AN IMMUNOCHEMICAL ANALYSIS OF GOLDFISH BRAIN PROTEINS USING ANTISERA RAISED AGAINST MEMBRANE FRACTIONS

by

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ABSTRACT

A purified or crude (P2) membrane fraction was isolated using the techniques of step sucrose gradient centrifugation and differential centrifugation respectively, to produce immunological probes with a specificity for goldfish brain membranes. These fractions were used as immunogens and injected into BALB/c mice according to 2 immunization schedules. The two groups of antisera obtained (anti-membrane and anti-P2) were analyzed through immunoblotting procedures. These 2 antisera both attach to a very large number of goldfish brain proteins as observed using this method. Nevertheless, both antisera share distinct reactivities in common against proteins with a molecular weight of 99Kd, 72Kd, 41Kd and 36Kd. Thus, these proteins might be highly immunogenic, compared to the ensemble of goldfish brain protoins present in the membrane fraction. In one series of experiments, many antibodies present in the anti-membrane and anti-P2 antisera have been affinity-purified by blot-elution and then analyzed with the immunoblotting technique. In the case of the anti-membrane antiserum, all of the affinity-purified antisera cross-reacted with a protein of 84Kd. A similar result was obtained with the anti-P2 antiserum, but this time all of the purified antibodies cross-reacted with a 72Kd protein which is common to the brain, skeletal muscles and viscera of the goldfish. P41 (an antibody purified for its affinity to a 41Kd protein) was immunohistochemically analyzed. The results obtained from the tissue and cellular distribution of the recognized antigens, suggest a general association with the cellular surface and the extracellular matrix. In a second series of experiments, the anti-P2 antiserum was consecutively absorbed with paraformaldehyde-fixed tissue sections of goldfish viscera and skeletal muscle. This "absorbed antiserum" was analyzed immunohistochemically and I was able to observe that in spite of the fact that the anti-P2 antiserum (in its native form), binds antigens with a widespread distribution in all tissues tested, the residual antibodies contained in the "absorbed antiserum" recognize preferentially the tectal pyramidal cells and the cerebellar Purkinje cells in the goldfish brain.

RESUME

Des fractions membranaires purifiées ou brutes (P2) , ont été isolées en utilisant respectivement, les techniques de centrifugation dans un gradient non-linéaire de sucrose et de centrifugation différentielle , dans le but de produire des sondes immunologiques ayant une spécificité pour les membranes du cerveau du poisson rouge. Ces fractions ont été utilisées en tant qu'immunogènes et injectées dans des souris BALB/c selon deux schédules d'immunisation. Les deux groupes d'antisérums obtenus (à savoir, anti-membranaire et anti-P2), ont été analysés à l'aide de procédures d'immunoblotting. Ces deux antisérums s'attachent chacun à un très grand nombre de protéines du cerveau du poisson rouge, tel qu'observé par cette méthode. Néanmoins, les deux antisérums ont des réactivitées distinctives en commun contre les protéines de poids moléculaire 99Kd, 72Kd, 41Kd et 36Kd. Donc, ces protéines pourraient être d'une grande immunogénicité, par rapport à l'ensemble des protéines des fractions membranaires du cerveau du poisson rouge. Dans le cadre d'une première série d'expérimentations, plusieurs des anticorps présents dans les antisérums anti-membranaire et anti-P2 ont été purifiés par leur affinité avec des protéines attachées au nitrocellulose (Western blot elution) et ensuite analysés à l'aide de la méthode d'immunoblotting. Dans le cas de l'antisérum anti-membranaire, tous les anticorps ont contre-réagi avec une protéine de 84Kd. Un résultat similaire a été obtenu avec l'antisérum anti-P2 mais cette fois-ci, tous les anticorps purifiés ont contre-réagi avec une protéine de 72Kd, qui est commune au cerveau, muscles squelettiques et viscères du poisson rouge. P41 (un anticorps purifié par son affinité à une protéine de 41Kd) a été analysé immunohistochimiquement et les résultats obtenus quant à la distribution tissulaire et cellulaire de l'antigène reconnu par cet anticorps, suggèrent une association générale avec la surface cellulaire et la matrice extracellulaire. Lors d'une seconde série d'expérimentations, l'antisérum anti-P2 a été consécutivement absorbé avec des tranches de muscle squelettique et de viscères fixées avec du paraformaldéhyde. Cet "antisérum absorbé" a été analysé immunohistochimiquement et de cette manière, j'ai pu observer que, en dépit du fait que l'antisérum anti-P2 (dans sa forme originaire) s'attache à des antigènes ayant une distribution étendue dans tous les tissus testés, les anticorps résiduels contenus dans "l'antisérum absorbé" reconnaissent d'une manière préférentielle les cellules pyramidales du tectum ainsi que les cellules de Purkinje du cervelet dans le cerveau du poisson rouge.

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TABLE OF CONTENTS

INTRODUCTION

Much of neurobiology through the 20th century has been concerned with the description of neuronal diversity on the basis of anatomical, physiological, pharmacological and developmental criteria (Purves and Lichtman 1985). The brain is an extraordinarily complex structure. This is reflected in the functional and anatomical diversity of its major composing units - neurons and glia, in the variety of cell classes of each of these units, and in the morphological complexity of individual neuronal and glial cells. Consequently, neurobiologists have studied the events occurring in the growing nervous system in order to unravel cellular processes (such as cell division, cell recognition, adhesion, migration, synaptogenesis and finally differentiation and cell death) which lead to successful targeting of neurons during neurogenesis and regeneration. The arrangement of neurons in 3-dimensional space, presumably directed by specific molecules, is crucial in the nervous system where the connections between neurons provide the basis for the ordered development and functional integrity of the organ. The mechanisms underlying neurite outgrowth in development and regeneration are thought to depend in part upon the availability of cell surface molecules that promote recognition and cellular adhesion (Rakic 1982, Lindner et al. 1983). Consequently, researchers have taken advantage of recent progress in immunotechnology and raised antibodies specific for molecules which are closely associated with the cell membrane. This has led, on the one hand, to the production of cell-specific markers which may allow cell-to-cell recognition during development; and on the other, to the discovery of a

variety of adhesion molecules (Edelman 1983) which are involved in such events as neural migration and axonal fasciculation and so are thought to play a key role in orchestrating the development of the nervous system.

In view of the importance of understanding the molecular basis of nervous system development and growth, the present thesis describes a project for which the starting goal was to obtain immunological markers for cell surface molecules in the goldfish brain, in the hope that these would recognize determinants potentially involved in the process of regeneration. Consequently, 2 different immunogens, namely a crude brain membrane fraction (P2), and a purified brain membrane fraction were prepared using the techniques of differential and step-sucrose gradient centrifugation respectively, and injected into mice to produce polyclonal antisera. The results presented in this thesis are concerned with the purification of these polyclonal antisera using absorption and blot elution techniques, and also with partial characterization (using immunoblotting and immunohisto-chemistry techniques) of the antigens recognized by the purified antibodies. The immunohistochemical results obtained demonstrate that after absorption of one of the antisera with heterologous tissues, two types of cells, namely the tectal pyramidal cells and the Purkinje cells of the goldfish brain are specifically recognized by the absorbed antiserum. Also, an antibody (P41) eluted from a Western blot, recognized an antigen apparently associated with the extracellular matrix and cell membrane in goldfish nervous and non-nervous tissues. These results provide a basis for future projects in the goldfish, which are discussed together with a critical analysis of the methods used and a comparison of the results obtained with those given in the literature.

THE IMMUNOLOGICAL APPROACH

One of the determining factors which allow one to raise specific antibodies is the nature of the immunogen used. Although some very interesting antibodies have been raised against homogenates of heterogeneous tissue such as spinal cord (Hockfield and McKay 1985, Hockfield 1987), cerebellum (Schachner et al. 1975, Lagenaur et al. 1980) and whole brain (MacPherson and Liakopoulou 1966, Bennett and Edelman 1968, Liakopoulou and Mac Pherson 1970, Schachner et al. 1976), without a well-defined immunogen, one faces difficulties in producing a reliable, minimally cross-reacting immunological marker. Nevertheless, immunological techniques have been used to isolate nervous-tissue-specific antigens (for a review see Mirsky 1982) and, in this attempt, a multiplicity of immunogen types have been used, ranging from crude organ homogenate (see above) to homogeneous nervous tissue cell populations (Goldschneider and Moscona 1972, Seeds 1975, Stallcup and Cohn 1976, Brockes et al. 1977, Martin 1977, Poduslo et al. 1977, Mallet et al. 1979, Hirn et al. 1982). The utility of the immunological approach then relies upon the ability to produce rather pure reagents (antibody probes) out of heterogeneous starting mixtures (nervous tissue) and thus, to maximize the efficiency of this approach, it is important to enrich the component or fraction of interest in the antigen in the first place.

The choice of the immunogen is, of course influenced by the degree of antibody specificity one wishes to obtain and by the purification steps one has the opportunity to employ once the polyclonal antiserum has been harvested. One of the favored purification procedures is absorption with

heterologous tissues. This depletes the antiserum of unwanted tissue cross-reactivities and thus can allow isolation of nervous-system specific antibodies (MacPherson and Liakopoulou 1966, Liakopoulou and MacPherson 1970, Goldschneider and Moscona 1972, Brockes et al. 1977). In addition, antisera may be absorbed with particular cell types, to obtain cell-type specificity (Schachner et al. 1976, Stallcup and Cohn 1976, Brockes et al. 1977) or even with subcellular fractions, to obtain subcellular specificity (DeRobertis et al. 1968, Mallet et al. 1979). Another approach to purifying antisera is to elute antibodies of interest either from antigens present on cells in culture (Mallet et al. 1979) or from antigens attached to nitrocellulose on immunoblots (Smith and Fisher 1984). Therefore, a more convenient application of the immunological approach can be made when the heterogeneity of the immune response generated against the available antigens is overcome by using antiserum purification procedures such as the techniques of absorption and elution.

Antiserum purification procedures can however be lengthy and deplete the stores of antibody available. Fortunately, most of the difficulties encountered due to heterogeneity of the immunological response and to the limited quantities of antibodies produced when raising polyclonal antisera (particularly in mice) can be overcome through the technique of monoclonal antibody production (Köhler and Milstein 1975). In this procedure, animals immunized with a particular antigen have their spleen cells (sites of antibody production) fused with myeloma cell lines in the presence of polyethylene glycol. The splenocyte/myeloma hybrids (hybridomas) are then serially diluted, cloned, and screened for the presence of the desired antibody reactivity using various methods (Köhler and Milstein 1975). The monospecific antibody produced in this manner can be replenished ad lib from these immortal cell lines. This technique of monoclonal antibody

production has been successfully applied to nervous system antigens (Eisenbarth et al. 1979, Lagenaur et al 1980, Hirn et al. 1982, Barnstable et al. 1983, Goridis et al. 1983, Grumet et al. 1984, Rathjen and Schachner 1984, Hockfield and McKay 1985, Williams et al 1985, Antonicek et al. 1987, Hockfield 1987, Rathjen et al 1987, Bier et al 1988) However, one advantage of polyclonal antibodies (when monospecific, ie recognizing only one protein) over monoclonal antibodies is the greater avidity of immune binding they show This is because, in a polyclonal antiserum, many different antibodies which may recognize many different sites (epitopes) on a single protein are likely to be present This advantage becomes obvious when, for example, a monoclonal antibody may not recognize a protein in its denatured form (such is the case when using immunoblotting techniques, whereby proteins are denatured in SDS), because it only recognizes an epitope in its native 3-dimensional conformation. In such a case, a polyclonal antibody would have a greater possibility for binding the protein in question because of its ability to recognize more than one epitope on the same protein. This phenomenon has often been observed and, in such cases, the use of a polyclonal antibody was favored (Stallcup et al. 1983, Rathjen and Schachner 1984, Williams et al. 1985, Jacob et al. 1986, Antonicek et al. 1987). Therefore, the decision on the nature of the immunological probe used is based mainly on two criteria, namely, specificity (usually better with monoclonal antibodies) and avidity (often better with polyclonal antibodies).

In conclusion, in using the immunological approach, the successful generation of useful antibodies can be hampered by the heterogeneity of the immune response when raising polyclonal antisera. To minimize this problem, it is essential to enrich the antigen of interest before immunization and/or to use purification methods once the polyclonal antiserum is

produced. For the characterization of nervous system proteins, monoclonal antibodies are favored because of their great specificity (therefore, no purification steps are needed) and their unlimited availability. However, sometimes their low avidity makes the use of polyclonal antibodies more relevant. A large body of discoveries in neurobiology have emanated from the immunological technology that has been developed, at an increasing pace, during the past 20 years. Therefore, in the following, a critical comparison of different protocols for isolation of cell surface antibodies is focused on.

MEMBRANE ISOLATION

Investigations on the role of cell surface molecules in characterizing cellular identity were initiated after the discovery, in 1963, by Koscielak, that blood group antigens could define the cell surface of red blood cells. Subsequently, the technique of immunizing with cell surface antigens was used to study liver cell determinants (see Perlmann et al 1964) and transplantation antigens (Kahan 1965, Kahan and Reisfield 1967, Shimada and Nathenson 1969, Smith et al. 1970, Yamane and Nathenson 1970). However, the first investigation about the serologic properties of antisera to cell surface determinants of the brain were those made by Shek and MacPherson (1971) who studied the immune response of rats to rat brain membrane fractions isolated using centrifugation techniques. They observed that low levels of antibodies appeared in the sera of rats injected with the nuclear and mitochondrial fractions of rat brain while rats injected with the microsomal (membrane) fraction produced the highest titers of antibodies. This original study showed then, that membrane preparations from the central nervous system can provide excellent immunogens. In taking advantage of that fact, the preparation of an enriched fraction from crude central nervous system tissue using centrifugation techniques, has been widely employed by researchers investigating the presence of surface markers in the central nervous system (DeRobertis et al 1968, Mickey et al. 1971, Shek and MacPherson 1971, Herschman et al. 1972, Bock et al. 1974, Milstein et al 1979, Lagenaur et al 1980, Barnstable et al. 1983, Grumet et al. 1984, Rathjen and Schachner 1984, Williams et al. 1985, Antonicek et al. 1987).

At the same time, other investigations have profited from the advents of immunotechnology by taking advantage of the fact that neurons are highly polarized cells (owing to their subcellular compartmentalization), and therefore, protein constituents can become localized to very different subcellular components such as the synaptic zone. Consequently, the techniques of differential, sucrose gradient or ficoll gradient centrifugation have been adapted by many anatomists and pharmacologists to isolate the synaptic end of neurons (synaptosome) for use as a relatively purified immunogen (DeRobertis et al 1968, Herschman et al 1972, Bock et al. 1974, Matthew et al 1982, Barnstable et al 1983, Williams et al 1985). In this technique, when synaptosomes derived from differential centrifugation are subjected to osmotic shock in hypotonic media, the synaptic membrane fraction can be collected in a relatively enriched form after gradient centrifugation while the mitochondria resist the osmotic lysis (Mickey et al. 1971, Jones and Matus 1974) For example, DeRobertis et al (1968) have raised rabbit antisera against synaptosomal membranes isolated (by isopycnic centrifugation) from the cat cerebral cortex They observed that, although several subfractions from the brain (myelin, synaptosomal membranes and mitochondria) had common antigens, the specificity of the antisera for synaptic membranes, could be improved by absorption with mitochondria and myelin They also showed, by electron microscope observations, that the antiserum, absorbed in this manner, could produce complement mediated lysis of the synaptosomal membrane, resulting in loss of axoplasm and synaptic vesicles. Similarly, Mickey et al (1971) raised a rabbit antiserum against synaptosomes, isolated by density gradient centrifugation from rat brain. They studied the cross-reactivity of the antiserum and observed that a major class of determinants is shared between brain mitochondria and synaptic membranes, that another major class is shared between myelin and synaptic membranes and that a third class is shared by

liver mitochondria and the various brain fractions. However besides these cross-reactivities, synaptosomes also displayed a unique class of determinants. These studies therefore show that some synaptosomal cell membrane determinants are immunologically distinct from determinants found in other subcellular fractions.

CELL ISOLATIONS

The methods mentioned above rarely define specific cells but rather specific molecules which may be found on different cell types in the central nervous system. Consequently a strategy that was developed is the isolation of whole cells derived from bulk isolation (Goldschneider and Moscona 1972; Pruss et al. 1977; Walker et al. 1979) with the aim of obtaining membrane-specific immunological markers which may also be cell specific. In this technique minced brain tissue is incubated in a trypsin solution in order to dissociate the individual cells which can then be used directly as immunogens. Among the original attempts at defining cell-specific surface components using this technique, immunological markers for the two major cell types in the central nervous system (neurons and glia) were produced. For example, Goldschneider and Moscona (1972), with the use of antisera prepared in rabbits against a suspension of live embryonic chick tissue cells followed by absorption with heterologous tissues, demonstrated the existence of brain specific embryonic chick brain cell surface antigens shared by both neurons and glia. However the purified antiserum also contained antibodies which would still react with all cells in the embryo, therefore indicating only partial absorption of the cross-reactive species. Similarly, using bulk isolated glia and neurons from rats, Stallcup and Cohn

1976) produced immunological probes against glia and neuron surface-specific components after absorption with a fetal packed washed cell lines of diverse origins (muscle and epithelial). Finally, Podusło et al. (1977) used both isolated rat brain neurons and lamb oligodendroglia to immunize rabbits. The antisera they produced were directed against cell-specific surface components of neurons and oligodendroglia respectively. These studies then demonstrated that the existence of differences in surface composition between neurons and glia cells could be used to generate antibodies specific to these cell types.

The cerebellum has often been investigated using immunological techniques to study cell surface antigens of particular cell types (see Schachner 1982 for a review). For example, a study in which development of the cerebellum was investigated is demonstrated by the work of Seeds (1976) who used reaggregated cells from 6-8d old mouse cerebella to raise polyclonal antibodies in rabbits. The interaction of the resulting antibodies with cerebellar cell surface components was assayed by immunohistochemistry. One interesting finding was that immunoreactivity of the mouse cerebellum with the antibody decreased with increasing age from embryo to adult, thus making the antigen a possible candidate for a developmentally-regulated molecule. Another example in the study of membrane specificities in the cerebellum is given by the elegant work of Margoliash et al. (1979), who raised polyclonal antibodies in rabbits directed against cultured rat cerebellar cell surfaces. The antiserum obtained was then purified by absorption with liver, heart and cerebrum from the rat. The remaining antibodies were then absorbed onto cultured cerebellar cells and eluted at pH 2.3. The resulting antibodies were specific for Purkinje cell membranes in the cerebellum. These experiments demonstrate that cell can be

differentiated from others in some structural components of the nervous tissue such as the cerebellum.

Alternatively, another method for the preparation of whole cells as immunogens involves the use of cell suspensions derived from cloned cell lines in culture (Seeds 1975, Stallcup and Cohn 1976, Brockes et al. 1977, Hirn et al. 1982) or tumor cell lines (Martin 1977) in the hope to produce cell-specific antisera. However, one objection to the use of that method is that cells in culture may either not be fully differentiated or may differentiate anomalously, and thus express antigens that are not characteristic of the normal differentiated cells of the nervous system (for a review, see Stallcup and Cohn 1979). Nevertheless, using a C-1300 ascites tumor cell line, Martin (1977) was able to produce a polyclonal antiserum which, after purification using the absorption method, could label proteins present in mouse brain (thus its name: mouse brain antigen-1, MBA-1) and to a lesser extent in kidney, but not in liver, lung, muscle, spleen or testes. Similarly, Brockes et al. (1977) raised an antiserum against a cell line derived from a tumor arising in the spinal cord and nerve roots in rats. After extensive absorption with rat liver and packed primary muscle cells from cultures, the antiserum specifically recognized the membrane of Schwann cells.

In conclusion, the production of antisera against cell membranes, synaptosomal membranes and whole cells can be used to raise antibodies specific for nervous tissue cellular and subcellular components. A general application of this technique has been undertaken in different fields of brain research. However, of particular interest are the membrane antigens which are involved in cellular adhesion. Investigations of these molecules are reviewed below.

CELL ADHESION MOLECULES

As already mentioned, one area of research in neurobiology, which has profited extensively from the immunological approach is the study of cell adhesion molecules (CAMs) involved in neural development (for recent reviews, see Edelman 1983, Goridis et al. 1983, Rutishauser 1983, Quarles 1984, Edelman 1985, Rutishauser and Goridis 1986, Rutishauser and Jessel 1988). Edelman, who won the Nobel prize in 1973 for his discoveries on the structure of antibodies, made use of his expertise with immunological techniques to raise an antibody against a molecule which is involved in neural cell aggregation and was therefore named the "neural cell adhesion molecule" or N-CAM. The polyclonal antiserum which was originally produced against this antigen, was used again later (Rutishauser et al. 1978, Hoffman et al. 1982) to purify N-CAM using an immunoaffinity column. In this procedure, an embryonic chick brain glycoprotein fraction was isolated using lectin affinity chromatography, and then passed over an anti-N-CAM antibody column to isolate the N-CAM antigen. The N-CAM was then used to produce monoclonal anti-N-CAM antibodies in large quantities in rabbits. Aggregation assays, originally developed by Huesgen and Gerish (1975) to study the mechanism of aggregation in slime molds in culture, were used to screen the different monoclonal antibodies produced (Rutishauser et al. 1978) In this assay, aggregated cells in culture are incubated with the monovalent antibody fragments (Fab) and disaggregation of the cells is an indicator that the antibody recognizes an epitope involved in aggregation, a so-called cell adhesion molecule Using this assay, 3 forms of N-CAMs were identified (Rothbard et al 1982), which differed in their sialic acid content. Interestingly enough, it appeared that the

expression of the different forms of N-CAM was developmentally regulated (Schlosshauer et al. 1984). In the early embryo, the sialic acid content of N-CAM is maximal (80% of sugar residues - Hoffman et al. 1982), whereas in the adult, N-CAM seems to have lost as much as two thirds of its sialic acid residues (Hoffman et al. 1982, Rothbard et al. 1982). This phenomenon was termed embryonic-adult conversion (E-A conversion) and to date, of all the CAM molecules that have been identified (see later), only N-CAM seems to undergo such a change during embryonic development. In this regard, it is of interest that a mutant mouse (staggerer), which shows gross disorganization in several regions of the central nervous system, fails to show the usual change in sialic acid content of N-CAM during maturation (Edelman and Chuong 1982). The adhesion properties of N-CAM were further investigated at the cellular level (Hoffman et al. 1982, Hoffman and Edelman 1983) and it was found that this molecule has 4 domains which are 1) a cytoplasmic domain at the $-NH_2$ terminal 2) a membrane-spanning domain, 3) a sugar moiety-bearing domain and 4) a binding site at the -COOH terminal of the molecule. This binding site seems to be specific for the attachment sites of N-CAM molecules found on other neurons which defines the binding pattern of N-CAM as homophillic (ie, self-binding). This was observed with the aid of an adhesion assay, whereby lipid vesicles were shown to aggregate only when they were bearing N-CAM molecules on their surface but not when devoid of N-CAM or when anti-N-CAM Fab fragments were present in the bathing medium (Hoffman and Edelman 1983). This suggested that the N-CAM molecule alone was sufficient for cell-cell adhesion and that no other adhesion molecule, and no unique N-CAM receptor molecule was needed for this event to take its course. These findings about the specific adhesive functions of N-CAM and the even more interesting ones pertaining to its differential developmental expression led other researchers to investigate the presence of other cell adhesion

molecules in the developing central nervous system of vertebrates. However, the first attempts in that direction only replicated the findings of Edelman and co-workers because, owing to N-CAM's high immunogenicity and prevalence at the cell surface, it is the easiest cell adhesion molecule to isolate, and D2 (Jørgensen and Bock 1974), NS-4 (Schachner et al. 1975) as well as BSP-2 (Hirn et al. 1981) all appeared to be structurally and functionally identical to N-CAM. Other attempts were successfully made however and the possibility that many such adhesion molecules could be playing a role in neurogenesis now seems likely. These molecules include the liver hepatocyte adhesion molecule (L-CAM - Bertolotti et al. 1980, Gallin et al. 1983), the neuron-glia adhesion molecules J1 (Kruse et al 1985), and Ng-CAM (Grumet and Edelman 1984, Friedlander et al. 1986, identical to L1 - Rathjen and Schachner 1984 - and NILE - Bock et al. 1985), the myelin-associated-glycoprotein (MAG - Sternberger 1979), the adhesion molecule on glia (AMOG - Antonicek et al. 1987), F-11 (Rathjen et al. 1987), Neurofascin (Rathjen et al. 1987). Curiously enough several of the cell adhesion molecules (eg. Ng-CAM, MAG and N-CAM, J1, but not AMOG - Antonicek et al. 1987) share a common epitope determinant recognized by HNK-1 (a mouse antibody raised against human lymphoblastoma and used as a marker for human lymphocytes with natural killer functions) and by L2, an antibody which appears to bind to an epitope similar to HNK-1 (Kruse et al. 1984, Martini and Schachner 1986). Consequently, a major epitope family, the L2/HNK-1 family, includes these cell adhesion molecules that possess a specific class of determinants bound by the L2 and the HNK-1 monoclonal antibodies, which might be related to their adhesive functions (Kruse et al. 1984, Keilhauer et al 1985) Of the cell adhesion molecules that have been functionally characterized to some extent, Ng-CAM, Neurofascin and AMOG are of special interest since their expression was also found to be developmentally regulated in varying amounts However,

these molecules do not appear in different forms through neurogenesis, as is the case with N-CAM. Ng-CAM is a molecule expressed on post-mitotic neurons, that carries out heterophilic binding with a putative receptor (so-called Gn-CAM - Grumet et al. 1984). In vitro assays with cerebellar explants incubated with anti-Ng-CAM antibodies, have indicated that the migration of the external granular cells towards the internal granular layer (their final location) is dependent at least in part on NgCAM mediated interaction (Lindner et al. 1986). A similar effect of the anti-AMOG antibody was observed (Antonicek et al. 1987), since AMOG, being expressed by glial cells, promotes attachment of cerebellar Bergmann glia to external granular neurons in a heterophillic manner. On the other hand, NgCAM and also Neurofascin seem to be molecules involved in neurite-neurite attachment at the level of fasciculation since monoclonal antibodies against Ng-CAM (Fischer et al. 1986) or Neurofascin (Rathjen et al. 1987) prevent formation of nerve bundles in vitro. From these types of results it has been suggested, then that during embryogenesis, the action of a variety of cell adhesion molecules upon neurites, directs their growth through different epochs of development.

Before the discovery of cell adhesion molecules, 2 major hypotheses had been formulated, concerning the harmonious development of the nervous system, and the correct growing of the neurons to their respective target cells. One of these hypotheses the so-called "resonance hypothesis", is somewhat sketchy in its formulation and deals with the fact that neurons have the potential to convey specific information, for example about a given modality, to their target and that only specific targets would have the possibility of processing this information. These qualities would make the neuron and the target cell "recognize" each other and would lead to the

establishment of a relevant connection, or synapse (Weiss 1924). Another hypothesis deals with the specificity of target cells in their chemical composition (or "address"), which would lead an axon to form contact according to the "recognition" of such a chemical specificity on its target. This hypothesis, the so-called "chemospecificity hypothesis", which was formulated by Sperry (1963) implied, in its most rigorous version, that all cells are different in terms of chemical composition and that this difference would mediate recognition and would be directed by genetic make-up.

Since, in the first instance, N-CAM, the first cell adhesion molecule to be fully characterized, is located on neural cells in the chick nervous system (but also on muscle cells and several other tissues - Rutishauser et al. 1978), and since it is a developmentally modulated molecule as well as a highly evolutionary-conserved molecule (Hoffman et al 1984), it has been suggested that it plays a major role (together with other cell adhesion molecules) in neurogenesis and therefore would be a prime candidate for influencing neuronal pathfinding. Consequently, a "regulator hypothesis" (Edelman 1983) has been proposed, whereby differential expression of various cell adhesion molecules would direct axons to their targets and the final result would again be the formation fo relevant synapses. The outcome of this hypothesis is that the complexities of neuronal development would rely on only a few gene products (the ones involved in the expression of the CAMs on various cells) but the involvment of epigenetic factors such as the induction of expression of different forms of CAMs (or different CAMs altogether), following mechanical adhesion at given stages of development, would play a key role in neuronal differentiation, pathfinding and synaptogenesis Credibility has been given to this hypothesis by the findings that substrate adhesion molecules (SAM's - Steinberg 1970, Lemmon et al 1982, Thiery et al 1982), which are components of the extra-

cellular matrix (laminin, proteoglycans, fibronectin, etc.), and also cell junctional molecules (CJM - Feldman et al. 1978) have been implicated in axonal routing during development (see Werz and Schachner 1988). These molecules are also found associated with cell adhesion molecules and the interaction between these different types of molecules (CAMs, SAMs and CJMs) may be the basis for the harmonious development of the nervous system.

CADHERINS

It has been reported that intercellular adhesion is due to at least two classes of mechanisms, one Ca^{++}-dependant and the other Ca^{++}-independent (Takeichi 1977, Urushihara et al. 1977, Ozaki et al. 1979). The Ca^{++}-dependent class of interactions is mediated by molecules termed Cadherins (for reviews see Takeichi et al. 1986, 1988). The cadherins are also distinguished from the cell adhesion molecules discussed in the previous section in that they are temperature as well as trypsin sensitive.

Cadherins can be divided into subclasses of different immunological specificity (Takeichi et al. 1985, See Takeichi et al. 1986, 1988 for reviews). For example, P-cadherin (placental cadherin) plays a role in uterine implantation of the fetus in mammals (Nose and Takeichi 1986) while E-cadherin (epithelial cadherin) is involved in adhesion of most epithelial cells derived from the ectoderm and endoderm. The latter molecule has been identified by many groups and evidence is given of its equivalence or cross-species analogy with L-CAM (Gallin et al. 1983), Arc-1 (Behrens et al. 1985), uvomorulin (Peyrieras et al. 1983), teratocarcinoma cadherin (Takeichi et al. 1981) and cell-CAM 120/80 (Damsky et al. 1983). Finally, the major cadherin found in the nervous system is N-cadherin (N-Cal-CAM - Bixby et

al. 1987, A-CAM - Volk and Geiger 1984).

All cadherin subclasses have distinct binding specificities but share a common structure with a Ca^{++}-attachment site close to the extracellular N-terminal region, a membrane-spanning domain and a cytoplasmic domain (about 80Kd) which is highly conserved among cadherins through evolution (Hatta et al. 1988, Takeichi 1988).

Cadherins are involved in cellular aggregation through homophillic binding (however, interactions could occur between different cadherin subclasses - Nose et al. 1988). They are present in every cell forming solid tissues indicating their general importance for cell-cell adhesion (Yoshida-Noro et al. 1984).

Interestingly, cadherins appear to be involved in segregation of tissues (Hatta and Takeichi 1986, Nose and Takeichi, 1986, Hatta et al. 1987), homing of migratory cells (Gallatain et al. 1983) and selective connection of neurons (Goodman et al. 1984, Takeichi et al. 1985) in mammals and birds. For example, Hatta and Takeichi (1986, and also Nose and Takeichi 1986 and Hatta et al. 1987) found that the expression pattern of each cadherin subclass in embryos is correlated with the segregation of cell layers. In neural tube formation, the patterns of N-cadherin expression appear to be complementary to those of E-cadherin (Hatta and Takeichi 1986) although there seems to be an early intermediate stage at which both molecules are expressed on the same cells (Hatta et al. 1987). Takeichi et al. (1986) proposed that the segregation of the neural tube from the overlying N-cadherin-negative (E-cadherin-positive) ectoderm resulted from homotypic adhesion that lead to the separation of these two ectodermal derivatives.

Subsequently, the loss of the N-cadherin molecule from the dorsal region of the neural tube is associated with the appearance and migration of neural crest cells. From these observations, it was therefore hypothesized that the termination of N-cadherin expression in the neural plate would release neural crest cells from their aggregation near the neural tube and thus permit their migration. Other such observations are given in the literature where transient expression of a cadherin subclass would give rise to developmental changes leading to cell migration or cell implantation at a final destination (for a review see Takeichi 1988).

Cadherins appear to exert their adhesive effect through actin-mediated morphological changes owing to the association of the molecule's cytoplasmic domain with cortical actin bundles. Evidence for this association is given by the observation that morphological changes occur in L cells (with little endogenous cadherin activity) when they are transfected with the E-cadherin gene (Nagafuchi and Takeichi 1988). It was also observed (Boller et al. 1985, Volk and Geiger 1986a,b, Hirano et al. 1987) that cadherins are components of intercellular adherens junctions, such as zonulae adherentes, which are known to be associated with actin bundles. By contrast, the neural cell adhesion molecule (N-CAM) is not concentrated at morphologically-specialized membrane structures. Hirano et al. (1987) studied the localization of cadherins and actin bundles in various culture cells by a double immunostaining method and found that cadherins present at the cell-cell boundary perfectly coincided with the cortical actin bundles. It is therefore possible that cadherins be involved in the contact mediated regulation of cell motility since actin is a major component of the cellular motility machinery (See Takeichi 1988).

The pattern of tissue distribution of N-cadherin is similar to that of N-CAM

(Edelman et al. 1983, Hatta et al. 1987). It was thus hypothesized (Hatta and Takeichi 1986) that many subtypes of adhesion molecules may be expressed on the same cells and that variations in the temporal expression and density distribution of either of these adhesion molecules would direct developmental events. Support to this hypothesis is given by the observation (Takeichi et al. 1979, Urushihara et al 1979, Brackenbury et al. 1981) that E-, N- and P-cadherin are overlapped in many kinds of cells in varying combinations. Such combinations of different types of cadherins must create a variety of adhesive specificity for cells Since Ca++-independent adhesion molecules such as N-CAM are co-expressed with cadherins, the combination of all these adhesion molecules should provide cells with a greater variety of adhesive specificity (see Edelman 1984, Takeichi 1988, for discussions).

As originally pointed out by Townes and Holtfreter (1955), the selective segregation or migration of cells can be produced by quantitative hierarchies of adhesion strengths in the form of preferences rather than absolute distinctions. At present, relatively few adhesive molecules have been sufficiently well characterized to permit an unambiguous analysis of their combined effect. However, studies in that direction are being elaborated in many laboratories.

GOLDFISH VISUAL SYSTEM REGENERATION

In the adult goldfish (as well as in other fish and amphibians), regeneration can spontaneously take place after transection of the optic nerve or tract. Since such a regenerative capacity seems to be lacking in

the central nervous system (CNS) of higher vertebrate species, the goldfish visual system has been the subject of much anatomical (Sperry 1948, 1963, Jacobson and Gaze 1965, Murray and Grafstein 1969, Gaze and Sharma 1970, Murray 1973, 1976, Lowenger and Levine 1988), pharmacological (Oswald et al. 1980, Kah and Chambolle 1983, Yoshida et al. 1983, Ross and Godfrey 1986), and biochemical (Murray and Grafstein 1969, Giulian et al. 1980, Maxwell and Elam 1980, Quitschke et al. 1980, Heacock and Agranoff 1982, Giulian et al. 1985, Perry et al. 1985, 1987, Schmidt 1987, Königstorfer et al. 1989, Yazulla et al. 1989) investigations.

This regenerative process includes first, formation of regenerative sprouts, then guidance of the growing axons over long distances through the optic nerve and tract, and finally, the successful matching of regenerating axon terminals with appropriate postsynaptic cells in the optic tectum.

The visual system of the goldfish therefore, is suitable for studying the phenomena of axonal growth and of cell interactions in the regenerating central nervous system of an adult vertebrate. The visual system of the goldfish is concerned with the transmission of information between the photoreceptor cells found in the retina, to the visual center, namely, the optic tectum where visual processing occurs (Sperry 1963). In that pathway, retinal ganglion cells act as intermediaries, gathering information from the photoreceptor cells and conveying it to the optic tectum via their long axons in the optic nerve and tract. After lesion of the optic nerve, the severed axons undergo Wallerian degeneration and are soon replaced by regenerating ganglion cell axons (Murray 1982). A number of changes occur at all levels in the ganglion cell (ie. soma, axon and synaptic ending), the final result being recovery of visual function within 1-2 months (Jacobson

and Gaze 1965, Grafstein and Murray 1969).

Morphological observations (Murray and Grafstein 1969) as well as radiolabeling experiments - where radioisotopes are injected into the eye and incorporated into newly synthesized proteins of the ganglion cells (Murray and Grafstein 1969, Guilian et al. 1980, Maxwell and Elam 1980, Benowitz et al. 1981, Heacock and Agranoff 1982, McQuarrie and Grafstein 1982, Chaknaborty et al. 1986, Perry et al. 1987), have shown that the following changes take place in the ganglion cell somata during optic axon regeneration. First, there is a change in the staining intensity of Nissl bodies (chromatolysis reaction) which imply protein synthesis since Nissl bodies are composed of ribosomes in association with which the bulk of cytoplasmic protein synthesis occurs (Murray and Grafstein 1969, Giulian annd Iwanij 1985). Second, cellular hypertrophy occurs (Murray and Grafstein 1969). Among the proteins that undergo changes in their synthesis rate during regeneration of the optic nerve are the structural components including microtubules (tubulin - Giulian et al. 1980, Quitschke et al. 1980, Heacock and Agranoff 1982, Perry et al. 1985), microfilaments (actin - Giulian et al. 1980, Quitschke et al. 1980, Heacock and Agranoff 1982, Perry et al. 1985) and neurofilaments (ON1, ON2 - Quitschke and Schechter 1983, Perry et al. 1985). Another protein with apparent molecular weight of 43Kd, shows the most marked increase in synthesis rate and was therefore named growth-associated protein 43 (GAP 43 - Skene and Willard 1981, Benowitz et al 1981, Heacock and Agranoff 1982, Perry et al 1985).

The regenerating ganglion cell axons grow in large numbers into optic nerve bundles. The growing axons from one eye then have to cross over at the level of the optic chiasm and , travel through the optic tract, to reach the tectum. It has been observed that, following optic nerve section,

oligodendroglia and multipotential glia proliferate in the optic tract (Guilian and Iwanij 1985, Guilian et al. 1985), and that this proliferation might be influenced by humoral factors released from the synaptic target sites in the optic tectum (Giulian and Iwanij 1985). This was suggested by an experiment where optic tract glia, in vitro, increased their amino acid incorporation in the presence of soluble factors from the optic tectum (Giulian and Iwanij 1985). During the first 3 months post-operation, axons in the optic nerve and tract increase in diameter (Murray 1976). These findings indicate that, even after recovery of sight (within 1-2 months post-operatively - Jacobson and Gaze 1965, Grafstein and Murray 1969), changes are still occurring in the visual pathway (Murray 1976, 1982).

In conclusion, the visual system of the goldfish, owing to its regenerative capacities and to its ordered organization, has proven to be a system of choice for carrying out investigations in neurobiology. These investigations involve concerns about axonal routing in neurogenesis, and the influence of soluble factors and cellular interactions leading to successful recovery of visual function.

MATERIALS and METHODS

ANIMAL CARE

GOLDFISH

The animals used in this study were adult Comet goldfish (Carassius auratus, Hartz Canada, Montreal). Their average length from snout to the base of the caudal fin was 6-7cm. The fish were fed once a day with TetraFin™ brand staple food enriched with lecithin (West Germany), and kept in aerated tanks filled with salted water (0.1% NaCl) at room temperature (18-24°C).

MICE

The mice used were 6 week old female BALB/c (Bar Harbor, Maine) kept in group cages not exceeding a density of 6 individuals/cage. The floor of the cage was layered with sawdust. Food (Purina™ rat chow) and water

supplies were available at all times. The mice were under the care of the McIntire building animal room technical staff. The cages were cleaned every week.

PROTEIN ASSAY

The bicinchoninic acid protein assay procedure (Sigma kit no. TPRO-52) was used to measure protein concentrations. In this assay, Cu (II) is reduced to Cu (I) by proteins, in a concentration-dependant manner. Bicinchoninic acid is a highly specific chromogenic reagent for Cu (I), forming a purple complex with an absorbance maximum at 562nm, therefore absorbance at 562nm is directly proportional to protein concentration.

In test-tubes, 2.5-15µl of proteins (either in phosphate buffered saline - PBS: 0.05M phosphate buffer pH5.4, 0.9% NaCl - or in sucrose) were added to 2mls of the reagent mixture (1 part of Copper (II) sulfate pentahydrate 4% solution to 50 parts of bicinchoninic acid solution -Sigma) and this mixture was diluted up with distilled H_2O to a final volume of 2.1mls. The tubes were successively incubated at 37°C for 30min and cooled to room temperature. The contents of the tubes were transfered to polystyrene phototubes and absorbance readings at 562nm were taken from a Perkin-Elmer (Lambda3) spectrophotometer. The absorbance of a no-protein blank was subtracted from the absorbance of the assay tubes to obtain the net absorbance due to protein. These absorbance results were matched to BSA (bovine serum albumin) standards to determine protein concentrations.

SUCROSE GRADIENT CENTRIFUGATION

The protocol used was a modification of the combined flotation-sedimentation density gradient centrifugation technique used by Jones and Matus (1974) to isolate synaptic plasma membranes from the rat brain.

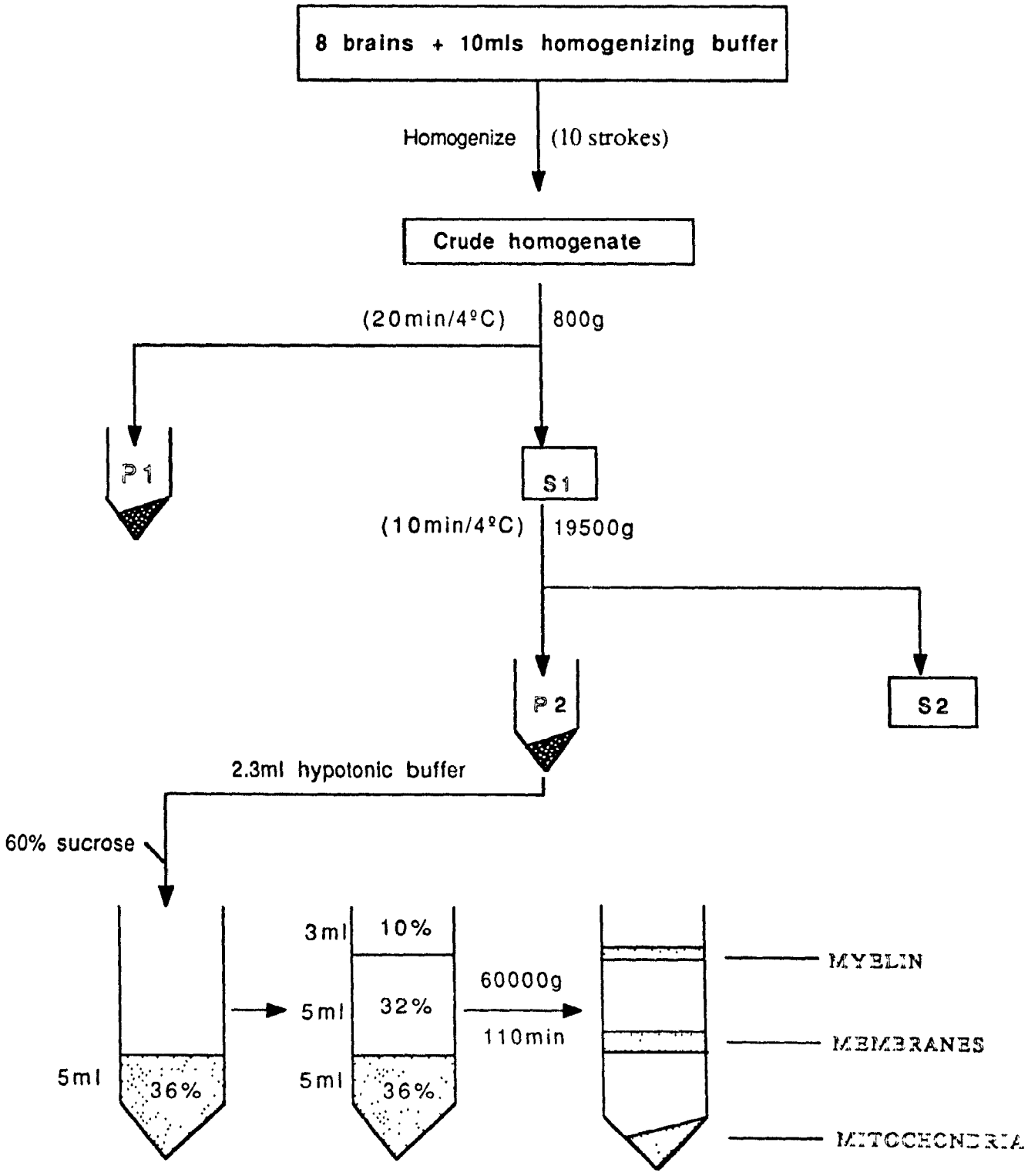
Eight brains (including vagal lobes) were dissected out of goldfish and ground (10 strokes) in a Potter-Elvehjem homogenizer in 1 ml of isotonic homogenizing buffer (4mM EDTA, 4mM Tris-HCl pH 7.4, 0.175M sucrose). Phenyl methyl sulfonyl fluoride (PMSF,100mM in isopropanolol), a proteolytic enzyme inhibitor was added at a concentration of 1% (v/v). The homogenate was then diluted about 10 times by adding 9mls of homogenizing buffer. The diluted homogenate was spun for 20 minutes at 800g (3200 RPM) in a table top microfuge at 4°C. The pellet (P1), containing unbroken cells as well as nuclei and membrane sheets (Graham 1987) was discarded, and the supernatant (S1) was spun for 10 minutes at 19500g (15200RPM) in a refrigerated centrifuge (Sorvall, model RC-5B, rotor model SA-600). The S2 supernatant, containing mostly peroxisomes, mitochondria, rough endoplasmic reticulum and Golgi apparatus (Graham 1987) was discarded. The P2 pellet, containing myelin, membranes and mitochondria (Graham 1987, Jones and Matus 1974) was subjected to centrifugation in a sucrose gradient (see flow chart below).

The P2 pellet was resuspended in 2.3ml hypotonic buffer (5mM Tris-HCl, pH8.1) at 0°C for 30 min, in order to break open sealed membrane vesicles . To this solution was added a 60% sucrose solution, in order to obtain a bottom sucrose layer (containing the P2 pellet in suspension) with a final

concentration of 36% (total volume of the bottom layer in each tube was 5mls). Then, 5mls of 32% sucrose and 3mls of 10% sucrose were overlayered successively to produce the gradient.

The gradient was spun for 110min at 60,000g (22,000 RPM), after which three fractions were harvested: the myelin fraction at the 10/32 % interface,

Flow chart for sucrose gradient centrifugation



the membrane fraction at the 32/36 % interface, and the mitochondrial pellet. The membrane fraction was then spun at 80000g (35,000RPM) for 3 hours in 6mls total volume of homogenizing buffer and the resultant membrane pellet was resuspended in 0.3ml of storing buffer (15mM Tris-HCl pH7.4, 0.25M sucrose, 15% glycerol).

ENZYME ASSAYS

OUABAIN-SENSITIVE Na^+,K^+-ATPASE

(plasma membrane marker)

The method used was modified from the protocols of Schimmel et al. (1973) and Holland et al. (1974). The ouabain-sensitive Na^+/K^+ ATPase content was determined in a 300µl reaction mixture (300mM imidazole-HCl pH7.5, 0.11M NaCl, 15mM KCl, 5mM NaN_3, 0.5mM EGTA, 4mM $MgCl_2$, 3mM ATP, 0.01% (v/v) Triton-X-100 and 5 or 10µg of sample proteins) at 4°C. The following reaction took place in the test tube.

$$ATP \xrightarrow[\text{enzyme, } Mg^{++}]{\text{Endogenous}} ADP + Pi$$

where the endogenous enzyme is Na^+, K^+-ATPase

The Na^+,K^+-ATPase activity was calculated as the difference in ATPase activity in the presence and absence of 1mM ouabain. Reactions were carried out at 37°C for 45min and stopped by adding an equal volume of 10% perchloric acid and 30µg of heat-activated charcoal (Fisher Scientific

Co.). After removal of protein by centrifugation (high speed on microfuge, for 3min at 4°C), inorganic phosphate content was determined by an assay (where molybdate which forms a complex with inorganic phosphate, is reduced with ascorbic acid) by taking 0.5ml of the supernatant and mixing it with a 1:6 ascorbate:molybdate solution (10% ascorbic acid, 0.42% ammonium molybdate.$4H_2O$ in 1N H_2SO_4, Ames 1966). Absorbance readings were taken in a spectrophotometer (Perkin-Elmer, Lambda 3) with polystyrene phototubes, at 820nm. Optical density readings of experimental results were matched to phosphate standards.

CYTOCHROME C OXIDASE ASSAY

(mitochondrial marker)

The enzyme activity was measured spectrophotometrically according to Sottocasa et al. (1967) by following the oxidation of reduced cytochrome C at 550nm. 0.6mM of Tuna fish Cytochrome C (Sigma chemicals co.) in 0.05M potassium phosphate buffer (pH7.4) was reduced by the addition of sodium dithionite crystals until a change of coloration, from dark red (oxidized form) to orange (reduced form) was obtained. In a 3ml cuvette, 0.1ml of 0.3% Triton-X-100 solution (v/v in buffer) and 0.1ml of protein were added to 2.7mls of potassium phosphate buffer. The reaction was started by adding 0.1ml of reduced cytochrome C. The following reaction took place in the cuvette:

$$\text{reduced Cytochrome C} \xrightarrow[\text{enzyme}]{\text{endogenous}} \text{oxidized Cytochrome C} + 1e^-$$

where the endogenous enzyme is cytochrome C oxidase

The decrease in optical density with time, was read from a chart paper recorder. The cytochrome C oxidase activity (µmoles/mg/min) was estimated according to the following formula:

$$c/time = (\Delta O.D.\ 550X3mls)/(18.5/mM * 1cm/\ mg\ protein)/time$$

where:

c: concentration µmole/mg

ΔO.D.550: variation in optical density at 550nm as a function of time

3mls: cuvette volume

18.5: extinction coefficient of cytochrome-C oxidase at 550nm

1cm: light path through the cuvette

2',3'-CYCLIC NUCLEOTIDE,3'-PHOSPHOHYDROLASE (CNPase)

(myelin marker)

The increase in absorbance at 340nm for the conversion of 2'3'cyclic NADP to 2'NADPH was determined spectrophotometrically according to the method by Hugli et al. (1973).

To start the conversion, 10-100µl of protein was added to a reaction mixture (5mM glucose-6-phosphate, 0.2M MES buffer pH6.0, 30mM $MgCl_2$, 0.05% (v/v) Triton-X-100, 0.25mM 2'3'cNADP and 0.4% (v/v) glucose phosphate dehydrogenase suspension (Boehringer Manheim Co.) in a 1ml final volume at room temperature. The following reactions took place in the cuvette:

$$2'3'cNADP \xrightarrow[\text{enzyme, Mg+}]{\text{Endogenous}} 2'NADP$$

$$2'NADP + G\text{-}6\text{-}P \xrightarrow{\text{GPD}} 2'NADPH + 6'PGL$$

Where:

the endogenous enzyme is 2',3'-cyclic nucleotide, 3'-phosphohydrolase (CNPase)

GPD: glucose phosphate dehydrogenase

6'PGL: phosphoglucono-δ-lactone

The rate of production of 2'NADPH was measured as U/mg protein on a paper chart recorder over 2-3min, according to the following formula:

$$\frac{\Delta A^{340}/6.22^{*}}{\text{volume protein/concentration protein}}$$

where ΔA^{340}: variation in absorbance over time at 340nm

*Extinction coefficient of NADPH at 340nm

ELECTRON MICROSCOPY

Following sucrose gradient centrifugation as described above, the different pellets (P1, P2, myelin, membranes, mitochondria) were incubated overnight at 4°C in fixative (0.1M phosphate buffer pH 7.4, 0.5%

paraformaldehyde, 0.0025% $CaCl_2$, 1.5% glutaraldehyde). After this fixation step, the pellets were rinsed in 0.1M phosphate buffer for 30min, 3 times consecutively, followed by post-fixation at 4°C (in the dark) in an osmium tetroxide solution (1% $Os0_4$, 1.5% potassium ferrocyanide in 0.1M phosphate buffer pH7.4). Then, a dehydration and embedding schedule was undertaken as follows: *1*- 10min rinse in 30% ethanol (4°C), *2*- 10min rinse in 50% ethanol (4°C), *3*- Quick rinse, then 20min incubation in 95% ethanol (room temperature), *5*- 2 X15min rinse in absolute ethanol (room temperature), *6*- 30min incubation in propylene oxide (4°C), *7*- 1hr incubation in 50:50 propylene oxide epoxy resin (49% EPON, 29% DDSA, 25% NMA, 2.3% DMP-30). The pellets were incubated overnight in 100% epoxy resin (4°C) and then placed under the care of Elizabeth Montgomery, the electron microscope technician who sectioned the epoxy resin blocks and stained them with lead citrate prior to mounting on gold grids. Magnifications up to 169,000 fold were achieved with good resolution from a Phillips 410 electron microscope. Pictures of representative fields were taken from each of the fractions used.

MOUSE IMUNIZATION

After collection of plasma membrane proteins by sucrose gradient centrifugation (for the membrane fraction) or by differential centrifugation (when crude membrane fraction - P2 - was used), the proteins were resuspended in PBS to obtain a final protein concentration of 0 5mg/ml.

The protein suspension was pulled through a 3cc syringe, together with an equal volume of Freund's complete adjuvant (Sigma Chemical Co.). The

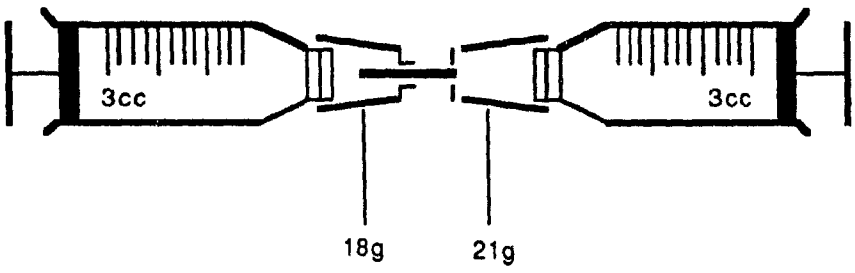
syringe was attached to another 3cc syringe by the combination of an 18 gauge needle (which was cut to a length of 3mm) and a 21 gauge needle (cut to 1cm) fit head to head into each other (see diagram below)

The emulsion was prepared by rapidly pushing the protein/adjuvant mix back and forth through the 2 syringes until the movement was opposed by the liquid density inside the syringes 200µl of the emulsion (50µg protein) was injected in the sub-cutaneous space in the back of 6 week-old female BALB/C mice using a 23G needle

Fourteen days after the primary injection, the mice were boosted by a similar injection in Freund's incomplete adjuvant (Sigma Chemical Co.). Fourteen days later, a final boost was carried out similarly, except that the mice were injected intraperitoneally

Three to 5 days after the second boost, the animals were bled from the retroorbital venous plexus (Weir 1973) under general anaesthesia (Avertin - 5% w/v tribromoethanol, 2.5% v/v tert-amyl alcohol - 0.3ml/20g) With practice, 250-300µl of blood could be recovered in this manner Subsequently, the blood was incubated in a closed eppendorf tube for 2hrs at room temperature, and then stored overnight at 4°C It was then centrifuged for 5min at 800g in order to pellet the blood clot which was carefully extracted from the tube, using a sterile needle The tube was then spun again at 14000g for 5min, the supernatant serum was transfered to a fresh tube, and 0.02% sodium azide was added (for prevention of bacteria growth). The antiserum was then stored at 4°C if it was to be used within a month, or at -20°C for longer storage times

Apparatus used for emulsion of a protein solution in Freund's adjuvant.



GEL ELECTROPHORESIS AND IMMUNOBLOTTING

PREPARATION OF SAMPLES

Tissue was either homogenized in phosphate buffered saline (PBS, 4°C) to obtain a crude preparation or centrifuged into a crude membrane (P2) or purified membrane fraction The extract was then dissolved in sample buffer (10% v/v glycerol, 5% ß-mercaptoethanol, 2 3% SDS, 0 0625M Tris-HCl pH6.8) to a final concentration equal to or larger than 1mg/ml Bromophenol blue (tracking dye) was added to a final concentration of 0 1%, after which the denatured protein solution was placed in a boiling-water bath for 10min

SDS-PAGE

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out as described by Laemmli (1970) for a discontinuous gel system. The proteins were resolved in a 10% polyacrylamide gel containing 33% of an acrylamide stock solution (30% acrylamide·N,N,methylene-bis-acrylamide 29 2:0 8) and 25% of lower gel buffer - LGB (1 5M Tris-HCl pH8.8, 0.4% SDS). Polymerization was initiated by the addition of ammonium persulfate (APS) and N,N,N',N'-Tetramethyl ethylendiamine (TEMED) to a final concentration of 0 03% w/v and 0 05% v/v respectively The gel was cast 10cm long and 1 5mm thick, overlayered with 50% isopropanol and allowed to polymerize for about 30min at room temperature . The stacking gel, containing 0 15% of 30% acrylamide stock, 24% upper gel buffer - UGB (0 5M tris-Hcl pH6 8, 0 4% SDS), 0 03% APS and 0 01% v/v TEMED, was layered (2cm) over the resolving gel after discarding the

isopropanol. Following polymerization of the stacking gel, the protein sample was loaded in individual lanes. The gels were calibrated with standard molecular weight markers (200Kd: myosin, 116Kd: b-galactosidase, 97Kd: phosphorylase b, 66Kd: bovine serum albumin, 43Kd: ovalbumin, 30Kd: soybean trypsin inhibitor, 22Kd: lysozyme - BioRad) or pre-stained molecular weight markers (84Kd: fructose-6 phosphate kinase, 58Kd: pyruvate kinase, 48.5Kd: fumarase - Sigma - these molecular weights were used only nominally since I observed that the 84Kd protein migrates fast and that the 58Kd and 48.5Kd proteins migrate slow, relative to the BioRad standards, probably due to some effect of prestaining the proteins) and was run at 3 Watts, constant power, in freshly-prepared running buffer (2.5mM Tris, 17.8mM glycine, 0.1% w/v SDS) for about 5hrs or at 55V, constant voltage, for 14-17hrs.

For shorter running times and smaller protein amounts, the mini-gel apparatus (Mighty Small II slab gel model SE 250, Hoefer Scientific instruments) was used. All steps and solutions were as mentioned above, with the exception that the resolving gel was cast 5cm long and 0.75mm thick. The gel was run at 3 Watts for about 1hr.

Either staining or protein transfer was carried out following electrophoresis.

COOMASSIE-BLUE STAINING

After the proteins were resolved, the gel was incubated in a Coomassie Blue solution (50% ethanol, 5% trichloroacetic acid, 7% acetic acid, 200µg/ml Coomassie-Blue R-250) overnight at room temperature on a

shaker (TekTator). Then, it was incubated for 1-2hrs in an ethanol-free Coomassie Blue solution, after which it was destained for at least 2hrs in 7% acetic acid.

PROTEIN TRANSFER

Gels to be used for protein transfer were lain over pre-wetted nitrocellulose paper cut to size, sandwiched in between 2 beds of pre-wetted 3MM paper and fit into a plastic transfer holder (Bio Rad) The transfer holder was placed into the transfer apparatus filled with cold ($0^{o}C$) transfer buffer (25mM Tris, 192mM glycine, 20% methanol) and the transfer was carried out for 30min in an ice bath at 1.2amps, constant current.

IMMUNOBLOTTING

The method described below for immunoblotting and visualization was adapted from Smith and Fisher (1984). After protein transfer, the blot was incubated in blocking buffer (0.05M PBS, 0 5% Tween-20, 3% BSA) for 1-2hrs at room temperature, after which it was rinsed twice in PBS/0.5% tween-20. Then, the blot was placed in a sealed plastic bag together with relevant antibody at a dilution of 1 500 (in blocking buffer) overnight at $4^{o}C$ Rinsing was then repeated, and the blot was challenged with an alkaline phosphatase-conjugated secondary antibody (goat anti-mouse - Zymed laboratories Inc.) at a dilution of 1:100 in blocking buffer for 2hrs at room temperature, and then again rinsed as before The blot was then rinsed in sodium-glycinate solution (50mM glycine-HCl pH9 6, 1N NaOH) and

incubated in a reaction mixture (95% v/v sodium glycinate solution, 0.33% pNBT, 0.17% 534-PO_4, 0.008 mM $MgCl_2$) until the labeled bands became visible. The blots were then immediately rinsed in PBS. When blots were processed together, for comparative purposes, they were reacted for identical times.

DIFFERENTIAL ABSORPTION

The proteins used as the absorbant were derived from a PBS crude extract, a crude membrane preparation (P2), or 4% paraformaldehyde-fixed tissue sections mounted on gelatin-coated slides. In the case of absorption with P2 or crude extract, the antiserum was introduced in the absorbent solution at a final dilution of 1:100 and incubated for 60min at room temperature on a nutator to allow antibody binding to take place. The antigen/antibody complexes were then pelleted by spinning down the incubation tube contents at 16000g for 20min in a tabletop microfuge. The supernatant, containing the free antibodies was transferred to a different tube and stored at 4°C. In the case of absorption with tissue sections, the antiserum was incubated at a dilution of 1:100 (containing 1% goat serum and 1% triton-X) with the fixed tissue sections for 2hrs at room temperature without shaking. The unbound antibody solution was recovered from the slides with a P200 pipetman., and stored at 4°C.

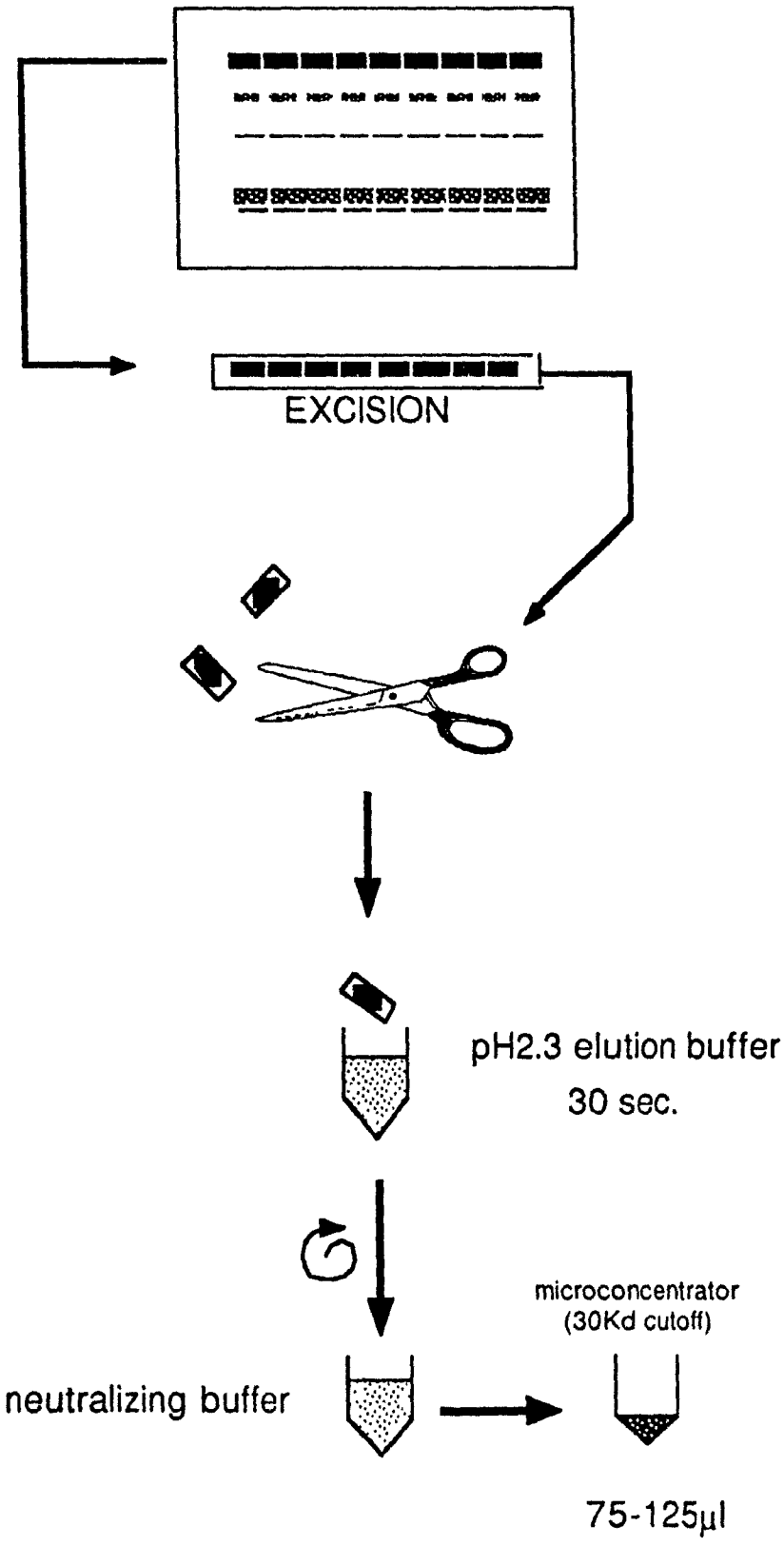
ANTIBODY ELUTION

Ten µg of proteins were loaded in each well of a mini-gel or 30µg of proteins in each well of a big slab gel The gel was run as described and the proteins were transferred onto nitrocellulose After the immunoblotting procedure, the antiserum reactivity was visualized by the alkaline phosphatase method. The reacted blot was rinsed briefly in 0 5% Tween/PBS, after which the protein bands of interest, spanning the width of the blot, were excised and cut into small ($\approx 2mm^2$) pieces (see diagram below).

The protocol used to elute antibody from the nitrocellulose pieces is derived from Smith and Fisher (1984). Nitrocellulose pieces were incubated for 30sec in 300µl of elution buffer (50mM glycine-HCl pH2.3, 500mM NaCl, 0.5% Tween-20, 100µg/ml BSA) which was then decanted and immediately neutralized with Na_2HPO_4 to a final concentration of 50mM The elution and neutralization steps could be repeated any number of times (usually 3) in order to obtain the maximum band-specific antibody yield Also, on occasion, serial elution was carried out after reprobing the antibody-depleted strips with fresh antiserum

The eluted antibody was then concentrated in the Centricon Micro-concentrator (Amicon Canada Ltd) The filtrate was discarded and the concentrate (proteins of molecular weight larger than 30Kd) - 75-100µl was reconstituted with PBS to a final volume of 200µl. These antibodies were stored at 4°C.

ANTIBODY ELUTION PROTOCOL



IMMUNOFLUORESCENCE

FRESH-FROZEN TISSUE

Goldfish were anaesthetized in a solution of 0.1% ethyl m-amino-benzoate (Sigma) in 0.1M phosphate buffer pH7.4 MS-222) for about 10min. They were then placed on the operating block on their back and the aorta was severed to let the blood flow out of the body. The fish were then turned to expose their dorsal side and the dorsal skull was cut with scissors and removed , after which the brainstem was cut right behind the vagal lobes (in order to impede any movement of the animal). The brain (encompassing the telencephalon, hypothalamus, optic tectum, cerebellum and vagal lobes) was removed and put in cold (4°C) PBS. When optic nerves were needed, they were also removed. For removal of the viscera, the animal was turned ventral side up and a large piece of body wall was removed in order to expose the visceral contents of the abdomen. Care was taken not to pierce the gall bladder.

The tissue was rapidly frozen on dry ice and then mounted onto a sectioning plate with embedding medium (Histo prep, Fisher scientific co.). The sectioning plate was then placed in the cryostat chamber and the tissue was allowed to equilibrate to the chamber's temperature (see below) for 10-15min.

PERFUSED TISSUE

The fish was deeply anesthetized (20-30min) in MS-222 and then placed on its side on the operation block. The 2 opercula were removed. Then, the

fish was placed ventral side up and a triangular piece of body wall overlying the heart was removed. The 2 feeder veins (common cardinal veins) were cut to provide an outflow path for the perfusate. Then, a 27 1/2 gauge needle (attached to the perfusion tubing) was inserted into the ventricle and the animal was perfused with about 8mls of PBS (until the outflow from the sinus venosus was clear). The perfusion tubing was then filled with 2% paraformaldehyde (pH7.4) which was allowed to flow through the animal's blood vessels for 20-30min at a speed of 1.2mls/min on a Model 203 peristaltic pump (Fisher scientific co.). When perfusion was over, the brain and/or optic nerves and viscera was carefully removed and incubated sequentially in 2% paraformaldehyde for 20min and in 20% sucrose (4°C) for 2hrs (or more).

The tissue was then frozen in carbonic ice followed by mounting on a sectioning plate, as described above.

SECTIONING

The tissue was sectioned in the cryostat chamber at -12°C (fresh frozen) or -20°C (formaldehyde-fixed) at a thickness of 12µm. Sections were mounted on gelatin/chrome alum-subbed slides by apposition. The mounted tissue was then postfixed with cold ethanol and acetone (2 minutes in ethanol followed by 30 seconds in acetone) in the case of fresh-frozen sections.

LABELING

The sections were incubated for 2hrs in 2% goat serum/PBS, then, overnight in the primary antibody mixture (1:500 mouse anti-goldfish

antibodies, 1% goat serum in PBS). [Note that in the case of perfused sections, 1% triton-X-100 was used in the primary and secondary antibody mixtures] The sections were then washed 3 times for 15min in PBS, after which they were incubated in the fluorescein isothiocyanate (FITC)-conjugated secondary antibody solution (1% goat anti-mouse/FITC - Zymed laboratories inc. - 1% goat serum in PBS). Washing was repeated as before.

The slides were then dipped for 1min in a Nuclear Yellow solution (90µl of nuclear yellow stock (3mg/ml) in 50mls distilled H_2O), after which they were washed as before, the rubber washer was discarded and the slides were mounted with a few drops of mounting medium (glycerol:carbonate buffer/1:1, pH9.0).

VISUALIZATION

After mounting, the edge of the cover slip was sealed with nail polish to prevent evaporation of the mounting medium and movement of the coverslip. The sections were viewed with oil immersion optics under blue light excitation with a Leitz I2 cube for FITC fluorescence, or under UV excitation with a Leitz A cube for nuclear yellow fluorescence, on a Leitz Dialux-20 microscope.

RESULTS

ANALYTICAL METHODS

ISOLATION OF IMMUNOGENS

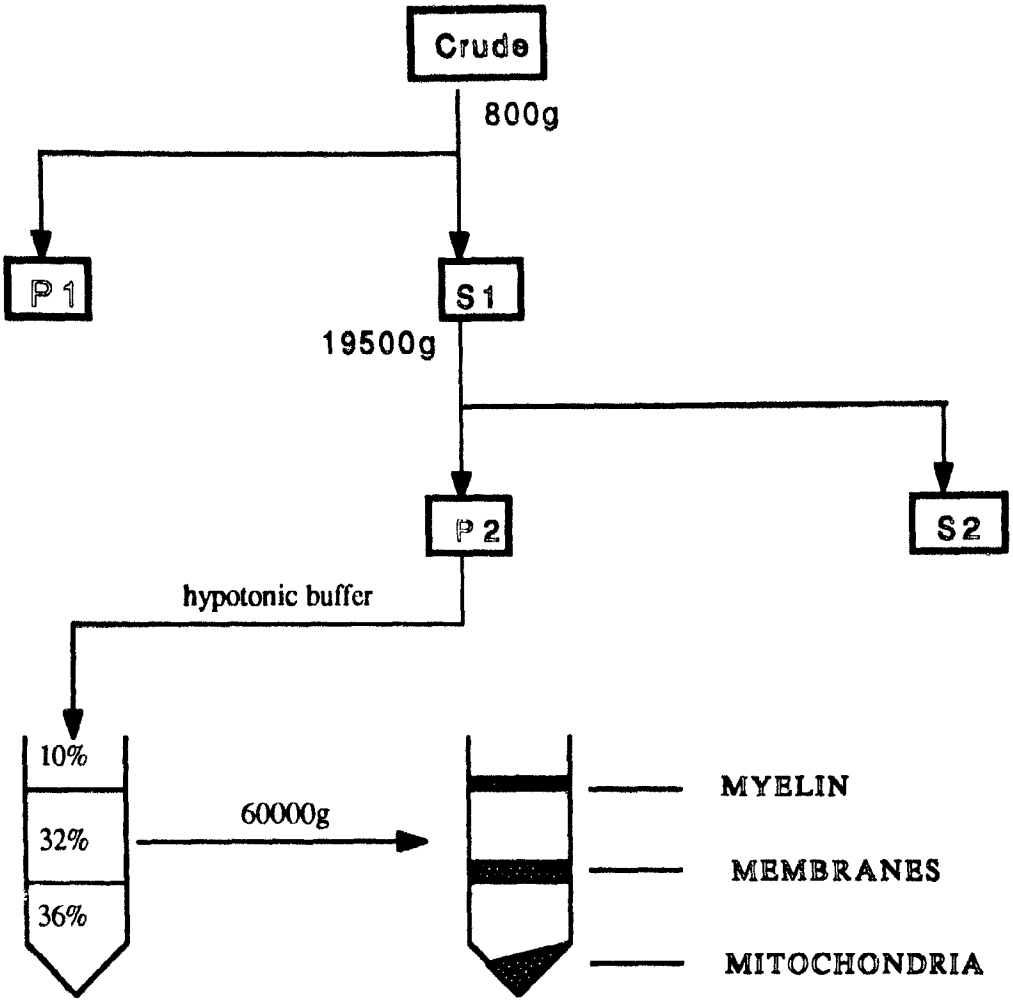
Two immunogens, with different purity levels were isolated with the techniques of differential or sucrose step gradient centrifugation, and used to produce polyclonal antibodies in mice, with the aim of obtaining brain cell membrane specific antibodies. Briefly,the first immunogen was isolated as follows: goldfish brains were homogenized and, following two-step differential centrifugation, a crude membrane pellet was obtained (figure 1A). This fraction corresponds to the second pellet obtained through the procedure and therefore will be referred to as the P2 pellet. The P2 pellet was embedded in epoxy resin and stained (as described in materials and methods) for electron microscopy visualization. Electron micrographs of this fraction indicate the presence of synaptosomes, mitochondria, membranes and myelin sheaths (figure 2). This crude membrane pellet was used as the immunogen to produce an antiserum which I will refer to as *anti-P2 antiserum*.

The second immunogen was a purified membrane fraction obtained by separating the crude membrane pellet through a three-step sucrose

Figure 1

A) Diagrammatic representation of the procedure carried out to harvest a purified goldfish brain membrane fraction.

B) SDS-PAGE of the various fractions obtained in the membrane purification procedure. Lane 1: mitochondrial pellet, lane 2: membrane fraction, lane 3: myelin fraction, lane 4: crude membrane pellet (P2), lane 5: S2, lane 6: S1, lane 7: P1, lane 8: crude homogenate. Molecular weight marker positions are indicated. Lane 2: dots from top to bottom: 99Kd, 84Kd, 72Kd, 58Kd and 41Kd (these correspond, in molecular weight, to the bands bound by the antibodies produced thereafter, that are referred to in the text). Molecular weight marker positions are indicated on the left by fructose-6-phosphate kinase: 84Kd, pyruvate kinase: 58Kd, fumarase: 48.5Kd (prestained markers); and on the right by phosphorylase b: 97Kd, bovine serum albumin: 67Kd,ovalbumin: 43Kd, and soybean trypsin inhibitor: 30Kd.


Crude
800g
P1
S1
19500g
P2
S2
hypotonic buffer
10%
32%
36%
60000g
MYELIN
MEMBRANES
MITOCHONDRIA

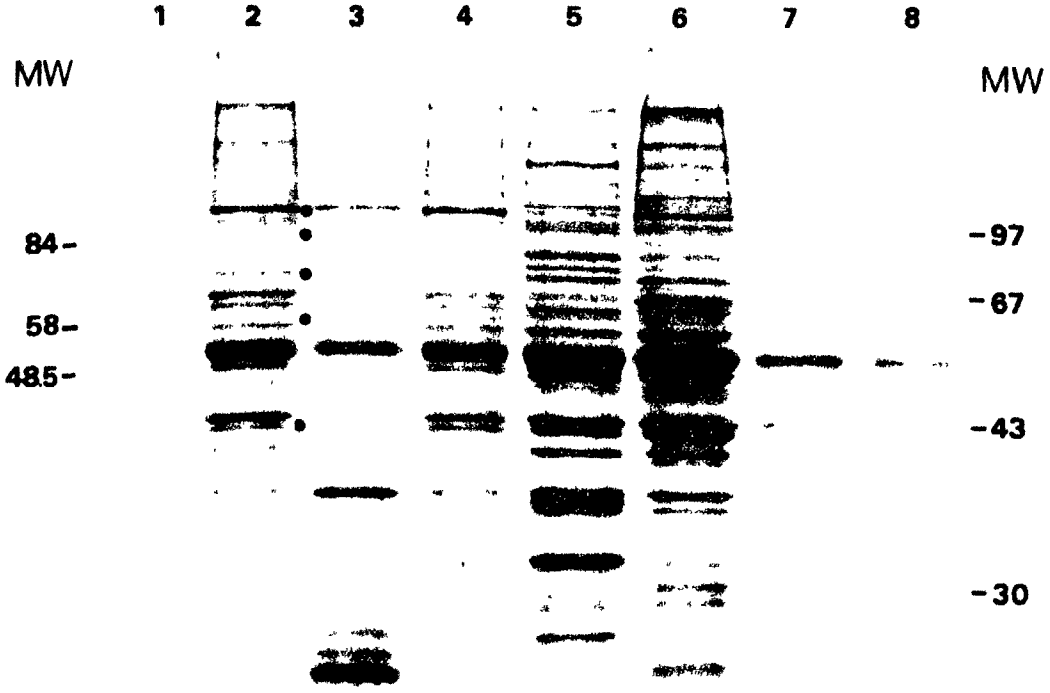

1
2
3
4
5
6
7
8
MW
MW
84-
58-
48.5-
-97
-67
-43
-30

Figure 2

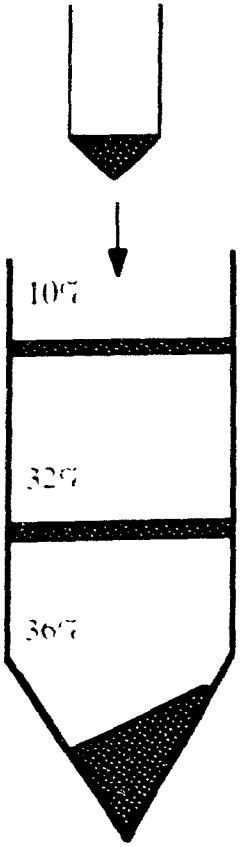
Representative electron micrograph of the crude membrane fraction (P2) derived from differential centrifugation of goldfish (*Carassius auratus*) brains. M; plasma membrane, mt: mitochondrion, ml: compact myelin sheath, s: synaptosome. Bar: 0.5μm.


M
mt
ml
s

gradient. Figure 1A shows a flow chart of the procedure The three main constituents of the P2 pellet, namely myelin, membranes (including synaptosomal membranes) and mitochondria could be separated by centrifugation through the 10/32/36 % sucrose layers, after submitting the P2 pellet to osmotic shock treatment in a hypotonic buffer solution (in order to break open the resealed plasma membranes) The membrane ghosts, which had resealed, were found at the 32/36 % sucrose interface, together with some sparse golgi stacks (figure 3) This membrane fraction, contained 43% of the total ouabain-sensitive Na^+,K^+ ATPase (a plasma membrane enzyme marker) activity (Table 1). The contamination of this fraction by myelin and mitochondria was minimal, accounting for only 3% of total cyclic nucleotide phosphohydrolase (a myelin enzyme marker) and 5% of total cytochrome-C oxidase (a mitochondrial enzyme marker) activity (Table 1). As a consequence, mitochondria could sometimes be observed in the membrane fraction (figure 4A) Sparse synaptosomes could also be observed, an example of which is shown in figure 4B. The synaptosomal contamination was not quantitatively examined but it was assumed that a proportion of the membranes in this fraction, would be synaptosomal in nature (Mickey et al 1971). The compact myelin sheaths, having a high lipid:protein mass ratio (Elam and Cancalon 1987), were found at the 10/32 % sucrose interface. The electron microscopic profile of this fraction (figure 5A) shows the compact myelin sheaths, mostly intact, together with other cell components which had not been altered (because of myelin sheath protection) during the membrane extraction procedure. This may explain the presence of high levels of the membrane and mitochondrial enzyme markers of 55% and 41% respectively (Table 1) However, most of the myelin was found in this fraction - 97% - (Table 1) Mitochondria, which were not extensively altered by osmotic shock treatment, kept a high density

Table 1

Summary compilation of the enzyme marker assays (CNPase- myelin marker, ATPase- plasma membrane marker, Cytochrome-C-oxidase- mitochondrial marker) showing the specific and total activity found in each of the indicated fractions as obtained from sucrose step gradient centrifugation of crude goldfish brain membranes (P2 fraction). These fractions were collected on the sucrose interfaces diagrammatically represented on the left.



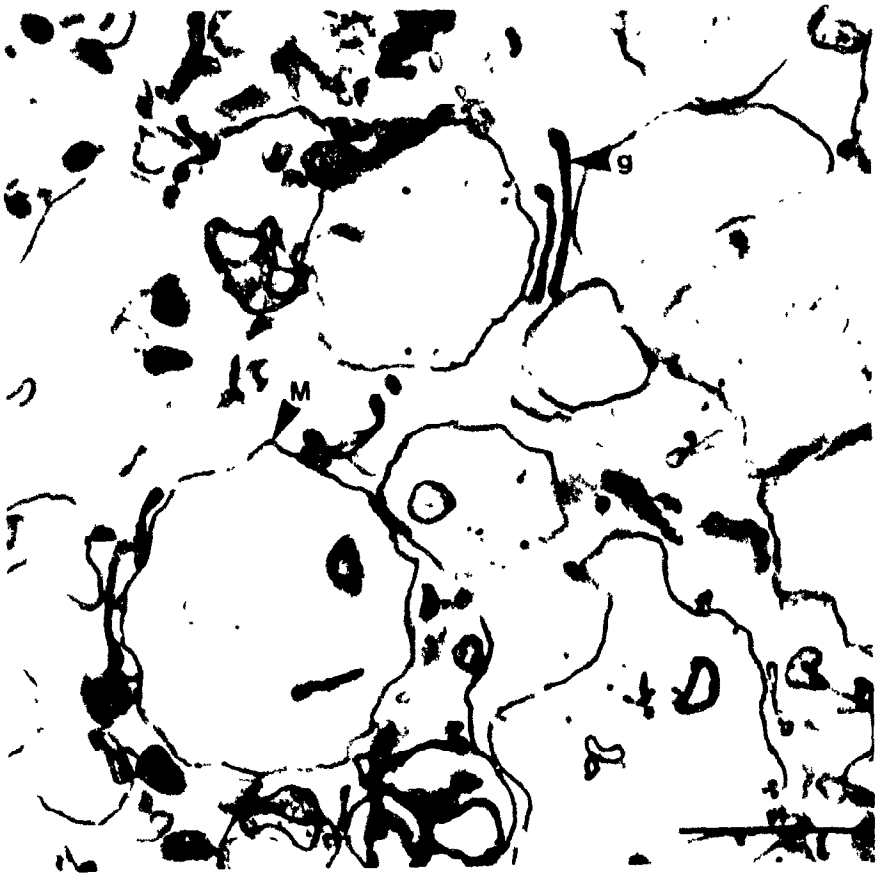
marker enzyme / fraction	CNPase activity		ATPase activity		Cytochrome-C oxydase activity	
	specific $X10^{-3}$ U/mg	total $X10^{-3}$ U	specific nmoles/mg PO_4	total nmoles PO_4	specific µmole/mg/min	total µmole/mg
P2	80	210	9.52	24.9	40	110
Myelin	220	63 (97)**	15.2	3.5 (55)	26	7.5 (41)
Membranes	12	2 (3)	16.7	2.7 (43)	6	0.9 (5)
Mitochondria	UD*	UD	1.09	0.14 (2)	93	10 (54)

* U.D. : Undetectable

** parentheses : % total activity

Figure 3

Representative electron micrograph of the goldfish brain membrane fraction collected from a 32/36 % sucrose interface following step gradient centrifugation of the crude membrane fraction (P2). M: resealed membrane ghosts, g: golgi stacks. Bar: 0.5µm.


g
M

and were harvested as a pellet at the bottom of the 36% sucrose layer (figure 5B). Fifty four percent of the enzyme marker activity was found in this fraction (Table 1). The membrane fraction was pooled and used as immunogen in a second group of mice to produce an antiserum which I will refer to as *anti-membrane antiserum*.

The degree of protein purification yielded throughout the membrane extraction procedures was followed electrophoretically by separating the different fractions obtained (through a 10% SDS-polyacrylamide gel after detergent solubilization), and qualitatively examining the variations in staining intensity of the different protein bands (figure 1B). The crude membrane (P2) fraction (figure 1B, lane 4) had essentially the same electrophoretic profile as the membrane fraction (figure 1B, lane 2).

DETERMINATION OF ANTISERUM REACTIVITIES

The two immunogens (P2 and purified membrane fraction) were each injected into 4 mice for the production of polyclonal antisera. In each cage, a fifth mouse was not injected and served as a non-immune control. In each group, the antisera harvested from all 4 mice differed only slightly on the basis of antibody titer and reactivity, as determined by Western blot analysis (figure 6). With blots of P2 proteins from the goldfish brain, the binding pattern of the anti-membrane antisera from mice 1-4 (figure 6A, lanes 1-4 respectively) were similar with respect to bands at approximate molecular weights of 99Kd, 72Kd and 41Kd. High molecular weight protein bands (molecular weights greater than 200Kd) were also stained. The variations in the antisera produced are summarized as follows: 1) mouse 1 produced an antibody against a protein migrating with approximate molecular weight of

Figure 4

Electron micrograph of the goldfish brain membrane fraction showing particulate contamination by mitochondria (A) and synaptosomes (B). M: resealed membrane ghosts, s: synaptosomes, mt: mitochondrion. Bar: 0.5µm.


mt
M


M
s
B

Figure 5

Representative electron micrograph views of the goldfish brain myelin (A) and mitochondrial (B) fractions. The myelin fraction was harvested at a 10/32 % sucrose interface following step gradient centrifugation of the crude membrane fraction (P2), and the mitochondrial fraction was recovered in the pellet below the 36% sucrose layer. ml: compact myelin sheaths, mt: mitochondrion. Bar: 0.5µm.


ml


mt
B

61Kd which was not found in the other antisera but, on the other hand, did not possess reactivities against 2 protein doublets at 84Kd and 58Kd found in the other 3 antisera; 2) antisera from mice 2 and 3 bound to a 36Kd protein which reactivity was not obtained with the antiserum from mouse 4. The non-immune control (figure 6A, lane 5) did not yield detectable reactivity with any of the goldfish brain P2 proteins.

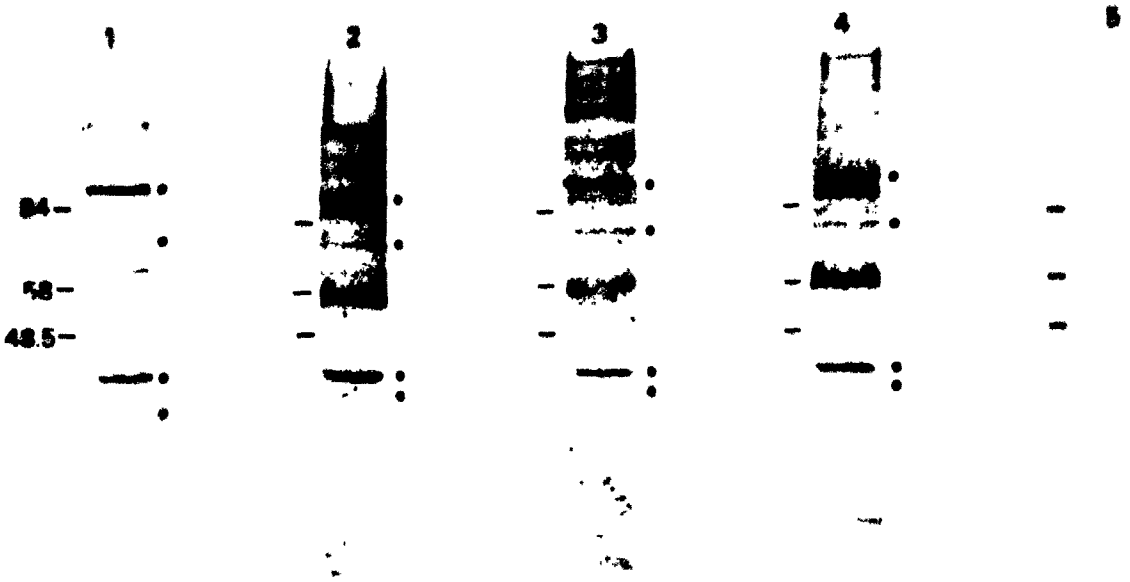
The anti-P2 antisera had a different reactivity spectrum as compared to the anti-membrane antisera (figure 6B). The major reactivity bands were observed at 72Kd and 41Kd and present in all antisera (lanes 1-4). The 4 mice injected with the P2 mixture showed similar reactivity spectra to each other, although mouse 1 yielded a much lower titer than the others (figure 6B, lane 1) with only reactivity to 3 protein bands from a crude goldfish brain extract, migrating to 72Kd, 41Kd and 36Kd approximate molecular weights. Antisera from mice 2, 3, and 4 were similar except that mouse 3 did not produce an antibody against a protein migrating as a broad band spanning molecular weights 56Kd-60Kd, and that mouse 4 produced an antibody against a single band migrating at 49Kd. These 3 antisera (ie. from mice 2, 3, and 4) were pooled to increase the total antiserum volume to be utilized in subsequent experiments. The final, pooled antiserum (figure 6B, lane 6) had a broad reactivity spectrum, summarized as follows: major reactivities at 72Kd and 41Kd and in the high molecular weight range (>200Kd), minor reactivities at 99Kd and 36Kd and a broad band reactivity around 90Kd and 58Kd. The pre-immune control, in this series, showed a weak reactivity at 72Kd.

Figure 6

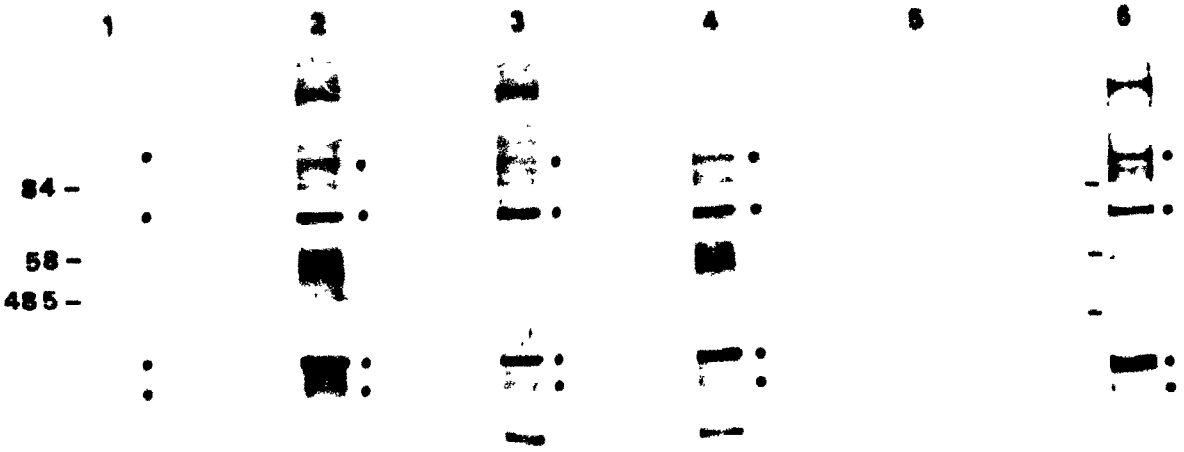
A) Immunoblotting patterns for each of the antisera obtained by injecting 4 mice with the same mixture of purified goldfish brain membranes. The antisera were reacted to goldfish brain crude membrane proteins. A control serum pattern is also shown (lane 5), in that case, the serum was obtained from a non-immunized control. Lanes 1-4: antisera obtained from mice 1-4 respectively. Dots on the right of each lane, from top to bottom: 99Kd, 72Kd, 41Kd and 36Kd. These correspond, in molecular weight, to the protein bands referred to in the text.

B) Immunoblotting patterns for each of the antisera obtained by injecting 4 mice with the same mixture of goldfish P2 proteins. The antisera were reacted to a crude homogenate of goldfish brain. Lanes 1-4: antisera obtained from mice 1-4 respectively. A non-immune control (obtained as described above) is also included (lane 5). Lane 6 represents the immunoblotting pattern of pooled sera from mice 2, 3 and 4. Indicated bands as in A.

A



B



PURIFICATION OF THE ANTISERA

Since the aim of this study was to obtain goldfish brain-specific antibody probes which would bind to cell membranes, and because the 2 types of antisera obtained (anti-P2 antiserum and anti-membrane antiserum) were shown to cross-react with heterologous organs (figure 7B and 9B, panel 1) and also because of their broad reactivity with the brain proteins themselves, antibody purification procedures were felt necessary.

Anti-membrane antiserum

Both the brain and optic nerve, are part of the central nervous system and therefore, they are expected to share a body of proteins in common. Thus, absorption of the anti-membrane antiserum with goldfish optic nerve crude membranes was undertaken in order to "fine-tune" the specificity of the antiserum towards brain proteins.The anti-membrane antiserum, when challenged to an optic nerve crude membrane immobilized onto nitrocellulose (figure 7A), was shown to cross-react with 3 protein bands of 123Kd, 55Kd and 41Kd approximate molecular weights. Of these 3 reactivities, the ones against the 41Kd and 123Kd protein bands were also present in the brain, however, the 55Kd protein may be optic nerve-specific because the antiserum did not bind to a brain protein at a corresponding molecular weight. After absorption, the resulting antiserum was however still reactive against the optic nerve proteins at 55Kd with some weak residual activity at 41Kd (figure 7B, lane 1). Such results might be due to the differential centrifugation technique used for P2 isolation, which cannot be 100% reproducible, thus, only a subtle variation in experimental conditions may possibly have excluded the 55Kd protein from the nerve P2 extract

used as absorbent. On the other hand, it was observed from these absorption results, that the antiserum could be depleted of some of its brain-directed reactivities (eg. the proteins with molecular weights greater than 100Kd and lower than 36Kd. Because this absorption procedure did not extensively purify the antisera, I tried a different procedure in which the major reactive antibodies were eluted from immunoblots (figure 8A) and the band-specific antibodies were then concentrated and reacted with P2 proteins on a Western blot (figure 8B). The bands which were eluted and concentrated independently were the ones directed against proteins at 99Kd, 84Kd, 72Kd, 58 Kd (broad band) and 41Kd (corresponding antibodies: m99, m84, m72, m58, and m41) (figure 8A). As demonstrated on figure 8B, the eluted antibodies all reacted with a protein doublet at 84Kd. Only 2 antibody elutions were successful in that, apart from the cross-reactivity with the 84Kd band, the antibody specificity was directed against the same protein band it was eluted from, these are the m99 and m72 antibodies (figure 8B, lanes 1 and 3 respectively) The m84 antibody only reacted with a protein doublet migrating to 84Kd. The other antibodies (m58 and m41) did not show any band-specific binding, although they did show cross-reactivity with the 84Kd doublet (figure 8B, lanes 4 and 5 respectively).

Anti-P2 antiserum

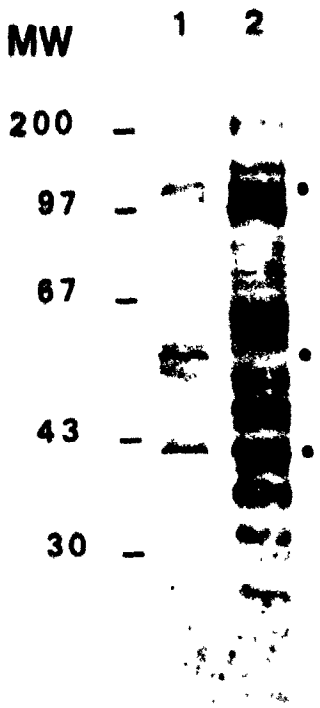
The anti-P2 antiserum had broad heterologous tissue reactivity when assayed with extracts of goldfish viscera (spleen, liver, intestines and mesentery) or skeletal muscle (figure 9B, panel 1). Therefore, it was absorbed with crude homogenates from viscera and muscle of which the electrophoresis patterns are shown in figure 9A. When the antiserum was

Figure 7

A) Immunoblotting pattern of the anti-membrane antiserum (from mouse 1) reacted to P2 proteins from goldfish optic nerves (lane 1) and brain (lane 2). Protein bands indicated (from top to bottom: 123Kd, 55Kd and 41Kd) are referred to in the text.

B) Immunoblotting pattern of the anti-membrane antiserum (from mouse 1) absorbed with a goldfish optic nerve crude membrane (P2) protein fraction, reacted to P2 protein spectrum from goldfish optic nerves (lane 1) and brain (lane 2). Protein bands indicated (from top to bottom: 99Kd, 72Kd, 41Kd and 36Kd) are referred to in the text.

A



B

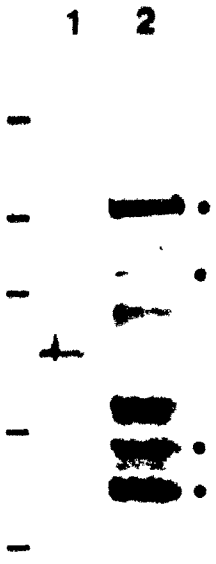
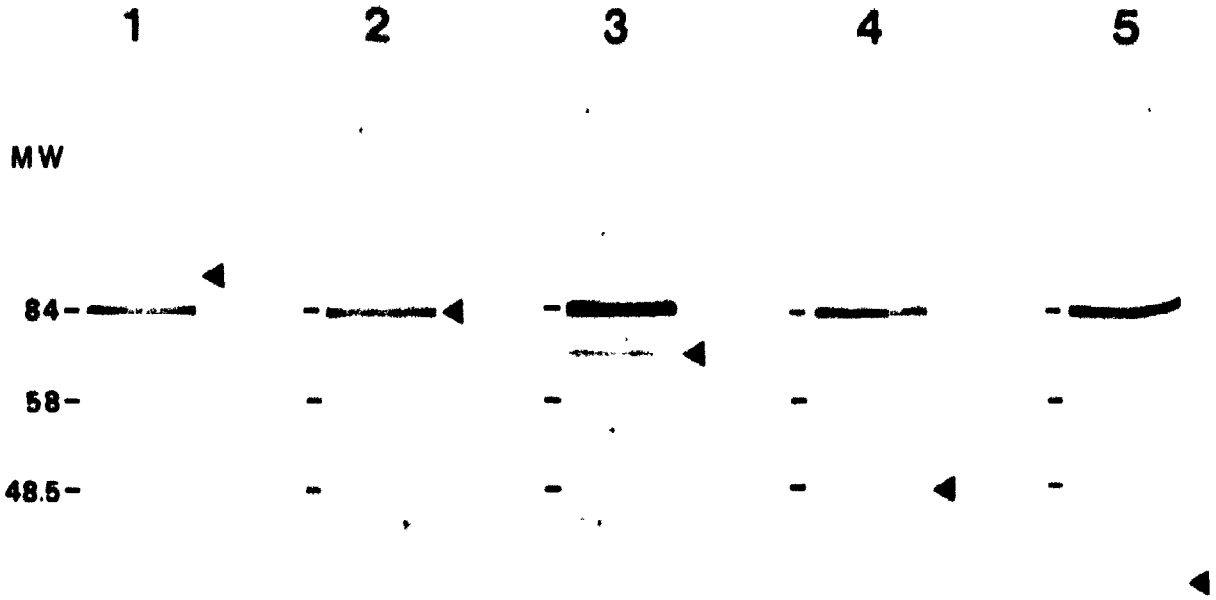


Figure 8

A) Immunoblotting pattern of the anti-membrane antiserum (from mouse 4) reacted to P2 proteins from goldfish brain. Arrows on the right point to the regions of the blots from which antibody was eluted. The numbers indicate the relative molecular weights at each level. These numbers were used to identify the eluted antibodies (eg, antibody m99 was eluted from the blot at the 99Kd relative molecular weight position).

B) Immunoblotting patterns of the antibodies eluted from a blot (as shown in A) and used to probe a goldfish brain P2 protein mixture. Arrows on the right of each lane indicate the position from which the antibodies were eluted for each band. Lanes 1-5: immunoblotting patterns of m99, m84, m72, m58 and m41 antibodies respectively.

B



absorbed with viscera, it was substantially depleted of immunoreactivity directed to proteins at molecular weights: 1) greater than 72Kd, 2) smaller than 36Kd, and 3) between 58Kd and 72Kd (figure 9B, panel 2). However, the absorption of the antiserum was not complete since it still reacted against the visceral 72Kd protein. When the antiserum was absorbed with a crude homogenate of skeletal muscle, antibodies against a muscle protein band just below 41Kd was also removed (figure 9B, panel 3). Once more, the absorption was not complete since muscle proteins (including the 72Kd protein) could still be immunostained following absorption (figure 9B, panel 3). When the antiserum was absorbed with crude brain homogenates, most reactivities disappeared except for residual binding of the 72Kd and 41Kd proteins, again suggesting incomplete absorption. Of the protein bands present in the brain but not in the muscle or viscera crude extracts, the reactivity spectrum of the anti-P2 antiserum could be shown (figure 10A) to delineate 3 bands with molecular weights of approximately 47Kd, 41Kd and 36Kd (corresponding antibodies: P47, P41 and P36). Antibodies bound to these protein bands were eluted, concentrated and then rehybridized onto a crude homogenate protein spectrum of brain, muscles and viscera (figure 10A and B). P41 seemed to be the most reactive antibody (figure 10B panel 3). However, this antibody greatly cross-reacted with the 72Kd protein found in the brain as well as muscles and viscera, and also minimally with other proteins with broad molecular weight distribution in all 3 tissues tested. These results are in sharp contrast to the elution results obtained with the anti-membrane antiserum (see earlier, figure 8), where a much lower level of cross-reactivity was observed in the brain tissue. This was further examined (figure 11). P41 was absorbed with the 41Kd protein band (bound to nitrocellulose) and it was observed that this treatment could completely deplete the eluted antibody of specific as well as non-specific reactivities (figure 11, panel 3). It was also found that the pre immune serum in this

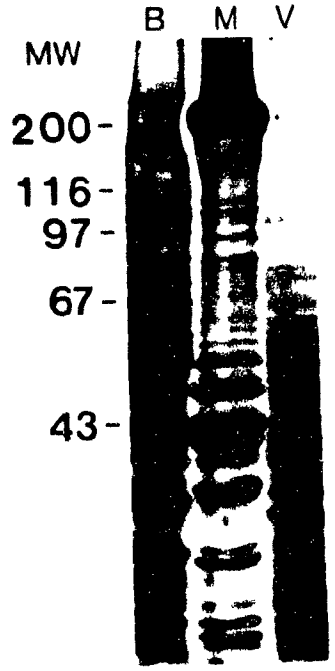
series bound weakly to some of the antigens, recognized by the immune sera (figure 11, panel 4). If such endogenous activity was also present in the immunized animal, it was absorbed out by the 41Kd protein (figure 11, panel 3).

Figure 9

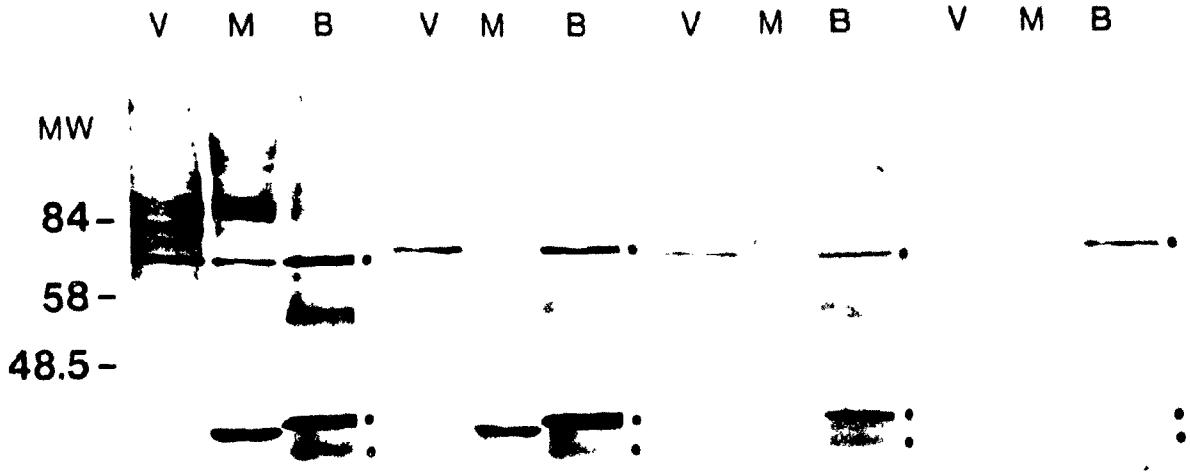
A) SDS-PAGE of a crude homogenate of goldfish brain (B), abdominal wall muscles (M) and viscera (V - liver, spleen, intestines and mesentery).

B) Immunoblotting pattern of the native anti-P2 antiserum (panel 1), the antiserum absorbed with a crude homogenate of goldfish viscera (panel 2), crude muscle homogenate (panel 3), and brain homogenate (panel 4). The various antisera were reacted with crude homogenates from goldfish viscera (V), muscles (M) or brain (B). Protein bands indicated by dots on the right (from top to bottom: 72Kd, 41Kd and 36Kd) are referred to in the text.

A



B



1 2 3 4

Figure 10

A) Immunoblotting pattern of the anti-P2 antiserum reacted with total goldfish brain proteins. Arrows on the right point to the levels from which antibodies were eluted. The antibodies are referred to by the relative molecular weight from which they were eluted (eg. P41).

B) Immunoblotting patterns of the antibodies eluted from the blot shown in A and used to probe a goldfish viscera (V), muscle (M) or brain (B) protein mixture. Arrows on the right of each lane indicate the position from which the antibodies were eluted for each band. Panel 1: immunoblotting pattern of native antiserum. Panels 2-4: immunoblotting pattern of P47, P41 and P36 antibodies respectively.

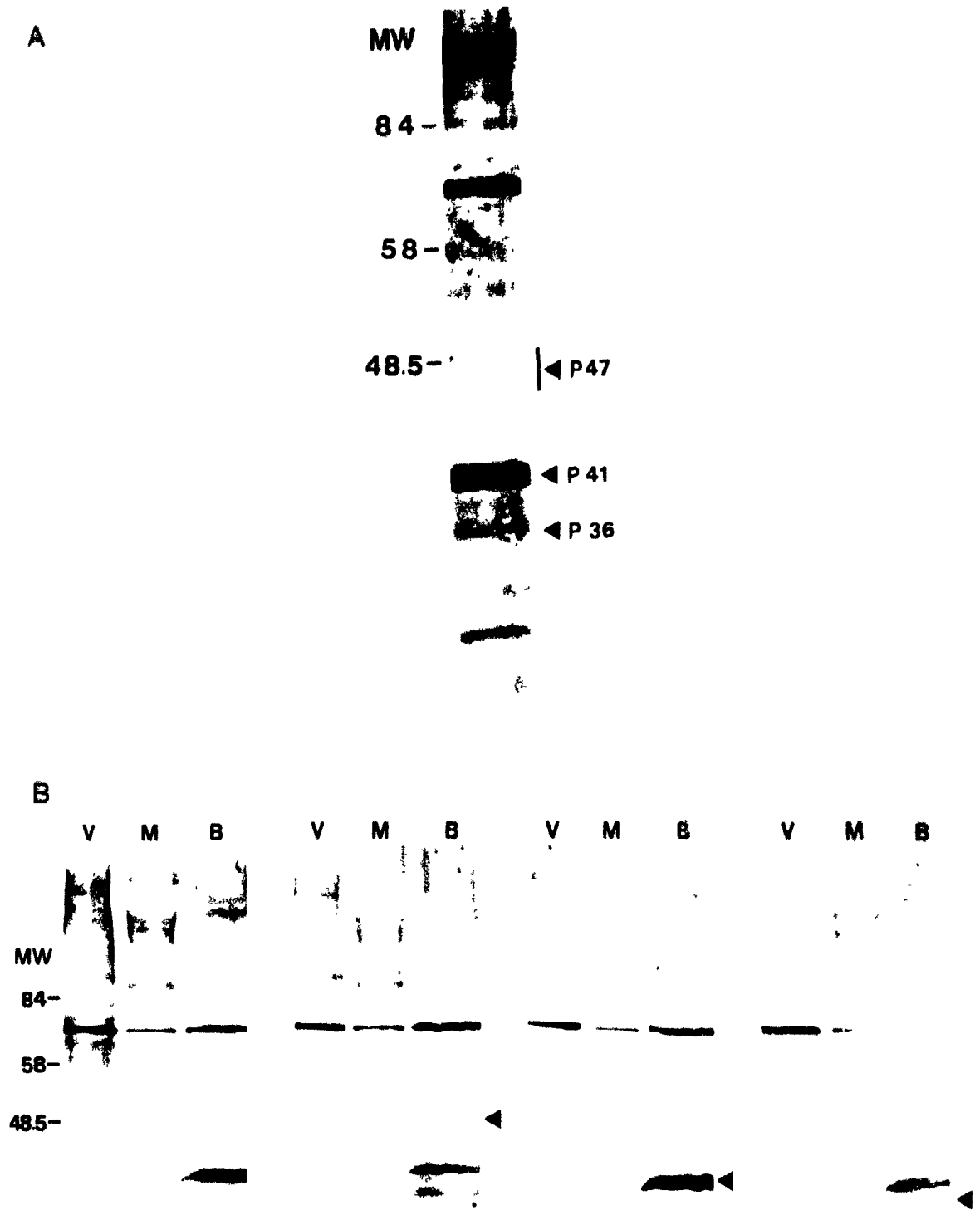
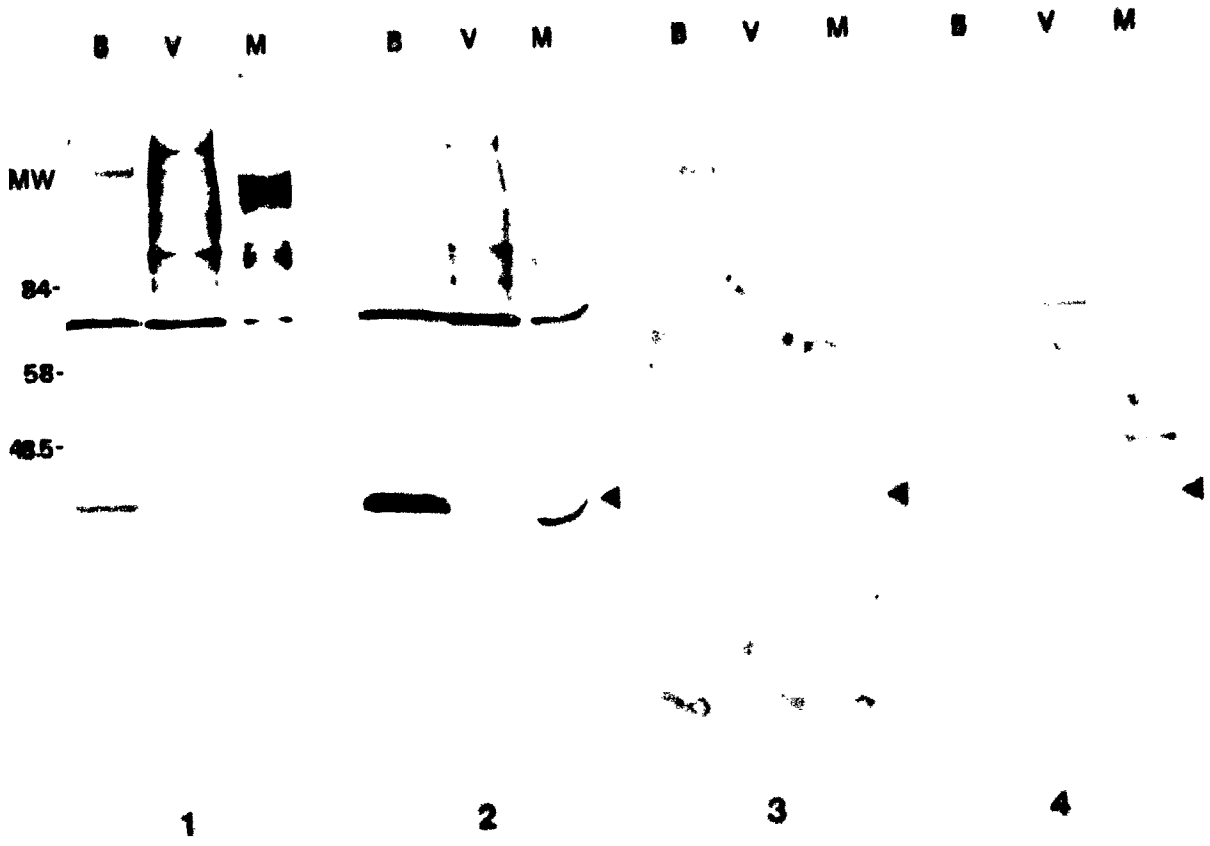

A
MW
84-
58-
48.5-
P47
P 41
P 36
B
V M B V M B V M B V M B
MW
84-
58-
48.5-
1
2
3
4

Figure 11

Immunoblotting patterns of the native anti-P2 antiserum (panel 1), the P41 antibody (panel 2), the P41 antibody absorbed with a purified protein derived from a nitrocellulose-immobilized 41Kd band strip (panel 3), and non-immune serum (panel 4). Arrows on the right indicate the position of the 41Kd protein. The various antisera were reacted with goldfish brain (B), viscera (V) and muscle (M) proteins.


B V M B V M B V M B V M
MW
84-
58-
48.5-
1
2
3
4

IMMUNOFLUORESCENCE LABELING WITH ANTI-P2 ANTISERUM

In order to assess the topographical distribution of the antigens recognized by the anti-P2 antiserum in its native and purified forms, coronal sections were prepared (12µm) from the 4% paraformaldehyde-fixed goldfish CNS (midbrain, forebrain, optic nerve, and retina) as well as from other tissues in the goldfish (intestines, liver, spleen, skeletal muscle, and testes). These were incubated in a 1:500 dilution of the antiserum solutions. For the CNS, the focus of interest was the optic tectum of the the midbrain (figure 12A and B). The optic tectum, in the goldfish as well as other teleost fish, forms the bulk of the midbrain roof (figure 12A) and is the largest visual center in the brain. Because of interest in visual processing, the anatomy of the goldfish optic tectum has been frequently studied (Ramòn 1899, Attardi and Sperry 1963, Sharma 1972, Meek and Schellart 1978, Romeskie and Sharma 1979,). Caudally, the valvulae cerebelli penetrate under the optic lobes. A representative cross-section is shown in figure 12C as an example of the coronal plane which was routinely prepared for immunohistochemical localization of brain antigens.

CONTROL

In order to properly interpret the immunohistochemistry results, the endogenous production, by mice, of anti-goldfish antibodies, had to be considered. The type of labeling routinely observed in the presence of the preimmune serum consisted of dull background labeling that was observed in all tissues tested (see figures 13-20). Aside from that type of labeling, the following tissues showed additional fluorescence in certain areas: 1)There

Figure 12

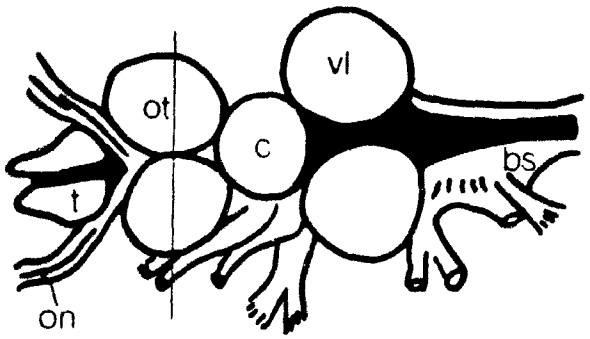
A) Dorsal view of the goldfish brain, showing major structures, of which the optic tectum (ot) was studied in particular.

B) Lateral view of the goldfish brain.

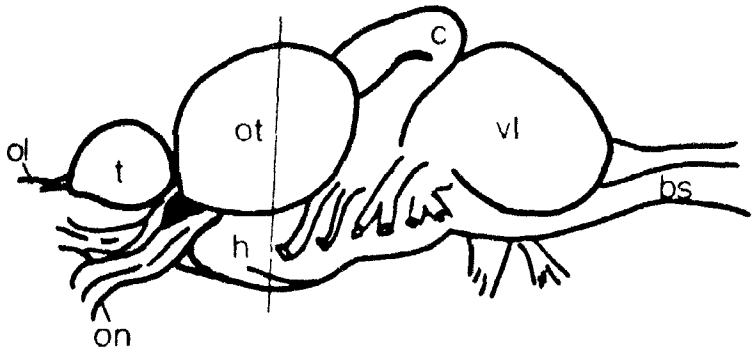
C) Transverse section through the mid-level of the optic tectum (the plane of section is indicated by the vertical line in A and B). The goldfish optic tectum (ot) is organized in a laminar fashion, it is bounded internally by the ependymal zone (e) which is adjacent to the tectal ventricle (tv), and externally, by the pia mater (pm). The valvulae cerebelli (VC) occupies the ventricular space at this level. The molecular (VCm) and granular (VCg) layers of the valvulae are indicated.

12

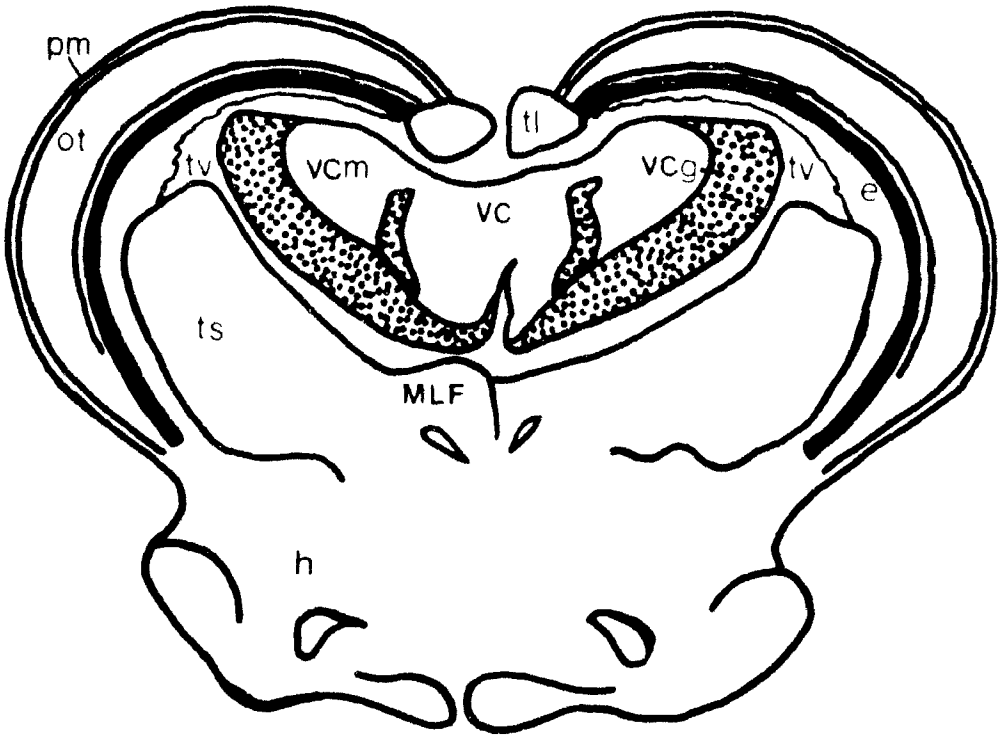
A



B



C



was weak labeling of blood vessels (figure 13A) . 2) The retina was labeled only in the photoreceptor cell area (figure 15A). 3) The spleen was stained in a dotted fashion and some sparse aggregations of the fluorescence, could be observed (figure 19A). The control labeling pattern was used as a baseline for negative signal and considered in determining positive fluorescence labeling in the following studies of mouse antisera. It has to be noted, however, that none of the immunohistochemical results were studied in a quantitative manner and therefore, subtraction of background labeling from positive immunolabeling was only qualitatively evaluated.

NATIVE ANTISERUM

When goldfish brain sections were stained with the native anti-P2 antiserum, labeling of blood vessels was seen in all parts of the tectum (figure 21B). The tectal laminae were not demarcated by the antiserum. A group of densely packed, large, round cells were also seen, adjacent to the tectal ventricle in the ependymal layer (figure 21B and C). In the telencephalon (figure 22), a homogeneously distributed population of intensely reactive, irregularly shaped profiles could be visible (arrow).

The remaining figures in this thesis (figures 13-50) show tissue sections reacted with various antibody preparations. Bound antibody was visualized using an FITC conjugated secondary antibody and viewed with blue excitation light and a Leitz I2 cube. In many instances sections were also counterstained with nuclear yellow which was visualized with UV excitation and a Leitz A cube.

Figures 13-20 are sections reacted with preimmune control serum. In all of these figures, the tissue is viewed both for FITC fluorescence (in A) and for nuclear yellow fluorescence (in B).

figure 13

Cross section through the valvulae cerebelli showing labeling of blood vessels (arrow). Bar: 48µm.

Figure 14

Longitudinal section through the optic nerve showing labeling of optic fiber bundles (arrows). Bar:48µm.

figure 15

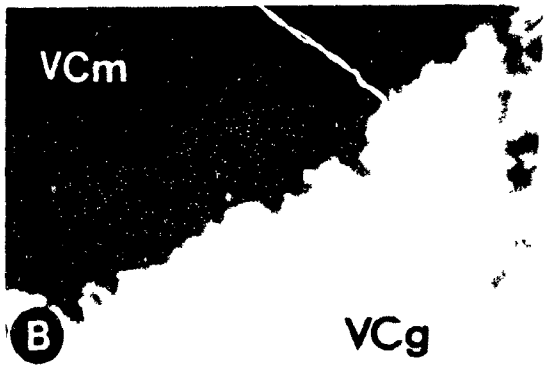
Cross section through the retina showing autofluorescence of outer segments (arrow). Bar: 48µm.

Figure 16

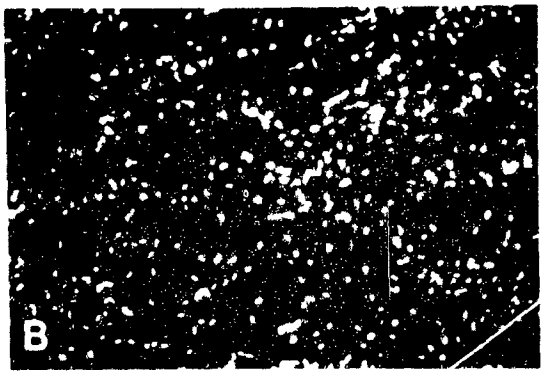
Cross section through the intestine showing labeling at the level of the epithelieum (arrows) and throughout the tissue. Asterisk: luminar space. Bar:48µm.

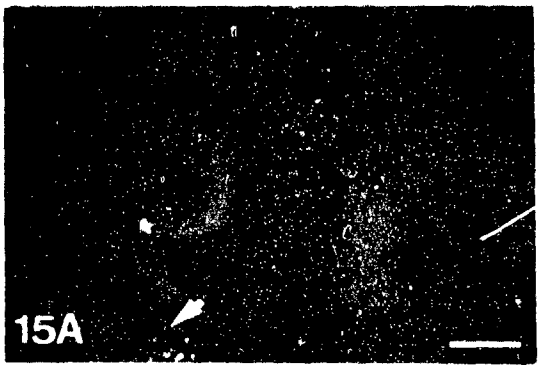
NOTE: Abreviations used with immunofluorescence figures are listed on page 106.

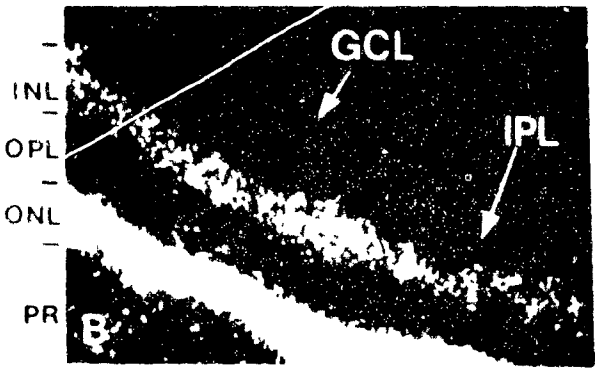

13A


VCm
B
VCg


14A


B


15A


GCL
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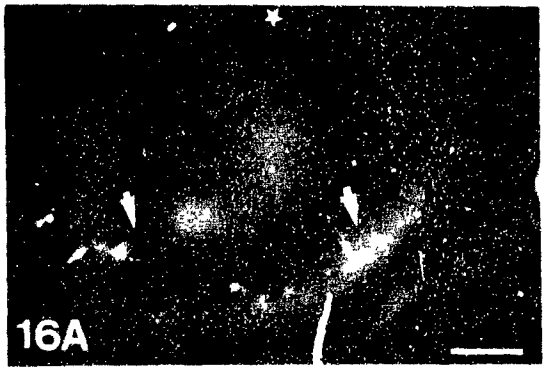

16A

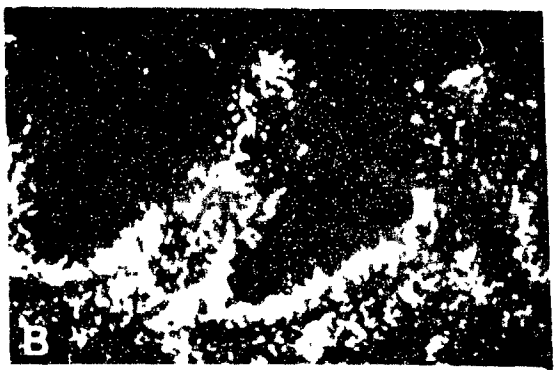

B

Figure 17

Longitudinal section through the skeletal muscle. Bar: 48µm.

Figure 18

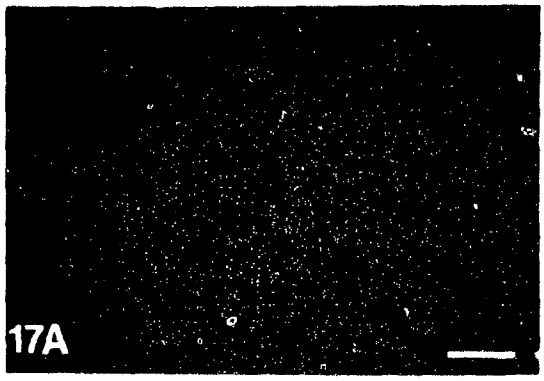
Cross section through the testes. Bar: 48µm.

Figure 19

Cross section through the spleen with dense staining of unidentified cell clusters (arrows). Bar: 48µm.

Figure 20

Cross section through the liver showing widespread labeling. Bar: 48µm.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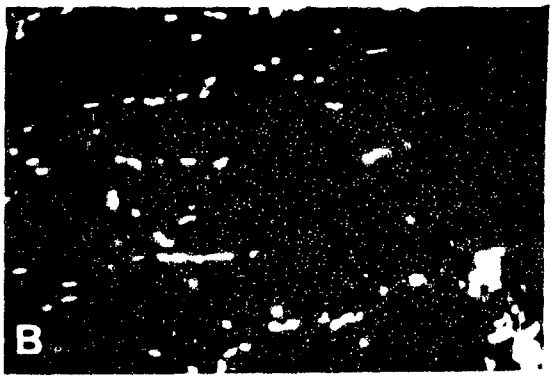
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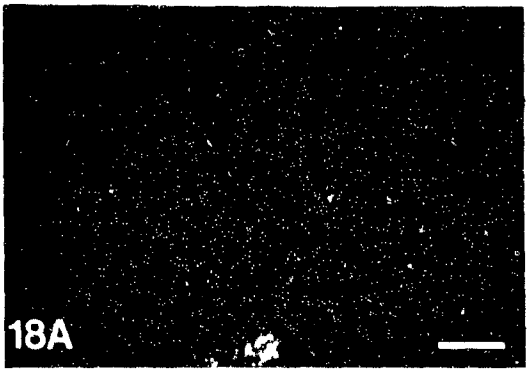
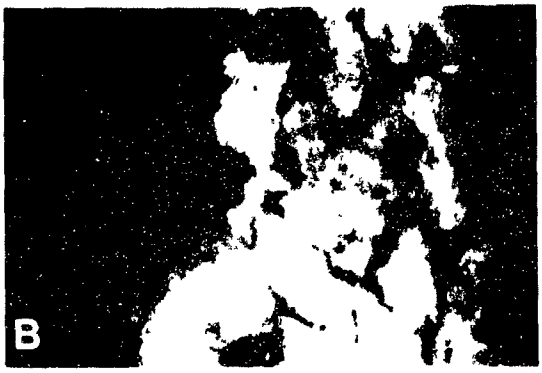

18A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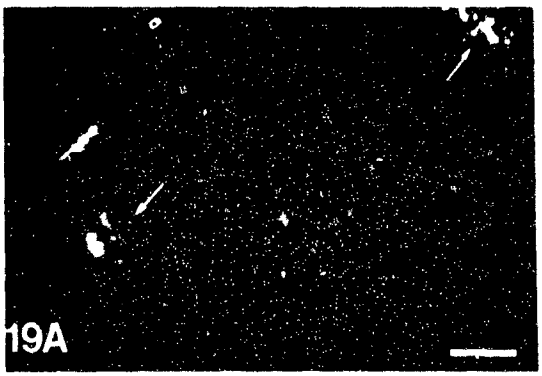
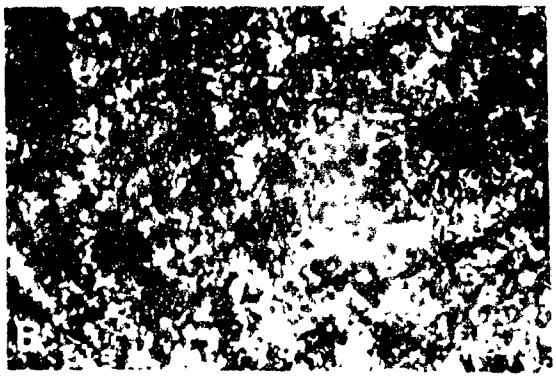
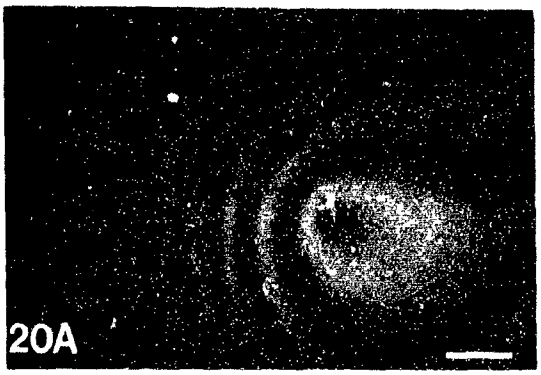
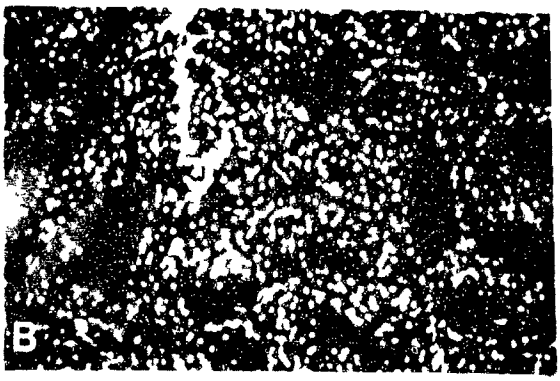

19A


B


20A


B

These may correspond to blood vessels. However, other types of profiles, namely small, filled, intensely stained structures, could also be observed (figure 22, arrowhead). This type of structure does not seem to morphologically correspond to a blood vessel. Other parts of the central nervous system were also widely immunostained. Two such examples are given in figures 23 and 24. All layers of the retina, except for the outer nuclear layer, were differently labeled by the native antiserum In the outer nuclear layer, only a sparse, intensely labeling cells of unidentified nature, could be observed. Also, it is noteworthy that, in the outer plexiform layer, the antiserum could define a cell population which is not uniformly distributed because the antigen recognized in the outer part of the layer is labeled densely (figure 23A, large arrow) as opposed to that in the inner part of the layer (which is labeled lightly). The optic nerve, was poorly labeled by the antiserum and had a very high background fluorescence (figure 24).

A considerable amount of cross-reactivity was directed to other tissues such as the intestines, liver, spleen, skeletal muscle as well as testes (figure 25-29). In the intestines(figure 25), immunolabeling at the level of the lamina propria was observed. In the liver, there were very highly stained varicosities which do not seem likely to correspond to hepatocytes, owing to their large, irregular shape (figure 26, arrow) but might be bile canaliculi. Within the spleen, the antiserum was distributed evenly and with low intensity, however, a more intense signal was observed at the tissue borders (figure 27). This may not be of importance since this type of result is a frequent consequence of uneven thickness in the tissue section. Of particular interest, a heavy labeling was seen on the muscle sections in the vicinity of the muscle fiber cell surface and extracellular matrix (figure 28, thick arrow). Intracellular material was also recognized by the antiserum (figure 28, thin arrow) and appeared as dotted strands in cross sections. It is

noteworthy that some muscle fibers had a higher diffuse intracellular background, that gave them a hazy appearance (figure 28, cell 1).were recognized by the antibody(ies) while other cells (figure 28, cell 2) had a clear background). In the testes, there was intense immunolabeling in the vicinity of the connective tissue spaces surrounding the seminiferous tubules (figure 29, arrow) however, sperm cells were lightly stained contributing to background observed within the seminiferous tubules. The patterns of immunofluorescence described above for the native antiserum reflects the one seen on blots of crude brain, muscles and viscera where a broad reactivity spectrum was observed in the brain, together with heterologous tissue cross-reactivity and high background (see earlier).

ABSORBED ANTISERUM

When the native antiserum was absorbed with either skeletal muscles or viscera, it tended to cause the detachment of tissue sections from gelatin-coated slides. This might be due to digestion of the fragile tissue mounted on the slides by enzymes present in the visceral organs used for absorption. Consequently, absorption of the antiserum was carried out by repeated incubations with 4% paraformaldehyde-fixed tissue sections mounted on slides (forming a lawn, see materials and methods). In this protocol, the native antiserum was absorbed with viscera (liver, spleen, mesentery, intestine) and skeletal muscles together. The immunohistochemical labeling patterns obtained when the antiserum was absorbed 5 consecutive times on a section-lawn, are shown in figures 30 and 31. In the optic tectum, well labeled neurons were seen in the superficial strata.

Figures 21-29 are sections reacted with the native anti-P2 antiserum.

Figure 21

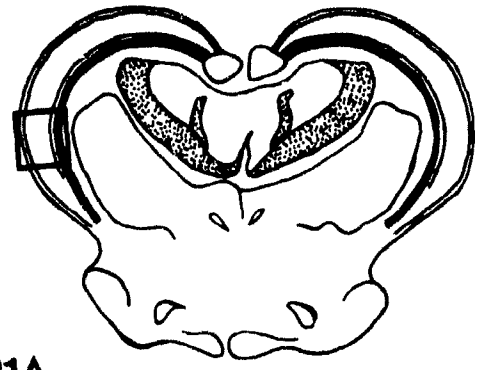
A) Cross section through the optic tectum outlining the area shown in B.

B) Cross-section through the tectum showing labeling of a blood vessel profile (arrow). Bar: 40μm.

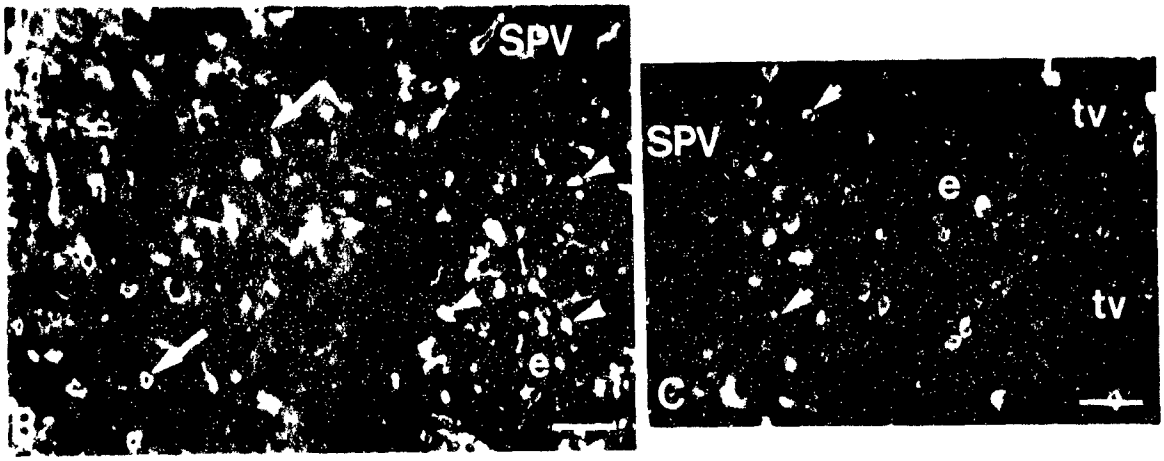
C) Higher magnification of the ependyma as shown in B, where intense labeling of cell-like profiles can be seen (arrows). Bar: 48μm.

Figure 22

Cross section through the forebrain showing labeling of blood vessel profiles (long arrows) and cell-like profiles (arrowheads). Note the high background reactivity in this structure. Bar: 48μm.



21A



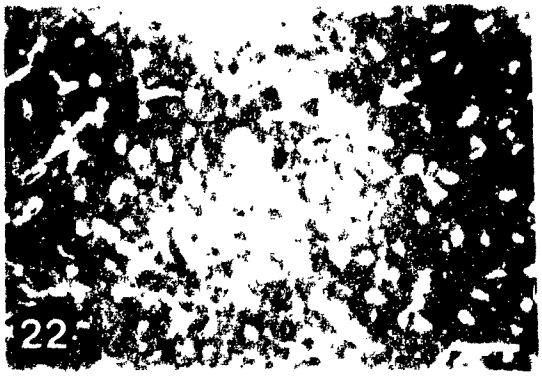


Figure 23

A) Cross section through the retina showing labeling of cells in the outer nuclear layer (short arrows) as well as labeling in the outer part of the outer plexiform layer (long arrows). Bar: 48µm..
B) Corresponding nuclear yellow fluorescence.

Figure 24

Longitudinal section through the optic nerve. Bar: 48µm.

figure 25

Cross section through the intestine showing labeling at the level of the epithelium (arrows). Asterisk: luminar space. Bar: 48µm.

Figure 26

Cross section through the liver showing dense labeling of unidentified profiles (arrow). Bar: 48µm.

Figure 27

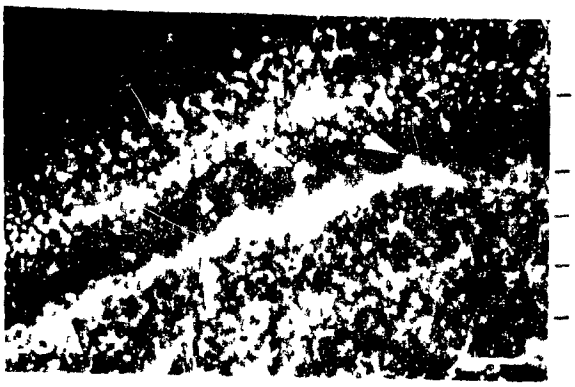
Cross section through the spleen with intense, widespread labeling. Bar: 48µm.

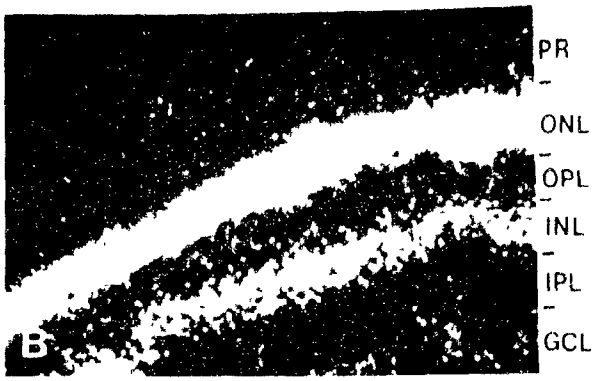
Figure 28

Cross section through skeletal muscle.The individual muscle cells are labeled at the level of the cell's periphery (thick arrow) and in the intracellular space (thin arrow). Also, some muscle cells (1) have a weak intracellular background labeling while others (2) have a low intracellular background. Bar: 48µm.

Figure 29

Cross section through the testis. The seminiferous tubules are surrounded by dense labeling (arrow - see also figure 50) Bar: 48µm.




PR
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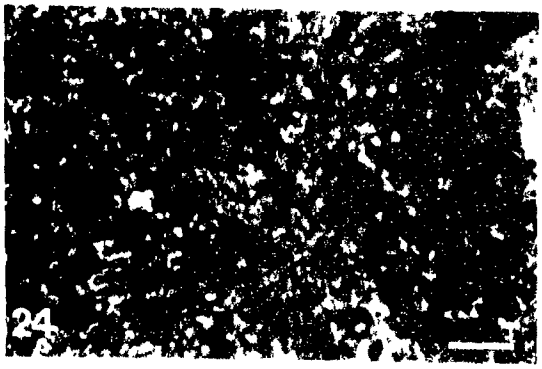
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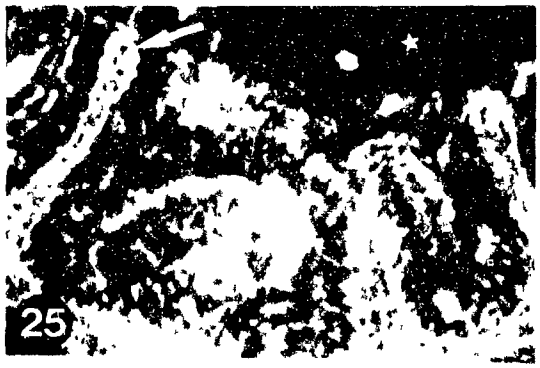
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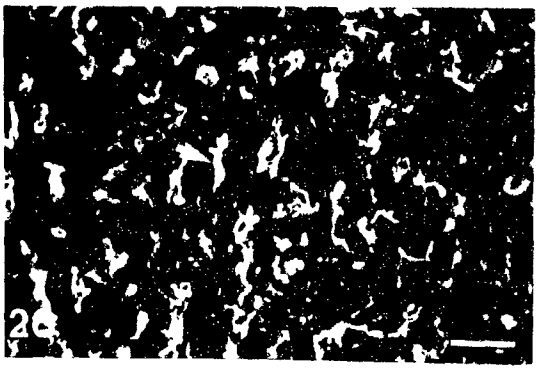
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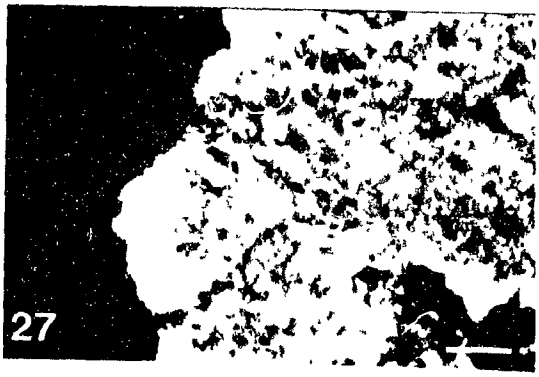
27


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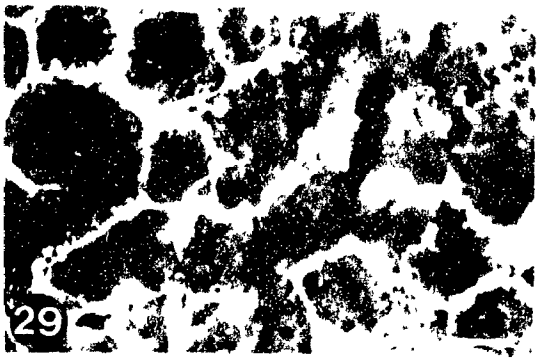
28


29

Figures 30-35 are sections reacted with anti-P2 antiserum absorbed with 4% paraformaldehyde-fixed viscera (liver, spleen, intestines and mesentery) and skeletal muscles. In figure 32, the antiserum was absorbed 9 times while in the others it was only absorbed 5 times.

Figure 32

A) Lateral view of the goldfish brain. The vertical line indicates the plane of section used in B.

B) Transverse section through the mid-level of the optic tectum. Area outlined corresponds to areas represented in C.

C) Montage of a transverse section through the tectum. Pyramidal cells having their cell bodies in the SFGS are labeled (arrows) Bar: 20μm.

D) High magnification of a pyramidal cell showing immunofluorescence labeling at the level of the dendritic arborizations (d), soma (s), axon (a) and axon collaterals (ac). Note that the stratum marginale (SM) is completely occupied by the dendritic arborization from the pyramidal cells (arrowheads) Bar: 10μm.

E) Corresponding nuclear yellow labeling pattern of the same area as in D showing a pyramidal cell nucleus (arrow).

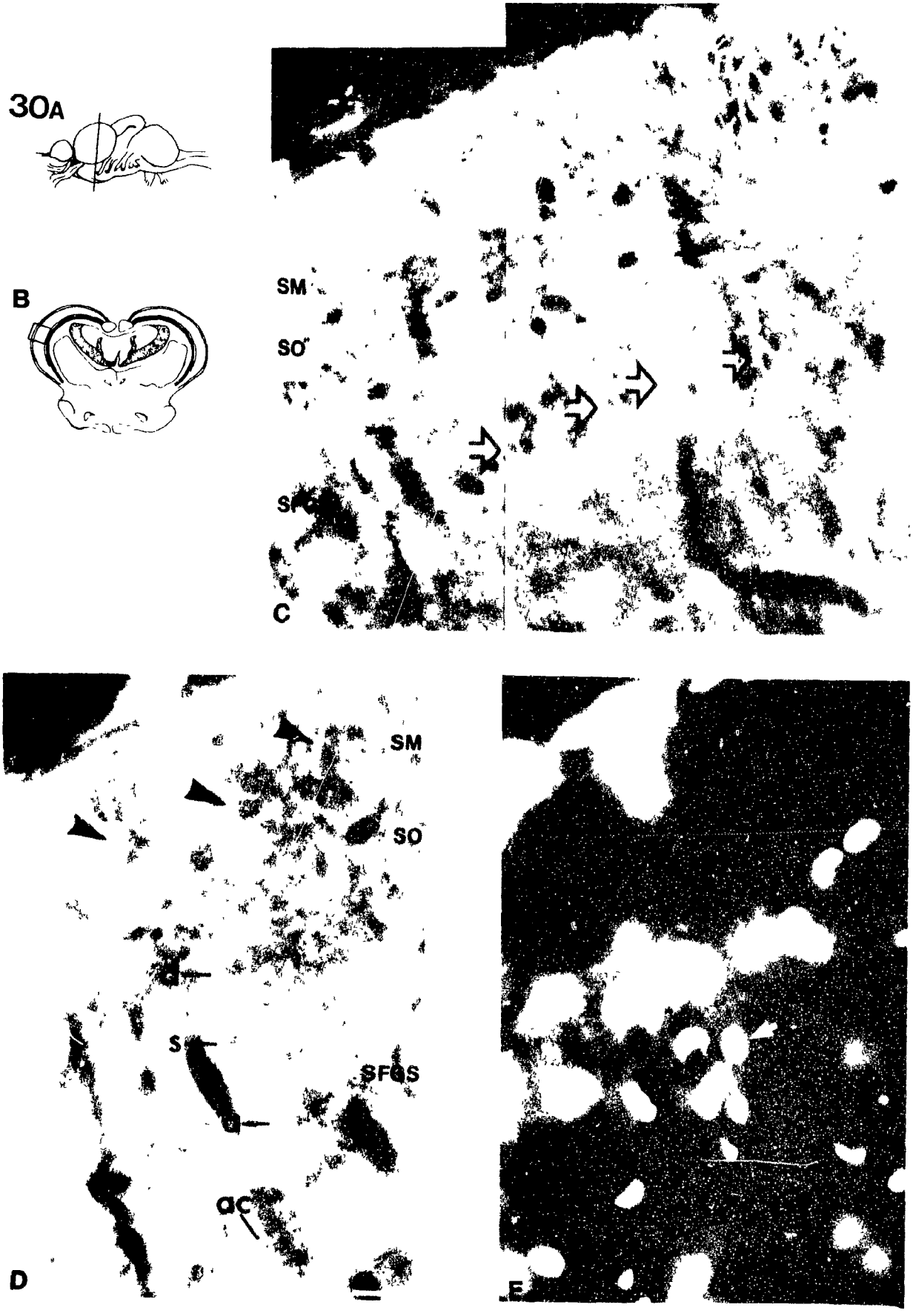

30A
B
SM
SO
S
C
SM
SO
S
SFOS
ac
D
E

Figure 31

A) Transverse section through the valvulae cerebelli showing the area represented in figure 31 B and C, and figure 32.

B) Transverse section of the valvulae cerebelli showing Purkinje cell staining (arrows). There is also dens labeling in the molecular layer, while the granular layer is only weakly labeled. Bar: 32µm.

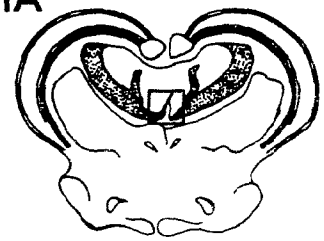
C) Corresponding nuclear yellow labeling pattern of the same area as in B showing the Purkinje cell nuclei (arrows).

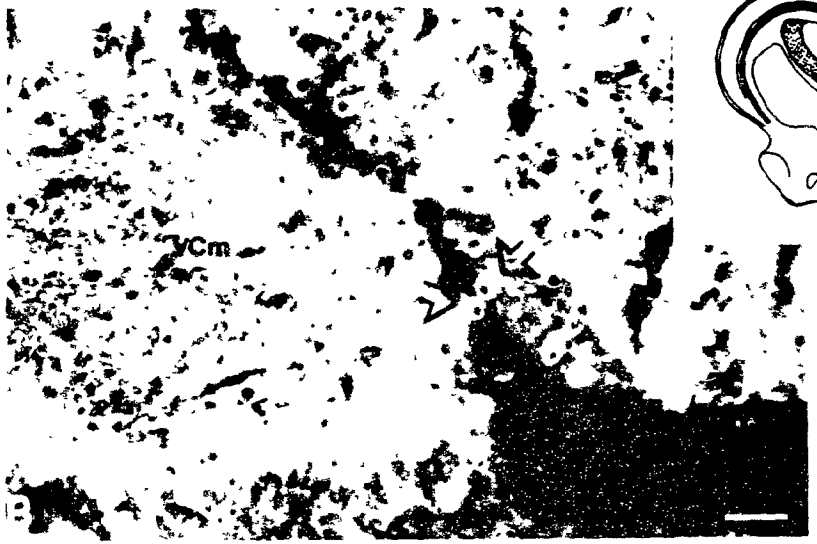
Figure 32

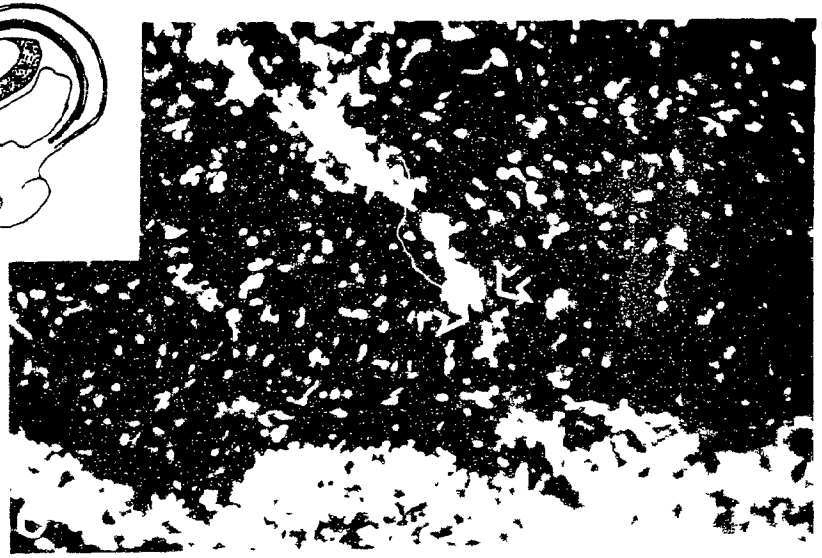
A) Transverse section as in 31B showing Purkinje cell staining (arrows). This serum was absorbed 9 times with viscera and muscle. Bar: 32µm.

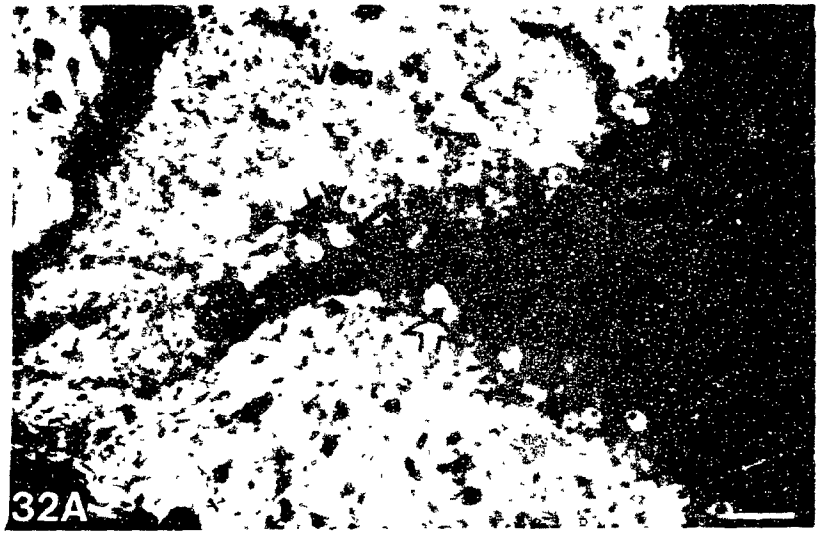
B) Corresponding nuclear yellow staining pattern of the same area as in A showing the Purkinje cell nuclei (arrows).

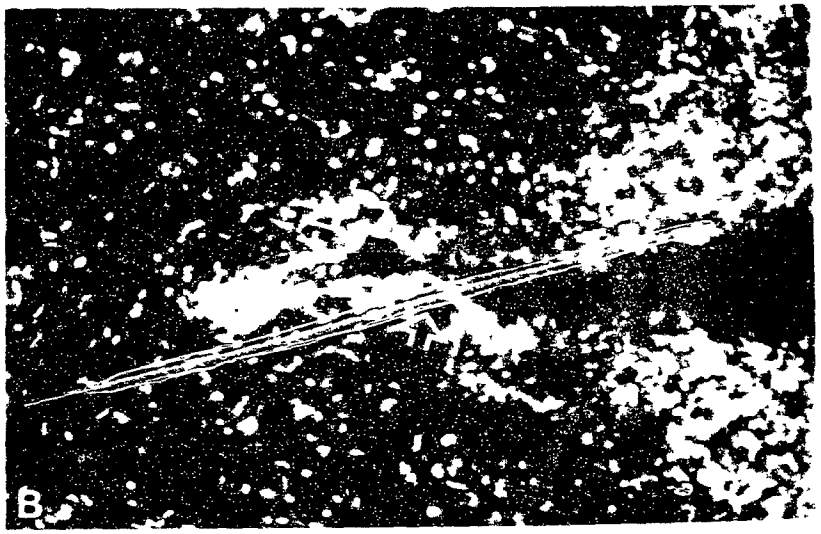
31A











These cells spanned the regions from the SFGS to the pial surface. Their somata are triangular or pyramidal in shape and are located in the SFGS (figure 30C and D). Thus, these cells were classified as pyramidal cells. There was no labeling of the large, ovoid nuclei of these cells which thus appeared as a transparent vacuole in the soma (figure 30E). The small dendritic processes of the tectal pyramidal cells are often densely packed, thus forming a highly fluorescent palisade of intermingled dendritic trees in the stratum marginali (SM, Figure 30D). In contrast to these dendritic spines, the main dendritic and axonal processes, as well as axon collaterals appear much thinner and weakly fluorescent. Elsewhere, in the midbrain, labeling is sparse, with the exception of the cerebellum where very large cells, also pyramidal in shape, were found at the borderline between the molecular and granular layers of the valvula. This cell type has a very characteristic morphology that make them easily recognizable as the Purkinje cells (figure 31 and 32).The dendritic arborization of the Purkinje cells, filling the molecular layer of the valvula cerebelli, was also immunostained. Again, for this type of cell, the large, round nuclei were unlabeled (figure 31C and 32B). When more consecutive absorptions were carried out (9 as opposed to 5), with the aim of enhancing the fluorescence signal, there was no significant improvement in the labeling intensity (figure 31B and 32A).

Using the absorbed antiserum, there was also labeling in the retina and optic nerve (figure 33 and 34). In the retina, the labeling was dispersed through all layers with the exception of the outer nuclear layer and the inner part of the outer plexiform layer (figure 33A arrow). Note that this labeling pattern was also observed with the native antiserum (figure 23A). The optic nerve (figure 34) showed copious background fluorescence, together with intense labeling of cell-like profiles (arrows). There was also labeling in the liver tissue (figure 35) but, this time, it appeared to be in the form of a

punctate signal (arrows) over the hepatic cell nuclei.

P41 ANTIBODY

Specificity of the P41 antibody was imunohistochemically tested following elution from immunoblot. The epitopes for this antibody were widely distributed in goldfish nervous (figures 37-42) and non-nervous(figure 43-46) tissues. In the optic tectum (figure 37), the labeling pattern of immunoreactive blood vessels was essentially similar to those observed with the native anti-P2 antiserum (figure 13A), although here, the background labeling seemed greatly reduced. This tendency was also observed in the forebrain (figure 39, arrows). This P41 antibody, again, similarly to the native antiserum, intensely labeled cells in the ependymal zone (figure 40). In the valvula (figure 38), there was no labeling of the Purkinje cells, but sparse labeling of varicose profiles in the granular and molecular layers (arrows). The optic nerve was stained very lightly (figure 41, arrows). The retina (figure 42) bound antibody in all of its layers except for the inner and outer nuclear layers. There was no labeling of cell-like structures in the outer nuclear layer, as opposed to the labeling pattern seen with the native antiserum (see figure 23A). The intestines and the liver were stained heavily (figures 43 and 44). The intestine showed intense immunolabeling of both the lamina propria , and the adventitia. In the liver varicose structures were intensely stained (figure 44, arrows). These 2 tissues, as compared to the native reactivity pattern, were stained in a better defined manner, owing to the great reduction in background fluorescence (cf figures 25 and 26). This tendency was also observed in 2 other tissues, namely muscle and testes (figure 45 and 46).

Figure 33

A) Cross section through the retina showing staining in the outer part of the outer plexiform layer (large arrow). There is also intense labeling of cells in the inner nuclear layer (small arrows). Bar: 40µm.

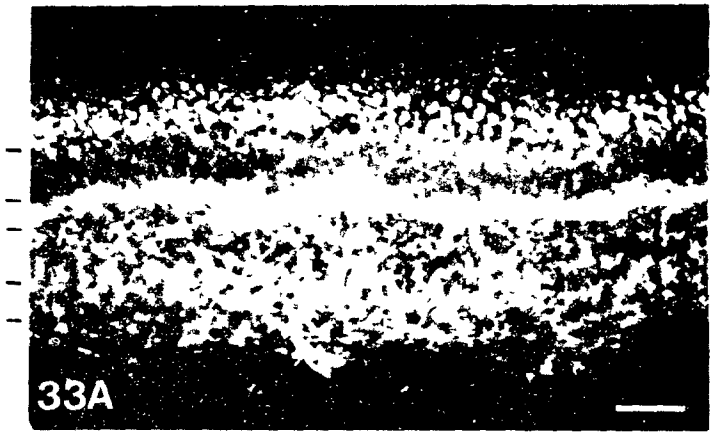
B) Corresponding nuclear yellow fluorescence. Arrow: ganglion cell.

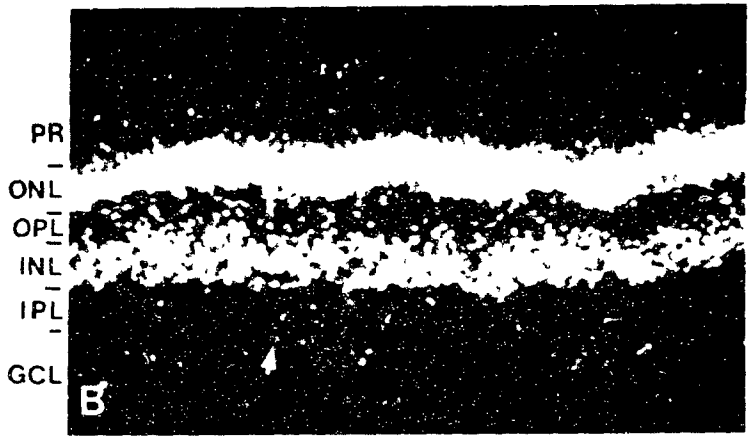
Figure 34

Longitudinal section through the optic nerve. Profiles of unknown nature are stained intensely (arrows). Bar: 24µm.

Figure 35

Cross section through the liver. Punctate labeling is seen homogeneously distributed across the tissue (arrows). Bar: 40µm.


33A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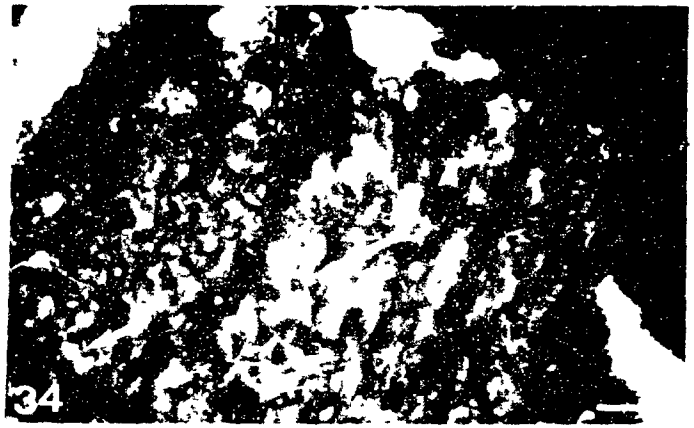
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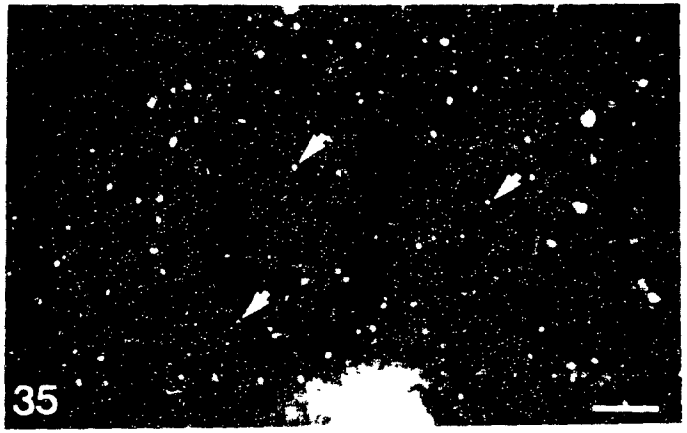

35

Figures 37-46 are sections reacted with the P41 antibody (obtained by blot elution, followed by concentration) used at a concentration of 1% (relative to its concentration in the native anti-P2 antiserum). Figures 37-40 and 42 have both FITC immunofluorescence (A) and corresponding nuclear yellow (B) labeling patterns.

Figure 36

Transverse section through the mid-level of the optic tectum showing the area depicted in figure 37 (square 1), and figure 38 (square 2).

Figure 37

Cross section through the optic tectum showing blood vessel labeling (arrow). Bar: 32µm.

Figure 38

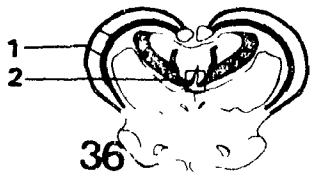
Cross section through the cerebellum. Purkinje cells are not labeled by this antibody, but instead, punctate profiles can be observed both in the granular and molecular layers of the valvulae (arrows). Bar: 48µm.

Figure 39

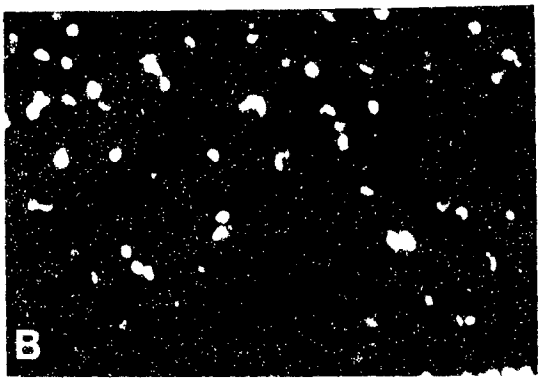
Cross section through the telencephalon showing blood vessel profiles (arrow). Bar: 48µm.

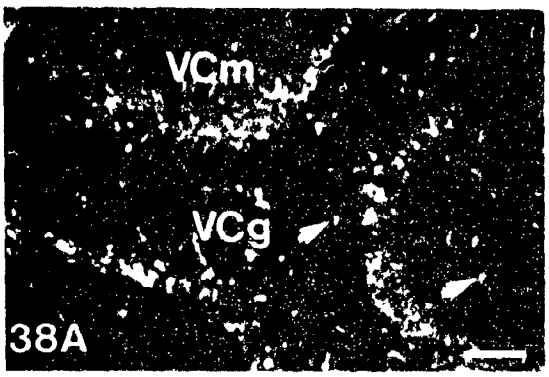
Figure 40

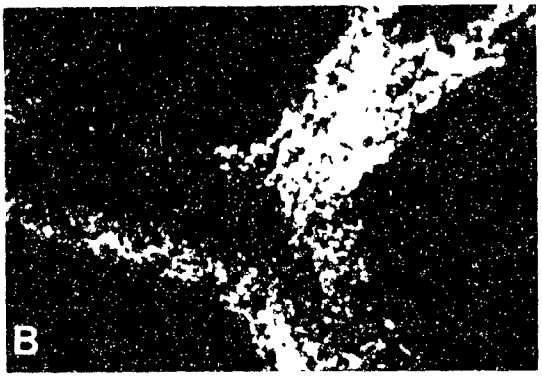
Cross section through the optic tectum in the ependymal layer area. Dense cell-like profile labeling can clearly be observed in this layer (arrows). Bar: 32µm.


1
2
36


37A


B


VCm
VCg
38A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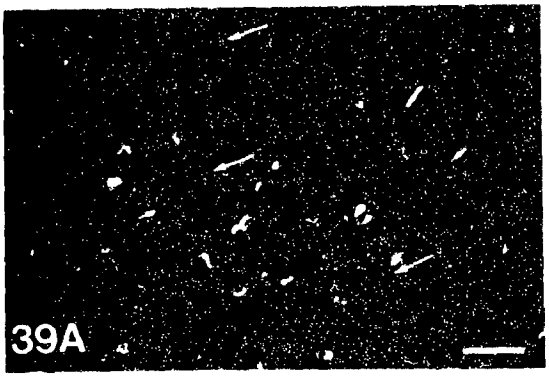
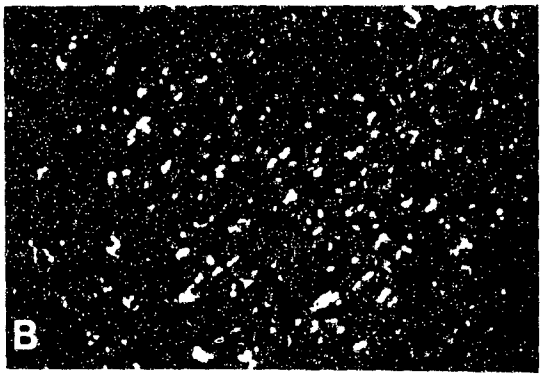
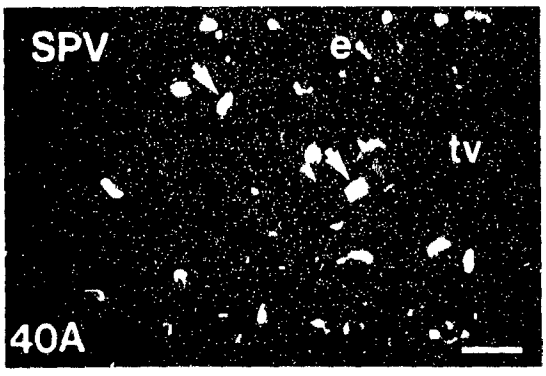

39A


B


SPV
e
tv
40A

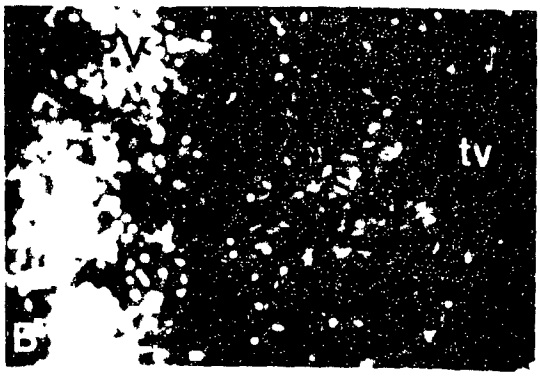

v
tv
B

Figure 41

Longitudinal section through the optic nerve showing labeling of unidentified profiles. Bar: 48µm.

Figure 42

A) Cross section through the retina. There is no staining in the outer and inner nuclear layers (layers 2 and 4 respectively). Bar: 48µm.

B) Corresponding nuclear yellow staining pattern of the same area as in B. Arrow: ganglion cell. Layer 1: photoreceptor cell layer, 2: outer nuclear layer, 3: outer plexiform layer, 4: inner nuclear layer, 5: inner plexiform layer, 6: ganglion cell layer.

Figure 43

Cross section through the intestines showing immunolabeling at the level of the lamina propria (long arrow) and the adventitia (short arrow). Asterisk: luminar space. Bar: 48µm.

Figure 44

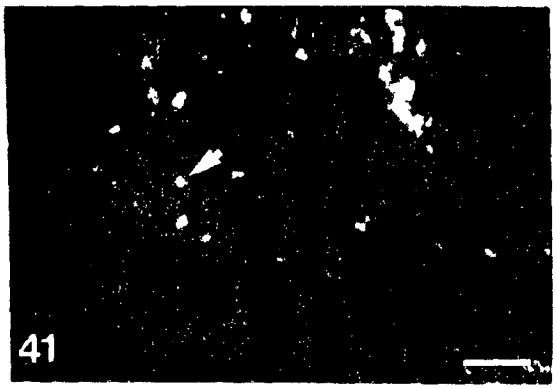
Cross section through the liver. Intense, widespread labeling of unidentified profiles is observed (arrows). Bar: 48µm.

Figure 45

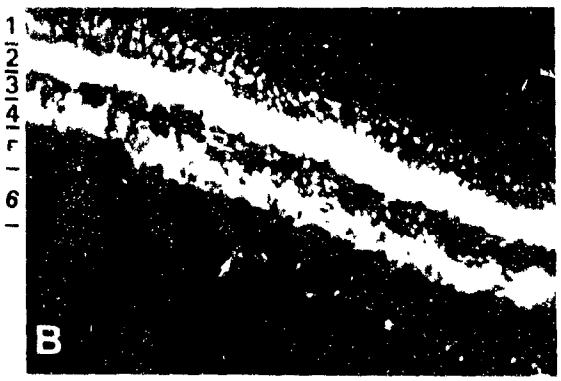
Cross section through the muscle showing labeling at the level of the cell surface and the extracellular matrix (arrow, see also figure 49),and intracellularly as well (arrowhead). Bar: 48µm.

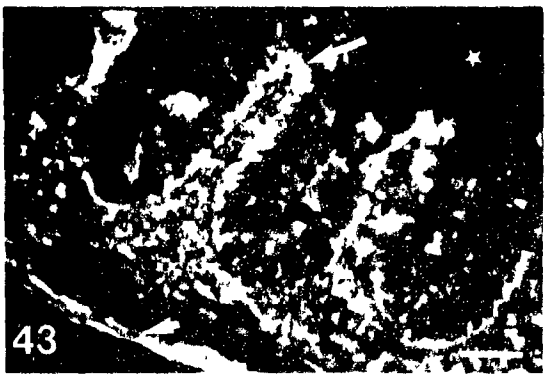
Figure 46

Cross section through testeshowing labeling at the level of the extracellular matrix where it invests the seminiferous tubule (arrow, see also figure 50). Bar: 48µm.


41


42A


B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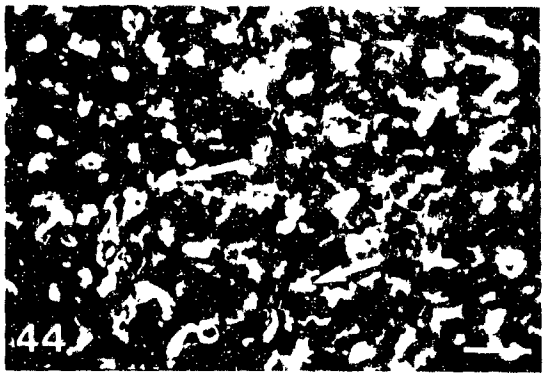
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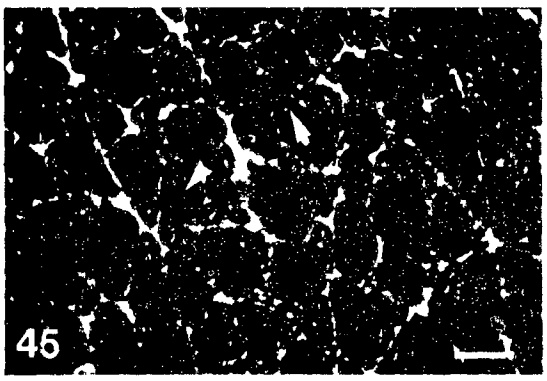
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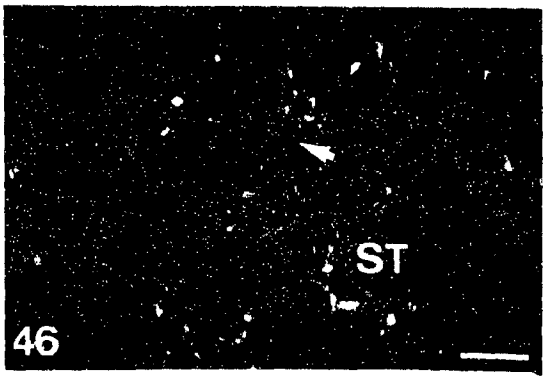

ST
46

Figures 47-50 are sections reacted with rabbit antibody directed against mouse laminin used at a dilution of 1:100 on ethanol/acetone-fixed tissue. Dr. R. L. Levine performed this experiment.

Figure 47

Cross section through the optic tectum showing labeling of blood vessels (long arrow) as well as pia mater (short arrow) . Bar: 40μm.

Figure 48

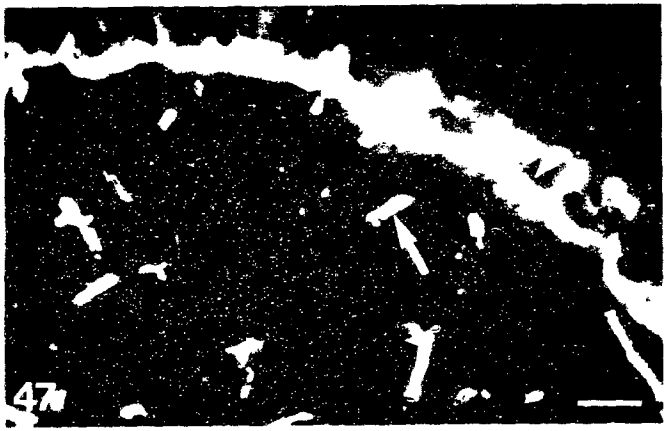
Longitudinal section through the optic nerve showing labeling of blood vessels (long arrow) as well as pia mater (short arrow) . Bar: 40μm.

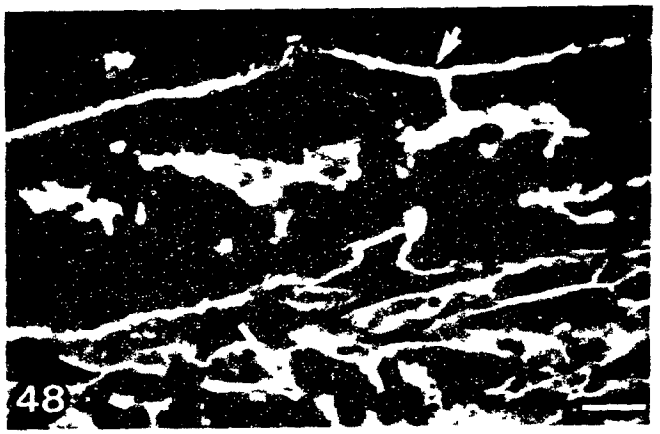
Figure 49

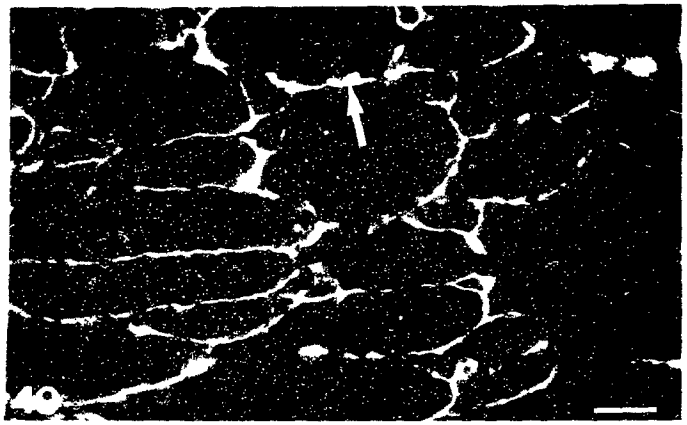
Cross section through the skeletal muscles where labeling of the extracellular matrix is seen (arrow). Bar: 40μm.

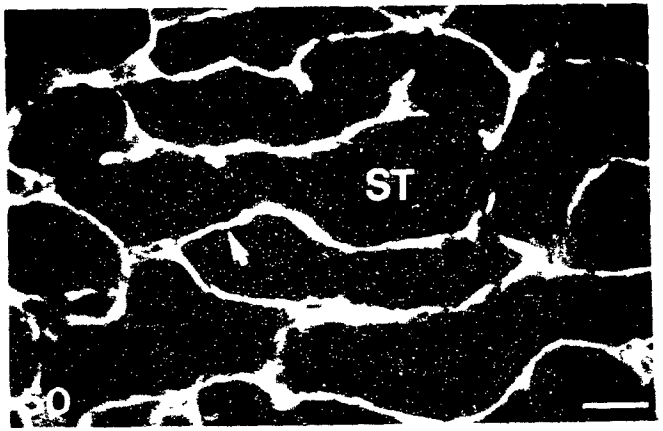
Figure 50

Cross section through the testes. The seminiferous tubules are surrounded by extracellular matrix which is densely labeled (arrow) Bar: 40μm.








ST

An example of such staining pattern obtained with laminin is shown in figures 47-50. There, the distribution of this extracellular matrix protein in muscles and testes corresponds to the one observed with the P41 antibody except for intracellular labeling of muscle cells with the latter (figure 45, arrowhead). However, due to the use of rabbit an .erum, conspicuous background fluorescence can be seen over the muscle fiber intracellular material and over the spermatic cells. In the optic tectum (figure 47), there is intense immunofluorescence labeling of the pia mater (short arrow) and over blood vessels (long arrow). This type of labeling is in contrast to the one obtained with the P41 antibody as it is better defined, more specific. In the optic nerve (figure 48) labeling is again observed in the pia mater (short arrow) as well as around blood vessels (long arrow).

LIST OF ABBREVIATIONS

bs: brain stem
c: cerebellum
e: ependymal zone
h: hypothalamus
MLF: nucleus of the medial longitudinal fasciculus
ol: olfactory nerve
on: optic nerve
ot: optic tectum
pm: pia mater
t: telencephalon
tl: torus longitudinalis
ts: torus semicircularis
tv: tectal ventricle
VCg: granular layer of the valvula cerebelli
VCm: molecular layer of the valvula cerebelli
vl: vagal lobe

GCL: ganglion cell layer
INL: inner nuclear layer
IPL: inner plexiform layer
ONL: outer nuclear layer
OPL: outer plexiform layer
PR: photoreceptor layer

SFGS: stratum fibroseum et griseum superficiale
SGC: stratum griseum centrale
SM: stratum marginale
SO: stratum opticum
SPV: stratum periventriculare

ST: seminiferous tubule

DISCUSSION

RESULTS OVERVIEW

In the hope of eliciting antibodies primarily directed at the surface of goldfish brain cells, I have isolated fractions of brain membranes and used them to immunize mice. For this purpose, two protocols were followed, in which the immunogen was either in the form of a crude membrane fraction (P2) isolated by differential centrifugation, or further purified by separating the crude membrane fraction through a 3-step sucrose gradient. These two immunogens had a more or less similar protein content (as determined electrophoretically with Coomassie Blue staining) however, contamination by myelin and mitochondria, assessed by electron microscopy, was considerable in the crude membrane fraction while it appeared negligible in the purified fraction

The antisera described in this thesis were studied with regard both to the spectrum of antibody reactivities they contained and to the distribution, in goldfish tissues, of the various antigens recognized by mouse antisera against the crude goldfish brain membrane preparation.

Immunoblotting analysis showed that each immunization protocol led to the production of similar antibodies in mice and that the two types of antisera (anti-P2 and anti-membrane) obtained had a broad range of reactivities to goldfish proteins in the brain and other organs. However, major immuno-

reactivity was seen at 99Kd and 41Kd with the anti-membrane antisera, and at 72Kd and 41Kd with the anti-P2 antisera. The native anti-P2 antiserum was assayed immunohistochemically on sections of various goldfish organs and it was observed that the broad reactivity seen on immunoblots was reflected in the results since in all tissues tested, there was widespread background reactivity as well as heavy labeling of various structures.

With the objective of depleting these antisera of non-brain cross-reactivities, absorption with heterologous organs was performed. The anti-membrane antiserum, when absorbed with a crude membrane preparation from the goldfish optic nerve, was depleted of background reactivities on immunoblots, (in the high - >100Kd - and low - <36Kd - molecular weight ranges) and major reactivities at 99Kd and 41kd were enhanced. However, the binding at 41Kd was also seen in the optic nerve after absorption, therefore suggesting incomplete absorption of the anti-membrane antiserum with optic nerve. Similarly, the anti-P2 antiserum was depleted of background reactivity when absorbed with goldfish viscera or skeletal muscle, although a major reactivity at 72Kd in the brain, which was also seen in the crude visceral and muscle extracts, still remained after absorption with these preparations, therefore once again suggesting incomplete absorption of the antiserum. Another absorption protocol was used with the anti-P2 antiserum, where paraformaldehyde-fixed sections of viscera and skeletal muscle were used as the absorbant. In that case, with indirect immunofluorescence microscopy, the absorbed antiserum was relatively specific for two types of cells, the tectal pyramidal cells and the cerebellar Purkinje cells in the fixed goldfish brain.

Various antibodies were eluted from immunoblots of both antisera. In the case of the anti-membrane antisera, five antibodies were eluted (at 99Kd,

84Kd, 58Kd, 41Kd and 36Kd), concentrated, and then reacted to blots containing crude brain membrane preparations. All of the antibodies eluted could react with a protein migrating to 84Kd and, apart from this cross-reactivity, two antibodies (against 99Kd and 72Kd protein bands) were successful in binding the protein they were eluted from. Similarly, three antibodies from the anti-P2 antiserum, were eluted (at 58Kd, 41Kd and 36Kd) and, all were shown to cross-react with a band at 72Kd. When the antibody eluted from the band at 41Kd (P41) was assayed immunohistochemically, it bound nervous as well as non-nervous tissues in a manner that suggest a general association with the cell surface and extracellular material.

COMPARISON OF THE 2 IMMUNIZATION PROTOCOLS USED

Based on the assumption that the purity of the immunogen is in direct relation with the purity of the antibody produced, the refinement of the fractions used to produce the antibody and characterize it was, of course, critical to the value and future usefulness of my experiments . Moreover, by using a purified immunogen (the membrane fraction), it was expected that less purification procedures would be needed in order to obtain the desired membrane-directed antibodies. A variety of methods have been used to isolate the immunogens with the aim of producing antibodies directed against synaptic membrane (DeRobertis et al. 1968, Mickey et al. 1971, Bock et al. 1974, Herschman et al. 1972, Williams et al. 1985, Grumet et al. 1984, Barnstable et al. 1983, Mallet et al. 1979), and as noted in these studies, even the best preparations are likely to be heavily contaminated by subcellular constituents other than membranes (eg. mitochondria) and also by non-neuronal material (eg. myelin membranes). My own observation,

using electron microscopy, that the crude and purified membrane fractions both contained mitochondria and golgi stacks are in agreement with these studies. Nevertheless, the purified membrane fraction, as opposed to the crude membrane fraction, contained only sparse mitochondria and no myelin fragments as assessed by electron microscopy. Moreover, it was shown by marker enzyme assays that contamination of the purified membrane fraction with mitochondria and myelin was respectively of only 5% and 3% of the total activity of these enzymes found in the gradient. Consequently, it appears that the membrane fraction I prepared was reasonably pure.

Examination of the crude and purified membrane proteins by gel electrophoresis and Coomassie Blue staining showed that both membrane preparations were similar with regard to their protein composition. Although there was a high degree of heterogeneity in the antigen population in both cases, a comparison of the protein spectrum in the membrane fraction with that of the S2 supernatant led to the observation that many protein bands had been lost through the purification procedure. These proteins present in the S2 supernatant but absent (or with a largely diminished relative concentration) in the crude or purified membrane fraction are likely constituents of the rough and smooth endoplasmic reticulum, lysosomes, golgi apparatus and soluble cytoplasmic proteins (for example, cytoskeletal proteins) (Graham 1987). Moreover, a comparison of the purified membrane fraction with the myelin fraction shows that low molecular weight proteins (<40Kd) - which are characteristic constituents of the myelin (Elam 1974, Roots et al. 1984) - are largely absent from the membrane fraction. Also, it is worth mentioning that a specific protein band migrating to an approximate molecular weight of 100Kd, was enriched in both membrane preparations.

This protein band might then well be attributed to the membrane fraction in the same manner as the subcellular distribution of membrane-specific marker enzymes are determined. Schlossauer, recently (1989) reviewed the different protocols used for isolation of purified membrane immunogens and observed that although the purification of membrane fractions using centrifugation techniques (as in the present investigation) has the advantage of simplicity and rapidity, this approach is insufficient in terms of enrichment of cell surface markers (eg. Na^+,K^+-ATPase, 5'nucleotidase enzyme markers). He reports that the heterogeneous nature of the membrane preparation might prevent the production of antibodies against some proteins of interest but with low antigenicity since the immune reaction would be shifted towards the production of antibodies only to the proteins with high antigenicity. Therefore this author makes the suggestion that membrane protein purification procedures through centrifugation techniques should be used in conjunction with lectin affinity chromatography (where the membrane preparation is passed over a lentil lectin column after which the glycoproteins retained by the column are eluted) and that known proteins of high antigenicity should be depleted from the glycoprotein mixture before injection. However, the problem that would obviously arise in using the methods suggested by Schlossauer (1989 - who was concerned with the isolation of rat brain membrane proteins) with the goldfish, would be the reduction in immunogen volume to a level too low for antibody production in mice unless extremely large quantities of goldfish brains were utilized (8 goldfish brains/immunization schedule were utilized in the present study).

Both types of antisera obtained in the present study, were shown by immunoblotting, to recognize many proteins in the goldfish brain. This type of broad immune reaction is not an uncommon property of heterologous antisera, and reactivities of this type have often been obtained by other

investigators when dealing with a minimally-purified immunogen (Golub 1971, Goldschneider and Moscona 1972, Engh et al. 1974)

The antisera obtained from the different mice within each immunization schedule (anti-membrane or anti-P2 antisera) had quite comparable reactivities. This was expected since the electrophoretic pattern of both immunogens (crude and purified membrane fractions) was similar. This similarity in the immune response suggests the presence of a few highly immunogenic determinants (or conversely a lack of immunogenicity in most antigens) in the immunogen protein pool. This may however be a disadvantage because, as already mentioned, some highly immunogenic determinants might be directing the production of antibodies. In that case, detection of particular proteins of interest would be hindered if they were of low immunogenicity (Schlosshauer 1989). Fortuitously, one of the advantages of obtaining largely similar antisera from different animals immunized with the same protein preparation, is that the antisera produced can be pooled and therefore larger quantities can be made available for immunological testing. This is obviously of great relevance when considering that mouse serum is harvested in such low quantities (250-300µl per bleed, routinely in our lab).

Comparison of the anti-P2 and anti-membrane antisera, revealed many antibody reactivities that might be common. It was observed that , while both the anti-membrane and anti-P2 antisera stained the 41Kd, 72Kd and 99Kd protein bands, only the reactivity against the 41Kd protein band appeared nervous system-specific, since it was absent when assayed against muscle or viscera proteins (but present when assayed against optic nerve) from the goldfish. Since the proteins with molecular weights (MW) of 41Kd and 72Kd, do not seem to be major goldfish brain proteins (there is a barely visible

protein population in the vicinity of these 2 molecular weights on SDS gelsstained with Coomassie Blue) it is interesting to find that they nonetheless react with the 2 sets of antisera. Therefore, it seems likely that they are amongst the most immunogenic proteins in my preparations. However, this conclusion has to be drawn with some restrictions owing to the uncertainty involved in accurately determining the molecular weight of proteins on a gel or blot. This difficulty might be reduced, on the other hand, by separation of the proteins in two dimensions, according to their isoelectric point as well as to their molecular weight , which would allow a more complete comparison of the antisera to be made (Dunbar 1987).

It is difficult, at this point, to determine if any of the proteins recognized by the antisera can be correlated to any known protein present in the goldfish brain. For example, it was suspected that the protein recognized at 99Kd (by both antisera) might be the Na^+,K^+-ATPase protein constituent of plasma membranes which has a MW of 100Kd in other species (Hobbs and Albers 1980). Such a protein might be expected to have a very low tissue specificity because all cell types possess this enzyme as a basic membrane constituent (since it is involved in maintenance of ionic balance between the inner and outer milieus - Hobbs and Albers 1980). This low tissue specificity may explain the widespread fluorescent labeling pattern of the native anti-P2 antiserum seen on all goldfish tissue sections tested. The protein with apparent MW of 41Kd seen on blots with both antisera. might be actin which has a MW of 43Kd (Zubay 1986). Similarly, this protein would be widely spread in its tissue distribution since all cells contain this cytoskeletal protein (Zubay 1986). In that case however, the possibility for recognition of actin is not likely since a relatively high level of contamination of the goldfish brain membrane fractions by actin would have had to occur and, as mentioned

earlier, most if not all of the cytoskeletal proteins would be expected to be present in the S2 supernatant. However, the possibility that some cytoskeletal proteins might be associated intracellularly with the cell membrane has to be borne in mind. Also, when the anti-P2 antiserum was reacted against blotted muscle proteins from the goldfish, low reactivity was observed in the viscinity of the 41Kd molecular weight. This indicates that the possibility for binding actin is little probable since this protein is expected to be found in great quantities in the skeletal muscle where it is involved in the mechanism of muscle contraction (Zubay 1986). Finally, suggestions for the correspondance of the protein with apparent MW of 36Kd bound on blots by both antisera include the 36Kd X protein originally described by Elam (1974) which is the major constituent of myelin in goldfish brains. This suggestion is based on the fact that a protein migrating to 36Kd on SDS gels, present in both immunogen preparations, was enriched in the myelin fraction. Alternatively, based on the fact that cells in the ependymal layer are heavily bound by the anti-P2 antiserum, as observed by fluorescence histochemistry, then an antibody might be recognizing components of glial ependymal cell surfaces, one of which (ependymin ß) has a MW of 37Kd (Shashoua 1976). However, a precise cellular and sub-cellular localization and a molecular characterization of the antigens bound by the anti-membrane and anti-P2 antisera would be needed to evaluate the significance of the similar and dissimilar features with other known proteins.

Obviously, the antisera, in their native form, were not reliable markers for goldfish brain membrane constituents, owing to their broad reactivities with heterologous tissues and with the brain tissue itself and thus had to be purified.

"ABOSRBED ANTISERUM"

To refine the reactivity pattern of my antisera they were absorbed with non-brain tissue of various origins. However, the problem in doing so was that the antisera were not available in large quantities and therefore, none of the absorptions could be run to completion because of the requirement for large antiserum volumes to carrying out the procedures. Nonetheless, brain reactivities were enhanced by the absorption procedures, and in the case of the anti-membrane antiserum, when it was absorbed with optic nerves, reactivity to a band at 99Kd (observed only in the brain and not in the optic nerve) was enhanced relative to others as observed by an immunoblotting assay. This protein could not be assumed, however, to be brain-specitic in the present study, because it might have been present in other tissues which were not tested. However, the observation that this protein was not present in the optic nerve extract rules out the possibility that it might be the Na^+,K^+-ATPase membrane protein. The reactivity against a band at 41Kd was also shown on immunoblots, to be enhanced relative to others when the anti-P2 antiserum was absorbed with muscles and viscera. This reactivitiy was the most prominent one from the absorbed anti-P2 antiserum and therefore, I wanted to ascertain the localization of this protein epitope in fixed goldfish tissues.

As was mentioned earlier (see results), a problem in trying to immunohistochemically localize the epitopes recognized by the absorbed antiserum (absorbed with fresh tissue) arose because it tended to cause fixed sections to detach from the gelatin-coated slides. This may have been due to enzymatic disruption of the attachment between the substrate (gelatin) and the goldfish tissue (Betz and Sakmann 1973). Becuase of this

problem, I tried absorbing the antiserum with fixed tissues, in which proteolytic enzymes from visceral tissues (used as absorbant) would have lost their effects. Moreover, it is very common (Hockfield and McKay 1985, Fambrough et al. 1982) that antigens recognized in their native form (or on a blot, ie. in their SDS-denatured form) are not recognized in tissue sections (ie. where the antigen is bound by fixative). Therefore, absorbing the anti-P2 antiserum with fixed tissues might be expected to give an optimal signal to noise ratio because, in this case, the antigens are encountered in the same configuration as they are in the indirect immunofluorescence assay which I used to verify the absorption results. One of the obvious disadvantages in using this time-consuming absorption method is that only small amounts of the antiserum can be utilized, leaving very limited quantities available for carrying out further analysis (eg. immunoblotting).

After partial absorption of the anti-P2 antiserum with paraformaldehyde-fixed skeletal muscles and viscera (including intestines, mesentery, liver and spleen), immunohistochemical analysis showed that there was recognition of two subsets of neurons, similar in their pyramidal shape, in the optic tectum and the cerebellum. These cells appear to correspond to the pyramidal cell in the tectum (Laufer and Vanegas 1974) and Purkinje cells in the cerebellum (Schaper 1893). The two cell types were labeled intracellularly in all subcellular compartments including cell soma (apart from the nucleus which was not labeled), dendrites and axon, and were preferentially recognized by the absorbed antiserum in the brain areas studied. Interestingly, many authors (see Vanegas 1983) have noticed a resemblance between the tectal pyramidal neuron and its presynaptic element (the marginal fibers) on the one hand, and the cerebellar Purkinje cells and parallel fibers on the other. In the optic tectum, the large pyramidal

cells, apart from receiving direct input from the retinotectal projection, receive a major input from the marginal fibers which run along the surface of the optic tectum in the stratum marginale and synapse onto the elaborate dendritic tree of these cells (Leghissa 1955) These marginal fibers are the axons of the granule cells located in the torus longitudinalis, which receives a major input from the valvulae cerebelli (Ito and Kishida 1978). In the cerebellum, the Purkinje cells receive a major input from the parallel fibers which run along the surface of the cerebellum in the molecular layer. These fibers are the axons of the cerebellar granule cells which lie deep to the Purkinje cell layer. The inputs to the granule cells of the cerebellum come from a variety of sources (via the cerebellar mossy fibers) including the vestibular centers, spinal cord and cerebrum (Kotchabhakdi 1976). Therefore, these two types of cells (the tectal pyramidal cell and the Purkinje cell), apart from having a common morphology, also have a similar connectivity pattern in two different parts of the goldfish brain. Moreover, the indirect connectivity between cerebellar cells (possibly via Purkinje cells since their axons constitute the only output from the cerebellum) and the tectal pyramidal cells, through the granule cells in the torus longitudinalis, may also provide a functional association between these two types of cells, which might be reflected in the molecules recognized by the absorbed antiserum.

It is possible that the absorbed antiserum, rather than recognizing a protein that is common to tectal pyramidal cells and Purkinje cells, could contain several antibodies that recognize different antigens which are not structurally related, but are distributed on only these two cell types. One way to determine the cell specificity of the absorbed antiserum (ie. Is it recognizing a common epitope or 2 or more unrelated epitopes on both types of cells?) would be by re-absorbing it with either the cerebellum

(which is devoid of tectal pyramidal cells) or the tectum (which is devoid of Purkinje cells). After absorption, residual binding of only one population of cells would correspond to the isolation of a cell-specific immunological probe. A similar approach was employed by Mallet et al. (1979) who absorbed an antibody against cerebellar membranes, onto cultured Purkinje cells. The binding antibodies were then eluted from the cell surfaces and, a Purkinje cell-specific antibody was isolated. In the present work, an attempt was made to absorb the anti-P2 antiserum more extensively, however, there did not appear to be any enhancement in labeling intensity, or in the specificity for either cell type in the goldfish brain. These results suggest that if the absorbed antiserum recognizes different antigens on both cell types, these are not likely to be common to the tissues used as absorbant and therefore, might qualify as central nervous system-specific antigens.

It should be noted that the immunolabeling pattern of both pyramidal cell species seems to differentiate cellular components while labeling the whole cell. For example, in both cases, the nuclei are devoid of label and, in the case of the tectal pyramidal cells, the dendritic arborization extending up to the minute dendrites, is labeled much more intensely than the other parts of the cell. Investigations have suggested that specific proteins may be found in certain subcellular components of the neurons (Levine 1965, Shek and MacPherson 1971, Jones and Matus 1974). Therefore, since in the protocol I used to produce the P2 immunogen, most, if not all of the cell nuclei are expected to be lost in the first pellet in the differential centrifugation procedure (Gray and Whittaker 1962), it is not surprising that the cell nuclei are not immunolabeled. The fact that the dendritic arborizations, of both cell types were intensely labeled, suggests either that the epitope is enriched in dendritic arborizations, that the absorbant used did not possess the epitopes that are usually found in dendrites, or that the dendritic antigen is

one of the most immunogenic in the P2 pellet.

Close inspection of the native antiserum, which must contain reactivities to pyramidal cells in the brain, does not indicate reactivities against these cell types but instead, diffuse reactivity patterns in most of the tissues tested. This suggests that the absorption procedures enhanced , the relative concentration of these antibodies manifold with respect to the crude serum. However, the antigens recognized by the absorbed antiserum, even though it is expressed strongly on 2 specific types of goldfish brain neurons and on retinal cells, and weakly, or not at all in other tissue tested (there is some light fluorescence in the liver and punctate type of labeling in the optic nerve) may not be as specific as it seems. For example, the destruction of some epitope by fixation could easily make such antigens appear as neuron-specific (for example if non-neuronal tissues were particularly sensitive to the chemical modifications involved in paraformaldehyde fixation). Thus, one must be cautious in interpreting such data based solely on immunohistochemical results. For this reason, it will be imperative in the future to determine the molecular weight of the antigens carrying the relevant epitopes and use this information to isolate them and raise more specific polyclonal antisera.

AFFINITY PURIFIED ANTIBODIES

Both antisera were reacted with immunoblots containing goldfish brain proteins and, antibodies specific to some protein bands were eluted, concentrated, and then re-assayed on blots. In both cases, the eluted

antibodies all cross-reacted with a protein band different from the one they were eluted from. For example, the eluted antibodies from the anti-membrane antiserum all cross-reacted strongly with a band migrating as a doublet with apparent molecular weight of 84Kd; a similar pattern was reproduced with the eluted bands from the anti-P2 antiserum, but this time, the cross-reactivity was directed to a band at 72Kd. These surprising results are difficult to interpret. These cross-reacting bands could not be breakdown products of a high molecular weight protein because, in some instances, the antibodies were eluted from a protein at a lower molecular weight than the one of the cross-reacting band. Therefore, I presently have no evidence suggesting the presence of degradation products and must provisionnaly conclude that the purified antiserum may not be protein-specific. Alternatively, an epitope common to many proteins might be recognized by the antibody. In that case, absorption with any of the cross-reacting proteins (which contain the epitope recognized by the antibody) should completely deplete the antibody and therefore, no further reactivity to any of the proteins should be observed. This was the case when the P41 antibody (an antibody present in the anti-P2 antiserum that was eluted from a blotted band at 41Kd) was absorbed with the affinity-purified 41Kd protein and then re-assayed on a blot containing brain, viscera and muscle proteins: no reactivity was obtained with any of the protein bands. These results suggest that at least one epitope recognized by the P41 antibody was also present on the 72Kd protein and that is probably the reason why cross-reactivity with this band was observed.

Whatever the epitopes recognized by the P41 antibody might be, there is little doubt that they are constituents of many goldfish proteins, such as the 41Kd and 72Kd brain proteins, the 72Kd visceral protein and the 72Kd and 36Kd muscle proteins since all of these proteins were shown, on

immunoblots, to cross-react with the P41 antibody. However, some of these proteins (for example, 72Kd in brain, muscle and viscera, 41Kd in brain and muscle) and also the 84Kd brain protein (which cross-reacts with all of the eluted antibodies from the anti-membrane antiserum) were bound by antibodies present in the pre-immune serum and thus are normally circulating antibodies (albeit of low titer) produced by mice. Moreover, these antibodies (present in the pre-immune serum) were produced in immunized BALB/c mice, primarily against these goldfish proteins which apparently share an epitope with each other (see above), and this might be due to a genetic tendency towards the production of this specific antibody in the BALB/c mouse strain (ie. BALB/c mice may be high responders for the epitope recognized). For example, in a number of cases where an antigen induces a strong immune response (such as the response to type C streptococcal carbohydrates in rabbits - see Roitt 1982), breeding experiments (see Moller 1978) have shown that the capacity to produce a specific B-cell clone is inherited and is linked to the genetic markers for the immunoglobulin constant region. On the other hand, the existence of antibodies against goldfish brain, muscle and viscera protein epitopes in the serum of a non-immunized mouse, as I observed, might be the consequence of autoimmunity in BALB/c mice. Many instances are known (see Roitt 1982) in which potential autoantigenic determinants are present on an exogenous cross-reacting antigen which provides the new carrier to provoke autoantibody formation. For example, some microorganisms carry determinants which cross-react with human proteins (see Glynn and Holborow 1964) and this may be an important way of inducing autoimmunity diseases in humans.

A very striking result of my investigations on the P41 antibody is that the antigens it recognizes are found in all tissues tested (such as optic nerve,

optic tectum, muscles, testes, intestines, liver and spleen) predominantly in areas which may indicate a general association with the extracelullar matrix components of these various tissues. Some studies suggest that the recognition of many proteins by epitope-specific antibodies is not an uncommon occurrence: the possibility for the existence of such shared antigenic determinants is easily imagined in view of the evidence that evolution has proceeded in part by gene duplication and then sequence divergence (Zubay 1986). Therefore, the relationship between non-nervous tissues and the brain tissue might be more than coincidental since, in addition to cell-type or tissue-specific antigens, shared classes of antigens may characterize the cells with a common lineage, such as neural retina or brain cells, another class of antigens may be shared, for example, by cells of mesenchymal origin (eg.: various connective tissues). An example of sequence homologies between different proteins is given with anti-intermediate filament (anti-IF), an antibody against all intermediate filament proteins (Pruss et al. 1981) which recognizes an epitope that these proteins have in common. In the goldfish, this antibody recognizes proteins at 145Kd, 80Kd, 58Kd, and 50Kd in the brain (Quitschke et al. 1985), while in mammalian brains it recognizes proteins at 200Kd, 150Kd, 70Kd and 50Kd (Hoffman and Lasek 1975). These correspond to both neurofillament subunits and gliofilament proteins. The anti-IF antibody is specific then for an antigenic determinant common to all intermediate filaments, which is conserved through evolution. This type of behaviour has also been observed with adhesion molecules that share a class of epitope determinants, namely the L2/HNK-1 class (Kruse et al. 1984).

The immunohistochemistry results, where the P41 antibody was assayed on different tissues, indicate that it may recognize antigens associated with

the extracellular matrix (ECM). In the muscle, the antigen recognized is distributed over the entire cell surface of myotubes. Some of the antigens are also associated with the blood vessels and the pial surface in the goldfish central nervous system. Finally, I observed an association with the epithelial lining of the seminiferous tubules and of the intestines (lamina propria). The recognition of extracellular matrix proteins by the P41 antibody might be indicated first by the similarity of labeling pattern in the goldfish skeletal muscle, optic nerve and tectum, with anti-laminin (laminin being an ECM protein). Moreover, Fambrough et al. (1982) have raised an antibody (#33) against chick muscle extracellular matrix which recognizes epitopes over the surface of myotubes in the same manner that P41 labels goldfish muscle cells. Their antibody also recognizes blood vessels in various chick tissues including brain (Fambrough et al. 1982). Secondly, the labeling pattern at the level of the seminiferous tubules with P41 can also be compared with that of laminin and is found in the epithelium (but not in association with sperm cell membranes) where the extracellular matrix is usually secreted (Wheater et al. 1982). Although P41 has a labeling pattern and distribution similar to these 2 antibodies against extracellular matrix (#33 and anti-laminin), the possibility that it might be recognizing a cellular membrane constituent, must still be borne in mind since Fambrough et al. (1982) remarked that the extracellular matrix (especially the basal lamina component of the extracellular matrix) is in close apposition to the cell membrane and that it is sometimes difficult, at the light microscopic level, to judge whether an antigen is present on the membrane or in the extracellular compartment.

Interestingly, cells in the ependymal zone of the goldfish optic tectum are also very densely stained with the P41 antibody. Cells in this area (ependymal cells) have been described in the goldfish (Schmidt and Lapp

1987, Königstorfer et al. 1989) as the cells involved in brain plasticity and also, it was found (Shashoua et al. 1986) that these cells bear on their surface, cell adhesion molecules (ependymins) that expresses the L2/HNK-1 (the family of proteins which express this epitope, includes other cell adhesion molecules such as L1, N-CAM and MAG, Kruse et al. 1984). Moreover, Werz and Schachner have recently (1988) demonstrated that components of the ECM can be implicated in cell-to-cell adhesion and that these are often found in association with cell adhesion molecules. Therefore, these results might add immunological support to the concept of common lineage between cells of different tissue origins through a shared epitope.

Immunohistochemical observations of the skeletal muscle demonstrated that purification of the P41 antibody (by blot elution) could deplete the native anti-P2 antiserum of background labeling in some muscle cells. This diffuse cell-specific background labeling with the native anti-P2 antiserum seen in about one half of the myofibers may be attributed to the recognition of myoglobin (or a protein associated with myoglobin) as it is known that the so-called red muscle fibers contain large amounts of myoglobin, as opposed to the white muscle fibers, and that both cell types are present in an individual skeletal muscle (Nauta and Feirtag 1986). However, the P41 antibody could still recognize, apart from the extracellular matrix (or membrane) component, an intracellular component in all muscle cells which, in a cross-section of the skeletal muscle, appeared as a punctate network over the sarcoplasm. This observation precludes the identification of the labeled antigen as a myofibril component (A, H, M and Z bands) because, in such a case, a homogeneous type of labeling of the sarcoplasm (see Leeson and Leeson 1976) would be expected.

A possibility which must be borne in mind is that many cells are capable of internalizing substantial portions of their plasma membranes (Heuser and Salpeter 1979). Therefore, another explanation for the intracellular location of the epitopes recognized by the P41 antibody in skeletal muscles is that it might be recognizing a protein constituent of the sarcoplasmic reticulum (which spans the whole cross-section of the muscle cell, as opposed to the Golgi apparatus or mitochondria for example that reside next to the nuclei), or even a protein being processed in the sarcoplasmic reticulum (ie. a pre protein - or pre-pro-protein, see Olsen 1983) for export to the cell periphery, from where it could be either expressed on the cell surface or secreted into the extracellular matrix pool. Support for this hypothesis is given by the observation of Fambrough et al. (1982) that intracellular pools of newly-synthesized fibronectin (an extracellular matrix protein) could be visualized after fluorescent labeling of permeabilized myotubes using a monoclonal antibody against fibronectin. These pools appeared to be localized in perinuclear granules (therefore presumably in the Golgi apparatus). Interestingly, labeling at the level of the sarcoplasmic reticulum, might suggest the possibility that the 72Kd protein recognized on blots after elution of the antibody binding to the 41Kd protein, could be a preprotein of the 41Kd protein final product.

Therefore, the intracellular labeling I observed in the skeletal muscle, might represent extracellular matrix (or membrane) labeling in this tissue because the P41 antibody might recognize both a final secreted (or expressed) protein, as well as an intracellular translation product being processed in the sarcoplasmic reticulum. However, a definitive decision in this regard will most likely require electron microscopy.

CONCLUSION

In conclusion, two immunological markers were obtained by purification of an antiserum against goldfish brain membranes. In the first instance, absorption of the antiserum with fixed skeletal muscle and viscera from the goldfish revealed a specificity for two types of pyramidal cells, namely the tectal pyramidal cells and the Purkinje cells, in the goldfish brain. The second immunological marker produced was obtained from blot elution of an antibody (P41) that binds a 41Kd goldfish brain protein. In that case, the antibody cross-reacted with a 72Kd protein present in goldfish brain, viscera and skeletal muscles and might therefore bind an epitope constituent of many proteins widely distributed throughout the goldfish nervous and non-nervous tissues. The immunolabeling pattern of the P41 antibody suggests a general association with extracellular matrix material, as well as specific binding to cells in the ependymal layer of the goldfish optic tectum, and to the intracellular muscle compartment.

It is not possible to definitely conclude, from these preliminary results, whether or not novel goldfish brain proteins have been defined. However, none of the previously-described antigens recognized by heterologous anti-goldfish antiserum bears similarity to the specificities revealed by the absorbed antiserum or the P41 antibody reported here (Benowitz and Shashoua 1977, Roots et al. 1984, Sharma et al. 1988). Therefore, this may be a first attempt at isolating tectal pyramidal cell and Purkinje cell specific markers as well as putative extracellular matrix markers in the goldfish.

The objective here, was to obtain an immunological probe against cell adhesion molecules in the goldfish brain. This is a difficult task to undertake when using minimally-purified immunogens to produce a polyclonal antiserum. Nevertheless, brain cell-specific reactivity was obtained with the absorbed antiserum. However, efforts still need to be made towards the complete absorption of the native antiserum in order to better define the tectal pyramidal cell and the Purkinje cell protein constituents recognized since it seems likely that new antisera might make it possible to distinguish each cell type from the other.

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