immunolabelling was obtained when immersion fixed (4% paraformaldehyde, 0.1% glutaraldehyde) IMHV and cerebellum samples were osmicated (1%), embedded in LR White and left unetched. Immunocytochemistry was then carried out using PBS buffer solution containing BSA (5%) and Tween 20 (1%) (pH7.4) throughout the procedure. R15 at a dilution factor of 1:10 gave the highest labelling density. No non-specific labelling was observed on the resin itself. The labelling of the nuclei was interpreted as partially non-specific since protein A/gold particles are known to have a tendency to adhere to cell nuclei. Pre-immune serum used in parallel with R15 did not recognize any antigen. Results of the EM study are shown in Figs 5.11-13. Medium density labelling of tissue was seen mainly on membranes of axons and dendrites with the occasional labelling of synaptic membranes and postsynaptic densities. Since it was difficult to precisely localize synaptic clefts on most micrographs, it was not possible to ascertain whether R15 differentially labelled symmetric and asymmetric synapses. However, R15 labelled only a subset of presynaptic and postsynaptic membranes which suggested a specific distribution of R15 antigen. There was also a small amount of label in the cytoplasm of axons, dendrites and boutons.

Figure 5.11 : Electron micrographs of IMHV from day-old chicks immunolabelled with R15 (panel a), and pre-immune serum (panel b).

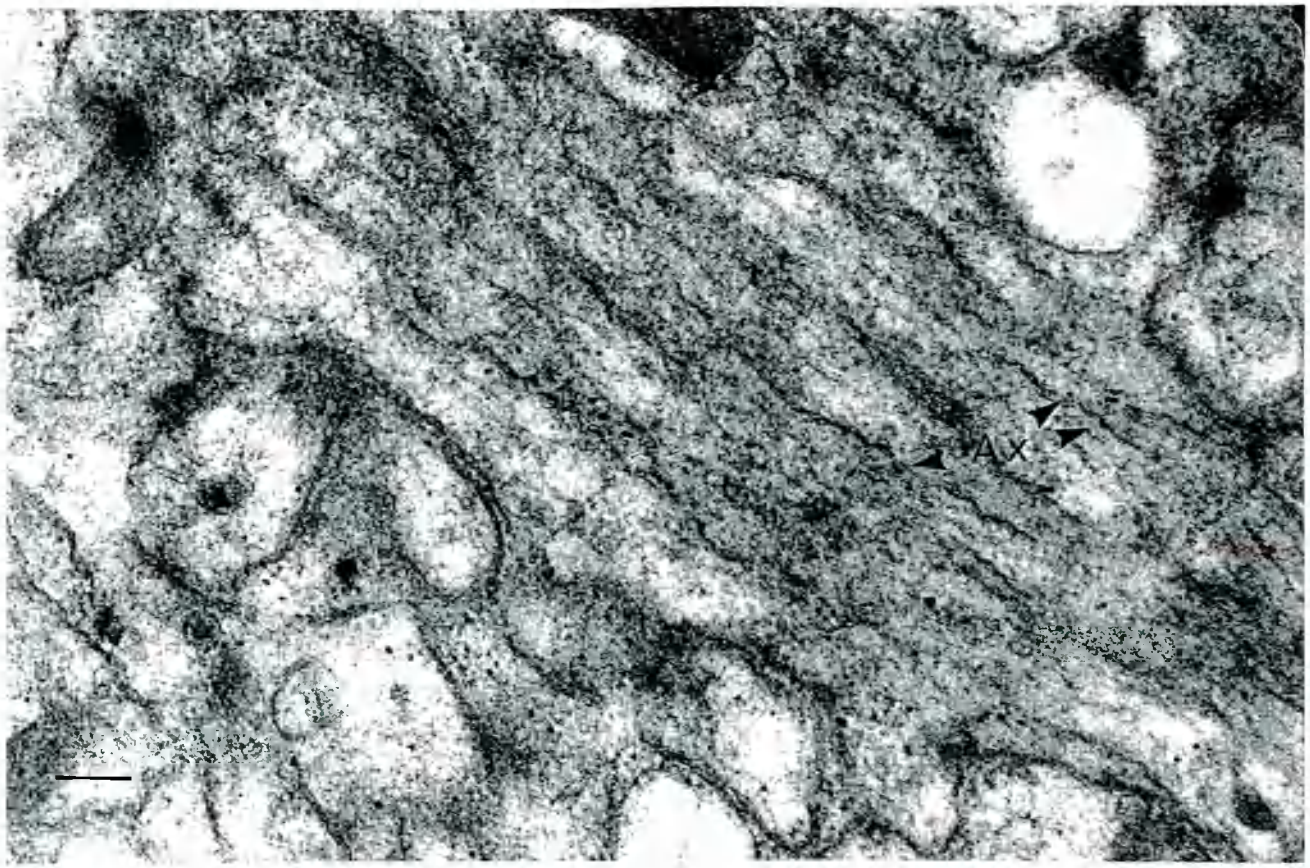
Protein A/gold label was seen on the membranes (M), synaptic membranes (SM), and occasionally cytoplasm (C). Results of pre-immune serum labelling suggested a high binding specificity for R15. Scale bar 200nm.



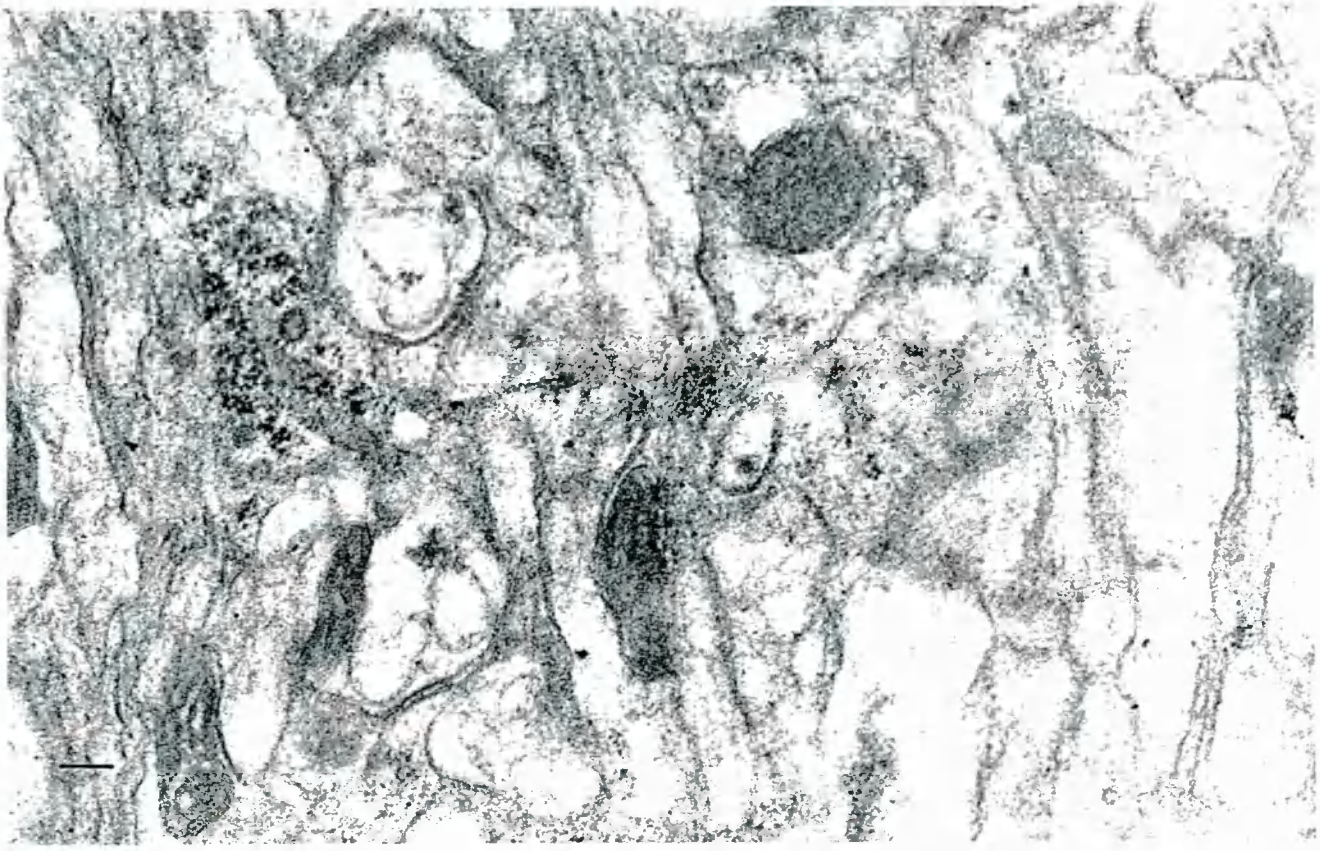
Panel a



Panel b

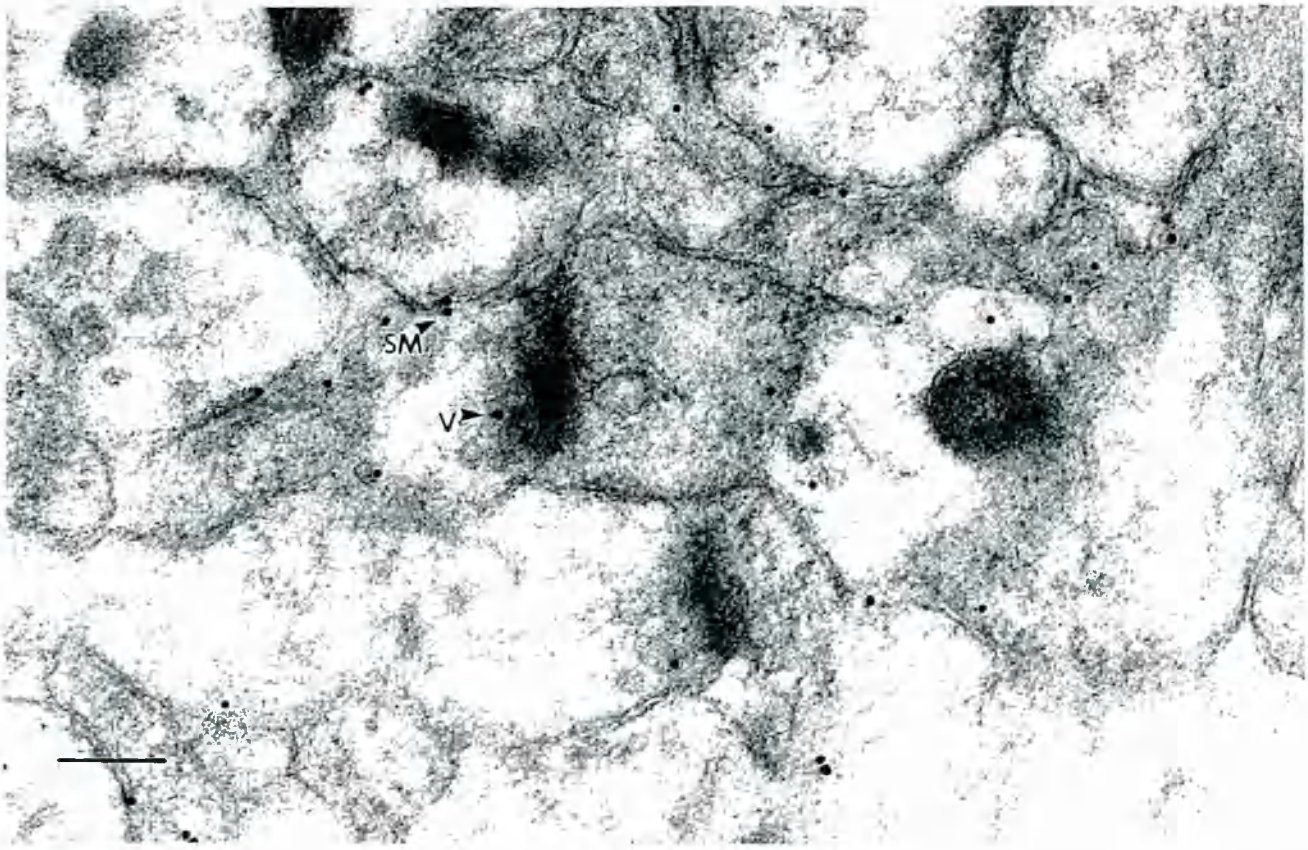


Panel a

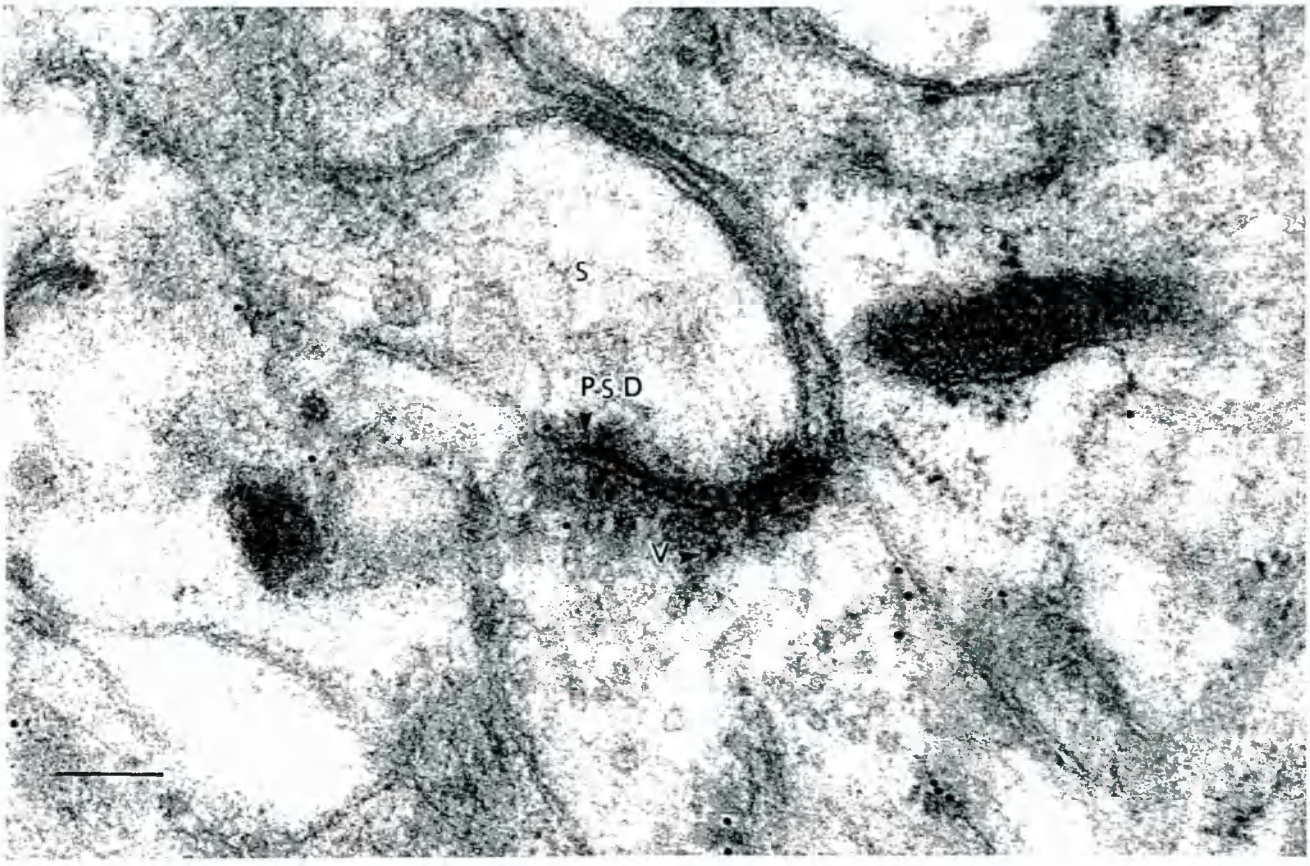


Panel b

Figure 5.12 : Electron micrographs of cerebellum from day-old chicks immunolabelled with R15 (panel a), and pre-immune serum (panel b). AX, axonal membrane; for other symbols see Figure 5.11. Scale bar 200nm.



Panel a



Panel b

Figure 5.13 : Electron micrographs of IMHV (panels a) and cerebellum (panel b) from day-old chicks immunolabelled with R15. The concentration of label in postsynaptic density (PSD) was evident. V, vesicle; SM, synaptic membrane; S, spine. Scale bar 200nm.

DISCUSSION

This study showed that the R15 was relatively a specific antibody recognizing a single protein component of Mr 60-62kD in SPM and PSD fractions. Comparison between patterns of UEA.I-binding proteins and R15 immunolabelling suggested that R15 antigen was fucosylated. Immunocytochemistry showed that the antibody was capable of labelling cell bodies in both cerebellum and forebrain. At EM level, the pattern of immunolabelling showed that R15 antigen was mainly membrane-bound and distributed through axons and dendrites. But more significantly, the antibody labelled specific presynaptic membranes and postsynaptic densities. This, in the light of its amnestic potency suggested that, after intracerebral injection, R15 targeted synaptic antigens, adhered to them, and consequently, disrupted their biological functions.

The patterns of R15 labelling at EM level seemed to be comparable with the labelling patterns of monoclonal antibodies Mab-gp50 and Mab-gp65 (Beesley et al., 1987), and those of monoclonal antibody 411B reported by Bullock and colleagues (1987).

Twenty-four hours after passive avoidance learning, bilateral intracerebral injection of purified R15 IgG rendered chicks amnesic when the antibody was injected 30min before, and possibly 30min after training. Bilateral injection of pre-immune serum given to trained birds 1hr and 10min before training, and 5hr after training did not cause amnesia at 24hr posttraining. The retention scores were 69%, 68%, and 100% respectively. It was, therefore, concluded that R15 specifically caused amnesia in birds that were injected 30min before training.

In the light of several reports that anti-brain antibodies caused convulsion and/or induced epileptiform neural activity (*in vitro*) or altered cortical EEG patterns (Jankovic, 1985), chicks injected with R15 were closely observed for possible

behavioural side-effects. No such effects were observed.

Equally important, R15 did not seem to alter chicks tendency to peck. However, these results only gave an indication of tendency to peck during training and not during the test; the antibody might have triggered a disinhibition mechanism by the time that chicks were tested for retention. One way to address this problem was by injecting two groups of water-trained birds with R15 and saline, 30min before training, and testing them 24hr later. Any discrepancies in percentage of pecking would then be attributable to the antibody. The effect of a polyclonal antibody (R14) on pecking was recently studied by Scholey (1991) who found no observable changes in chicks behaviour. This antibody was raised to chick forebrain homogenate in a similar way to R15.

R15 antibody at 10μg/hemisphere induced partial amnesia; during retention testing 58% of MeA birds pecked at the bead. However, the dose response effect of R15 needs to be investigated and could be done by bilateral injection of different concentrations of R15 at 30min before training and recording of birds' retention 24hr later.

The time-course of amnesia induction by R15 warrants further investigation. In the above study, birds given bilateral injections of R15, were tested 24hr after training. However, amnesia may become manifest sometime earlier than 24hr. Knowledge of the time-course of R15 activity would indicate whether it inhibits neural mechanisms involved in short, intermediate, or long-term memory. For example, in contrast to anti-N-CAM antibody that induced amnesia when rats received the injection between 6-8hr posttraining (Nolan et a., 1987a), the brain specific anti-protein 2 antibody rendered the animals amnesic when it was injected minutes before training (Nolan et al., 1987a; Doyle et al., 1990). This indicated that these antibodies interfered with different processes involved in memory formation and consolidation.

ELISA showed that in all four brain regions, R15 antigenicity increased significantly 6hr after passive avoidance learning, but by 24hr there was a significant drop in R15 absorption in MeA chicks. Further investigation would show the exact timecourse of the turnover rate of R15 antigen. This can be achieved by measuring R15 absorption 1hr, 3hr and 12hr after training. It is evident, however, that R15 antigen is significantly expressed during the first wave of glycosylation which occurs immediately after training to 6hr posttraining time (Zamani & Rose, 1990). It seems that R15 antigen undergoes an early phase of up-regulation followed by another phase of down-regulation. It might, therefore, function as a regulatory molecule such as membrane-bound receptor or enzyme. Cell surface glycosyltransferases that are themselves glycosylated have been identified whose expression is necessary for normal cellular functions such as intercellular contact (e.g., Sadler, 1984).

The use of rather large areas of the forebrain for ELISA might have masked possible localized changes such as in the IMHV, LPO and PA. Absorption analysis of these structures would show whether variations in immunogenicity of the R15 antigen after training is concentrated in any specific brain structure(s), and also whether the effect is lateralized. Results from above study indicated that 6hr after training R15 absorption of left forebrain base were significantly higher than those of right. However, a similar trend was observed in W-trained birds. Therefore, unless more absorption analyses are carried out on specific brain structures the question of lateralization remains equivocal.

In order to ascertain whether passive avoidance training results in differential expression of R15 antigen between presynaptic membranes and postsynaptic densities quantitative protein A/gold immunolabelling could be used. Results from such experiments would also show whether posttraining expression of R15 antigen is lateralized in different brain structures.

Considering the results from Chapter 4, it is likely that antibody R15 recognizes one of the immunogenic epitopes of the 62-80kD glycoprotein band. It showed a significantly increased fucosylation in LPO 6hr after training, and a significant drop 24hr later. However, no such increase was observed in the IMHV 6hr after training. No data is available from LPO at 24hr posttraining time.

There are contradictory reports concerning the half-life of IgG molecules in the brain. It is thought that they remain intact for at least several hours after injection and slowly diffuse to other regions. Recently, Scholey (1991) studied the rate of diffusion of a purified IgG in the chick brain and found that, 1hr after injection of the antibody into the IMHV, by far the greatest amount of IgG was still detectable in this same structure. No data is available at later time-points. It is therefore possible that several hours after injection, IgG diffuses to other areas, such as LPO, which are implicated in memory formation and recall (Patterson et al., 1990). In parallel with the IMHV, LPO shows similar neurochemical, molecular and morphological changes consequent upon training (see Chapter 1). This structure was also implicated in our fucose double-labelling study (see Chapter 4). The direct injection of R15 into LPO may determine whether its antigen plays a role in the formation of memory (or recall) shortly after injection.

In conclusion, the results reported here further illustrate that neuroimmunological techniques are powerful tools in studying neural mechanisms underlying cognitive processes. The use of monoclonal antibodies to target highly specific epitopes make these techniques doubly powerful. Recently, Jork and associates (1991) investigated the role of a particular antigen in rats trained on a brightness discrimination task. They used a monoclonal antibody that recognizes a specific oligosaccharide chain containing a fucose(α1->2)galactose linkage and found that it impaired retention, but not learning, of the task. This further emphasized the important role that certain classes of

fucosylglycoproteins play in neural processes involved in memory formation and recall. It remains to be seen whether R15 recognizes an oligosaccharide chain or a polypeptide chain epitope. In principal, *in vitro* application of exo- and endo-glycosidases such as chick α-fucosidase and endo-glycosidase-H in combination with immunoblotting techniques will elucidate this point.

In order to ensure that the mechanism of action of R15 IgG is specific for neural events involved in memory processing, it is important that it is tested for state-dependency. If R15 function is not state-dependent, intracerebral injection of the antibody 23.5hr after training (i.e., 30min before test) will not lead to increased avoidance. This experiment must be carefully controlled. Thus, four groups of MeA birds would be required in this experiment. Each group is given two sets of injections, one 30min before training, and the other 23.5hr after training. Group A is given saline injections on both occasions, group B, saline and R15 injections respectively, group C, R15 on both occasions, and group D, R15 and saline injections respectively. If R15 function is not state-dependent, the retention scores of birds in groups C and D tested 24hr after training should be identical (both groups should be amnesic). Further, if birds in group B showed a significant decrease in their retention (i.e., they pecked at the dry bead), it may be argued that the antibody inhibited recall mechanisms since, by 24hr posttraining, neural processes involved in memory consolidation are expected to be completed.

CHAPTER SIX

Identification And Characterization Of Glycoproteins Using Specific Probes And Two-dimensional Gel ELectrophoresis

CHAPTER SIX

IDENTIFICATION AND CHARACTERIZATION OF GLYCOPROTEINS USING SPECIFIC PROBES AND TWO-DIMENSIONAL GEL ELECTROPHORESIS

INTRODUCTION

In previous chapters the role of fucosylated glycoproteins in neural mechanisms underlying information processing was studied (see Chapters 3, 4, & 5). The majority of these molecules are also extensively glycosylated with other types sugar molecules such as, mannose, galactose, N-acetylglucosamine (GlcNAc), N-acetylgalactosamine (GalNAc), and N-acetylneuraminic acid (NANA). In most cases the intact carbohydrate chain structure of these macromolecules is essential for a full manifestation of glycoprotein function *in situ*. It is therefore appropriate to try and analyse the oligosaccharide contents, sequence and structure of these macromolecules. One such method is to use lectins.

Lectins and their application

Lectins are proteins or glycoproteins (Mr 8.5kD-400kD) that have special binding affinities for membrane bound glycoconjugates (for a review see Sharon & Lis, 1989). They are found across almost all living species, from micro-organisms (viruses, protozoa, bacteria) to plants (grains and legumes) and animals (both invertebrates and vertebrates). Lectins are not, however, distributed uniformly across species. For example, most invertebrate lectins have specific affinity for NANA (e.g., horseshoe crab contains Limulus polyphemus).

Although lectin binding is sugar specific, they may require the presence of other sugars in the carbohydrate chain of the substrate or have different affinity for the same sugar depending on its position in the chain. For example, two mannose specific lectins from pea and lentil require the presence of a fucose linkage to the innermost asparagine-linked GlcNAc residue of the oligosaccharide chain. Binding of the lectin concanavalin A (Con. A) to mannose is, on the other hand, not affected by the presence of fucose. Instead, it has about 130-fold stronger binding with the trisaccharide mannoseα3(mannose α6)mannose, than with methyl α-mannoside. Most lectins require metallic ions such as Mn^{2+} and Ca^{2+} for activity (e.g., Con. A). Lectins are widely used in clinical research laboratories because they offer many advantages including high specificity, stability and formation of reversible linkages (see Sharon & Lis, 1989).

Lectins have been used to show that many neuronal membrane receptors are glycosylated [e.g., Acetylcholine receptor and opiate receptors (Richter et al., 1991)], and that the cell membrane is fluid such that it allows its protein/glycoprotein constituents to form clusters. Lectins have also provided evidence for the presence of glycoproteins on intracellular membranes. They are located mainly on the non-cytoplasmic (luminal) surface of subcellular organelles such as mitochondria, rough endoplasmic reticulum, Golgi apparatus and nuclear envelope. A recent and rapidly growing application of lectins in the area of neuroanatomy is their use as tracers for mapping neuronal connections. The general methodology is based on the injection of the lectin into a specified site in the nervous system such as a region of neuronal terminals. The lectin is then taken up *in vivo* and transported axonally either to the axonal terminal or the nerve cell body. The lectin is usually conjugated with horse radish peroxidase (HRP), or radioisotopes. Phaseolus Vulgaris Leuko agglutinin, an anterograde tracer is, however, used non-conjugated. Visualization is carried out by the

us of biotinilated anti-phaseolus antibody and horseradish peroxidase-conjugated avidin.

Two-dimensional gel electrophoresis

One other powerful way of identifying and characterizing neuronal proteins/glycoproteins is two-dimensional polyacrylamide gel electrophoresis (2-D PAGE). It has been claimed that this technique enables the detection of up to 1100 different solubilised protein species on a single gel (O'Farrell, 1975). It this study, it was decided to exploit the properties of lectins and the power of the 2-D PAGE technique simultaneously in order to characterize in more detail those glycoproteins that exhibited significant fucose incorporation after passive avoidance learning.

METHODS

Glycoprotein visualization

In order to increase the sensitivity of labelling, proteins resolved by both one- and two-dimensional PAGE were transferred to a high protein-binding nitrocellulose paper (by Western blotting). This procedure was based on methods described by Towbin et al. (1979), Hawkes (1982), Gordon-Weeks and Harding (1983), and Symington et al. (1981). After PAGE, gels were placed into transfer buffer which consisted of Tris (25mM, pH8.3), glycine (192mM), and methanol (20%) for up to 30min in order to allow the gels to shrink. They were then placed in a sandwich consisting of plastic frames, sponge sheets, filter papers, and nitrocellulose paper. This experimental set-up is shown in Fig. 6.1. Care was taken to remove air bubbles trapped between the gels and nitrocellulose papers. All sandwich components were kept wet throughout the procedure. The transfer of proteins took place under 200mA constant current for 1.5hr for mini gels and 4hr, for large gels. The Western blot equipment was kept in an ice-

water mixture during this period. At the end of the run the unbound surface on nitrocellulose paper (i.e., the area of the paper that did not contain transferred proteins) was blocked by either Tris buffered saline (TBS)(pH7.4), containing Ca^{2+} (1mM) and Mg^{2+} (1mM), and Tween20™ (0.05% v/v), when identifying fucosylglycoproteins, or TBS containing Ca^{2+} (1mM), Mg^{2+} (1mM), Tween20 (0.05%), and bovine serum albumin (BSA) (3% w/v), when identifying mannosylated glycoproteins. When identifying sialylated glycoproteins, Mg^{2+}, Mn^{2+}, and Ca^{2+} (1mM each) were added to the TBS. Blots were blocked for at least 1hr at room temperature while being gently agitated on a rocker. Block solutions were then replaced with fresh block solutions containing lectin. Blots were incubated for at least 3hr at room temperature on the rocker. They were then washed 3x20min in block solution and overlaid with either horseradish peroxidase (HRP)-conjugated anti-Ulex Europaeus type I (UEA.I) antibody or HRP-labelled avidin (see Table 6.1). Blots were then incubated overnight at 4°C.

The final staining step was preceded by 3 washes as described above. The substrate used for HRP was diaminobenzidine tetrahydrochloride (DAB). It was dissolved fresh in TBS (0.05% w/v) and H_2O_2 (0.015% v/v) was added to the solution just before visualization. Colour developed within 5min. The reaction was quenched by washing the blots with double distilled water (DDW) several times. Blots were then air-dried and prepared for photography.

In some experiments anti-glycoprotein antibodies such as anti-N-CAM antibodies were used. Here, skimmed milk powder solution (5% w/v) was added to all wash, block and overlay solutions except the last wash before visualization.

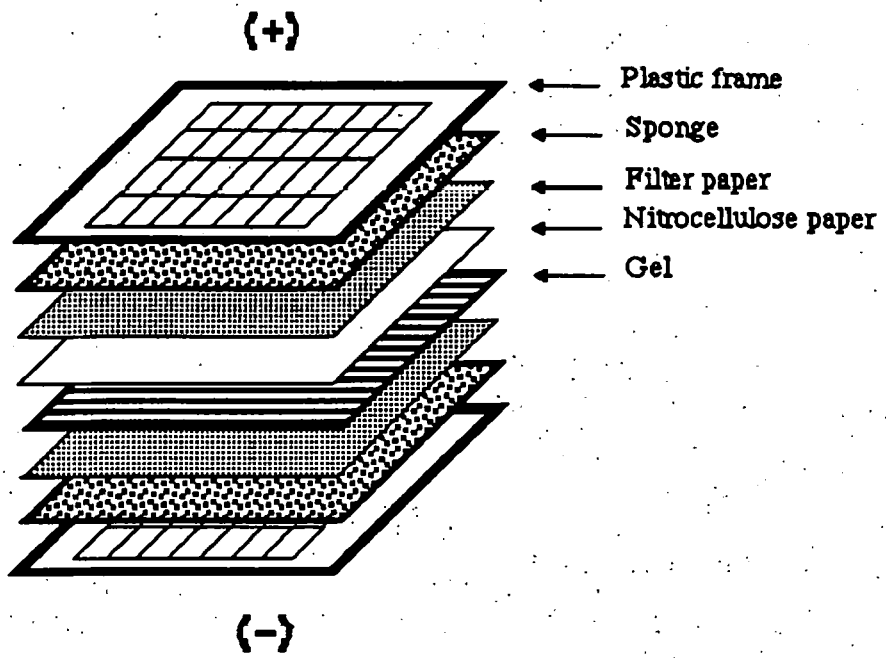


Fig. 6.1 : A schematic diagram of Western blot sandwich.

\+ positive electrode; _ negative electrode.

Two dimensional gel electrophoresis

Two-dimensional sodium dodecyl sulphate polyacrylamide gel electrophoresis (2-D PAGE) was carried out using the techniques described by O'Farrell (1975), O'Farrell et al. (1977), and Rodnight et al. (1988).

1. First dimension : Protein samples to be resolved by 2-D PAGE were denatured using a urea-based lysis buffer containing urea (9.5M), lysine (12.5mM), Nonidet P-40 (NP-40) (5% v/v), bromophenol blue (BPB) (0.005%), 2-β-mercaptoethanol (2%), and ampholines (1.6% pH range 5-8 and 0.4% pH range 3-10). Samples were not heated because temperatures above 45°C cause the decomposition of urea. The final protein concentration in the lysis buffer was 2mg/ml.

Isoelectric focusing (IEF) gel solutions for the first dimension consisted of acrylamide (3.5% w/v), ampholines (1.6% pH range 5-8 and 0.4% pH range 3-10), urea (9.5M) and *N,N'*-methylenebisacrylamide (0.12%) and *N,N,N',N'*-tetramethylethylene-diamine (TEMED) (0.08%). This mixture was degassed and aliquots were stored at -20°C under N_2. NP-40 (4% v/v) was added after degassing to prevent frothing. Ammonium persulfate (0.1% final concentration) was used to catalyse acrylamide polymerization. The IEF solution was drawn into glass rods of 2.6mm internal diameter with a syringe. Polymerization was carried out at 37°C.

Before running, the IEF gels were loaded with 20µl of a "prerun" solution consisting of 9.5M urea, 2% NP-40, 2% ampholines (1.6 pH 5-7 and 0.4% pH 3-10), and 5% 2-β-mercaptoethanol., They were run at 200volts, 300 volts, and 400volts each for 15min using 50mM degassed NaOH as upper tank buffer and 25mM H_3PO_4 as lower tank buffer. At the end of the run the prerun buffer in IEF rods was replaced with 20-100µl of solubilised sample solution. Rods were then loaded with 20-40µl of overlay buffer

containing 8M urea, 1% ampholines (0.8% pH 5-8 and 0.2% pH 3.5-10), and 5% NP-40. IEF rods and the upper tank were then filled with upper tank buffer. Isoelectric focusing was carried out at 400volts for a total of 6000vhr overnight. The potential difference was then increased to 1000volts. The total vhr did not exceed 14000. At the end of the run rods were marked and kept at-20°C pending the second dimension run (see Fig. 2.3c for a schematic drawing of IEF setup).

Measurement of pH gradient : IEF gels were laid on a piece of parafilm and cut in 10mm sections. Each section was thoroughly mixed with 2ml of degassed deionized double distilled water (DDDW) for 10min and its pH measured using a conventional electronic pH meter.

2. Second dimension : The standard one-dimensional PAGE equipment was used during this phase with one modification. The top side of the inner gel slab was bevelled so that the isoelectric focusing gels could be firmly placed between the gel slabs (see Fig. 2.3a). In order to maximize sample recovery no equilibration was carried out, but an upper tank buffer with a high content of SDS (2%), as described under one-dimensional electrophoresis (Chapter 2), was used for the first 30min of electrophoresis. Rods were thawed out under warm running water for a few seconds and their contents slowly ejected onto the top of the gel slabs. Care was taken to remove air bubbles trapped between the IEF gel and the slab gel. IEF gels were fixed in position with 1% agarose solution made in high SDS upper tank buffer containing 0.006% BPB. After 10min gels were run at 20mA/gel constant current for 30min in high SDS buffer. At the end of this run the upper tank buffer was drained through the tap (see Fig 2.3a), and replaced with normal running buffer which consisted of Tris buffer (25mM, pH8.3) containing 192mM glycine and 0.1% SDS. Standard molecular weight markers were added to the well made on the corner of gels, and gels were run at 20mA/gel overnight until the dye front reached the bottom of the gels.

Protein silver staining on 2-D gels

Silver staining was carried out using essentially the methods described by Wray et al. (1981). One of the advantages of these methods is that silver staining may be carried out on previously Coomassie blue-stained proteins.

All solutions and buffers were made in DDDW. Polyacrylamide gels were prepared and proteins run as described before. Coomassie blue stained gels were washed 3 times in 50% methanol and DDDW alternately for at least 3hr. Non-stained gels were merely soaked in the methanol solution for 2hr and then washed twice in DDDW. Gels were then transferred into a 10% glutaraldehyde solution, soaked for 30min, and then washed twice in DDDW for 40min. Gels were transferred into freshly prepared silver staining solutions for 15min and shaken continuously. The stain solution was made by slowly adding 4ml of a 20% silver nitrate solution to 21ml of NaOH solution (0.36%) containing 1.4ml of 35% NH_4OH stock solution. Continuous shaking during stain solution preparation ensured solution uniformity. Absolute alcohol was then added to make 100ml of stained solution. After staining, gels were washed 3 times as before and developed for 10min using a freshly prepared developing solution. This solution was made by mixing 1ml of citric acid (1%) and 100µl of formaldehyde (38%) with 20ml of ethanol and making it up to 200ml with DDDW. Gels were quickly washed twice and transferred into a methanol solution (50%) containing 10% acetic acid to quench staining. The dark background was removed by immersion in Kodak Rapid Fixer™. Using this method protein spots in the nanogram range were detectable.

Protein silver staining on nitrocellulose blots

In order to enhance the contrast, Western-blotted glycoproteins visualized by DAB were silver stained. The method was based on a chemical reaction involving DAB, rather than proteins, which is the case when silver staining is carried out directly on gels. All reagents for silver staining were provided in a kit purchased from Amersham

International plc. UK (DAB enhancement kit, RPN.1174). The method was found to be quick and sensitive, and provided reproducible results using a minimum amount of reagents.

Passive avoidance training

Where specified, chicks were trained on the one-trial passive avoidance paradigm. Three groups were trained, one on W- and two on MeA-coated beads. Fifteen minutes after training, one of the MeA-trained groups was given bilateral intracerebral injections of 2DGal (10μmol/10μl/hemisphere). Birds were tested 6hr after training and those that responded accurately sacrificed and their forebrains prepared for analysis. The training and testing procedure was carried out blind.

Primary Agent	Dilution	Block/Wash	Secondary Agent	Dilution
Biotin-ConA	1:800	#	Avidin	1:500
UEA.I	1:150	§	HRP-Anti-UEA	1:250
Biotin-MAA	1:300	¥	Avidin	1:500
Biotin-SNA	1:500	∂	Avidin	1:500
Ependymin	1:200	¶	HRP-Anti-Rabbit	1:250
Anti-N-CAM^{R}	1:400	¶	HRP-Anti-Rabbit	1:300
Anti-N-CAM^{G}	1:2000	¶	HRP-Anti-Rabbit	1:250
Anti-N-CAM^{B}	1:1000	¶	HRP-Anti-Rabbit	1:250

Table 6.1 : Glycoprotein specific lectins and antibodies used to identify chick brain glycoconjugates. #, Block/wash solution, TBS containing, Tween20, Ca^{2+}, Mg^{2+}, and BSA (pH7.4); §, Block/wash solution, TBS containing Tween20, Ca^{2+}, Mg^{2+} (pH7.4); ¶, Block/wash solution, TBS containing Tween20, Ca^{2+}, Mg^{2+}, Milk powder (pH 7.4); ¥, Block/wash solution, TBS containing Tween20, Ca^{2+}, Mg^{2+}, Mn^{2+}, BSA (pH 7.4); ∂, Block/wash solution, TBS containing Tween20, Ca^{2+}, Mg^{2+}, Mn^{2+}, (pH 7.4). R, G, B, gifts from Drs Regan, Goridis, and Bock.

Four brain regions, left and right forebrain roof, and left and right forebrain base, were quickly dissected and stored at -20°C overnight. SPM and PSD fractions were prepared and protein contents of each sample estimated. Samples were then prepared for either 1-D or 2-D PAGE. Before subcellular fractionation, forebrain regions obtained from three chicks belonging to the same training group were combined. This was to obtain adequate tissue for subcellular preparation and had the added advantage of providing more reliable patterns of glycoproteins on gels.

RESULTS

Application of lectins on 1-D gels

1. Characterization of fucosylglycoproteins : Ulex Europaeus type I (UEA.I) extracted from gorse seeds binds specifically to terminal fucose residues. UEA.II, found in the same seed, is specific for GlcNAc. In this study UEA.I was used to detect the presence of fucose containing glycoconjugates in subcellular fractions of the four different chick forebrain areas described above.

Figs. 6.2a&b show the patterns of UEA.I labelling of SPMs and PSDs from all three groups of trained chicks. About 30 protein bands were observed in each sample, some either strongly or exclusively labelled in SPM lanes and some in PSD lanes.

The specificity of UEA.I affinity for fucose was tested by running a parallel Western blot whose UEA.I solution had been previously mixed with fucose. Thus, 1M fucose was added to UEA.I made in block solution and was gently shaken overnight using a vertical turn-table. The solution was spun at $10.6kg_{max}$ in a Microcentaure bench centrifuge for 30min and the supernatant used for Western blotting. Also, the non-

specific protein labelling by anti-UEA.I-antibody was tested by running a lectin labelling procedure in the presence and absence of the lectin. Results are shown in Fig. 6.3a&b.

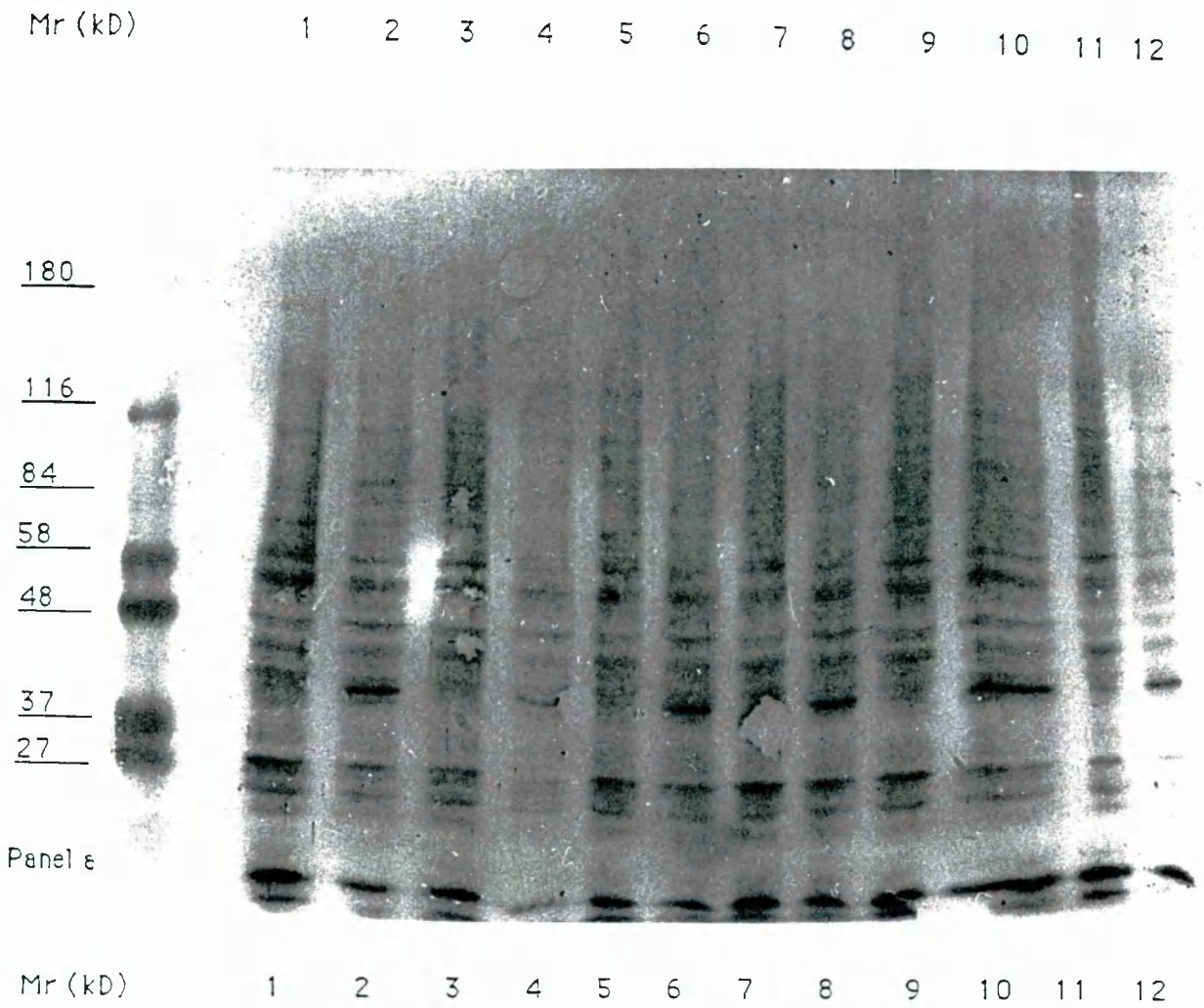
As is shown in Fig. 6.2, the 180kD band was almost exclusively labelled in PSD samples, whereas the 40kD band was seen only in SPM samples. Protein bands indicating 150kD, 84kD and 50kD were more densely labelled in SPM samples and those indicating 120kD, 100kD, and the 60-62kD doublet more densely labelled in the PSDs.

Comparison between W-trained and MeA-trained birds revealed that, in the PSD samples from forebrain base, the 62kD band of MeA-birds were relatively more densely labelled than that of W-birds. The MeA birds that were given 2DGal injections showed similar trends to W birds. No clear visual differences were observed in forebrain roof samples.

2. Characterization of mannosylglycoproteins : In order to analyse mannose containing glycoproteins the lectin Concanavalin A (Con A) was used. Its affinity for mannose is especially high when the sugar is combined with two other molecules in the form of the trisaccharide mannoseα3(mannoseα6)mannose. Figs. 6.4a&b show a typical Western blot pattern on which Con A was used to identify mannosylated proteins.

Fig. 6.2 : Western blotted UEA.I-labelled fucosylglycoproteins in forebrain roof (panel a) and forebrain base (panel b).

From left to right, tracks 1-4 are samples from W birds; 5-8, are samples from MeA birds injected with 2DGal; 9-12, are samples from MeA birds. Odd-numbered tracks are PSDs and even-numbered tracks are SPMs. Standard molecular weight markers are shown in the far left column.


Mr (kD)
1 2 3 4 5 6 7 8 9 10 11 12
180
116
84
58
48
37
27
Panel a
Mr (kD)
1 2 3 4 5 6 7 8 9 10 11 12
180
116
84
58
48
37
27
Panel b

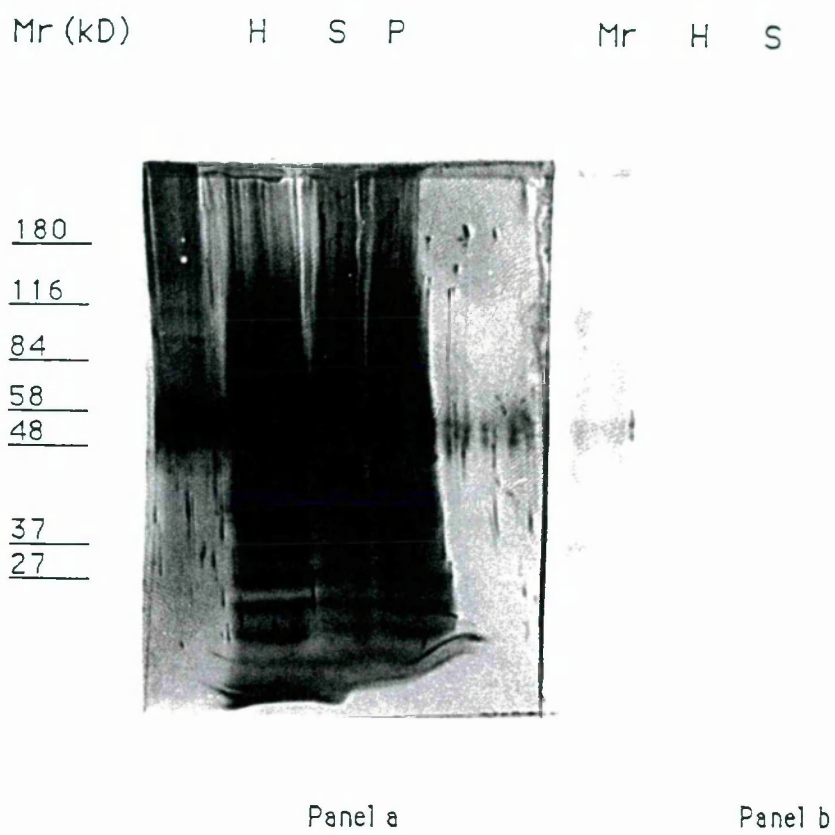


Fig. 6.3a&b : Western blots of UEA.I-labelled fucosylglycoproteins in the absence (panel a) and presence (panel b) of 1mM fucose.

Chick forebrain was used to prepare the samples. Mr, standard molecular weight markers; H, homogenate; S, SPMs; P, PSDs. Equal quantities of protein samples, the lectin UEA.I and anti-UEA.I antibody were used in both blots.

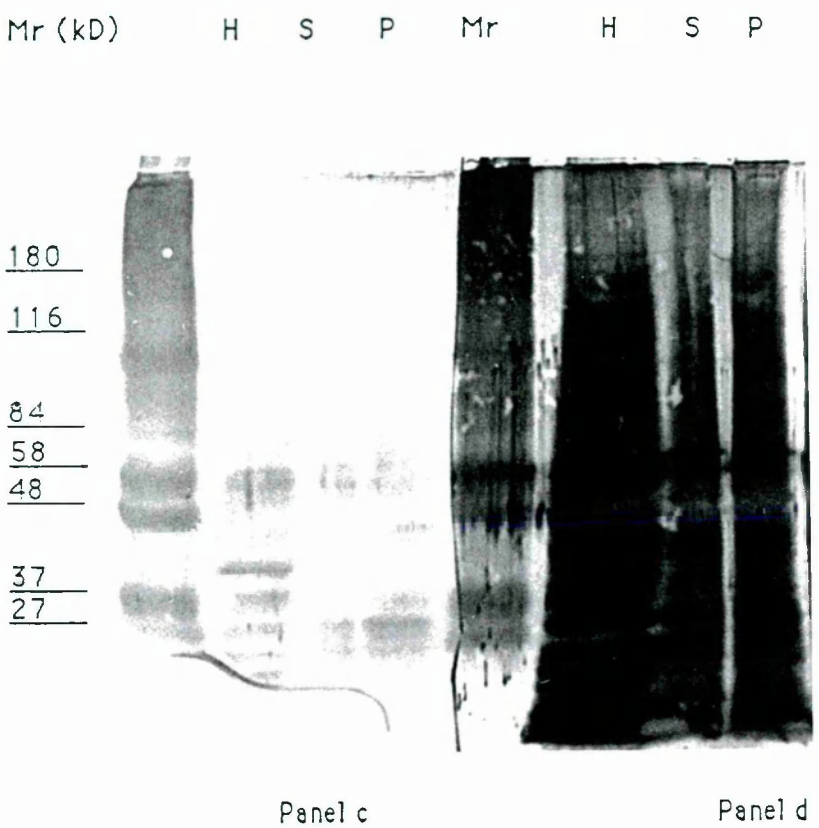


Fig. 6.3c&d : Western blots of fucosylglycoproteins in the presence (panel c) and absence (panel d) of the lectin UEA.I.

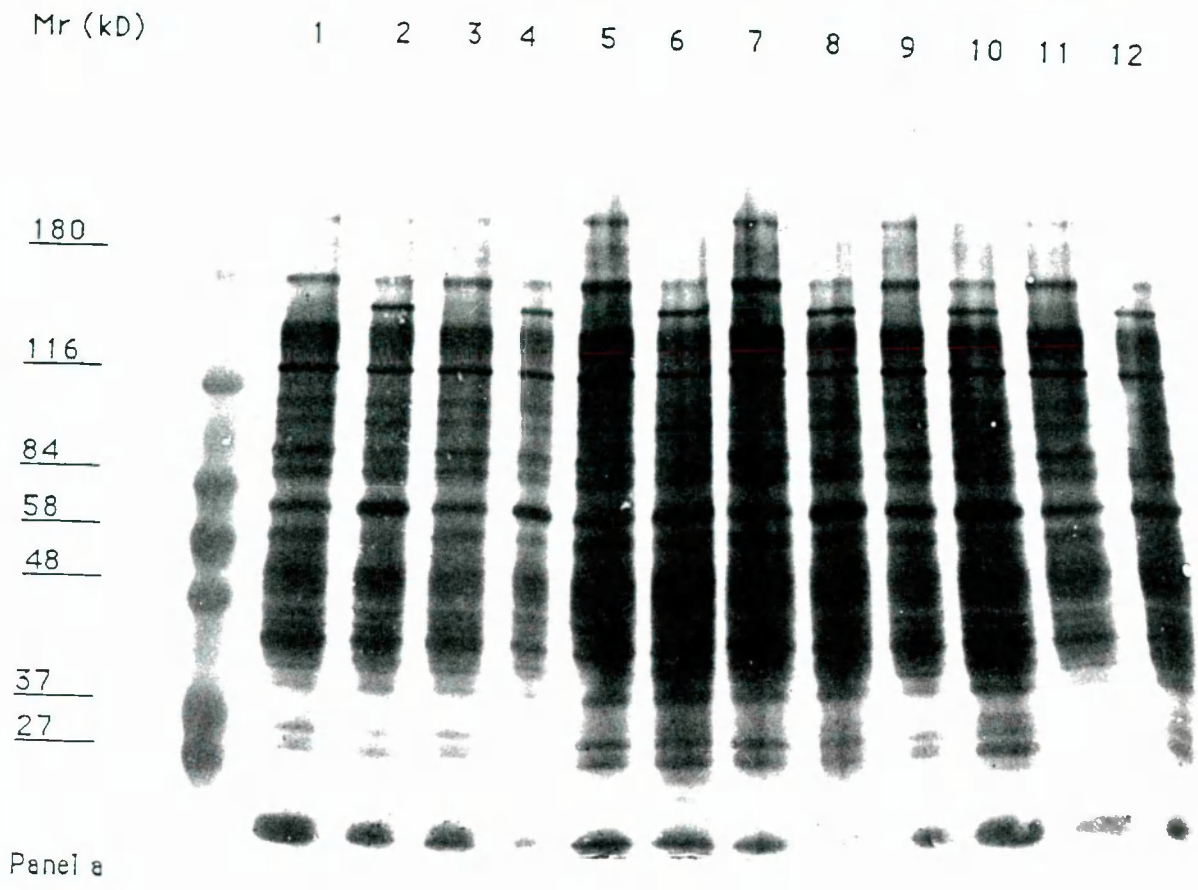
Chick forebrain was used to prepare the samples. Mr, standard molecular weight markers; H, homogenate; S, SPMs; P, PSDs. Equal quantities of protein samples and anti-UEA.I antibody were used in both blots.

The affinity specificity of Con A in this system was tested in a parallel study where α-D-Methyl mannoside (0.5M) was added to all wash, block, and overlay solutions. Results showed that protein bands in the control blot were hardly labelled (Fig. 6.5). It was, therefore, concluded that the labelling patterns in test blots were due to Con A-mannose complex formation. This experiment also showed that avidin, in the absence of biotin, did not bind protein samples (Fig. 6.4).

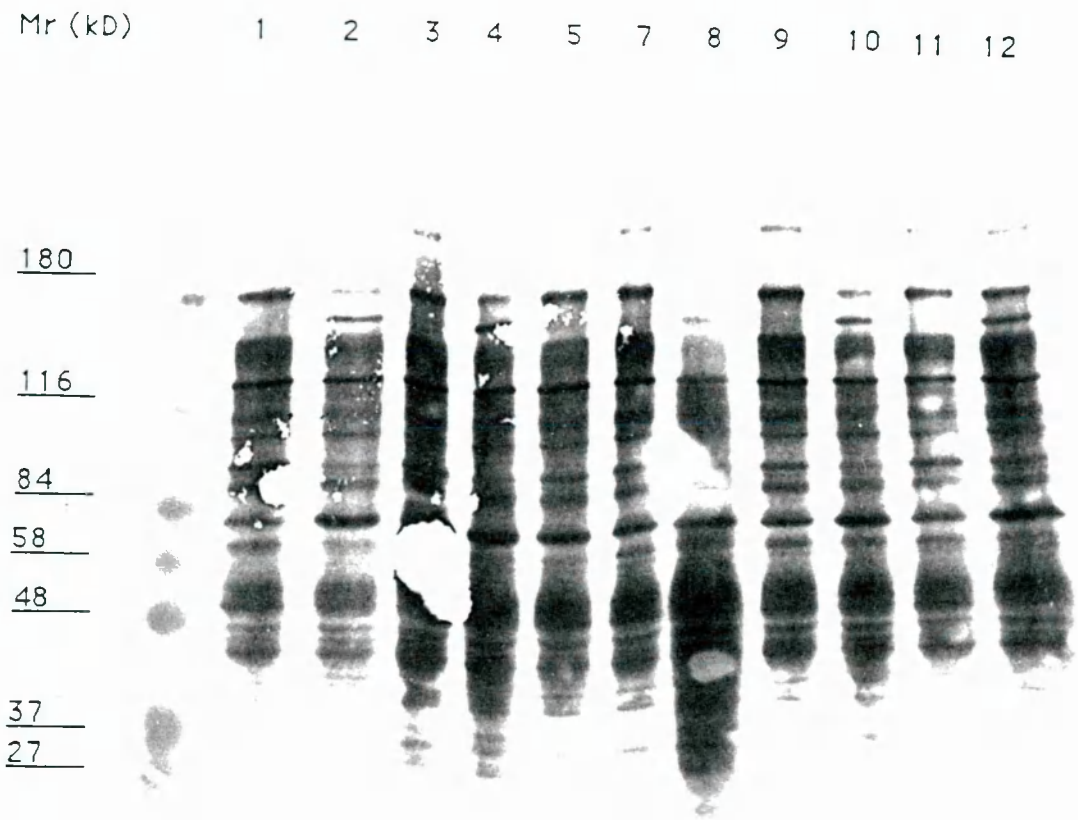
After Con A labelling 26 glycoprotein bands were distinguished in SPM and PSD samples and, as with UEA.I patterns, a number of mannose containing proteins were either exclusively or relatively more densely labelled in one or other of the subcellular fractions. Thus, protein components of Mr 220, 180, 93 and 62kD were PSD enriched and protein components of Mr 160 and 71kD were found to be SPM enriched. The 120kD and 50kD component were equally labelled in SPM and PSD samples. As far as passive avoidance learning was concerned, no differences were visible by eye between trained and control samples.

Fig. 6.4a&b : Western blots of Con A-labelled mannosylglycoproteins in forebrain roof (panel a) and forebrain base (panel b) samples.

From left to right, tracks 1-4, samples from W birds; 5-8, samples from MeA birds injected with 2-DGal injections; tracks 9-12, samples from MeA birds. Odd-numbered tracks are PSDs and even-numbered tracks are SPMs. Mr, standard molecular weight markers.



Panel a



Panel b

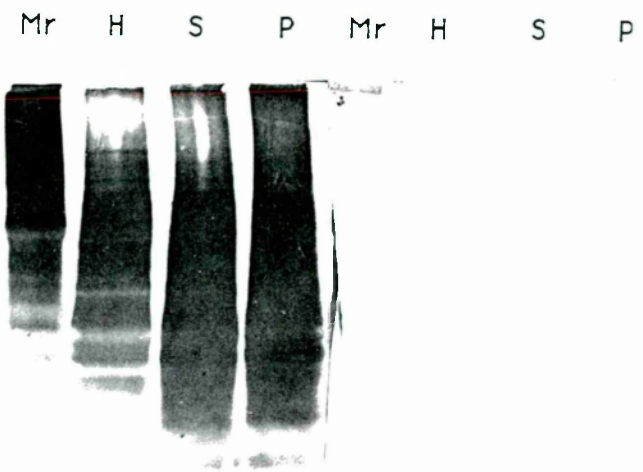
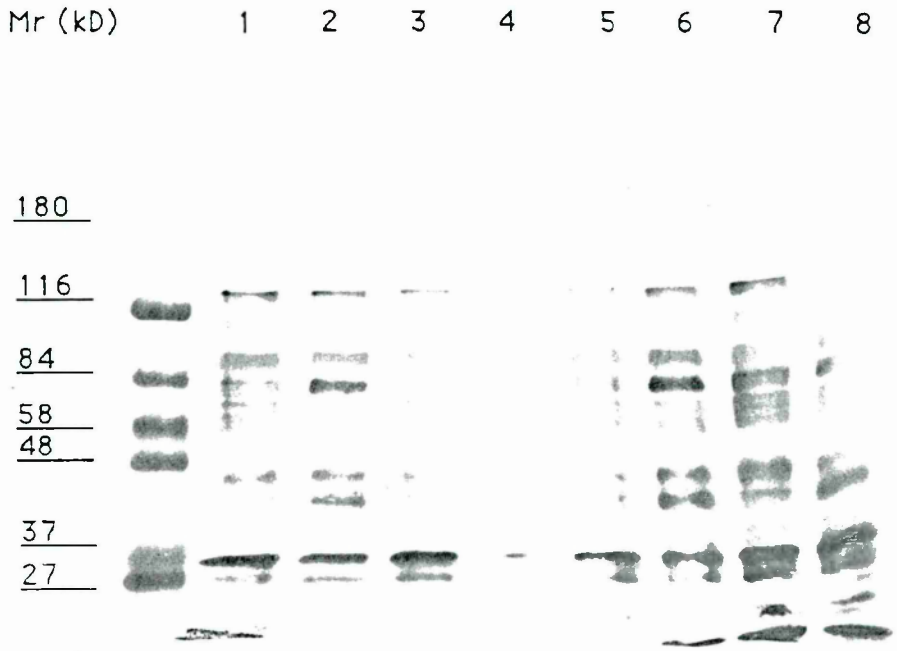


Fig. 6.4c&d : Western blots of Con A-labelled mannosylglycoproteins in the absence (panel c) and presence (panel d) of α-D-methylmannoside (500mM).

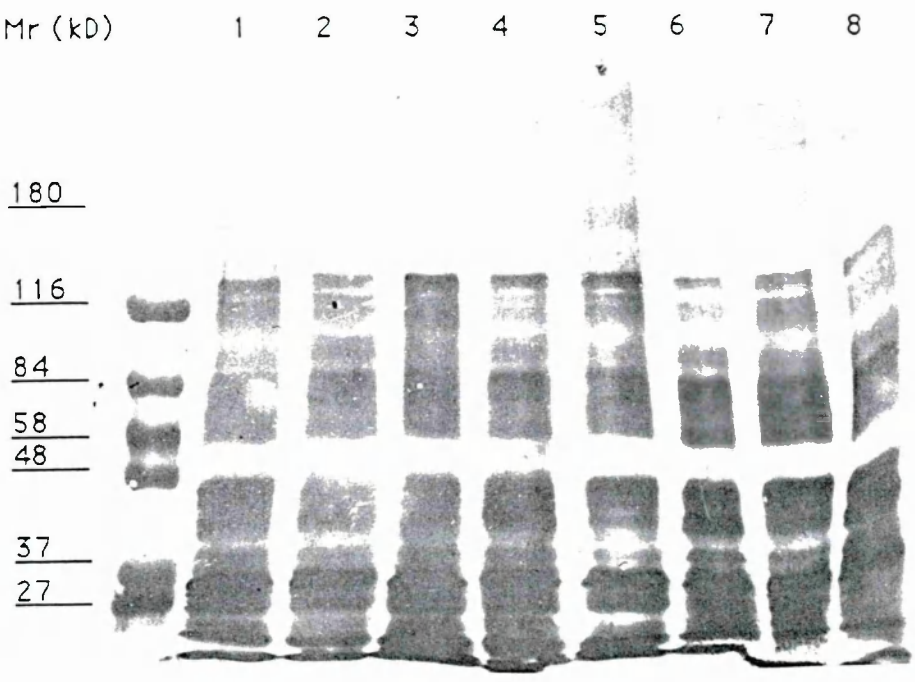
Chick forebrain was used to prepare the samples. Mr, standard molecular weight markers; H, homogenate; S, SPMs; P, PSDs. Equal quantities of protein samples, Con A, and avidin were used in both blots.

Fig. 6.5 : Western blots of glycoproteins containing the NANA(α2-3)galactose linkage detected using the lectin MAA.

Panel a, forebrain roof samples; panel b, forebrain base samples. From left to right, tracks 1-4, samples from W birds; tracks 5-8, samples from MeA birds. Odd-numbered tracks are PSDs and even-numbered tracks are SPMs. Mr, standard molecular weight markers.



Panel a



Panel b

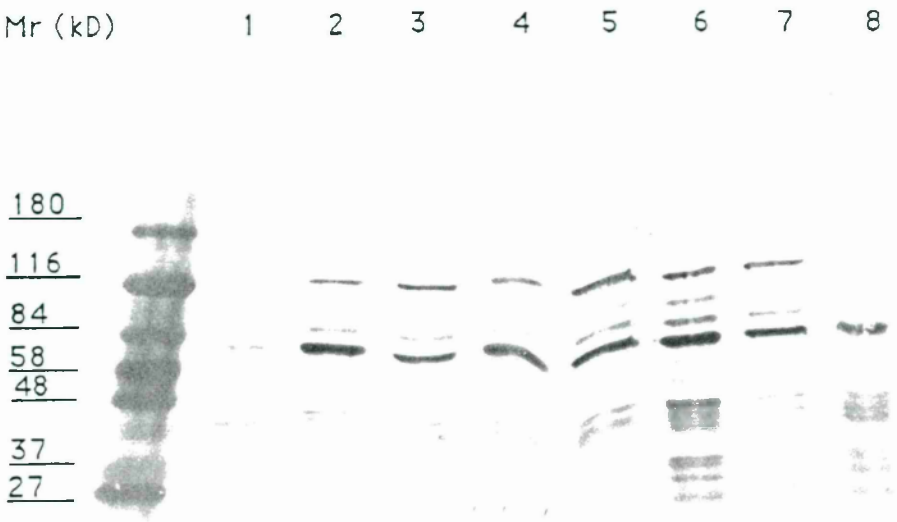
3. Characterization of sialic acid containing glycoproteins : Two lectins with high affinities for different sialic acid (NANA)-sugar complexes were chosen for this study. Maackia amurensis (MAA), specific for terminal NANA(α2-3)galactose, and Sambucus Nigra bark extract specific for terminal NANA(α2-6)galactose or NANA(α2-6)GalNAc. However, since NANA(α2-6)galactose is absent in neural tissues (Regan, 1991), the labelled patterns obtained in this study were considered to be due to NANA(α2-6)GalNAc. Further, It was noted from the outset that neither lectin recognized homopolymers of sialic acids present in N-CAM molecules (see Regan, 1991 for a review of N-CAM structures and functions). This does not, however, rule out the recognition of N-CAM by MAA and SNA since, apart from NANA homopolymers, N-CAM molecules contain galactose, fucose, mannose, GalNAc, and GlcNAc (Hoffman et al., 1982).

The MAA labelling of SPM and PSD samples revealed up to 17 densely stained glycoprotein bands (Figs. 6.5a&b). Protein components of Mr 220 and 180kD from both SPM and PSD samples were, however, weakly but equally labelled. The 84kD component was strongly labelled in SPMs, and the labelling of 45kD component was almost exclusive to presynaptic fractions. Protein components of Mr 116-120 and 62kD were evenly labelled between pre- and postsynaptic fractions. Finally, MAA did not recognize the 50kD glycoprotein component.

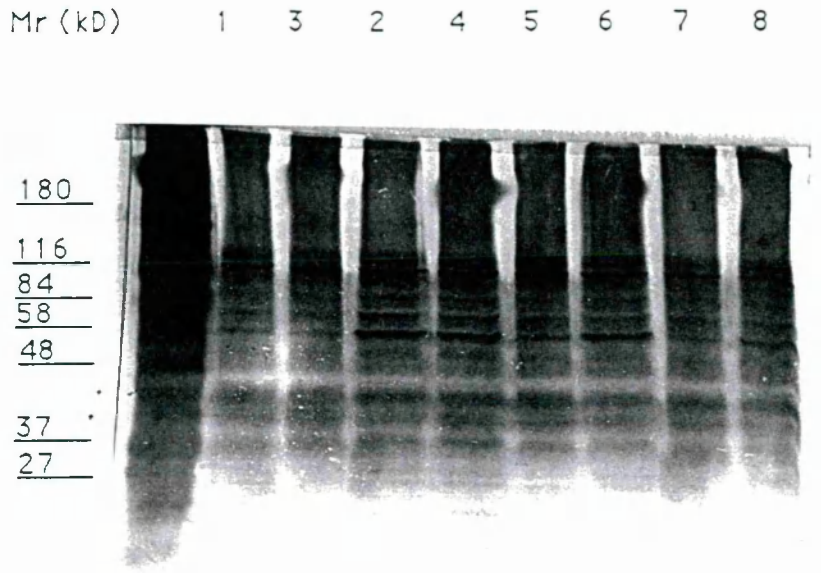
Using the lectin SNA more than 19 protein components were labelled (Figs. 6.6a&b). Compared with MAA, SNA recognized more high molecular weight components in subcellular structures. However, similarly to MAA, the 84kD band was labelled more densely in SPMs than in PSDs, Also the protein components of Mr 45 and 37kD followed the labelling pattern of the 84kD component. Furthermore, 116-120 and 62kD components were equally labelled in SPMs and PSDs. Like MAA, SNA did not recognize the 50kD glycoprotein component. No differences in the intensity of labelling

Fig. 6.6 : Western-blots of glycoproteins containing the NANA(α2-6)GalNAc linkage detected using the lectin SNA.

Panel a, forebrain roof samples; panel b forebrain base samples. From left to right, tracks 1-4, samples from W birds; tracks 5-8, samples from MeA birds. Odd-numbered tracks are PSDs and even-numbered tracks are SPMs. Mr, standard molecular weight markers.



Panele



Panel b

were detected between samples obtained from W- and MeA-trained chicks using either of NANA specific lectins.

Identification of chick brain glycoproteins by specific antibodies

Polyclonal anti-N-CAM and anti-ependymin antibodies raised against isolated rat and goldfish neural glycoproteins respectively were tested against chick antigen and assessments made of their distribution in different structures and subcellular fractions.

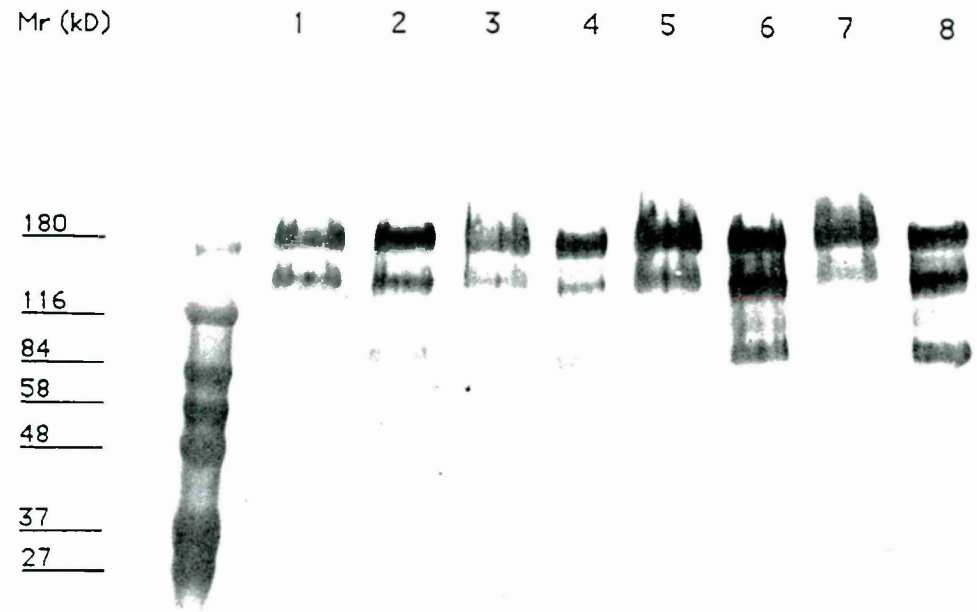
Anti-N-CAM antibodies : Three different polyclonal antibodies to N-CAM were used in this study; they were generously provided by Dr C. Regan (University College, Dublin), henceforth referred to as N-CAM^{R}; Dr C. Goridis (Centre D'Immunologie Inserm, Marseille) (N-CAM^{G}); and Professor E. Bock (University of Copenhagan, Denmark) (N-CAM^{B}). As with the lectin labelling study, SPM and PSD samples from forebrain roof and base of W and MeA birds were separated, Western-blotted and immunolabelled with these antibodies.

Anti-N-CAM^{R} antibody recognized three protein bands in SPMs and two in PSDs; these were 180, 140 and 84kD in the former and 180 and 140kD in the latter fractions respectively (Figs. 6.7a&b). N-CAM120 was not recognized by this antibody. The intensity of N-CAM140 labelling was stronger in SPM than PSD samples.

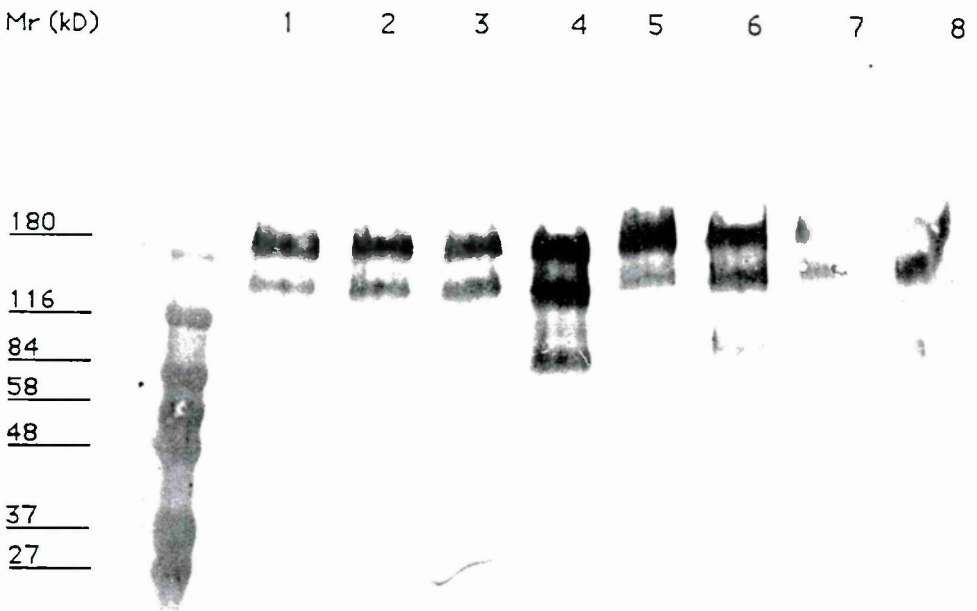
Unlike anti-N-CAM^{R}, anti-N-CAM^{G} recognized four protein components, of Mr 180, 140, 84, and 52-55kD (Fig. 6.8). Interestingly, the 84kD component was virtually exclusive to SPM samples. This showed that both anti-N-CAM^{R} and anti-N-CAM^{G} antibodies recognized the same protein at 84kD region.

Fig. 6.7 : Immunolabelling of chick brain N-CAM by anti-N-CAM^R antibody.

Panel a, forebrain roof samples; panel b, forebrain base samples. From left to right, tracks 1-4, samples from W birds; tracks 5-8, samples from MeA birds. Odd-numbered tracks are PSDs and even-numbered tracks are SPMs. Mr, standard molecular weight markers.



Panel a



Panel b

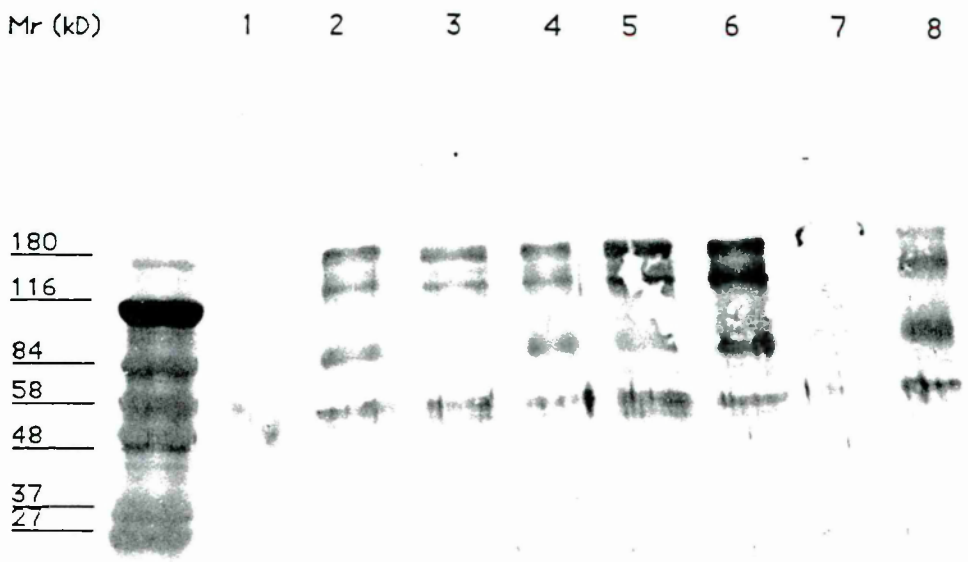


Fig. 6.8 : Immunolabelling of chick brain N-CAM by anti-N-CAM^{G} antibody.

From left to right, tracks 1-4, samples from W birds; tracks 5-8, samples from MeA birds. Odd-numbered tracks are SPMs and even-numbered tracks are PSDs. Mr, standard molecular weight markers.

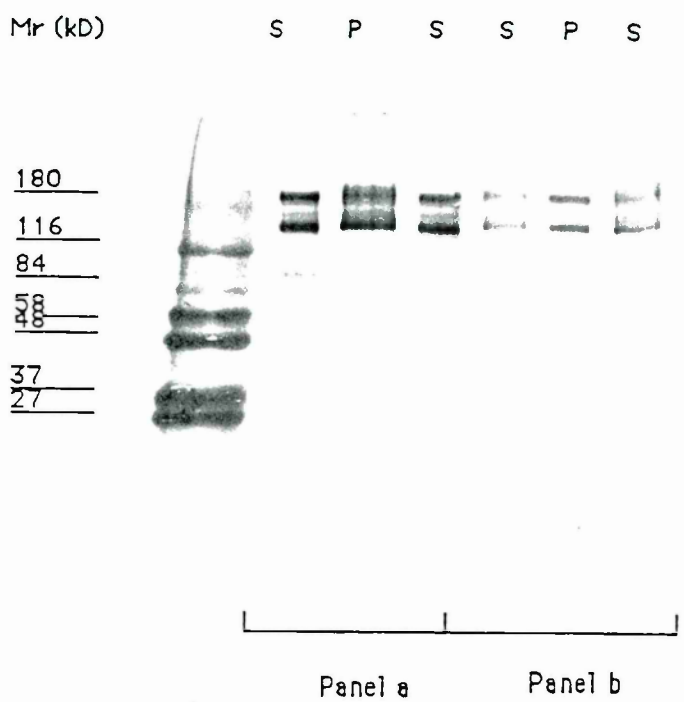


Fig. 6.9 : Immunolabelling of chick brain N-CAM by anti-N-CAM^{B} antibody.

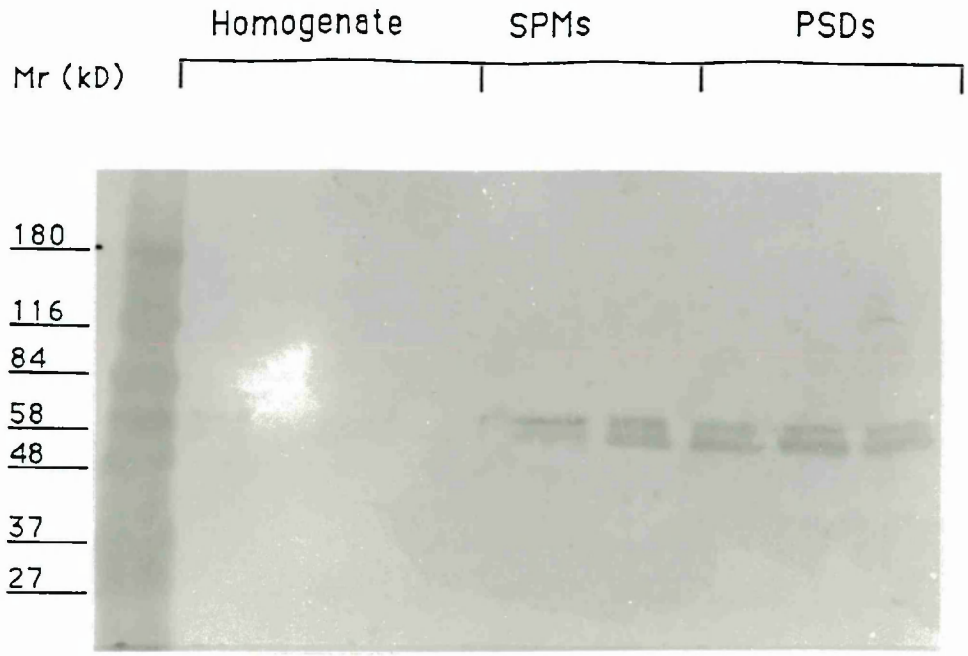
Panel a, immunoblotting with 1:500 dilution of the antibody; panel b, immunoblotting with 1:1000 dilution of the antibody. S, SPMs; P, PSDs; Mr, standard molecular weight markers.

The pattern of immunolabelling obtained from anti-N-CAM^B was similar to that of anti-N-CAM^R (Fig. 6.9). Protein components of Mr 180 and 140kD, and also an SPM specific 84kD band were labelled by this antibody. No training-related changes were observed in any brain regions by any anti-N-CAM antibodies.

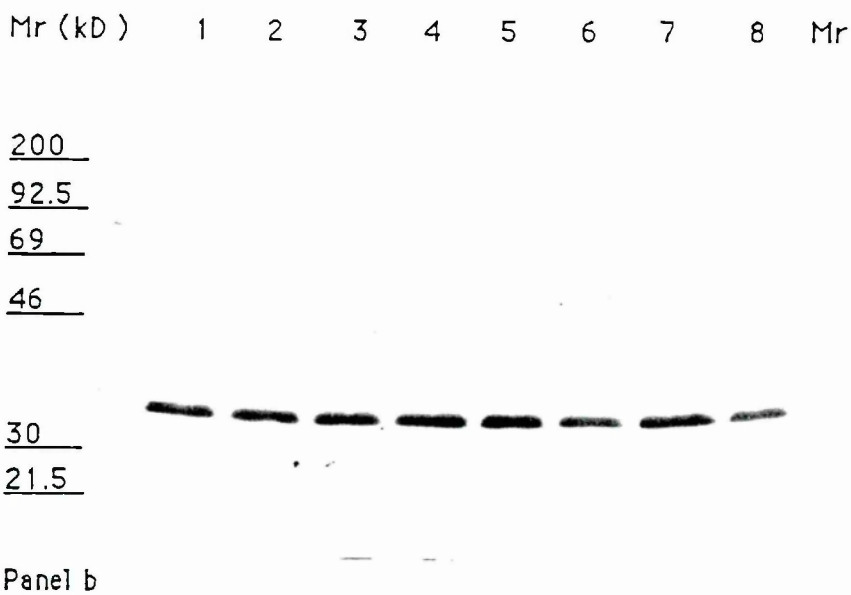
Anti-EPENDYMIN antibody : The polyclonal anti-ependymin antibody was a generous gift from Dr R. Schmidt (Goethe-Universität, Frankfurt). When homogenate, SPM and PSD samples were tested, a doublet of relative molecular weight 58-62kD was labelled by this antibody (Fig. 6.10). The antibody only weakly reacted with its antigens in the PSD samples. Since monomers of ependymin have relative molecular weights of 31 and 37kD (Schmidt & Marktscheffel, 1990), the doublet labelled by the antibody was apparently the results of dimerization, which is reported to take place in the presence of low Ca^{2+} concentrations *in vivo* and *in vitro* (Shashoua et al., 1990). The 58kD bands were probably deglycosylated dimers [deglycosylated monomers have Mr 28kD (Schmidt et al., 1990)] and the 62kD bands glycosylated form of the 31kD monomer. It is also possible that glycosylated monomers of ependymin in chick brain have slightly different molecular weights.

Characterization of glycoproteins by gel electrophoresis

The technique of 2-D PAGE was used to analyse fucosylglycoproteins of chick brain. Figs. 6.11a&b show patterns of chick forebrain proteins (homogenate) resolved on a 2-D gel and stained with Coomassie blue. Part (a) shows the pattern of proteins resolved in a 5-15% linear gradient gel, and part (b) shows the resolution of an identical sample in a linear 10% gel. The 5-15% gel revealed over 85 well-resolved distinct polypeptide spots. In the 10% gel, on the other hand, only about 70 polypeptide spots were distinguished. However, the linear gel provided a good quality of resolution in the medium-to-low molecular weight range (45kD and below).



Panel a



Panel b

Fig. 6.10 : Immunolabelling of chick brain ependymin by anti-ependymin antibody. Panel a, from left to right, tracks 1&2, homogenates; 3-5, SPMs; 6-8, PSDs; Panel b, tracks 1-4 SPMs; tracks 5-8 PSDs. Mr, standard molecular weight markers. Subcellular preparations were carried out either in the absence (panel a) or the presence (panel b) of 2.5mM Ca^{2+}.

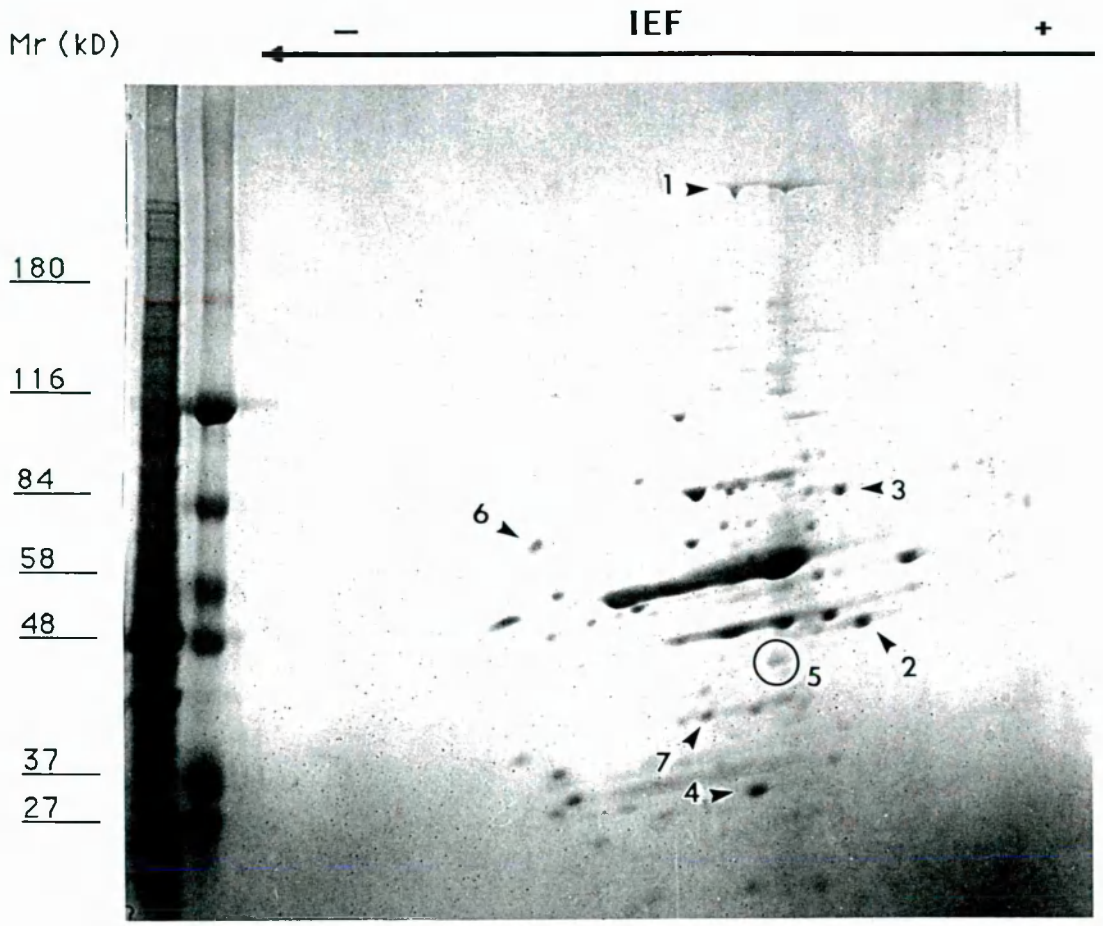


Fig. 6.11a : Two-dimensional pattern of chick forebrain proteins resolved in a 5-15% polyacrylamide linear gradient gel stained with Coomassie blue. The approximate isoelectric focusing range is shown on top of the diagram. The first track shows a one-dimensional separation of the same protein sample. The second track shows standard molecular weight markers. Possible identities of protein spots numbered in the figure are as follows: 1, microtubule-associated protein-2 (MAP-2); 2, B-50 [protein kinase C (PKC) substrate]; 3, 82-87kD PKC substrate; 4, DARP32 (32-35kD cAMP dependent protein kinase substrate); 5, 40kD of Ca^{2+}/calmodulin protein kinase substrate; 6, synapsin 1; 7, α-subunit of pyruvate dehydrogenase (adapted from Rodnight et al., 1985; Rodnight et al., 1988). IEF range is shown by + and – (pH5-11).

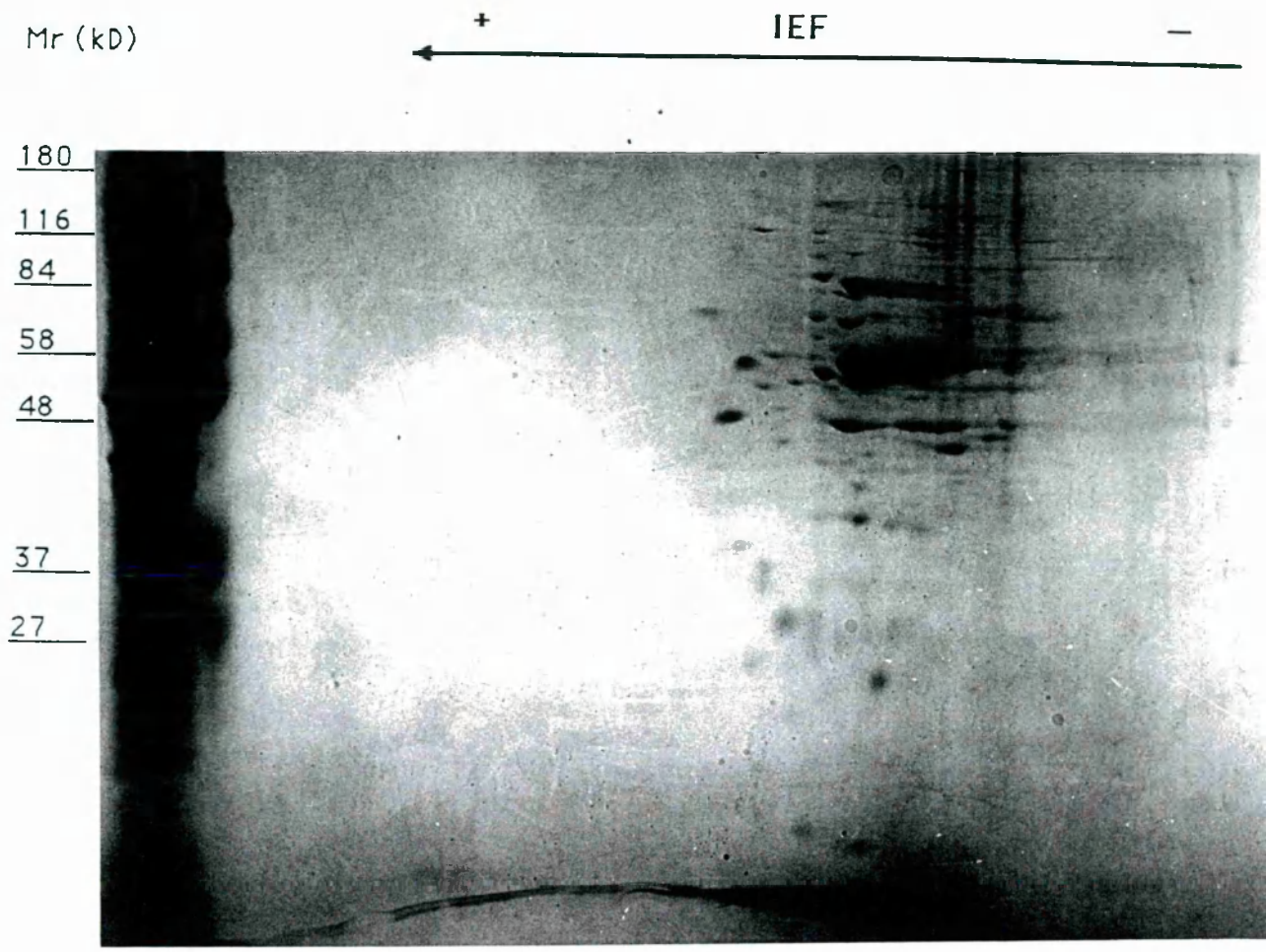


Fig. 6.11b : Two-dimensional pattern of chick forebrain proteins resolved in a 10% linear polyacrylamide gel and stained with Coomassie blue. The approximate isoelectric focusing range is shown on top of the diagram. The first track shows the one-dimensional separation of the same protein sample. The second track shows standard molecular weight markers. IEF range is shown by + and – (pH5-11).

Fig 6.12 demonstrates the two-dimensional pattern of homogenate polypeptides visualized by silver staining. Over 150 uniformly distributed components, with their IEF values ranging between pH5-10, were distinguished on the gel.

The two-dimensional patterns of subcellular fractions were also studied. Resolution of SPM and PSD samples revealed over 50 protein spots in Coomassie blue stained gels (Fig. 6.13).

SPM and PSD samples resolved by 2-D PAGE were Western-blotted and lectin-labelled with UEA.I. In order to enhance the staining, blots were silver stained. Figs 6.14a&b show typical patterns of fucosylglycoproteins obtained from these samples. Over 55 components were observed in each SPM and PSD sample.

In the region of 180kD, a broad band of neutral-to-acidic glycoprotein was recognized by UEA.I. This was thought to be the major 180kD component of PSDs reported in other independent studies (Gurd, 1989, 1991). The area 100-120kD contained seven neutral-to-acidic spots which included one protein band. Eight spots were distinguished in the region of 62-80kD and these had a broader IEF range. The 50kD region contained a very broad basic protein band, probably the major PSD phosphoprotein, a subunit of Ca^{2+}/calmodulin dependent protein kinase II, which has been reported in a number of studies (e.g., Kennedy et al., 1983). Weakly stained acidic and a neutral glycoprotein spots were also labelled in the 50kD area.

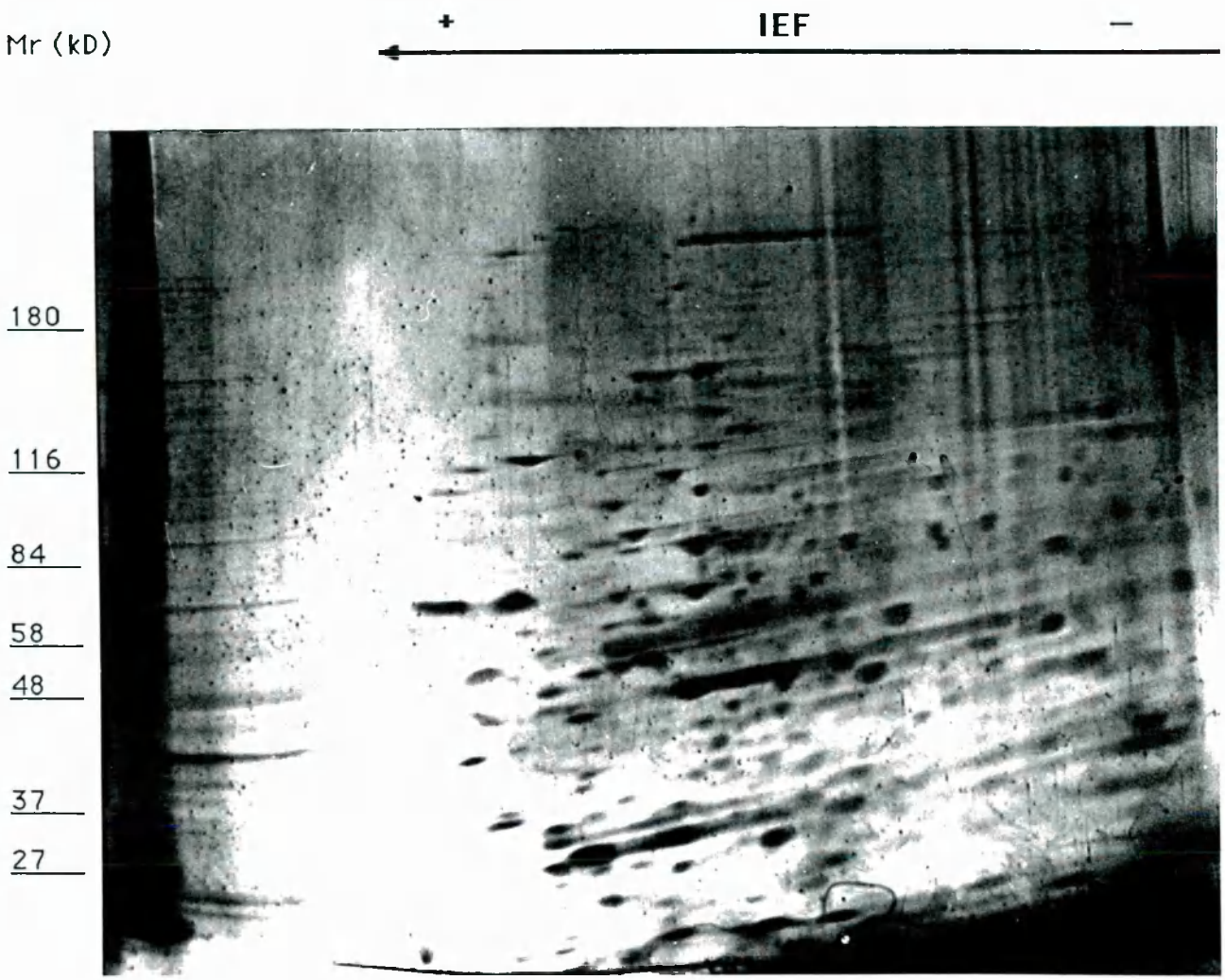


Fig. 6.12 : Two-dimensional pattern of chick forebrain proteins resolved in a 5-15% linear gradient polyacrylamide gel and silver stained. The approximate isoelectric focusing range is shown on top of the diagram. Mr, standard molecular weight markers. IEF range is shown by + and – (pH5-11).

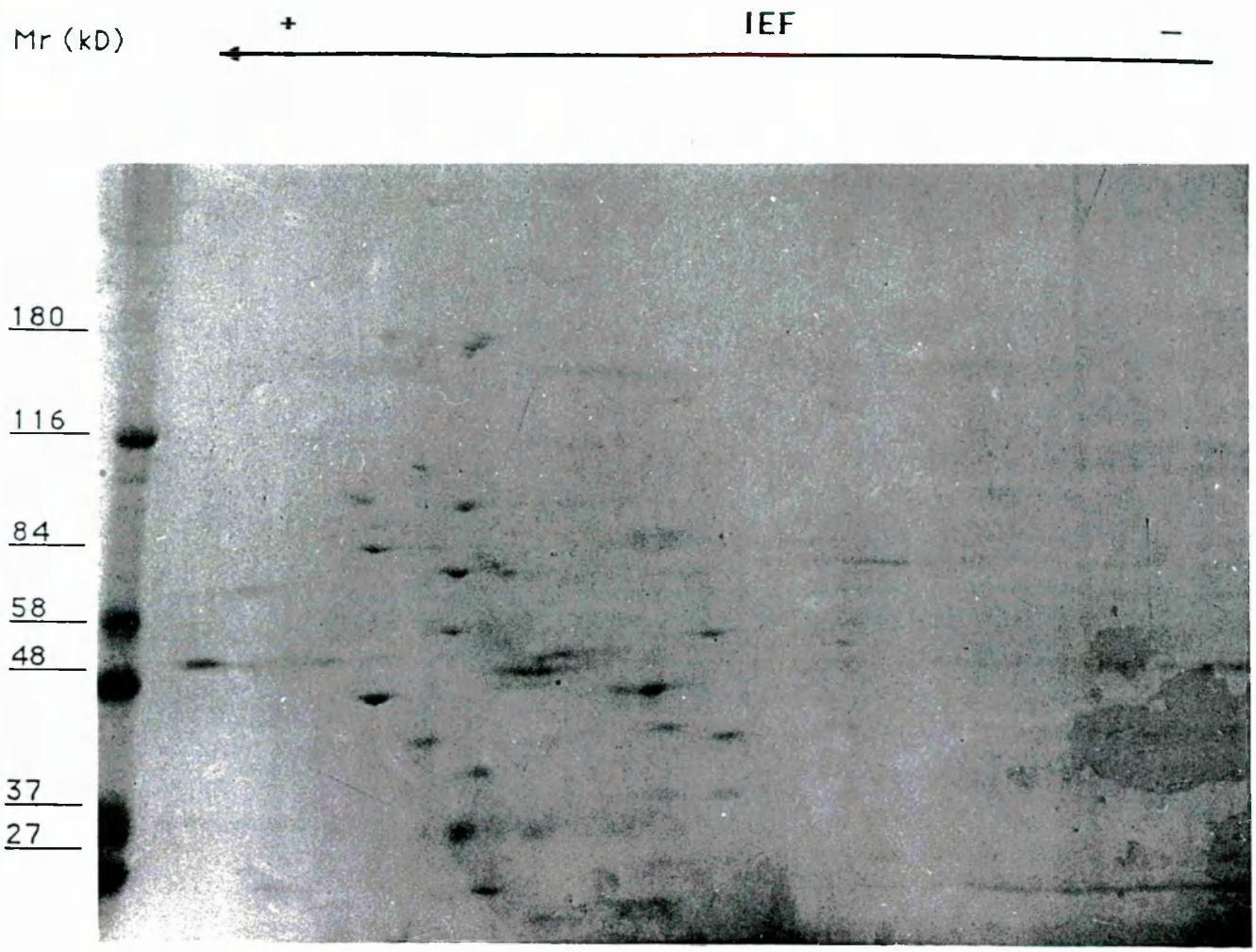
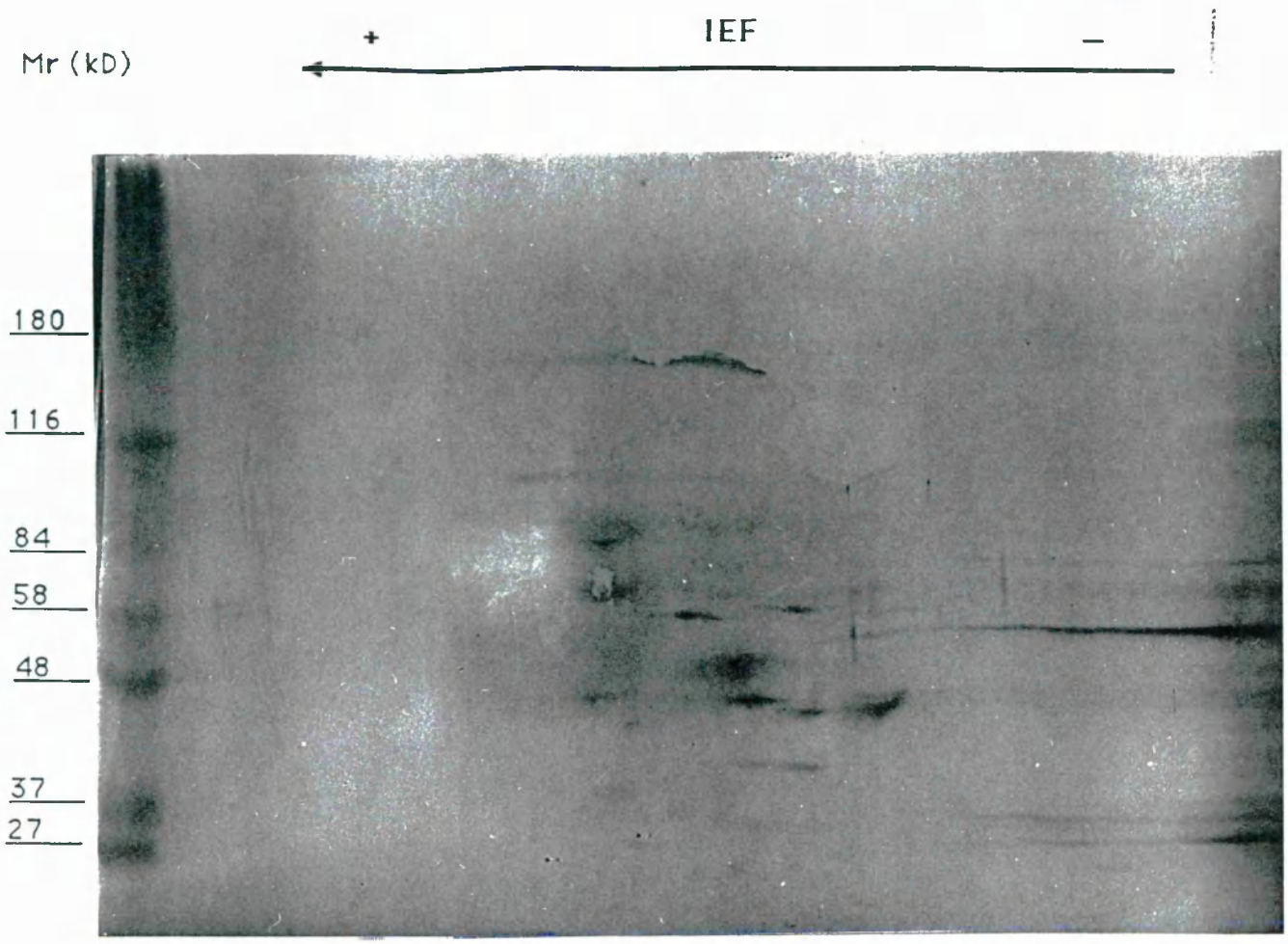
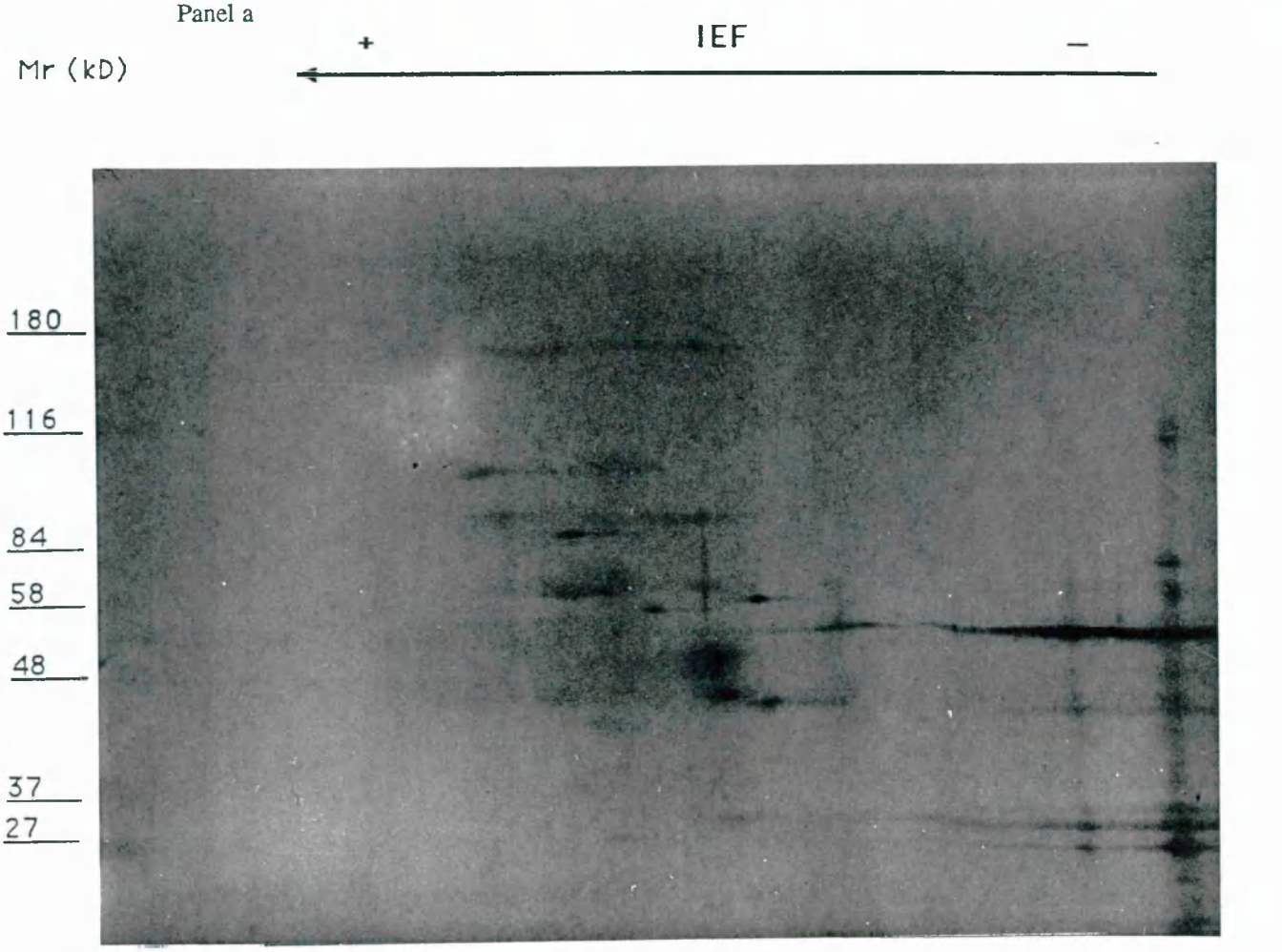


Fig. 6.13 : Two-dimensional pattern of chick forebrain SPM proteins resolved in a 5-15% linear gradient polyacrylamide gel stained with Coomassie blue. The approximate isoelectric focusing range is shown on top of the diagram. The one-dimensional column shows standard molecular weight markers. IEF range is shown by + and – (pH5-11).

Fig. 6.14 : Two-dimensional patterns of fucosylglycoproteins in SPMs (part a) and PSDs (part b) of day-old chick forebrain resolved in a 5-15% linear gradient gel. Samples were Western-blotted and silver stained as described in the Methods section. The lectin UEA.I was used to label fucose. The approximate isoelectric focusing range is shown on top of the diagram. The one-dimensional track shows standard molecular weight markers. IEF range is shown by + and – (pH5-11).



Panel a



Panel b

When comparing protein patterns between SPMs and PSDs, it was found that they generally shared the same composition, but the relative intensity of lectin labelling differed for a few molecules (in this study equal quantities of samples were used to facilitate simple comparison). Lectin labelling of a neutral 42-45kD doublet was found to be enhanced in SPM blots and similar trend was observed for a 37kD neutral protein SPM spot. In contrast, a relatively acidic glycoprotein spot of Mr 80kD was found to be more densely labelled in the PSD blots.

DISCUSSION

Various techniques are currently used to analyse neural glycoproteins. The most common ones include immunocytochemistry, lectin binding using either gels or Western blots, radioisotopic sugar uptake, antibody affinity column chromatography, lectin affinity column chromatography and ELISA. In this study, analysis of glycoproteins was carried out by the combination of Western blotting and 2-D PAGE techniques. Antibodies with known antigenic specificity in other species were also used. The presence of N-CAM180 and N-CAM140 in chick was confirmed using three independently-produced polyclonal antibodies raised against rat N-CAM. This suggested that N-CAMs are highly conserved molecules.

All N-CAMs are variations on a basic structure and differ mainly in three aspects :

A. The degree of sialylation : In polyacrylamide gels N-CAMs from adult rat brain contain low levels of NANA (known as N-CAML, Mr 120-180) In embryonic brain, on the other hand, N-CAMs are heavily sialylated (N-CAMH, Mr 400kD) (Hoffman et al., 1982),

B. The mode of membrane attachment : Whereas N-CAM180 and 140 are integral membrane proteins, the attachment of N-CAM120 is effected by a glycosylated form of

phosphatidylinositol linkage (Rutishauser, 1989),

C. The presence of a cytoplasmic domain : Unlike N-CAM120, N-CAM180, contains a large cytoplasmic polypeptide domain; N-CAM140, on the other hand, has a smaller cytoplasmic domain (Regan, 1991).

No N-CAM84 is reported in the literature. However, the molecular weight of the N-CAM140 polypeptide backbone is 90kD. It is, therefore, possible that the 84kD component recognized by anti-N-CAM antibodies is the deglycosylated epitope of N-CAM140. The immunodetection of this antigen exclusively in SPMs may imply that the process of subcellular fractionation and, in particular, the use of the detergent N-octylglucoside during preparation of PSDs resulted in detachment of this antigen from PSDs. However, in a pilot study octylglucoside was added to one SPM suspension, mixed and spun at $10kg_{max}$ for 30min and both supernatant and pellet immunolabelled by $N\text{-}CAM^{R}$. The 84kD component was labelled in supernatant but, no epitope was labelled in PSDs, indicating that octylglucoside did not precipitate the 84kD epitope. This is still not irrefutable proof that N-octylglucoside, or any other reagents used during the subcellular preparation, do not cause the cleavage of this antigen from PSDs. The use of alternative detergents may also help in assessing whether this epitope is exclusively localized in SPM. However, N-octylglucoside is actually milder than other commonly-used detergents.

As noted before the application of lectins by various techniques remains amongst the most effective ways of studying glycoconjugates. Here, various lectins with different carbohydrate affinity were used on Western blots for the following purposes :

A. To obtain a general pattern of glycoconjugates of chick brain and assess their subcellular localization,

B. To examine the carbohydrate contents of those glycoproteins that had previously

been found to show increased fucose incorporation after passive avoidance learning but ceased to do so in the presence of 2DGal (see Chapters 3, 4 and 5),

C. To analyse in detail fucosylglycoproteins of chick forebrain using the technique of 2-D PAGE, making special note of the regions on the gels that correspond to molecular weights of fucosylglycoproteins implicated in learning ("memorins"!),

D. To examine whether lectin labelling on Western blots is a sufficiently sensitive technique to detect relatively small changes in carbohydrate content, and in particular fucose, of oligosaccharide chains after passive avoidance training.

A. Chick forebrain glycoconjugates : The number of identified neural glycoconjugates has increased steadily. Table 6.2 illustrates only some of the better-characterized glycoconjugates of the nervous system. A number of these molecules are reported to be neuron specific and the rest have been also localized in other tissues such as the liver. The majority of these molecules are membrane-bound and the rest have been identified as extracellular components. In chick brain, some thirty fucosylglycoproteins have been detected in SPM and PSD samples (e.g., Gurd & Mahler, 1974; Zanetta et al., 1977). The majority of these molecules had high mannose contents (that is, they were N-type glycoproteins). Over 17 of them contained the NANA(α2-3)galactose linkage and over 19 contained the NANA(α2-6)GalNAc oligosaccharide linkage.

Results from the UEA.I labelling of fucosylglycoproteins were in agreement with those from the radioisotope studies reported in previous chapters. That is, some 30-35 components of SPMs and PSDs were found to incorporate labelled fucose. In independent studies in other species a similar number of glycoconjugate components have been reported (e.g., Gurd, 1989; also see Table 6.2).

Glycoconjugate	Location	Reference
gp230	SPM/PSD/SJ	Gurd, 1989
gp180 ϕ	PSD mainly	Gurd, 1989
gp145, gp116, gp110, gp45-50	SPM/PSD/SJ	Gurd, 1989
Acetylcholine (nicotinic & muscarinic)	nerve terminal	Hucho, 1986; Merlie et al., 1982 Rauth et al., 1986
Glutamate binding protein	nerve terminal	Michaelis, 1975
Opiate receptor	nerve terminal	Gioannini et al., 1982
Na^+/K^+ ATPase (55kD)	synapse	Dahl and Hokin, 1974
Synaptophysin/p38* ϕ (40kD)	vesicle	Rehm et al., 1986
Thy-1 (17-18kD; 25kD by PAGE)	PSD	Hooghe-Peters and Hooghe, 1982 Bernard et al., 1983 Lappuke et al., 1987
SV2 (75-110kD)*	SPM and vesicle	Buckley and Kelly, 1985
N-CAM* ϕ (180, 140, 120kD)	synapse	Regan, 1991; Schachner, 1992 Doyle et al., 1990; Bock, 1991
MAG (100kD)	myelin	Quarles et al., 1973
D2*	synapse	Jørgensen and Møller, 1980
CNSgp130*	synapse	Moss, 1986
gp50*	synapse	Beesley et al., 1987
gp65*	synapse	Hill et al., 1982
gp58*, gp62*	synapse	Mahadik et al., 1982
L1*	synapse	Rathjen and Schachner 1984
F-10-44-2*	synapse	McKenzie et al., 1982
BSP-2*	cell surface	Rougon et al., 1982
BSP-3*	cell surface	Hirn et al., 1988
NS-4*	cerebellum	Schnitzer and Schachner, 1981
Purkinje cell gp*	cerebellum	Reeber et al., 1981
A2B5*	SPM	Eisenbarth et al., 1979
MRC OX-2*	cell surface	Barclay and Ward, 1982
F-3-87-8*	cell surface	Nolan et al., 1987b, Lakin et al., 1983
Ependymin* (37 and 31kD)	ECM	Shashoua et al., 1990 Schmidt and Shashoua, 1981
Laminin* (900kD, A 400, B1 215, B2 205)	ECM	Manthorpe et al., 1983 & 1988
Fibronectin* (450kD, 220 & 220)	ECM	Stewart and Pearlman, 1987
Cytotactin* (1000kD)	ECM	Grumet et al., 1985
Integrin* ϕ (a & b 140) (chick)	ECM receptor	Cohen et al., 1986 & 1987
Amyloid β-protein precursor*	Extracellular	Doyle et al., 1990

Table 6.2 : Some glycoproteins identified and localized in the nervous system. *, studied immunocytochemically; ϕ, phosphorylated; ECM, extracellular matrix. They are not necessarily brain specific.

B. Training related glycoproteins : Fucosylglycoproteins of Mr 180, 100-120, 62-80, 50, and 37kD were found to show increased fucose incorporation at different times after passive avoidance learning. Lectin labelling showed that these molecules, contained various other carbohydrates in addition to fucose. Table 6.3 shows the relative intensity of lectin labeling of these glycoproteins. N-CAM 180 and 140 were also assessed since the 180kD fucosylglycoprotein band shared the same molecular weight as N-CAM180. However, it remains to be seen whether they in fact are the same molecule.

This analysis provided a qualitative measure of glycoproteins carbohydrate contents. No probes to label GlcNAc were used. Therefore in future studies a full analysis of the sequence and structure of the oligosaccharide chains of these molecules should be carried out. As mentioned out in the introductory chapter, both N-type and O-type glycoproteins potentially possess highly complex oligosaccharide structures. Without a full analysis of these structures, a clear understanding of the biological function of neural glycoconjugates will not be possible.

C. Detailed analysis of fucosylglycoproteins : The limitations of one-dimensional gel electrophoresis are well understood. 2-D gel electrophoresis, on the other hand, has theoretically the capacity for resolving over 5000 polypeptides per sample (O'Farrell, 1975). In Chapters 3 and 4 the 1-D PAGE technique was used to identify 7 broad training-related glycoprotein components. 2-D PAGE was used to examine how many glycoproteins, of what isoelectric focusing values, were detectable in each of these components.

	Relative carbohydrate labelling			
Mr (kD)	fucose	mannose	NANA(α2-3)galactose	NANA(α2-6)GalNAc
180	++	+++	+	--
100-120	++	+++	++	++
62-80	++	++	+++	++
62	++	++	+++	+
55	+	+++	--	+
50	++	++	--	--
41	++	+	++	--
37	++	++	++	+
33	+++	++	+++	+
N-CAM180	++	+++	+	--
N-CAM140	++	++	--	--

Table 6.3 : Relative carbohydrate contents of chick brain glycoproteins. Assessment was carried out by visual comparison of corresponding Western blots. +, the lowest visible label; +++, the highest visible label; --, carbohydrate labelling absent.

In order to prevent "charge heterogeneity" due to artefactual charge modification of proteins which leads to the production of multiple spots of the same protein, tissue samples were prepared fresh and solubilised in lysis buffer before being stored at -20°C (see O'Farrell, 1975).

In the present study, between 150 and 200 silver-stained spots were detectable which, compared with Coomassie blue stained gels, provided an increase in sensitivity of 2-3 fold. However, probably the use of more protein containing incorporated [^{35}S]amino acid residues per gel would significantly increase the detection power of the system. Gels may also be over exposed to X-ray films in order to detect very small or lightly labelled proteins.

2-D PAGE combined with immunological techniques may be used to analyse in detail fucosylated antigens such as N-CAM or R15. In a pilot study, the antibody R14 (Scholey, 1991) was used to immunoblot PSDs antigens resolved on a two-dimensional gel. Several protein spots of various IEF values were labelled by the antibody.

Lectin labelling revealed over 55 fucosylglycoproteins in SPMs and PSDs. This was almost twice the number of glycoproteins detected in one-dimensional gels. Training-related fucosylglycoproteins of Mr 180, 100-120, 62-80, 50, and 37kD each contained a number of glycoprotein spots of different IEF values. Further, comparison between SPM and PSD patterns revealed that glycoprotein spots of Mr 42/45kD (doublet) and 37kD were enriched in SPMs whereas the 80kD component was enriched in PSDs.

D. The effect of learning on fucosylation : Lectin labelling was used to monitor changes in fucose incorporation into neural glycoproteins after passive avoidance

training. However, the densitometric analysis of the resulting Western blots did not detect any significant differences. This may have been due to the relatively small increases in fucose uptake seen in samples from MeA birds (see Chapter 4). However, the use of radiolabelled fucose showed small but significant increases in protein fucosylation 6hr and 24hr after training (see Chapter 4). It was concluded that the technique of lectin labelling was not sensitive enough to detect consistent but small variations in precursor uptake.

The results reported in this chapter seem encouraging. The combined techniques of two-dimensional gel electrophoresis and lectin binding were used to further characterize training-related glycoproteins. The two-dimensional pattern of antigen/s recognized by R15 needs to be identified. Also, the technique of 2-D PAGE could be used to raise polyclonal antibodies to identified proteins that were identified as showing increased fucosylation and to examine their role both during and after training.

CHAPTER SEVEN

General Discussion And Future Perspectives

CHAPTER SEVEN

GENERAL DISCUSSION AND FUTURE PERSPECTIVES

The approach taken in these investigations was based on a large body of results reported around 1980 (e.g., Burgoyne & Rose, 1980; Sukumar et al., 1980) indicating the involvement of glycoproteins in the formation of memory, both in chicks and mammals. Since then, more details regarding the role of fucose and its processing enzymes have emerged (e.g., Lössner & Rose 1983; Popov et al., 1983) and memory-related cellular cascades involving the activation of fucokinase and fucosyltransferase have been investigated (e.g., Matthies, 1989). Several models of neural plasticity that to a greater or lesser extent involve glycoproteins have been developed (e.g., Bock, 1991; Rose, 1991b; Schachner, 1992; Schmidt et al., 1990, 1991).

One of the early hypotheses that postulated a role for glycoproteins was developed by Margolis and Margolis (1970). According to them, glycoproteins play an important role in information storage, and this hypothesis implies that (i), during ontogenesis, glycoproteins may provide the information that facilitates the establishment of appropriate interneuronal connections, and (ii), in mature organisms, nerve impulses may alter the configuration or structure of glycoproteins that are secreted by neurons and deposited on the cell surface (presumably at the nerve terminals). As a result, the transmission capability of the synapses may alter and, thus potentially influence the future behaviour of the organism.

In his model for memory, Brunngraber (1972) has suggested that short term memory (STM) may result from rapid conformational changes in the macromolecular structure

of the neuronal membrane and glycoproteins. Long term memory (LTM), on the other hand, as also suggested by Margolis and Margolis (1970), is considered to be a consequence of an alteration of the neuronal membrane as a result of deposition of glycoproteins whose heteropolysaccharide chains alter in structure following neuronal activity.

In the "Signpost" theory of information processing forwarded by Bogoch (1974), glycoproteins are believed to be "responsible for the recognition required to establish specific brain circuitry, and that their disorders result in disordered function of brain circuitry and consequently, disorders of thought, mood, and behaviour". Glycoproteins can thus pave the way for the flow of information and establishment of communication in the nervous system. Neural glycoproteins show much heterogeneity, as well as changes with development and pathology. Their concentration and turnover also correlate with certain types of behaviour and training. "These are all consistent with the notion that they are directly involved in (the mechanisms of) learning and memory" (Bogoch 1968). For more discussion on the function of glycoproteins in the formation of brain circuitry see Bogoch (1969, 1972), and Matthies (1980).

A more classical description of the involvement of glycoproteins in the formation and consolidation of memory is given by Matthies (1982, 1989). There are said to exist neural connections concerned with unconditioned (UCS) and conditioned stimuli (CS). In addition, there are neural connections involved in motivational and emotional states. "Firing of CS neurons induces short term facilitation, which may be prolonged and enhanced by action potentials evoked by UCS, so that a further CS activation would also induce action potential during the limited time of *intermediate* storage" (Matthies, 1982). These events may then lead to the formation and transportation of fucosylglycoproteins to the synaptic junction of CS through activation of a second messenger system that involves dopaminergic neurons. The synapse is now

permanently activated and stimulation of CS would then always be sufficient to trigger unconditioned response in the form of an action potential (Matthies, 1982).

The "mnemon hypothesis" developed by Young (1965) although not seeming to point directly to the possible involvement of adhesion molecules during memory formation, is one of the early models of dynamic neural networks requiring an important role for glycoproteins. Accordingly, each particular piece of synaptically connected neural tissue has a certain number of potentially reinforcable connections. Those that do not lead to reward are suppressed or eliminated selectively until the remaining functional networks are stabilized, thus constituting a mnemon (Young, 1965).

This hypothesis may be extended in terms of reward and punishment, since in order to survive, a system needs to continuously evaluate the outcome of its behavioural response in a changing environment. For the network to develop, therefore, it is conceivable that the neural connections responsible for either reward or punishment are reinforced whereas the remaining connections would be suppressed. After all, continual addition of new glycoproteins and/or increased synapse formation in a confined volume of the brain does not seem feasible. Evidence for synapse loss or regression during neural development and imprinting has been presented by a number of researchers (e.g., Cowan et al., 1984; Scheich, 1987). However, to date, there have been no reports of specific glycoproteins that show deglycosylation during memory formation. As described in Chapter 4, certain chick brain glycoproteins were found to show a drop in fucose incorporation at various times after training. However, it is not known whether this phenomenon is indeed a reflection of neural regression induced by learning. Since much of the literature points to increased posttraining protein glycosylation, our interest was focused on the identification of those glycoproteins that showed training-related increased fucose incorporation. The above hypotheses imply that both configurational changes and synthesis of glycoproteins are phenomena that

take place during both short and long-term memory formation. The results agree with there being a critical role of glycoproteins in the neural processing of learning and memory.

One point remains unresolved; that is, whether there exist neural mechanisms which are involved in guiding the newly synthesized glycoproteins to specific and appropriate synaptic terminals responsible for processing particular types of information (for instance, passive avoidance learning). It seems unlikely that during the formation of each memory-related neuronal trace, the nervous system becomes engaged in redundant (and energy consuming) synthesis and distribution of glycoproteins to all available dendritic branches only to be used by a sub-set of specific dendritic spines and axonal boutons.

In order to resolve this problem, a possible mechanism may be suggested interrelating the neural events occurring during memory formation and consolidation. It involves three distinct mechanisms taking place and leading to the incorporation of glycoproteins at the synapses. Firstly, upon neural stimulation, triggered as a result of behavioural training or chemical or electrical induction, pico-second posttranslational modification of existing membrane-bound glycoproteins takes place. This is by activation of membrane-bound fucosylating enzymes and helps to transiently increase the synaptic efficacy. Secondly, persistent neural spike activity may trigger the incorporation of existing glycoproteins at neural terminals into the membrane. Finally, *de novo* synthesis of glycoproteins is initiated as a result of the activation of the second messenger systems which, in turn, are initiated during the first phase of neuronal events. A proportion of these newly synthesized glycoproteins may then be incorporated into the membrane as part of the consolidatory memory processes. In addition, the concentration of intracellular glycoproteins transported axonally to the

nerve terminals is stabilized once again.

There is now much evidence that long-term memory formation/consolidation involves incorporation of sugars like fucose into neural glycoproteins. They include results of a series of studies using the one-trial passive avoidance task in chick (Sukumar et al., 1980; McCabe & Rose, 1985), and a step-down passive avoidance task and a brightness discrimination task in rats (Popov et al., 1982). The activities of fucokinase and fucosyltransferase have been shown to alter both in chick and rat from minutes to 9hr after training (Lössner & Rose 1983; McCabe, 1985; Popov et al., 1983). Protein fucosylation takes place only when the process of memory formation proceeds unhindered. Thus, when chicks are made amnesic by the immediate application of transcranial subconvulsive electroshocks, no increased fucosylation occurs (Rose & Harding, 1984).

The involvement of glycoproteins in neural processes leading to memory formation was addressed in this thesis by a number of experimental approaches. They included competition experiments for characterization of the role of 2-deoxy-D-galactose *in vivo* and *in vitro*, study of fucosylation changes consequent upon chicks learning a passive avoidance task, detailed analysis of these glycoprotein molecules by raising polyclonal antibodies against them, and the use of various immunological techniques and two-dimensional gel electrophoresis to further characterize the glycoproteins.

The role of 2-deoxy-D-galactose : According to one of the criteria set out by Rose (1981a,b, 1983), if a neural event is necessarily involved in processes of memory formation then its inhibition will lead to amnesia. The administration of 2-deoxy-D-galactose (2DGal), has been shown to cause amnesia both in rat and chick (Jork et al., 1986; Rose & Jork, 1987). The amnestic effect of 2DGal is long lasting. However, 4hr after injection of 2DGal, the incorporation of fucose, injected 1hr after 2DGal

administration, is not inhibited, although there is a 68% inhibition 2hr after 2DGal injection (Rose & Jork, 1987). This short period of the 2DGal inhibitory effect is almost paralleled by its amnestic effect. If injection of 2DGal precedes training by 3.5hr, no amnesia is observed when MeA chicks are tested 1hr after training (Rose & Jork, 1987). However, birds show some degree of amnesia when 2DGal is injected either 1.5hr or 1hr before training, whereas they show clear amnesia if they are given a 2DGal injection just before and after training (Rose & Jork, 1987). The amnestic effect of 2DGal in chicks injected 30min after training falls to the level of birds that are injected with 2DGal over 1hr before training; that is, about 50% of chicks show amnesia.

These data suggest that 2DGal starts to inhibit fucosylation shortly after injection. However, our results show (Chapter 3) that simultaneous injections of fucose and 2DGal led to almost no inhibition of fucosylation, but if the injection of 2DGal preceded that of fucose by 1hr, there was a significant inhibition of fucose incorporation (a time-gap of 30min between the two sets of injections is assumed to lead to some degree of inhibition). Considering that training chicks on the passive avoidance task leads to a small but significant increase in fucosylation, it may therefore be concluded that 2DGal needs to inhibit only a small proportion of fucose incorporation to cause amnesia.

The effect of 2DGal is lateralised for the passive avoidance task; that is, birds given unilateral injections of 2DGal into the right hemisphere show amnesia 3hr after training (Barber & Rose, 1991). It is generally believed that 2DGal inhibits fucosylation of those glycoproteins that show increased fucose incorporation after training (Bullock et al., 1990b). A number of neural receptors such as opiate receptors are fucosylated and their function may be affected by 2DGal (Richter et al., 1991).

Detailed analysis of fucosylglycoproteins has revealed that there are two waves of fucosylation following training (Popov et al., 1980 and 1983). The electrophoretic analysis of hippocampal tissue from rats trained on a brightness discrimination task showed a significant increase (25-30%) in medium and high molecular weight membrane-bound fucosylglycoproteins 7-9hr after training (Popov et al., 1976, 1980). Also, the time-course of the activity of fucokinase and fucosyltransferase in rats followed that of fucosylation (Popov et al., 1983). If 2DGal inhibits training-related protein fucosylation when injected around the time of training, it can be used to show whether there is a second wave of protein fucosylation in chick. Indeed, when MeA birds were given bilateral intracerebral injections of 2DGal and tested 24hr after training, those that received the inhibitor 5-7hr after training showed amnesia (see Figure Ap.1 in Appendix; Zamani & Rose, 1990). These results suggest that, as in the rat, there is a second period of protein fucosylation in the chick as a consequence of memory formation. The results of training-related fucose incorporation reported in Chapter 4 suggested that the LPO is an important region during the second period of increased glycoprotein accumulation. It would be interesting to address the following points. Firstly, is the amnestic effect of 2DGal lateralised 5-7hr after training? Secondly, how long after injection do chicks become amnesic? And thirdly, what structure in the forebrain does 2DGal affect in order to cause amnesia?

The first question may be addressed by injecting the experimental hemisphere with inhibitor 5-7 after training and testing the birds at a specified posttraining time, for example 24hr. The rate at which 2DGal starts to cause amnesia can be examined by testing the birds from shortly after to 24hr after injection and training. If it were found that chicks are amnesic hours, but not minutes, after injection, it may be difficult to clearly answer the third question, since it is expected that once introduced into the forebrain 2DGal starts to diffuse rather quickly. Barber and Rose (1991) found that 1hr

after unilateral injection, the main bulk of radioactivity due to [^{3}H]2DGal is found in the same hemisphere where it was injected. However, the precise rate of diffusion of 2DGal within the injected hemisphere has not been explored. This could be determined by injecting and measuring specific activity of [^{3}H]2DGal in different brain regions such as the IMHV, hippocampus and LPO using the brain mould described in Chapter 2. If 2DGal had a fast rate of diffusion, galactose could be injected into the control hemisphere of chicks when the unilateral effect of 2DGal was to be investigated. The use of galactose is suggested since, when injected simultaneously with galactose and 2DGal, MeA birds do not show amnesia (Rose & Jork, 1987). This is because galactose and 2DGal compete for the same position in the oligosaccharide chains of glycoproteins. Also, in order to demonstrate whether the injection of 2DGal into LPO causes amnesia during 7-9hr posttraining, the IMHV of MeA birds may be injected with galactose while LPO receives 2DGal and the recall recorded at a specified time after training. The induction of amnesia would be in agreement with the results from lesion studies showing that posttraining ablation of the LPO renders trained chicks amnesic (Gilbert et al., 1991). As a result of a series of studies that consisted of lesions to the IMHV and LPO of trained-chicks at various pre- and posttraining time-points (Patterson et al., 1990; Gilbert et al., 1991), Rose (1991a&b) suggested that information processing takes place at specific times and in specific structures, and lesioning of any one or more of these structures (at specified times) either leads to amnesia or re-allocation of information processing to other structures (Rose, 1991a&b, also see part 2 of Chapter 1 for a brief review of these studies).

The results reported in Chapter 4 of this thesis suggest that the LPO may be the site of the second wave of fucosylation. Also, different biochemical events may take place in the IMHV and LPO, since different glycoproteins were found to be involved in these regions and at different times after training.

Fucose and memory retention : The injection of fucose into rat hippocampus improves retention of the shuttle box avoidance and brightness discrimination tasks (Wetzel et al., 1982). What such exogenous sources of fucose do in the brain is, however, a matter of debate. It has been suggested that it may stimulate glycoprotein synthesis, or inhibit lysosomal degradation of the polypeptide backbone of glycoproteins, or inhibit fucosidase (Wetzel et al., 1982; Zatz & Barondes, 1970). Circumstantial evidence from a number of studies suggests that fucose administration may have similar effects in the chick. For example, 7% of MeA chicks that were given bilateral intracerebral injections of fucose (20µmole) pecked at a dry bead 30min and 1hr after training as compared with 11% of saline injected birds (Rose & Jork, 1987). Similar results have also been reported by Bullock et al. (1991). The effect of fucose on memory processing, however, merits further investigation. Groups of chicks could be trained with normal and diluted MeA (5-10%; see Rosenzweig et al., 1991), and tested at different time-points thereafter. The experimental group would be injected with fucose and the control group with saline 30min before training. A quiet control group would also be used to monitor the effect of fucose injection on chicks' behaviour (e.g., assuming pecking activity at the test time). If fucose is to improve learning and/or retention, the percentage of pecking by the experimental group should be comparable with the group of chicks trained on undiluted MeA. In addition, it would be interesting to carry out a dose response test with fucose.

It is also possible to use a weak aversant instead of diluted MeA. For example, in a series of studies by Bourne et al. (1991), quinine was used in parallel with MeA. It was found that chicks trained on quinine had a shorter retention time than MeA birds. The retention levels in both groups were similar 45min after training, but the percentage of quinine-trained birds avoiding the bead at test dropped progressively thereafter and by 24hr posttraining they were not significantly different from W-trained birds. The accompanying fucose incorporation was much lower in quinine birds than in MeA-

trained birds. It is possible the quinine and diluted MeA affect olfactory/gustatory systems in qualitatively different ways.

Approach *vs* aversion learning : Since training chicks with W-coated beads may be regarded as an approach learning (water being a positive reinforcer) comparing neurobiological changes between this type of learning and that of MeA (avoidance) learning may raise certain problems. In mammals it is known, for example, that different neural systems are involved in appetitive and aversive learning tasks, and other structures such as the amygdala may be differentially involved in a number of appetitive and aversive learning paradigms (e.g., Carlson (1986) Chapter 14). Furthermore, the incorporation of fucose and expression of an immediate early gene *c-fos* has been shown to increase in both W and MeA birds, albeit to a lesser extent in W than in MeA birds (Anokhin et al., 1991; Sukumar et al., 1980).

Whether the increase in fucose incorporation into the W group simply reflects basal developmental changes or is directly related to learning-induced neurobiological changes, may be addressed by including other control groups. It is therefore proposed to use a control group consisting of chicks undergoing pre-training with one extra pre-training bead presentation, whilst others groups are trained on W and MeA. Appropriate neurochemical assessments would then be carried on all groups. The results would indicate whether water training leads to similar trends of neurochemical events to MeA training, and whether these events occur during the same time-window and/or in the same brain structures.

Despite its many advantages as a behavioural training paradigm, the passive avoidance task also presents certain disadvantages. For example, it does not delineate the strength of memory and, when amnestic agents are used, it does not clearly show the degree of

amnesia caused in individual birds. However, it does provide a measure of population learning or amnesia. The use of different colour beads at testing allows the investigator to determine whether birds are able to discriminate between a neutral and aversant bead. When using interventive methods, it may be appropriate to use other training paradigms in addition to the passive avoidance task, since different learning tasks exploit different abilities and probably cause neurobiological changes in different brain structures. Among the tasks available is the appetitive pebble floor food discrimination paradigm (e.g., Rogers et al., 1974, 1977), visual and auditory imprinting (Horn, 1985; Scheich, 1987), and perceptual learning (Vallortigara et al., 1990a&b). The cumulative results of these studies would shed more light on the specific function of particular drugs (e.g., 2DGal) and their behavioural effects.

The pebble floor task presents certain problems. While searching for food, chicks receive sensory information about the food and the characteristics of the floor via their feet. This input may manifest itself into additional neurochemical changes which consequently confuse the results. A modification to the pebble floor design is proposed to try to avoid this problem. Thus, food and sand could be scattered on a narrow platform (about 2.5cm) on one side of the pen ensuring that chicks are unable to climb onto it but could see and reach the food mixed with sand and learn the task (see Fig. 7.1). The effect of 2DGal on learning the pebble floor task merits investigation, since this study would show whether this inhibitor can affect an appetitive task.

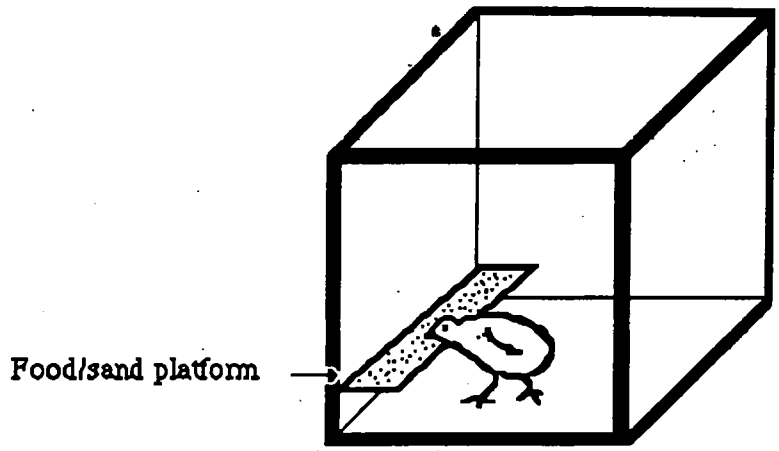


Figure 7.1 : A schematic diagram of the modified pebble floor training paradigm. Chicks are placed in the pen individually and the extent of their learning is measured by the percentage of correct pecks per unit time.

Exploiting immunological techniques : Immunological methods are powerful tools for investigating neurochemical events during development, learning and memory formation. In Chapter 5, the production of a specific polyclonal antibody (R15) was discussed. Its antigen is localized in pre- and postsynaptic structures. R15 was found to render chicks amnesic if injected during a specified time-window and the immunoreactivity of its antigen was affected by training, showing an increase at 6hr and a drop at 24hr posttraining.

Further study would help to characterize the role of this antibody and may be summarized as follows:

A. The time course of R15 immunogenicity could be further investigated at 1hr, 3hr,

and about 9hr after training, and the results compared with the present data. This would show whether R15 immunoreactivity peaks at a particular time-point, most probably around the immediate posttraining period, or whether it shows a second wave of immunoreactivity at 5-7hr after training.

B. ELISA could be used to assess the regional and subcellular distribution of R15 in both control and trained chicks. Structures such as IMHV and LPO and subcellular fractions such as SPMs and PSDs are of particular interest.

C. The effect of training on R15 immunoreactivity could be assessed by the technique of immunoelectron microscopy. The use of protein A-gold particles allows relative quantification.

D. Despite earlier results from posttraining R15 immunoreactivity, it is not obvious whether these variations in the forebrain are necessary for the processing and storage of memory. Bilateral and/or unilateral injections of the antibody into different structures such as IMHV and LPO would help resolve this problem. However, the existence of asymmetry in the visual projections in the chick (mainly in males) (Adret & Rogers, 1989; Boxer & Stanford, 1985) should be considered when interpreting the results.

E. R15 has been shown to cause amnesia for the passive avoidance paradigm in chick. It would be interesting to investigate its role in other training paradigms such as the modified pebble floor task.

F. Immunoseparation of R15 antigen using samples that have already incorporated radiolabelled fucose would show more clearly whether the antigen is a fucosylated glycoprotein. A similar method was recently used by Doyle and associates to examine training-related alterations in sialylation of N-CAM (Doyle et al., 1991).

G. The use of R15 in other animals such as rat would show whether similar glycoproteins are involved in learning and memory formation regardless of species. As yet there is no evidence of R15 cross-reactivity with antigens from other species.

The procedure for the production of new antisera can be modified in order to achieve

highly specific antibodies. Thus, proteins from purified SPMs and PSDs can be run over an UEA.I affinity column (e.g., Zanetta, 1977), extracted fucosylglycoproteins resolved electrophoretically and different bands corresponding to a small range of specified molecular weight bands cut out from the gels and prepared for antibody production.

The work reported in Chapter 5 was part of a comprehensive immunotechnological approach to neural plasticity being employed in the BBRG. Recently, another polyclonal antibody (R14) raised from chick PSDs by Scholey (1991) showed similar behavioural effects to those of R15. Further, it was shown to cross-react with one neural antigen from rat. The immunoreactivity of R14 antigen increased 24hr after tetanic stimulation of rat hippocampus. R14 was shown to be amnestic when chicks are tested 24hr after passive avoidance training and injections given 30, 15, or 10min before training. However, the amnesia was not evident 1hr or 3hr posttraining (injections at 5-7hr posttraining were not included). The results of such an experiment would show whether the R14 antigen plays a role in information processing during the second wave of protein fucosylation (see Chapter 4; Zamani & Rose, 1990).

The role of N-CAM in neural plasticity is currently under study. An anti-N-CAM antibody has been found to affect both waves of memory processing and consolidation. Thus, injection of this antibody around the time of training or at 6hr, but not 3hr, posttraining, rendered chicks amnesic (Scholey, personal communication). These results rather contrasted with those of Doyle et al. (1990) in which the antibody rendered rats amnesic if injected 8-10hr after training but not at other time-points. Furthermore, rats exhibited amnesia 48hr posttraining as opposed to the 24hr shown in chicks. The second wave of protein fucosylation in rats is shown to occur between 7-9hr following training whereas in the chick it seems to happen around 5-7hr after training (Popov et al., 1983).

Other analytical techniques : The lectin binding study (Chapter 6) produced a qualitative measure of chick brain glycoproteins that are involved in memory formation and of their sugar contents. Con.A, UEA.I, MAA, and SNA showed that the molecules contain varying amount of mannose, fucose, NANA(α2-3)galactose, and NANA(α2-6)GalNAc (see Table 6.3). Further, the glycoprotein of Mr 180kD was found to contain similar amounts of these sugars to N-CAM180. It was therefore concluded that glycoprotein180 was probably identical to N-CAM. Various anti-rat N-CAM antibodies cross-reacted with chick brain N-CAM180 and showed similar mobility on gels to this glycoprotein.. The use of lectins could determine whether these glycoproteins contain other sugars, such as GlcNAc. It is assumed that almost all N-type glycoproteins contain GlcNAc, since, as it was seen in Chapter 1, it is the first sugar in the assembly of the common carbohydrate core of N-type glycoproteins. Functional characterization of glycoproteins requires detailed knowledge about the oligosaccharide structures of these molecules. As was pointed out in Chapter 1, N-type and O-type glycoproteins can potentially possess highly complex structures. Without a full analysis of these structures a clear understanding of the biological functions of these molecules may not be possible. Until now, relatively little information has been available about the oligosaccharide structures of synaptic glycoproteins. Krusius et al. (1978) have identified five O-linked sugar chains on SPMs. Analyses of N-linked oligosaccharides have led to the identification of triantennary complex chains (between 60-80% of the total sugars), biantennary structures (21%), and high mannose chains (13-16%) (e.g., Krusius et al., 1978). But, almost no hybrid oligosaccharides have been found to be associated with synaptic glycoproteins (Gurd, 1989). Identification and characterization of the processing enzymes involved in oligosaccharide assembly and understanding of their mode of function will offer more insight into glycoprotein involvement in memory formation and consolidation.

Using two-dimensional gel electrophoresis, antigens labelled with antibodies may be eluted from gels and their amino acid and carbohydrate sequences analysed. This would, however, require a highly specific antibody. Alternatively, specific antibodies can be used in expression libraries to identify clones which express such antigens and consequently to characterize the antigens themselves (e.g., Gurd et al., 1991).

In summary, a number of neurochemical and immunological approaches were used in this project to identify and analyse the role of glycoproteins involved in memory formation and consolidation. Our interest was mainly focused on fucosylated glycoproteins. This was based on published results implicating the role of these glycoproteins in neural plasticity in different species. We showed that the amnestic sugar 2-deoxy-D-galactose inhibited fucose incorporation in a dose-response manner both *in vivo* and *in vitro*. The *in vitro* pattern of protein inhibition at synaptic level suggested two differential glycoprotein synthesis and distribution across the synaptic cleft. These glycoproteins were identified by gel electrophoresis using purified synaptic plasma membranes (SPMs) and postsynaptic densities (PSDs).

In subsequent studies, training-related fucosylglycoproteins were identified and the timecourse of their expression as well as their sub-cellular and anatomical distribution were examined. An *in vitro* increase in the rate of fucosylation was found in the right forebrain base, containing the LPO, 4hr after passive avoidance training. Separation of these glycoproteins by gel electrophoresis revealed a significant increase in fucose incorporation into a 110-120kD glycoprotein component of the forebrain base.

Encouraged by these findings, the identification and characterization of training-related glycoproteins were extensively studied using the double-labelling technique. Subsequently, a number of glycoproteins showing significant increase in fucosylation either 6hr or 24hr after training, or at both posttraining time-points, were identified *in*

vitro. Anatomical and sub-cellular investigations localized these glycoproteins mainly in SPMs and PSDs of LPO. In SPMs, glycoproteins of Mr 50, 41, and 33kD, and in PSDs, those of Mr 150-180, 100-120, 62-80, 55, and 33kD showed increased fucosylation at different time-points after training. Several other proteins showed significant decreases in fucosylation during this period.

In order to further characterize the role of glycoproteins identified above, a series of immunological studies were carried out. A polyclonal antibody R15 was raised against chick forebrain and it recognized a 60-62kD synaptic glycoprotein. Behavioural studies revealed it to render MeA-trained birds amnesic if it was intracerebrally administered 30min before training. The immunocytochemical studies showed that R15 labelled cell bodies, as revealed by light microscopy, and presynaptic membrane and postsynaptic densities, by electronmicroscopy. The immunoreactivity of the R15 antigen increased 6hr after training, whereas it was significantly decreased after 24hr.

Using specific lectins, the R15 antigen was found to contain fucose, mannose, NANA, galactose, and GalNAc. The sugar content of other glycoproteins that were found to be involved in memory formation were also examined as shown in Fig. 6.3 of Chapter 6. Further analysis of neural glycoproteins were carried out using the technique of 2-D PAGE. It revealed over 55 fucosylated glycoproteins in SPMs and PSDs, three of which were located in the region of R15 antigen (60-62kD).

Finally, specific antibodies whose antigens have been implicated in learning and memory formation were used to examine whether their immunoreactivity changed as a result of passive avoidance training. Anti-N-CAM antibodies and anti-ependymin antibody showed cross reactivity with chick antigens from SPMs and PSDs. However, immunoblotting did not show training-induced related changes.

Results presented here are found to be in agreement with the current understanding of the role of neural glycoproteins in learning and memory formation and strongly support attempts for detailed structural and genetic analyses of these glycoproteins. Such results would extend our understanding of the relationship between glycoproteins and neurobiological events involved in learning and memory formation.

APPENDIX

APPENDIX

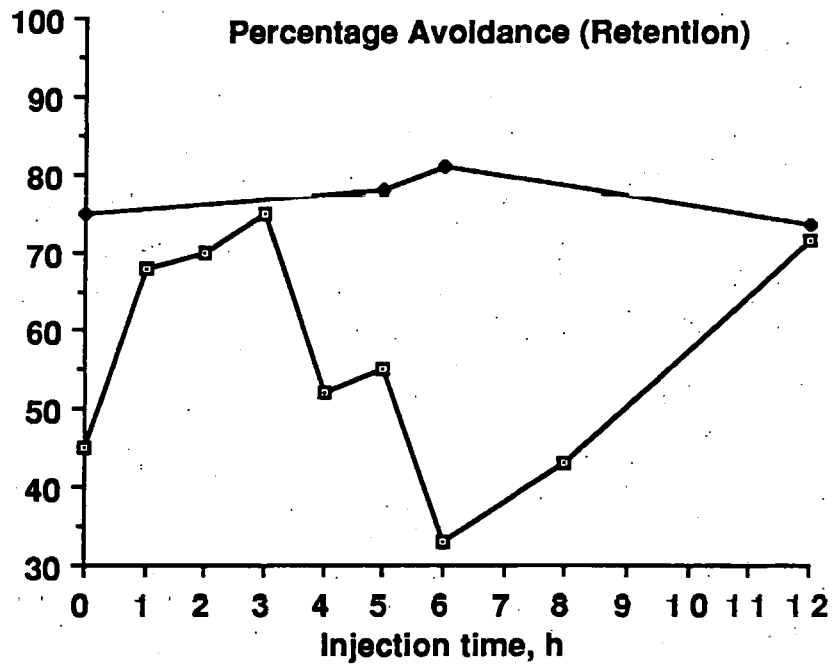


Figure Ap. 1 : Timecourse Of 2DGal induced amnesia for passive avoidance training. Day-old MeA-trained chicks were injected with either 0.9% saline or 10μmol/10μl of 2DGal. The graph shows the percentage retention in chicks tested 24h after training. Bold squares representing saline-injected chicks and dotted squares 2DGal-injected birds. The number of chicks tested at each time-point were 12, 19, 16 and 19, for saline-injected birds, and 31, 19, 10, 16, 21, 33, 18, 23 and 21, for 2DGal-injected birds respectively.

PUBLICATIONS ARISING FROM THIS PROJECT

Zamani M.R., Bullock S., Jork R. & Rose S.P.R. (1989) Fucose incorporation into glycoproteins, effects of 2-deoxy-D-galactose. *Neuroscience Letters, S36*, 60.

Bullock S., Potter J., Zamani M.R. & Rose S.P.R. (1989) Use of the amnestic agent 2-deoxy-D-galactose in identifying chick brain fucosylglycoproteins involved in learning & memory formation. *Neuroscience Letters, S36*, 60.

Zamani M.R. & Rose S.P.R. (1990) Timecourse of 2-deoxy-D-galactose induced amnesia for passive avoidance training in chick. Open Network Conference, Neural Mechanisms of Learning & Memory (ESF Scientific Networks), London 3-6 April 1990, p 5-12

Zamani M.R. & Bullock S. (1990) Effect of 2-deoxy-D-galactose on fucose incorporation into brain glycoproteins. *Biochemical Society Transactions, 18(3)*, 417-418.

Bullock S., Zamani M.R. & Rose S.P.R. Characterisation and localisation of pre- and postsynaptic glycoproteins of the chick forebrain showing changed fucose incorporation following passive avoidance training. *Journal of Neurochemistry* (in press).

Zamani M.R. & Bullock S. (1991) Identification of a neural glycoprotein that is involved in memory formation in chick. Avian Learning & Plasticity, a satellite meeting of the European Neuroscience Association at the Open university, Milton Keynes, 6-8 September.

Davies H.A., Zamani M.R., Brown J.Y. & Bullock S.(1991) Immunolocalisation of a protein involved in passive avoidance learning in chick. Avian Learning & Plasticity, a satellite meeting of the European Neuroscience Association at the Open university, Milton Keynes, 6-8 September.

Zamani M.R. & Bullock S. (in preparation) An investigation into protein Glycosylation in chick brain by two-dimensional gel electrophoresis.

Zamani M.R. & Bullock S. (in preparation) The anti-chick brain antibody R15 recognizes a 60 kD synaptic glycoprotein.

Zamani, M.R. & Bullock S. (in preparation) R15, an antibody that recognizes a 60 kD synaptic glycoprotein, produces amnesia in chicks given one-trial passive avoidance training.

REFERENCES

REFERENCES

Abdel-Latif, A.A. (1986) Calcium-mobilizing receptors, polyphosphoinositides and the generation of second messengers. Pharmac. Rev., 38, 227-72.

Adret, P., Rogers, L.J. (1989) Sex difference in the visual projections of young chicks: a quantitative study of the thalamofugal pathway. Brain Res., 478, 59-73.

Aleksidze, N., Potenska, A., Murphy, S., Rose, S.P.R. (1981) Passive avoidance training in the young chick affects forebrain a-Bungarotoxin and serotonin binding. Neurosci. Lett., S7, 244.

Ali, S., Bullock, S., Rose, S.P.R. (1988a) Phosphorylation of synaptic proteins in chick forebrain: changes with development and passive avoidance training . J. Neurochem., 50, 1579-87.

Ali, S., Bullock, S., Rose, S.P.R. (1988b) Protein kinase C inhibitors prevent long-term memory formation in the one-day-old chick. Neurosci. Res. Commun., 3, 133-9.

Ambron, R.T., Goldman, J.E., Schwartz, J.H. (1974) Axonal transport of newly synthesized glycoproteins in a single identified neuron of Aplysia california. J. Cell. Biol., 61, 665-75.

Ando, S., Yu, R.K. (1979) Isolation and structural study of a novel fucose-containing-disialoganglioside from human brain . New York: Academic Press.

Anokhin, K.V., Mileusnic, R., Shamakina, I.Y., Rose, S.P.R. (1991) Effects of early experience on c-fos gene expression in the chick forebrain. Brain Res., 544, 101-7.

Anokhin, K.V., Rose, S.P.R. (1991) Learning induced increase of immediate early gene messenger RNA in the chick forebrain. Eur. J. Neurosci., 3, 162-7.

Arce, A., Maccioni, H.J., Caputto, R. (1971) The biosynthesis of gangliosides. Biochem. J., 121, 483-93.

Aroson, N., De-Duve, C. (1968) Digestive activity of lysosomes. J. Biol. Chem, 243, 4564-73.

Autilio, L.A., Appel, S.H., Pettis, P., Gambetti, P.L. (1968) Biochemical studies of synapses *in vitro* I. Protein synthesis. Biochem., 7, 2615-22.

Barber, A., Gilbert, D.B., Rose, S.P.R. (1989) Glycoprotein synthesis is necessary for memory of sickness-induced learning in chicks. Eur. J. Neurosci., 1, 673-7.

Barber, A.J., Rose, S.P.R. (1991) Amnesia induced by 2-Deoxygalactose in the day-old chick: lateralisation of effects in two different one-trial learning tasks. Behav. Neural Biol., 56, 77-88.

Barclay, A.N., Ward, H.A. (1982) Purification and characterization of membrane glycoproteins from rat thymocytes and brain. Eur. J. Biochem., 129, 447-58.

Bateson, P.G.G., Rose, S.P.R., Horn, G. (1973) Imprinting lasting effects o uracil incorporation into chick brain. Science, 181, 576-8.

Bateson, P.P.G. (1966) The characteristics and context of imprinting. Biol. Rev., 41, 177-220.

Bateson, P.P.G., Horn, G., Rose, S.P.R. (1972) The effects of early experience on regional incorporation of precursors into RNA and protein in the chick brain. Brain Res., 39, 449-65.

Bateson, P.P.G., Horn, G., Rose, S.P.R. (1975) Imprinting: correlation between behavior and incorporation of [14C]uracil into chick brain. Brain Res, 84, 207-20.

Bateson, P.P.G., Wainwright, A.P. (1972) The effects of prior exposure to light on imprinting process in domestic chicks. Behaviour, 42, 279-90.

Beesely, P.W., Palandino, T., Gravel, C., Hawkes, R., Gurd, J.W. (1987) Characterisation of gp50, a major glycoprotein present in rat brain synaptic membranes, with a monoclonal antibody. Brain Res., 408, 232-8.

Bekesi, J.G., Winzler, R.J. (1967) The metabolism of plasma glycoproteins. J. Biol. Chem., 242, 3873-9.

Benett, G., Di Giamberadino, L., Koenig, H.L., Droz, B. (1973) Axonal migration of protein and glycoprotein to nerve endings. II. Radioautographic analysis of the renewal of glycoproteins in nerve endings of chick cilliary ganglion after intracerebral injection of [^{3}H]fucose and [^{3}H]glucosamine. Brain Res., 60, 129-46.

Benowitz, L. (1972) Effects of forebrain ablations on avoidance learning in chicks.

Arch. Biochem. Biophys., 147, 753-61.

Benowitz, L. (1980) Functional organization of the avian telencephalon . New York: Plenum Press.

Berger, E.G., Buddecke, E., Kamerling, J.P., Kobata, A., Paulson, J.C., Vliegenthart, J.F.G. (1982) Structure, biosynthesis and functions of glycoprotein glycans. Experientia, 38, 1129-62.

Bernard, C.C.A., Gibbs, M.E., Hodge, R.J., Ng, K.T. (1983) Inhibition of long-term memory formation in the chicken by anti-chick Thy-1 antibody. Brain Res. Bull., 11, 111-6.

Berridge, M. (1986) Second messenger dualism in neuromodulation and memory. Nature, 323, 294-5.

Beyer, T.A., Sadler, J.E., Rearick, J.I., Paulson, J.C., Hill, R.L. (1981) Glycosyl transferases and their use in assessing oligosaccharide structure and structure-function relationships. Adv. Enzymol., 52, 23-175.

Bock, E. (1991) Cell-cell adhesion molecules. Biochem. Soc. Transact., In press,

Bogoch, S. (1968) The biochemistry of memory . New York: Oxford University Press.

Bogoch, S. (1969) Future of the brain sciences . New York: Plenum Press.

Bogoch, S. (1972) Brain glycoproteins and inter-cell recognition: tay-sachs' disease and intraneuronal recognition. In B. W. Volk, & S. M. Aronson (Ed.),

Sphingolipids, sphingolipidosis and allied disorders (pp. 127-49). New York: Plenum Press.

Bogoch, S. (1974) Glycoproteins and brain circuitry: The signpost theory in normal memory function and in the regressive states of brain tumors and the psychoses. Biol. Psychiatry, 9, 73-88.

Bondareff, W., Pysh, J.J. (1968) Distribution of the extracellular space during postnatal maturation of rat cerebral cortex. Anat. Rec., 160, 773-80.

Bondy, S.C. (1971) Axonal transport of macromolecules. Protein migration in the CNS. Exp. Brain Res., 13, 127-34.

Bourne, R.C., Davies, D.C., Stewart, M.G., Csillag, A., Cooper, M. (1991) Cerebral glycoprotein synthesis and long-term memory formation in the chick (gallus domesticus) following passive avoidance training depends on the nature of the aversive stimulus. Eur. J. Neurosci., 3, 243-8.

Boxer, M.I., Stanford, D. (1985) Projections to the posterior visual hyperstriatal region of the chick: an HRP study. Exp. Brain Res., 57, 494-8.

Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of proteins utilizing the principle of protein-dye binding. Anal. Biochem., 72, 248-54.

Bradley, P., Davies, D.C., Horn, G. (1985) Connections of the hyperstriatum ventrale of the domestic chick (Gallus domesticus). J. Anatom., 140, 577-89.

Bretscher, M.S., Raff, M.C. (1975) Mammalian plasma membranes. Nature, 258, 43-7.

Brunngraber, E.G. (1972) Chemistry and metabolism of glycopeptides derived from brain glycoproteins. Adv. Med. Biol., 25, 17-49.

Brunngraber, E.G., Aguilar, V., Aro, A. (1969) Chromatography on calcium hydroxylapatite of glycoproteins solubilised by triton X-100 from particulates prepared from rat brain. Arch. Biochem. Biophys., 129, 131-41.

Büchsel, R., Hassels-vischer, B., Tuber, R., Reutter, W. (1980) 2-deoxy-D-galactose impairs the fucosylation of glycoproteins of rat liver and morris hepatoma. Eur. J. Biochem., 11, 445-53.

Buckley, K., Kelly, R.B. (1985) Identification of a transmembrane glycoprotein specific for secretory vesicles of neural and endocrine cells. J. Cell Biol., 100, 1284-94.

Bullock, S., Csillag, A., Rose, S.P.R. (1987) Synaptic vesicle proteins and acetylcholine levels in chick forebrain nuclei are altered by passive avoidance training. J. Neurochem., 46, 340-8.

Bullock, S., De Graan, P.N.E., Oestreicher, A.B., Gispen, W.H., Rose, S.P.R. (1990a) Identification of a 52 kD chick brain membrane protein showing changed phosphorylation after passive avoidance training. Neurosci. Res. Commun., 6, 181-6.

Bullock, S., Gordon-Weeks, P.R., Csillag, A. (1988) Preparation and characterisation

of a monoclonal antibody to an antigen highly enriched in chic brain postsynaptic densities. J. Neurochem., 51, 442-50.

Bullock, S., Potter, J., Rose, S.P.R. (1991) Incorporation of fucose into chick brain glycoproteins: effects of the amnesic agent 2-deoxygalactose. In H. J. Conradt (Ed.), Protein glycosylation: Cellular, biotechnological and analytical aspects. GBF monographs (pp. 53-6). Braunschweig: VCH Publishers.

Bullock, S., Potter, J.A., Rose, S.P.R. (1990b) Effects of the amnesic agent 2-deoxygalactose on incorporation of fucose into chick brain glycoproteins. J. Neurochem., 54, 135-42.

Burchuladze, R., Potter, J., Rose, S.P.R. (1991) Memory formation in the chick depends on membrane-bound protein kinase C. Brain Res., 535, 131-8.

Burgoyne, R.D. (1989) A role for membrane-inserted protein kinase C in cellular memory? TIBS, 14 May, 87-88.

Burgoyne, R.D., Rose, S.P.R. (1978) Transient increase in [^{3}H]fucose incorporation following first exposure of dark-reared rats to light. Life Sci., 23, 269-74.

Burgoyne, R.D., Rose, S.P.R. (1980) Subcellular localization of increased incorporation of [^{3}H]fucose following passive avoidance learning in the chick. Neurosci. Lett., 19, 343-8.

Campagne, M.V., Oestricher, A.B., En Henegouwen, V., Gispen, W.H. (1990) Ultrastructural double localization of B-50/GAP43 and synaptophysin (p38) in the neonatal and adult rat hippocampus. J. Neurocytol., 19, 948-61.

Caputto, R., Maccioni, H.J., Arce, A., Cumar, F.A. (1976) Biosynthesis of brain gangliosides. Adv. Exp. Med. Biol., 71, 27-44.

Carbonetto, S. (1984) The extracellular matrix of the nervous system. TINS, 7, 382-387.

Carlson, N.R. (1986) Anatomy of learning. In Physiology and behaviour (pp. 542-96). Boston: Allyn & Bacon Press.

Caroni, P., Carlson, S.S., Schweitzer, E., Kelly, R.B. (1985) Presynaptic neurons may contribute a unique glycoprotein to the extracellular matrix at the synapse. Nature, 314, 441-3.

Casagrande, V.A., Harting, J.K. (1975) Transneuronal transport of tritiated fucose and proline in the visual pathways of tree shrew Tupaia glis. Brain Res., 96, 367-72.

Cherkin, A., Lee-Teng, E. (1965) Interruption by halothane of memory consolidation in the chick. Fed. Proc., 24, 328-9.

Cicero, T.J., Cowan, W.M., Moore, B.W., Suntzeff, V. (1970) The cellular localization of the two brain specific proteins, S-100 and 14-3-2. Brain Res., 18, 25-34.

Cocchia, D., Michetti, F., Donato, R. (1981) Immunochemical and immunocytochemical localization of S-100 antigen in normal human skin. Nature, 294, 85-7.

Coffey, J.W., Miller, O.N., Sellinger, O.Z. (1964) The metabolism of L-fucose in the rat. J. Biol. Chem., 239, 4011-8.

Cohen, J., Burne, J.F., McKinlay, C., Winter, J. (1987) The role of laminin and the laminin/fibronectin receptor complex in the outgrowth of retinal ganglion cell axons. Dev. Biol., 122, 407-18.

Cohen, J., Burne, J.F., Winter, J., Bartlett, P. (1986) Retinal ganglion cells lose response to laminin with maturation. Nature, 322, 465-7.

Cowan, W.M., Fawcett, J.W., O'Leary, D.M.D., Stanfield, B.B. (1984) Regressive events in neurogenesis. Science, 225, 1258-65.

Crick, F. (1984) Memory and molecular turnover. Nature, 312, 101.

Croiset, G., Heijnen, C.J., de Wied, D. (1990) Passive avoidance behavior, vasopressin and the immune system. Neuroendocrinology, 51, 156-61.

Dahl, J.L., Hokin, L.E. (1974) The sodium-potassium adenosine triphosphatase. Ann. Rev. Biochem., 43, 327-56.

Davies, D.C., Taylor, D.A., Johnson, M.H. (1988) The effects of hyperstriatal lesions on one-trial passive-avoidance learning in the chick. J. Neurosci., 8, 4662-6.

Davis, H.P., Squire, L.R. (1984) Protein synthesis and memory: A review. Psychol. Bull., 96, 518-59.

Davis, J.L., Pico, R.M., Cherkin, A. (1982) Memory enhancement induced in chicks by L-prolyl-L-lecyl-glycineamide. Pharmacol. Biochem. Behav., 17, 893-6.

Dawson, G., Stoolmiller, A.C. (1976) Comparison of the ganglioside composition of established mouse neuroblastoma cell strains grown *in vivo* and in tissue culture. J. Neurochem., 26, 225-6.

Dette, B.A., Wesemann, W. (1978) On the significance of sialic acid in high affinity 5-hydroxytryptamine uptake by synaptosomes. Hoppe-Seyler's Z. Physiol. Chem., 359, S. 399-406.

DiFiglia, M., Roberts, R.C., Benowitz, L.I. (1990) Immunoreactive GAP43 in the neuropil of adult rat neostriatum: Localization in unmyelinated fibers, axon terminals, and dendritic spines. J. Comp. Neurol., 302, 992-1001.

Donowicz, M.S., Kivatinitz, S.C., Caputto, B.L., Caputto, R. (1988) Synthesis and translocation of gangliosides and glycoproteins during urethane anesthesia. J. Neurochem, 50, 1369-74.

Donowicz, M.S., Kivatinitz, S.C., Caputto, B.L., Caputto, R. (1988) Synthesis and translocation of gangliosides during urethane anesthesia. J. Neurochem., 29, 939-45.

Dowson, G., Hancock, L.W.1. (1989) Inborn errors of complex carbohydrate catabolism. In M. R.U., & M. R.K. (Ed.), Neurobiology of glycoconjugates (pp. 421-49). New York: Plenum Press.

Doyle, E., Bruce, M.T., Breen, K.C., Smith, D.C., Anderton, B., Regan, C.M.

(1990) Intraventricular infusions of antibodies to amyloid-b-protein precursor impair the acquisition of a passive avoidance response in the rat. Neurosci. Lett., 115, 97-102.

Doyle, E., Nolan, P.M., Bell, R., Regan, C.M. (1991) Hippocampal NCAM180 transiently increases sialylation during the acquisition and consolidation of a passive avoidance response in the adult rat. J. Neurosci. Res. (in press).

Duband, J.L., Rocher, S., Chen, W.T., Yamada, K.M., Thiery, J.P. (1986) Cell adhesion and migration in the early vertebrate embryo: location and possible role of the putative fibronectin receptor complex. J. Cell. Biol., 102, 160-78.

Dudai, Y. (1989) The neurobiology of memory. Concepts, findings, trends. New York: Oxford University Press.

Dunlop, D.S., Van Elden, W., Lajtha, A. (1975) Optimal conditions for protein synthesis in incubated slices of rat brain. Brain Res., 99, 303-18.

Dunlop, D.S., Van Elden, W., Lajtha, A. (1977) Developmental effects on protein synthesis rates in regions of the CNS *in vivo* and *in vitro*. J. Neurochem., 29, 939-45.

Durand, P.B., Barrone, C., Della, C. (1969) Fucosylated glycoconjugates of the nervous system. J. Pediat., 75, 665-9.

Dutton, G.R., Barondes, S.H. (1970) Glycoprotein metabolism in developing mouse brain. J. Neurochem., 17, 913-20.

Edelman, G.M. (1986) Cell adhesion molecules in the regulation of animal form and

tissue pattern. Ann. Rev. Cell Biol., 2, 81-116.

Edelman, G.M. (1987) Neural Darwinism: The theory of neuronal group selection. New York: Basic Books Inc.

Edelman, G.M., Murray, B.A., Mege, R.M., Cunningham, B.A., Gallin, W.A. (1987) Cellular expression of liver and neural cell adhesion molecules after transfection with their cDNAs results in specific cell-cell binding. PNAS (USA), 84, 8502-6.

Eisenbarth, G.S., Walsh, F.S., Nirenberg, M. (1979) Monoclonal antibody to a plasma membrane antigen of neurons. PNAS (USA), 76, 4913-7.

Finne, J., Krusius, T., Margolis, R.K., Margolis, R.U. (1979) Novel mannitol-containing oligosaccharides obtained by mild alkaline borohydride treatment of a chondroitin sulfate proteoglycan from brain. J. Biol. Chem., 254, 10295-300.

Fitzgerald, M. (1990) c-fos and the changing face of pain. TINS, 13, 439-440.

Flexner, J.B., Flexner, L.B. (1967) Restoration of expression of memory lost after treatment with puromycin. PNAS (USA), 57, 1651-4.

Flowers H.M. (1981) Chemistry and biochemistry of D- and L-fucose. Adv. Carbohyd. Chem. Biochem., 39, 279-345.

Forman, D.S., Grafstein, B., McEwen, B.S. (1972) Rapid axonal transport of fucosyl glycoproteins in the goldfish optic system. Brain Res., 48, 327-42.

Fu, S.C., Gurd, J.W. (1983) Developmental changes in the oligosaccharide composition of synaptic junctional glycoproteins. J. Neurochem., 41, 1726-34.

Gammon, C.M., Goodrum, J.F., Toews, A.D., Okabe, A., Morell, P. (1985) Axonal transport of glycoconjugates in the rat visual system. J. Neurochem., 44, 376-87.

Gaston, K. (1980) Evidence for separate and concurrent avoidance learning in the two hemispheres of the normal chick brain. Pharmacol. Biochem. Behav., 28, 129-37.

Gibbs, M.E., Barnett, J.M. (1976) Drug effects on successive discrimination learning in young chicks. Brain Res. Bull., 1, 295-9.

Gibbs, M.E., Gibbs, C.L., Ng, K.T. (1978) A possible physiological mechanism for short-term memory. Physiol. Behav., 20, 619-27.

Gibbs, M.E., Ng, K.T. (1977) Psychobiology of memory formation. Biobehav. Rev., 1, 113-36.

Gibbs, M.G., Ng, K.T. (1976) Memory formation: a new three phase model. Neurosci. Lett., 2, 165-9.

Gigg, J. (1991) An investigation of the neurophysiological correspondents of learning and memory in two forebrain regions of the day-old chick. Ph. D. Thesis, The Open University, England,

Gilbert, D., Patterson, T.A., Rose, S.P.R. (1991) Dissociation of brain sites

necessary for registration and storage of memory for a one-trial passive avoidance task in the chick. Behav. Neurosci., 105, 553-61.

Ginsberg, V., Neufeld, E.F. (1969) Complex heterosaccharides of animals. Ann. Rev. Biochem., 38, 371-88.

Gionnini, T., Foucaud, B., Hiller, J.M., Hatten, M.E., Simon, E.J. (1982) Lectin binding of solubilised opiate receptors: Evidence for their glycoprotein nature. Biochem. Biophys. Res. Commun., 105, 1128-34.

Goodrum, J.F., Stone, G.C., Morell, P. (1989) Axonal transport and intracellular sorting of glycoconjugates. In R. U. Margolis, & R. K. Margolis (Ed.), Neurobiology of glycoconjugates (pp. 277-309). New York: Plenum Press.

Gordon-Weeks, P., Hading, S. (1983) Major differences in the concanavalin A binding glycoproteins of postsynaptic densities from rat forebrain and cerebellum. Brain Res., 277, 380-5.

Greenogh, W.T., Hwang, H.N.S., Gorman, C. (1985) Evidence for active synapse formation or altered postsynaptic metabolism in the visual cortex of rats reared in complex environments. PNAS (USA), 82, 4549-52.

Grumet, M., Hoffman, S., Crossin, K.L., Edelman, G.M. (1985) Cytotactin, an extracellular matrix protein of neural and non-neural tissues that mediates glia-neuron interaction. PNAS (USA), 82, 8075-9.

Gurd, J.W. (1989) Glycoproteins of the synapse. New York: Plenum Press.

Gurd, J.W. (1991) Glycoproteins of the postsynaptic density: biochemical and

immunochemical characterization of PSD-GP180. Biochem. Soc. Abstr., (Oct. issue).

Gurd, J.W., Bissoon, N., Kelly, P.T. (1983) Synaptic junctional glycoproteins are phosphorylated by cAMP-dependent protein kinase. Brain Res., 269, 287-96.

Gurd, J.W., Gordon-Weeks, P., Evans, W.H. (1982) Biochemical and morphological comparisons of post-synaptic densities prepared from rats, hamsters and monkey brains by phase partitioning. J. Neurochem., 39, 1117-24.

Gurd, J.W., Mahler, H.R. (1974) Fractionation of synaptic membrane glycoproteins by lectin-affinity chromatography. Biochemistry, 13, 5193-8.

Gurney, M.E. (1984) Suppression of sprouting at the neuromuscular junction by immune sera. Nature, 307, 546-8.

Gustavsson, S., Ohlson, C., Karlsson, J.O. (1982) Glycoproteins of axonal transport: Affinity chromatography on fucose-specific lectins. J. Neurochem., 38, 852-5.

Haddad, A., Smith, M.D., Hercovics, A., Nadler, M.I., Leblond, C.P. (1971) Radioautographic study of *in vivo* and *in vitro* incorporation of [^{3}H]fucose into thyroglobulin by rat thyroid follicular cells. J. Cell. Biol., 49, 856-82.

Hakamori, S.I. (1981) Glycosphingolipids in cellular interaction differentiation and oncogenesis. Ann. Rev. Biochem., 50, 733-64.

Halzonetis, T.D., Georgopoulos, K., Greenberg, N.E., Leder, P. (1988) c-jun dimerizes with itself and with c-fos, forming complexes of different DNA

binding affinities. Cell, 55, 917-24.

Hambley, J.W., Rogers, L.J. (1979) Retarded learning induced by intracerebral administration of amino acids in the neonatal chick. Neuroscience, 4, 677-84.

Hart, G.W., Haltiwanger, R.S., Holt, G.D., Kelly, W.G. (1989) Glycosylation in the nucleus and cytoplasm. Ann. Rev. Biochem., 58, 841-74.

Hart, G.W., Holt, G.D., Haltiwanger, R.S. (1990) Nuclear and cytoplasmic glycosylation: novel saccharide linkages in unexpected places. In R. A. Bradshaw, & M. Purton (Ed.), Proteins: Form and function (pp. 219-226). Cambridge: Elsevier Trends Journals.

Hawkes, R. (1982) Identification of concanavalin A-binding proteins after sodium dodecyl sulfate-gel electrophoresis and protein blotting. Anal. Biochem., 123, 143-6.

Herschman, H.R., Cotman, C., Matthews, D.A. (1972) Serologic specificities of brain subcellular organelles: 1. Antisera to synaptosomal fractions. J. Immunol., 108, 1362-9.

Hess, E.H. (1959) Imprinting. Science, 130, 133-41.

Hill, I.E., Selkirk, C.P., Hawkes, R.B., Beesley, P.W. (1988) Characterization of novel glycoprotein components of synaptic membranes and postsynaptic densities, gp55 and gp65, with a monoclonal antibody. Brain Res., 461, 27-43.

Hirn, M., Pierres, M., Deagostini-Bazin, H., Hirsch, M.R., Goridis, C., Ghandour,

M.S., Langley, O.K., Gombos, G. (1982) A new brain cell surface glycoprotein identified by monoclonal antibody. Neuroscience, 7, 239-50.

Hoffman, S., Edelman, G.M. (1983) Kinetics of homophilic binding by embryonic and adult forms of the neural cell adhesion molecule. PNAS (USA), 80, 5762-6.

Hoffman, S., Sorkin, B.C., White, P.C., Brackenbury, R., Mailhammer, R., Rutishauser, U., Cunningham, B.A., Edelman, G.M. (1982) Chemical characterization of a neural cell adhesion molecule purified from embryonic brain membranes. J. Biol. Chem., 257, 7720-9.

Hooghe-Peters, E., Hooghe, L. (1982) The Thy-1 glycoprotein on nerve cells in culture. J. Neuroimmunol., 2, 191-200.

Horn, G. (1985) Memory, imprinting, and the brain. Oxford: Oxford University Press.

Horn, G., Horn, A.L.D., Bateson, P.P.G., Rose, S.P.R. (1971) Effects of imprinting on uracil incorporation into brain RNA in the "split brain" chick. Nature, 229, 131-2.

Horn, G., McCabe, B.J. (1984) Predispositions and preferences. Effects on imprinting of lesions to the chick brain. Anim. Behav., 32, 288-92.

Horn, G., McCabe, B.J., Bateson, P.P.G. (1979) An autoradiographic study of the chick brain after imprinting. Brain Res., 168, 361-73.

Horn, G., McCabe, B.J., Cipolla-Neto, J. (1983) Imprinting in the domestic chick: the role of each side of the hyperstriatum ventrale in acquisition and retention. Exp. Brain Res., 53, 91-8.

Horn, G., Rose, S.P.R., Bateson, P.P.G. (1973) Monocular imprinting and regional incorporation of tritiated uracil into the brain of intact and "split brain" chicks. Brain Res., 56, 227-37.

Howard, K.J., Rogers, L.J., Boura, L.A.L. (1980) Functional lateralisation of the chicken forebrain revealed by use of intracranial glutamate. Brain Res., 188, 369-82.

Hubbard, S.C., Ivatt, R.J. (1981) Synthesis and processing of asparagine linked oligosaccharides. Ann. Rev. Biochem., 50, 555-83.

Hucho, F. (1986) The nicotinic acetylcholine receptor and its ion channel. Eur. J. Biochem., 158, 211-26.

Hughes, R.C. (1983) Glycoproteins . Bristol: Arrowsmith Ltd.

Hyden, H., Lange, P. (1971) Brain-cell protein synthesis specifically related to learning. PNAS (USA), 65, 898-904.

Jankovic, B.D. (1985) From immunoneurology to immunopsychiatry: Neuromodulating activity of anti-brain antibodies. Internat. Rev. Neurobiol., 26, 249-314.

Jankovic, B.D., Rakic, L., Janjic, M., Mitrovic, K., Ivanus, J. (1968) Effect of intraventricular injection of anti-brain antibody on defensive conditioned

reflexes. Nature, 218, 270-1.

Järnefelt, J., Finne, J., Krusius, T., Panvala, H. (1978) Protein bound oligosaccharides of cell membranes . TIBS, 3, 110-4.

Jentoft, N. (1990) Why are proteins O-glycosylated? TIBS, 15, 291-4.

Johnson, M.H., Horn, G. (1987) The role of a restricted region of the chick forebrain in the recognition of individual conspecifics. Behav. Brain Res., 23, 269-75.

Jørgensen, O.S., Mogensen, J., Divac, I. (1987) The N-CAM D2-protein as marker for synaptic remodelling in the red nucleus. Brain Res., 405, 39-45.

Jørgensen, O.S., Møller, M. (1980) Immunocytochemical demonstraition of the D2 protein in the presynaptic complex. Brain Res., 194, 419-29.

Jork, R., De Graan, P.N.E., Van Dongen, C.J., Zwiers, H., Matthies, H. (1984b) Dopamine-induced changes in protein phosphorylation and polyphosphoinositide metabolism in rat hippocampus. Brain Res., 291, 73-81.

Jork, R., Grecksch, G., Matthies, H. (1982b) Apomorphine and glycoprotein synthesis during consolidation. Pharmacol. Biochem. Behav., 17, 11-3.

Jork, R., Grecksch, G., Matthies, H. (1986) Impairment of glycoprotein fucosylation in rat hippocampus and the consequences on memory formation. Pharmacol. Biochem. & Behav., 25, 1137-44.

Jork, R., Schmidt, M., Lössner, B., Matthies, H. (1984a) Dopamine-stimulated L-fucose incorporation into brain proteins is related to an increase in fucokinase

activity. Biomed. Biochim. Acta, 43, 261-70.

Jork, R., Schmidt, S., Schulzeck, S., B., L., Popov, N., Matthies, H. (1982a) Mechanisms of dopamine induced changes in hippocampal glycoprotein metabolism. Pharmacol. Biochem. Behav., 17, 203-7.

Jork, R., Schnurra, I., Smalla, K.H., Grecksch, G., Popov, N., Matthies, H. (1989) 2-deoxy-galactose mediated amnesia is related to an inhibition of training-induced increase in rat hippocampal glycoprotein fucosylation. Neurosci. Res. Commun., 5, 3-8.

Jork, R., Smalla, K.H., Karsten, R., Gresksch, G., H.L., R., Matthies, H. (1991) Monoclonal antibody specific for histo-blood group antigens H (type 2 & 4) interferes with long-term memory formation in rats. Neurosci. Res. Commun., 8, 21-7.

Jourdian, G.W. (1979) Biosynthesis of glycosaminoglycans. In R. U. Margolis, & R. K. Margolis (Ed.), Complex carbohydrates of nervous tissue (pp. 103-26). New York: Plenum Press.

Kanfer, N.J., Richards, R.L. (1967) Effects of puromycin on the incorporation of radioactive sugars into gangliosides *in vivo*. J. Neurochem., 14, 513-8.

Karpiak, S.E., Graf, I., Rapport, M.M. (1978) Antibodies to GM1 ganglioside inhibit a learned avoidance response. Brain Res., 151, 637-11.

Karpiak, S.E., Graf, L., Rapport, M.M. (1976a) Antisera to brain gangliosides produces recurrent epileptiform activity. Science, 194, 735-7.

Karpiak, S.E., Rapport, M.M. (1975) Behavioural changes in two-month old rats following prenatal exposure to antibodies against synaptic membranes. Brain Res., 92, 405-13.

Karpiak, S.E., Rapport, M.M. (1979) Inhibition of consolidation and retrieval stages of passive-avoidance learning by antibodies to gangliosides. Behav. Neural Biol., 27, 146-56.

Karpiak, S.E., Serokosz, M., Rapport, M.M. (1976b) Effects of antisera to S-100 protein and to synaptic membrane fraction on maze performance and EEG. Brain Res., 102, 313-21.

Karten, H.J., Hodos, W. (1967) A stereotaxic atlas of the brain of the pigeon (columba livia) . Baltimore: Johns Hopkins Press.

Kaufman, R.L., Ginsberg, V. (1968) The metabolism of L-fucose by HeLa cells. Exp. Cell Res., 50, 127-32.

Kennedy, M.B., Bennet, M.K., Erondu, N.E. (1983) Biochemical and immunochemical evidence that the major postsynaptic density protein is a subunit of a calmodulin-dependent protein kinase. PNAS (USA), 80, 7357-61.

Kiang, W.L., Margolis, R.U., Margolis, R.K. (1981) Fractionation and properties of a chondroitin sulfate proteoglycan and the soluble glycoproteins of brain. J. Biol. Chem., 256, 10529-37.

Kishimoto, Y. (1983) Ceramides and cerebrosides (2nd edition). New York: Plenum Press.

Kleitman, N., Wood, P., Johnson, M.I., Bunge, R.P. (1988) Schwann cell surfaces but not extracellular matrix organized by Schwann cells support neurite outgrowth from embryonic rat retina. J. Neurosci., 8, 653-63.

Kobiler, D., Fuchs, S., Samuel, D. (1976) The effect of anti-synaptosomal plasma membrane antibodies on memory. Brain Res., 115, 129-38.

Koch, C., Poggio, T. (1983) A theoretical analysis of electrical properties of spines. Proc. R. Soc. Lond. B., 218, 544-77.

Kornfeld, R., Kornfeld, S. (1985) Assembly of asparagine-linked oligosaccharides. Annu. Rev. Biochem., 54, 631-64.

Koshaka, S.I., Takamatsu, K., Aoki, E., Tsukada, Y. (1979) Metabolic mapping of chick brain after imprinting using [14C]2-deoxyglucose technique. Brain Res., 172, 539-44.

Kossut, M., Rose, S.P.R. (1984) Differential 2-deoxyglucose uptake into chick brain during passive avoidance training. Neuroscience, 12, 971-7.

Kozarsky, K., Kingsley, D., Krieger, M. (1988) Use of mutant cell line to study the kinetics and function of O-linked glycoprotein of low density lipoprotein receptors. PNAS (USA), 85, 4335-9.

Krayanek, S. (1980) Structure and orientation of extracellular matrix in developing chick optic tectum. Anat. Rec., 197, 95-109.

Krayanek, S., Goldberg, S. (1981) Oriented extracellular channels and axonal

guidance in the embryonic chick retina. Dev. Biol., 84, 41-50.

Krug, M., Jork, R., Reymann, K., Wagner, M., Matthies, H. (1991) The amnesic substance 2-deoxy-D-galactose suppresses the maintenance of hippocampal LTP. Brain Res., 540, 237-42.

Krusius, T., Finne, J., Margolis, R.U., Margolis, R.K. (1978) Structural features of synaptosomal mitochondrial, and soluble glycoproteins of rat brain. Biochem., 17, 3849-54.

Krusius, T., Reinhold, V.N., Margolis, R.K., Margolis, R.U. (1987) Structural studies on sialylated and sulfated O-glycosidic mannose-linked oligosaccharides in the chondroitin sulfate proteoglycan of brain. Biochem. J. 245, 229-234.

Laemlmi, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 227, 680-5.

Lakin, K.H., Allen, A.K., Farbre, J.W. (1983) Purification and preliminary biochemical characterization of the human and rat forms of the central nervous system-specific molecule F3-87-8. J. Neurochem., 42, 385-94.

Landis, S.C. (1983) Neuronal growth cones. Ann. Rev. Physiol., 45, 2183-7.

Lappuke, R., Bernard, C.C.A., Gibbs, M.E., Ng, K.T., Bartlett, P.F. (1987) Inhibition of memory in the chick using a monoclonal anti-Thy-1 antibody . J. Neuroimmunol., 14, 317-24.

Ledeen, R.W. (1978) Ganglioside structure and distribution: are they organized at the

nerve ending? J. Supramol. Struct., 8, 1-17.

Ledeen, R.W. (1983) Gangliosides (2nd edition). New York: Plenum Press.

Lee, G., Aloj, S.M., Brady, R.O., Kohn, L.D. (1976) The structure and function of glycoprotein hormone receptors. Biochem. Biophys. Res. Commun., 73, 370-7.

Letourneau, P.C. (1983) Axonal growth and guidance. TINS, 6, 451-5.

Levin, B.E. (1977) Axonal transport of [^{3}H]fucosyl glycoproteins in noradrenergic neurons in the rat brain. Brain Res., 130, 421-32.

Lisman, J.E. (1985) A mechanism for memory storage insensitive to molecular turnover: a bistable autophosphorylating kinase. PNAS (USA), 82, 3055-7.

Lorenz, K.Z. (1981) The foundations of ethology. New York: Springer-Verlag Press.

Lössner, B., Bullock, S., Rose, S.P.R. (1988) 411B: a monoclonal postsynaptic marker for modulations of synaptic connectivity in the rat brain. J. Neurochem., 51, 385-90.

Lössner, B., Rose, S.P.R. (1983) Passive avoidance training increases fucokinase activity in right forebrain base of day-old chicks. J. Neurochem., 41, 1357-63.

Maccioni, A.H.R., Gimenez, M.S., Vaputto, B.L., Caputto, R. (1974a) Labelling of the gangliosidic fraction from brains of chickens exposed to different levels of stimulation after injection of [^{3}H]glucosamine. Brain Res., 73, 503-11.

Maccioni, H.J.F., Arce, A., Landa, C., Caputto, R. (1974b) Rat brain microsomal gangliosides. Biochem. J., 138, 291-8.

Mahadik, S.P., Korenovsky, V., Ciccarone, V., Laev, H. (1982) Synaptic membrane antigens in developing at brain cerebral vortex and cerebellum. J. Neurochem., 39, 1340-7.

Maier, V., Scheich, H. (1983) Acoustic imprinting leads to differential 2-deoxy-D-glucose uptake in the chick forebrain. PNAS (USA), 80, 3860-4.

Manthorpe, M., Engvall, E., Ruoslahti, E., Longo, F.M., Davis, G.E., Varon, S. (1983) Laminin promotes neural regeneration from cultured peripheral and central neurons. J. Cell Biol., 97, 1882-90.

Manthorpe, M., Hagg, T., Engvall, E., Varon, S. (1988) Laminin-like immunoreactivity in adult rat brain neurons . Soc. Neurosci. Abs., 14.

Margolis, R.K., Margolis, R.U. (1970) Sulfated glycopeptides from rat brain glycoproteins. Biochemistry, 9, 4389-96.

Margolis, R.K., Margolis, R.U. (1975) Disposition of fucose in the brain. J. Neurochem., 19, 1023-30.

Margolis, R.K., Margolis, R.U. (1979a) Complex Carbohydrates of Nervous tissue. New York: Plenum Press.

Margolis, R.K., Margolis, R.U. (1979b) Structure and distribution of glycoproteins and glycosaminoglycans. New York: Plenum Press.

Margolis, R.K., Margolis, R.U. (1983) Glycoproteins and proteoglycans. In L. Lajtha (Ed.), Handbook of Neurochemistry (pp. 177-204). New York: Plenum Press.

Margolis, R.K., Margolis, R.U. (1989) Structure and localization of glycoproteins and proteoglycans. New York: Plenum Press.

Margolis, R.U., Margolis, R.K. (1972) Sulfate turnover in mucopolysaccharides and glycoproteins of brain. Biochem. Biophys. Acta, 264, 426-31.

Mason, R.J., Rose, S.P.R. (1987) Lasting changes in spontaneous multi-unit activity in the chick brain following passive avoidance training. Neuroscience, 21, 931-41.

Mason, R.J., Rose, S.P.R. (1988) Passive avoidance learning produced focal elevation of bursting activity in the chick brain: Amnesia abolishes the increase. Behav. Neural Biol., 49, 280-92.

Matsui, Y., Lombard, D., Hoflack, B., Harth, S., Massarelli, R., Mandel, P., Dreyfus, H. (1983) Ectoglycosyltransferase activities at the surface of cultured neurons. Biochem. Biophys. Res. Commun., 113, 446-53.

Matsui, Y., Lombard, D., Massarelli, R., Mandel, P., Dreyfus, H. (1986) Surface glycosyltransferase activities during development of neuronal cell cultures. J. Neurochem., 46, 144-50.

Matthews, M.A., Narayanan, C.H., Siegenthaler-Matthews, D.J. (1982) Inhibition of axoplasmic transport in the developing visual system of the rat. III. Electron

microscopy and Golgi studies of retino-fugal synapses and post-synaptic neurons in the dorsal lateral geniculate nucleus. Neuroscience, 7, 405-22.

Matthies, H. (1978) Learning and memory. In C. Dumont (Ed.), Advances in Pharmacology and therapeutics (pp. 117-35). New York: Oxford University.

Matthies, H. (1980) Pharmacology of learning and memory. TIPS, 1, 333-6.

Matthies, H. (1982) Plasticity in the nervous system - an approach to memory research. In C. J. Marsan, & H. Matthies (Ed.), Neuronal plasticity and memory formation. International brain research organization monograph series (pp. 1-13). New York: Raven Press.

Matthies, H. (1989) In search of cellular mechanisms of memory. Prog. Neurobiol., 32, 277-349.

Mc Kibbin, J.M. (1978) Fucolipids. J. Lipid Res., 19, 131-47.

McCabe, B.J., Cipolla-Neto, J., Horn, G., Bateson, P.P.G. (1982) Amnesic effects of bilateral lesions placed in the hyperstriatum ventrale of the chick after imprinting. Exp. Brain Res., 48, 13-21.

McCabe, B.J., Horn, G., Bateson, P.P.G. (1981) Effects of restricted lesions of the chick forebrain on the acquisition of filial preferences during imprinting. Brain Res., 205, 29-37.

McCabe, N.R. (1985) Training and fucose metabolism in chick brain. Ph.D., The Open University, England.

McCabe, N.R., Rose, S.P.R. (1985) Passive avoidance training increases fucose incorporation into glycoproteins in chick forebrain slices *in vitro*. Neurochem. Res., 10, 1083-95.

McCabe, N.R., Rose, S.P.R. (1987) Increased fucosylation of chick brain proteins following training: effects of cycloheximide. J. Neurochem., 48, 538-42.

McKenzie, J.L., Dalchau, R., Fabre, J.W. (1982) Biochemical characterization and localization in brain of a human brain-leucocyte membrane glycoprotein recognised by a monoclonal antibody. J. Neurochem., 39, 1461-6.

McKibbin, J.M. (1978) Fucolipids. J. Lipid Res., 19, 131-47.

Merlie, J.P., Sebbane, R., Tizartos, S., Lindstrom, J. (1982) Inhibition on glycosylation with tunicamycin blocks assembly of newly synthesized acetylcholine subunits in muscle cells. J. Biol. Chem., 257, 2694-701.

Merlie, J.P., Sebbane, R., Tzartos, S., Lindstrom, J. (1982) Inhibition of glycosylation with tunicamycin blocks assembly of newly synthesized acetylcholine receptor subunits in muscle cells. J. Biol. Chem., 257 , 2694-701.

Michaelis, E.K. (1975) Partial purification and characterization of a glutamate-binding protein from rat brain. Biochem. Biophys. Res. Commun., 65, 1004-12.

Mileusnic, R., Rose, S.P.R., Tillson, P. (1980) Passive avoidance learning results in region specific changes in concentration of and incorporation into colchicine-binding proteins in the chick forebrain. J. Neurochem., 34, 1007-15.

Milner, B., Corkin, S., Teuber, H.-L., (1968) Further analysis of the hippocampal

amnesic syndrome: 14-year follow-up of HM. Neropsychologia, 6, 215-34.

Montreuil, J. (1984) Spatial conformation of glycans and glycoproteins. Biol. Cell, 51, 115-31.

Morell, P., Quarles, R.H., Norton, W.T. (1989) Myelin formation, structure, and biochemistry. New York: Raven Press.

Morgan, I.G., Austin, L. (1968) Synaptosomal protein synthesis in a cell-free system. J. Neurochem., 15, 41-7.

Morre, D.J., Kartenbeck, J., Franke, W.W. (1979) Membrane flow and interconversions among endomembranes. Biochim. Biophys. Acta, 559, 71-152.

Morris, R. (1985) Thy-1 in developing nervous tissue. Dev. Neurosci., 7, 133-60.

Morrison, I.M. (1986) Glycolipids and gangliosides. Carbohydrate chemistry (pp. 578-618). Kent: Whitstable Litho Ltd.

Moss, D.J. (1986) Characterization of a soluble trypsin fragment of gp130: a neuronal glycoprotein associated with the cytoskeleton. J. Cell Biochem., 32, 97-112.

Murakami, k., Gordon-Weeks, P.R., Rose, S.P.R. (1986) Isolation of post-synaptic densities from day-old chicken brain. J. Neurochem., 46, 340-7.

Nagai, Y., Iwamori, M. (1980) Brain and thymus gangliosides: their molecular diversity and their function in cell surface membranes. Mol. Cell Biochem., 29, 81-90.

Nakabeppu, Y., Ryder, k., Nathans, D. (1988) DNA binding activities of three murine jun proteins: stimulation by fos. Cell, 55, 907-15.

Nevis, A.H., Collins, G.H. (1967) Electrical impedance and volume changes in brain during development. Brain Res., 5, 57-85.

Nieto-Sampedro, M., Bussineau, C.M., Cotman, C.W. (1981) Optimal concentration of Iodonitrotetrazolium for the isolation of junctional fractions from rat brain. Neurochem. Res., 6, 307-20.

Nolan, P.M., Bell, R., Regan, C.M. (1987a) Acquisition of a brief behavioural experience in the presence of neuron-specific and D2-CAM/N-CAM-specific antisera. Neurochem. Res., 12, 619-24.

Nolan, P.M., Bell, R., Regan, C.M. (1987b) Acquisition of a brief behavioural experience is inhibited by the brain specific monoclonal antibody, F3-87-8. Neurosci. Lett., 79, 346-50.

O'Farrell, P.H. (1975) High resolution two-dimensional electrophoresis of proteins. J. Biol. Chem., 250, 4007-21.

O'Farrell, P.Z., Goodman, H.M., O'Farrell, P.H. (1977) High resolution two-dimensional electrophoresis of basic as well as acidic proteins. Cell, 12, 1133-42.

Ochoa, E.L.M., Bangham, A.D. (1976) N-acetylneuraminic acid molecules as serotonin binding sites. J. Neurochem., 26, 1193-8.

Ohlson, C., Karlsson, J.O. (1983) Glycoproteins of axonal transport: Polypeptides interacting with the lectin from Aleuria aurantia. Brain Res., 264, 99-104.

Ott, U., Odermatt, E., Engel, J., Furthmayr, H., Timpl, R. (1982) Protease resistance and conformation of laminin. Eur. J. Biochem., 123, 63-72.

Palade, G. (1975) Intracellular aspects of the process of synthesis. Science, 189, 347-348.

Parekh, R.B., Tse, A.G.D., Dwek, R.A., Williams, A.F., Rademacher, T.W. (1987) Tissue-specific N-glycosylation, site-specific oligosaccharide patterns and lentil lectin recognition of rat Thy-1. EMBO, 6, 1233-44.

Patel, S.N., Stewart, M.G. (1988) Changes in the number and structure of dendritic spines, 25h after passive avoidance training in the chick, Gallus domesticus. Brain Res., 449, 34-46.

Patterson, T.A., Alvarado, M.C., Rosenzweig, M.R., Bennett, E.L. (1988) Time courses of amnesia development in two areas of the chick forebrain. Neurochem. Res., 13, 643-7.

Patterson, T.A., Alvarado, M.O., Warner, I.T., Bennet, E.L., Rosenzweig, M.R. (1986) Memory stages and brain asymmetry in chick learning. Behav. Neurosci., 100, 850-9.

Patterson, T.A., Gilbert, D.B., Rose, S.P.R. (1990) Pre- and post-training lesions of the intermediate medial hyperstriatum ventrale and passive learning in the chick. Exp. Brain Res., 80, 189-95.

Patterson, T.A., Rose, S.P.R., Bradley, P.M. (1989) Anisomycin and amnesia in the chick: State-dependent effects are not present with intracranial injections. Dev. Brain Res., 49, 173-8.

Patterson, T.A., Rosenzweig, M.R., Bennet, E.L. (1987) Amnesia produced by anisomycin in an appetitive task is not due to conditioned taste aversion. Behav. Neural Biol., 47, 17-26.

Paulson, J.C. (1989) Glycoproteins: what are the sugar chains for? TIBS, 14, 272-6.

Persohn, E., Schachner, M. (1990) Immunohistological localization of the neural adhesion molecules L1 and N-CAM in the developing hippocampus of the mouse. J. Neurocytol., 19, 807-19.

Pfenninger, K.H. (1973) Synaptic morphology and cytochemistry. Prog. Histocem. Cytochem, 5, 1-85

Pohle, W., Acosta, L., Krug, M., Rüthrich, H., Matthies, K.H., Matthies, H. (1986) Incorporation of [^{3}H]fucose in hippocampal structures after tetanization and conditioning by perforant path stimulation. Adv. Biosci. , 59, 105-8.

Pollack, L., Atkinson, P.H. (1983) Correlation of glycosylation forms with position in amino acid sequence. J. Cell Biol., 97, 293-300.

Popov, N., Matthies, H. (1986) Effects of carbohydrate precursors of glycomacromolecules on retention performance of a learning task in rats. Adv. Biosci., 59, 229-32.

Popov, N., Pohle, W., Ruhrich, H.L., Schulzeck, S., Matthies, H. (1976) Timecourse and disposition of fucose radioactivity in rat hippocampus: a biochemical and microautoradiographic study. Brain Res., 101, 283-93.

Popov, N., Schmidt, S., Schulzeck, R., Jork, R. (1983) Changes in activities of fucokinase and fucosyltransferase in rat hippocampus after acquisition of a brightness discrimination reaction. Pharmacol. Biochem. Behav., 19, 43-7.

Popov, N., Schulzeck, S., Pohle, W., Matthies, H. (1980) Changes in the incorporation of [^{3}H]fucose into rat hippocampus after acquisition of a brightness discrimination reaction: an electrophoretic study. Neuroscience, 5, 161-7.

Popov, N., Schulzeck, S., Pohle, W., Matthies, H. (1982) Alterations in rat hippocampal glycoproteins after acquisition of a brightness discrimination reaction. In C. A. Marsan, & H. Matthies (Ed.), Neural plasticity and memory formation, International brain research organization monograph series (pp. 193-7). New York: Raven Press.

Presper, K.A., Basu, N., Basu, S. (1978) Biosynthesis *in vitro* of fucose-containing glycosphingolipids in human neuroblastoma IMR-32 cells. PNAS (USA), 75, 289-93.

Quarles, R.H., Brady, R.O. (1971) Synthesis of glycoproteins and gangliosides in developing rat brain. J. Neurochem., 18, 1809-20.

Quarles, R.H., Everly, J.L., Brady, R.O. (1973) Evidence for the close association of

a glycoprotein with myelin in rat brain. J. Neurochem., 21, 1177-91.

Radin, N.S. (1983) Sulfatides. In L. A. (Ed.), Handbook of Neurochemistry (pp. 163-178). New York: Plenum Press.

Raedler, E., Raekdler, A., Feidhaus, S. (1981) Varying expressions of lectin receptors within embryonic cell layers of murine cerebral cortex. Anat. Embryol., 162, 21-8.

Rahman, H. (1982) Correlations among neuronal ganglioside metabolism, Bioelectrical activity, and memory formation in teleost fishes. In C. A. Marsan, & H. Matthies (Ed.), Neuronal plasticity and memory formation, International brain research organisation monogaph series (pp. 203-13). New York: Raven Press.

Rahman, H. (1984) Memory formation by means of molecular facilitation in synapses with Ca2+-ganglioside-modulation. Japan J. Neuropsychopharmacol., 6, 383-91.

Ralph, T., Shwarz, T., Datema, R. (1982) The lipid pathway of protein glycosylation and its inhibitors: the biological significance of protein-bound carbohydrates. Adv. in Carbohydrate Chem. & Biochem, 40, 287-379.

Rambourg, A., Leblond, C.P. (1967) Electron microscope observations on the carbohydrate-rich cell coat present at the surface of cells in the rat. J. Cell Biol., 32, 27-53.

Rathjen, F.G., Schachner, M. (1984) Immunocytological and biochemical

characterization of a new neuronal cell surface component (L1 antigen) which is involved in cell adhesion. EMBO J., 3, 1-10.

Rauth, J.J., Lambert, M.P., Cho, N.J., Chin, H., Klein, W.L. (1986) Glycoprotein properties of muscarinic acetylcholine receptors from bovine cerebral cortex. J. Neurochem., 46, 23-32.

Reeber, A., Vincendon, G., Zanetta, J.P. (1981) Isolation and immunohistochemical localization of a Purkinje cell specific glycoprotein subunit from rat cerebellum. Brain Res., 229, 53-65.

Regan, C.M. (1991) Regulation of neural cell adhesion molecule sialylation state. Int. J. Biochem. , 23, 513-23.

Rehm, H., Wiedemann, B., Betts, H. (1986) Molecular characterization of synaptophysin, a major calcium-binding protein of synaptic vesicle membrane. EMBO, 5, 535-41.

Reichner, H., Witebsky, E. (1934) Die serologische Unterscheidung zwischen grauer und weiβer Substanz des Nervensystems. Z. Immunitaetsforsch., 18, 410-32.

Richards, C.D. (1983) Actions of general anaesthetics on synaptic transmission in the CNS. Br. J. Anaesth., 55, 201-7.

Richter, P., Pohle, W., Grecksch, G., Smalla, K.H., Jork, R., Matthies, H. (1991) Enhanced development of morphine tolerance in rats treated with 2-deoxy-D-galactose. Psychopharmacology, 104, 279-83.

Robinson, H.C., Telser, A., Dorfman, A. (1966) Studies on biosynthesis of the

linkage region of chondroitin sulfate-protein complex. PNAS (USA), 56, 1859-66.

Rodnight, R., Trotta, E.E., Perrett, C. (1985) A simple and economical method for studying protein phosphorylation *in vivo* in the rat brain. J. Neurosci. Method, 13, 87-95.

Rodnight, R., Zamani, R., Tweedale, A. (1988) An investigation of experimental conditions for studying protein phosphorylation in micro-slices of rat brain by two-dimensional electrophoresis. J. Neurosci. Methods., 24, 27-38.

Rogers, L.J., Drennan, H.D., Mark, R.F. (1974) Inhibition of memory formation in the imprinting period: irreversible action of cycloheximide in young chickens. Brain Res., 79, 213-33.

Rogers, L.J., Oettinger, R., Szer, J., Mark, R.F. (1977) Separate chemical inhibitors of long-term and short-term memory: contrasting effects of cycloheximide and ethacrynic acid on various learning tasks in young chickens. Proc. R. Soc. London, 196, 171-95.

Rose, S.P.R. (1981a) Criteria for investigation the biochemistry of memory formation. In R. Rodnight, H. Bachelard, & W. Stahl (Ed.), Chemisms of the brain (pp. Chapter 25). New York: Churchill Livingstone.

Rose, S.P.R. (1981b) What should a biochemistry of learning and memory be about? Neuroscience, 6, 811-21.

Rose, S.P.R. (1983) Towards a biochemistry of memory formation. In A.-A. C.

Caputto R. (Ed.), Neural transmission in learning and memory (pp. 211-19). New York: Raven Press.

Rose, S.P.R. (1985) Passive avoidance training in the chick: A model for the analysis of the cell biology of memory storage. In D. L. Alkon, & C. D. Woody (Ed.), Neural mechanisms of conditioning (pp. 283-97). New York: Plenum Press.

Rose, S.P.R. (1989) Glycoprotein synthesis and postsynaptic remodeling in long-term memory. Neurochem. Int., 14, 299-307.

Rose, S.P.R. (1991) How chicks make memories: the cellular cascade from c-fos to dendritic remodeling. TINS, 14, 390-7.

Rose, S.P.R. (1991b) Biochemical mechanisms involved in memory formation in the chick. In R. J. Andrew (Ed.), Neural and behavioural plasticity, the use of the domestic chick as a model. (pp. 277-305). Oxford: Oxford University Press.

Rose, S.P.R., Bullock, S., Jork, R. (1987) Glycoprotein synthesis is necessary for memory formation in the chick. Neuroscience, 22, S187.

Rose, S.P.R., Csillag, A. (1985) Passive avoidance training results in lasting changes in deoxyglucose metabolism in left hemisphere regions of the chick brain. Behav. Neural Biol., 44, 315-24.

Rose, S.P.R., Harding, S. (1984) Training increases [^{3}H]fucose incorporation in chick brain only if followed by memory storage. Neuroscience, 12, 663-7.

Rose, S.P.R., Jork, R. (1987) Long-term memory formation in chick is blocked by 2-

deoxygalactose, a fucose analogue. Behav. Neural. Biol., 48, 246-58.

Rosenzweig, M.R., Bennett, E.L., Martinez, J.L., Beniston, J.D., Colombo, P.J., Lee, D.W., Patterson, T.A., Schulteis, G., Serrano, P.A. (1991) Stages of memory formation in the chick: findings and problems. In Andrew R. J. (Ed.), Neural and behavioural plasticity, the use of the domestic chick as a model. (pp. 389-408). Oxford, New York, Tokyo: Oxford University Press.

Rostas, J.A.P., W.N., L., Jeffrey, P.L. (1981) Glycosyltransferase activities in chick brain synaptic functions. Neurosci. Lett., 24, 155-60.

Roth, S., McGuire, E.J., Roseman, S. (1971) Evidence for cell-surface glycosyltransferase. J. Cell. Biol., 51, 536-42.

Rothman, J.F., Fine, R.E. (1980) Coated vesicles transport newly synthesized membrane glycoproteins from endoplasmic reticulum to plasma membrane in two successive stages. PNAS (USA), 77, 780-4.

Rougon, G., Deagostini-Bazin, H., Hirn, M., Goridis, C. (1982) Tissue- and developmental stage-specific forms of a neural cell surface antigen linked to differences in glycosylation of a common polypeptide. EMBO J., 1, 1239-44.

Rouser, G., Kritchevsky, G., A.N., S. (1978) Lipid composition of the brain and its subcellular structures. In C. G. Tedeschi (Ed.), Neuropathology, Methods and Diagnosis (pp. Chapter 26). Boston: Little, Brown and Company.

Routtenberg, A. (1982) Memory formation as a posttranslational modification of brain proteins. New York: Raven Press.

Rutishauser, R. (1989) Polysialic acid as a regulator of cell interactions. In R. U. Margolis, & R. K. Margolis (Ed.), Neurobiology of glycoconjugates (pp. 367-79). New York: Plenum Press.

Rutishauser, U., Jessell, T.M. (1988) Cell adhesion molecules in vertebrate neural development. Bethesda: The American Psychological Society.

Sadler, J.E. (1984) Biosynthesis of glycoproteins: formation of O-linked oligosaccharides. In V. Ginsburg, & P. W. Robbins (Ed.), Biology of carbohydrates (pp. 199-342). New York: John Wiley & Sons.

Sajonev, D. (1987) N-glycans of synaptic glycoproteins in rat brain. MSc. Thesis, University of Toronto, Canada.

Saraswatti, S., Bucchawat, B.K. (1970) Transfer of N-acetylneuraminic acid to neuroaminidase-treated alkaline phosphatase from sheep brain. Biochem. Biophys. Acta, 212, 170-2.

Savaki, H. (1977) Changes in rat brain gangliosides following active avoidance conditioning. Pharmacol. Biochem. Behav., 7, 7-12.

Schachner, M. (1992) Neural recognition molecules and their influence on cellular functions. New York: Raven Press.

Schachter, H. (1978) Subcellular distribution of glycoconjugates. In P. W. Horowitz M.I. (Ed.), The glycoconjugates New York: Academic Press.

Scheich, H. (1987) Neural correlates of auditory filial imprinting. J. Comp. Physiol.,

161, 605-19.

Schliebs, R., Rose, S.P.R., Stewart, M.G. (1985) Effect of passive avoidance training on *in vitro* protein synthesis in forebrain slices of day-old chicks. J. Neurochem., 44, 1014-28.

Schmidt, F.O. (1964) Neurosci. Res. Prog. Bull., 2, 43-75.

Schmidt, R. (1987) Changes in subcellular distribution of ependymins in goldfish brain induced by learning. J. Neurochem., 48, 1870-8.

Schmidt, R. (1990) Molecular biological and biochemical analysis of glycoprotein synthesis related to plastic changes in the goldfish CNS. VIII International Neurological symposium, Magdeburg.

Schmidt, R., Marktscheffel, P. (1990) Molecular characterisation of neuronal substrate adhesion molecules implicated in plasticity of the CNS. In N. Elsner, & G. Roth (Ed.), Brain-perception, cognition (pp. 441-52). Stuttgart: Thieme Press.

Schmidt, R., Piront, M.-L. (1985) Participation of specific glycoproteins in memory formation. Biol. Chem. Hoppe Seyler, 336, 127.

Schmidt, R., Rother, S., Schlingensiepen, K.H., Brysch, W. (1991) Neural plasticity depending on a glycoprotein synthesized in goldfish leptomeninx. Prog. Brain Res.

Schmidt, R., Rother, S., Schwerdtfeger, W.K. (1990) Glycoproteins of the extracellular matrix mediate plasticity in the CNS. Exp. Brain Res., 19, 17-28.

Schmidt, R., Shashoua, V.E. (1981) A radioimmunoassay for ependymins b and g. Two goldfish brain proteins involved in behavioral plasticity. J. Neurochem., 36, 1368-77.

Schmidt, S., Staak, S., Richter, P., Grecksch, G., Popov, N., Jork, R. (1989) Amnestic action of 2-deoxy-D-galactose is not related to an uridylate trapping effect. Neurosci. Res. Commun., 5, 2-5.

Schnitzer, J., Schachner, M. (1981) Expression of Thy-1, H-2 and NS-4 cell surface antigens and tetanus toxin receptors in early perinatal and adult mouse cerebellum. J. Neuroimmunol., 1, 429-56.

Scholey, A. (1991) Immunological investigations into synaptic plasticity. Ph. D. Thesis, The Open University, England,

Schwartz, R.T., Datema, R. (1982) The lipid pathway of protein glycosylation and its inhibitors. In H. D. Tipson R.S. (Ed.), Advances in carbohydrate chemistry and biochemistry (pp. 287-380). New York: Academic Press.

Scott, J.P. (1962) Critical periods in behavioral development. Science, 138, 949-58.

Seifert, W., Hollmann, M., Wierazko, A. (1986) A functional role for ganglioside GM1 in synaptic transmission and synaptic plasticity of the rat hippocampus. Adv. Biosci., 59, 247-53.

Sharon, N., Lis, H. (1989) Lectins. London and New York: Chapman and Hall.

Shashoua, V. (1986) The role of brain extracellular proteins in learning and memory.

In D. Alkon, & C. D. Woody (Ed.), Neural mechanism of conditioning (pp. 459-62). New York: Plenum Press.

Shashoua, V.E. (1983) The role of specific brain proteins in long-term memory formation. In S. P. L. Morrison A.R. (Ed.), Changing concepts of the nervous system (pp. 681-716). New York: Academic Press.

Shashoua, V.E. (1985) The role of brain extracellular proteins in neuroplasticity and learning. Cell Molec. Neurobiol., 5, 183-207.

Shashoua, V.E., Hesse, G.W. (1989) Classical conditioning leads to changes in extracellular concentrations of ependymin in goldfish brain. Brain Res., 484, 333-9.

Shashoua, V.E., Hesse, G.W., Milinazzo, B. (1990) Evidence for the *in vivo* polymerization of ependymin: a brain extracellular glycoprotein. Brain Res., 522, 181-90.

Shashoua, V.E., Moore, M.E. (1978) Effect of antisera to b and g goldfish brain proteins on the retention of a newly acquired behaviour. Brain Res., 148, 441-9.

Shtark, M.B., Gainutdinov, K.L., Khichenenko, V.I., Shevchuk, E.V., Starostina, M.V. (1987) Biological effects of antibrain antibodies. Electrophysiological, immunochemical and behavioural aspects. Ann. NY Acad. Sci., 496, 405-15.

Simpson, D., Thorne, D.R., Loh, H.H. (1976) Sulfated glycoproteins, glycolipids, and glycosaminoglycans from synaptic plasma and myelin membranes: Isolation and characterization of sulfated glycopeptides. Biochem., 15, 5449-52.

Smith, D.F., Keppler, D.O.R. (1977) 2-Deoxy-D-galactose metabolism in ascites hepatoma cells results in phosphate trapping and glycolysis inhibition. Fur. J. Biochem., 73, 83-92.

Snider, M.D. (1984) Biosynthesis of glycoproteins: formation of N-linked oligosaccharides. New York: John Wiley & Sons.

Sonino, S., Ghidoni, R., Galli, G., Tettamanti, G. (1978) On the structure of a new fucose containing ganglioside from pig cerebellum. J. Neurochem., 31, 947-56.

Specht, S.C., Grafstein, B. (1977) Axonal transport and transneuronal transfer in mouse visual system following injection of [^{3}H]fucose into the eye. Exp. Neurol., 54, 352-68.

Spiro, R.G. (1963a) Study of carbohydrates of glycoproteins. Meth. Enzymol., 28, 3-26.

Spiro, R.G. (1963b) Characterization of carbohydrate units of glycoproteins. Meth. Enzymol., 28, 27-46.

Stanojev, D. (1982) N-Glycans of synaptic glycoproteins in rat forebrain. MSc Thesis, University of Toronto, Canada

Stanton, P.K., Sarvey, J.M., Moskal, J.R. (1987) Inhibition of the production and maintenance of long-term potentiation in rat hippocampal slices by a monoclonal antibody. PNAS (USA), 84, 1684-8.

Starling, J.J., Keppler, M. (1977) Metabolism of 2-deoxy-D-galactose results in the accumulation of 2-deoxy-D-galactose 1-phosphate in brain. J. Neurochem., 29, 603-4.

Stellner, K., Hakomori, S.I. (1973) Enzymatic conversion of "H1-glycolipids" to A or B-glycolipid and deficiency of these enzyme activities in adenocarcinoma. Biochem. Biophys. Res. Commun., 55, 439-45.

Steward, O., Falk, P.M. (1985) Polyribosomes under developing spine synapses: Growth specialization of dendrites at sites of synaptogenesis. J. Neurosci. Res., 13, 75-88.

Steward, O., Falk, P.M. (1986) Protein-synthetic machinery at postsynaptic sites during synaptogenesis: A quantitative study of the association between polyribosomes and developing synapses. J. Neurosci, 6, 412-23.

Stewart, G.R., Pearlman, A.L. (1987) Fibronectin-like immunoreactivity in the developing cerebral cortex. J. Neurosci., 7, 3325-33.

Stewart, M.G. (1991) Changes in dendritic and synaptic structure in chick forebrain consequent on passive avoidance learning. In R. J. Andrew (Ed.), Neural an behavioural plasticity (pp. 305-28). Oxford: Oxford University Press.

Stewart, M.G., Rose, S.P.R., King, T.S., Gabbot, P.L.A., Bourne, R. (1984) Hemispheric asymmetry of synapses in chick medial hyperstriatum ventrale following passive avoidance training. Brain Res., 12, 261-269.

Stryer, L. (1981) Biochemistry (2nd edition). San Francisco: Freeman & Company.

Sturgess, J.M., Minaker, E., Mitranic, M.M., Moscarello, M.A. (1973) The incorporation of L-fucose into glycoproteins following in the golgi apparatus of rat liver and in serum. Biochem. Biophys. Acta, 320, 123-32.

Sukumar, R., Rose, S.P.R., Burgoyne, R.D. (1980) Increased incorporation of [3]fucose into chick brain glycoproteins following training on a passive avoidance task. J. Neurochem, 34, 1000-6.

Sutton, B.J., Phillips, D.C. (1983) The three dimensional structure of the carbohydrate within the Fc fragment of immunoglobulin G. Biochem. Soc. Trans., 11, 130-2.

Suzuki, K. (1981) Chemistry and metabolism of brain lipids. Boston: Little, Brown & Co.

Symington, J., Green, M., Brackmann, K. (1981) Immunoautoradiogaphic detection of proteins after electrophoretic transfer from gels to diazo-paper: analysis of adenovirus. PNAS (USA), 78, 177-81.

Tischmeyer, W., Kaczmarek, L., Strauss, M., Jork, R., Matthies, H. (1990) Accumulation of c-fos mRNA in rat hippocampus during acquisition of a brightness discrimination. Behav. Neural Biol., 54, 165-71.

Tobias, J.M. (1964) A chemically specific molecular mechanism underlying excitation in nerve: A hypothesis. Nature, 203, 13-7.

Towbin, H., Staehelin, T., Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some

applications. PNAS (USA), 76, 4350-4.

Towsend, R.R., Li, Y.T., Li, S.C. (1979) Brain glycosidases. In R. U. Margolis, & R. K. Margolis (Ed.), Complex carbohydrates of nervous tissue (pp. 127-38). New York: Plenum Press.

Vallortigara, G., Zanforlin, M., Compostella, S. (1990b) Perceptual organization in animal learning: Cues or objects? Ethology, 85, 89-102.

Vallortigara, G., Zanforlin, M., Pasti, G. (1990a) Geometric modules in animals' spatial representations: a test with chicks (Gallus domesticus). J. Comp. Psychol., 104, 248-54.

Vogt, P.k., Bos, T.J. (1989) The oncogene jun and nuclear signalling. TIBS, 14 May, 172-5.

Volk, B.W., Aronson, S.M. (1972) Sphingolipidosis and allied disorders. Adv. Expt. Med. & Biol. Vol. 19. Sphingolipids, (Proceedings of the symposium on sphingolipidosis & allied disorders held in Brooklyn NY Oct. 25-27 1971) New York: Plenum Press.

Weinberg, R.A. (1985) The action of oncogenes in the cytoplasm and nucleus. Science, 230, 770-6.

Wetzel, N., Popov, N., Lössner, B., Matthies, H. (1982) Effect of L-fucose on memory retention in rats. In C. A. Marsan, & H. Matthies (Ed.), Neural plasticity and memory formation, International brain research organization monograph series (pp. 197-200). New York: Raven Press.

Wherret, L.R., Hakomori, S.I. (1973) Characterization of a blood group B glycolipid accumulation in the pancreas of a patient with Fabry's disease. J. Biol. Chem., 248, 3046-51.

Wilkinson, J.F. (1957) Pathway of the synthesis of fucose from glucose in klebsiella aerogenes. Nature, 180, 995-7.

Williams, A.F., Barclay, A.N. (1988) The immunoglobulin superfamily--Domains for cell surface recognition. Ann. Rev. Immunol., 6, 381-406.

Wray, W., Boulikas, T., Wray, V.P., Hancock, R. (1981) Silver staining of proteins in polyacrylamide gels. Anal. Biochem., 118, 197-203.

Yang, H.J., Hakomori, S.I. (1971) A sphingolipid having a novel type of ceramide and lacto-N-fucopentaose III. J. Biol. Chem., 246, 1192-200.

Young, J.Z. (1965) The organization of a memory system. Proc. R. Soc. Lond. [Biol.], 163, 285-320.

Zamani, M.R., Rose, S.P.R. (1990) Timecourse of 2-deoxy-D-galactose induced amnesia for passive avoidance training in chick. Proceedings of Open Network Conference on Neural Mechanisms of Learning and Memory, London, 3-6 April: 5-12.

Zanetta, J.P., Dontenwill, M., Meyer, A., Roussel, G. (1985) Isolation and immunohistochemical localization of a lectin-like molecule from the rat cerebellum. Dev. Brain. Res., 17, 233-43.

Zanetta, J.P., Reeber, A., Ghandour, M.S., Vincendon, G., Gombos, G. (1977) Synaptosomal plasma membrane glycoproteins. ii. Isolation of fucosyl-glycoproteins by affinity chromatography on the Ulex europaeus lectin specific for L-fucose. Brain Res., 138, 317-28.

Zanetta, J.P., Roussel, G., Ghandour, M.S., Vincendon, G., Gombos, G. (1978) Postnatal development of rat cerebellum: massive and transient accumulation of concanavalin A binding glycoproteins in parallel fiber axolemma. Brain Res., 142:301-319,

Zatz, M., Barondes, S.H. (1970) Fucose incorporation into glycoproteins of mouse brain. J. Neurochem., 17, 157-63.

Zatz, M., Barondes, S.H. (1971) Particulate and solubilised fucosyltransferases from mouse brain. J. Neurochem., 18, 1625-37.

Zieske, J.D., Bernstein, I.A. (1982) Modification of cell surface glycoproteins: addition of fucosyl residues during epidermal differentiation. J. Cell Biol., 95, 626-31.