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# Distribution of a brain-specific extracellular matrix protein in developing and adult zebrafish

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# Distribution of a brain-specific extracellular matrix protein in adult zebrafish.

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**Running Title:** Brain-specific ECM protein in teleosts

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# SUMMARY

A monoclonal antibody (IgG) that recognizes a 53 kDa zebrafish brain protein was isolated and used to characterize the distribution of this protein in zebrafish. (1) The antigen was found only in the brain and not in any other tissues such as muscle, dermis, and cartilage. Within the brain, the antibody recognized fibrous structures in the extracellular matrix (ECM) outside neuronal cells but did not stain either neuronal bodies or glial cells. (2) The pattern of antigen distribution is not perineuronal, as the density of the antigen at the periphery of the cells was practically identical to that of the empty intercellular spaces. Therefore, this monoclonal antibody does not recognize the perineuronal glycocortex. (3) The antigen is distributed only in limited areas of the brain, namely in the periphery of the forebrain, the hypothalamus, the optic tectum, the interpeduncular nucleus, the cerebellum and the ventricular rim of the medulla. In the optic tectum, the antibody strongly stained the most superficial layer, and in the cerebellum, it stained the molecular but not the granular layer. The expression of the K-54 antigen does not depend on scar formation nor on regeneration of the brain as it exists in the normal adult zebrafish brain. These patterns of distribution are very different from those of other typical brain ECM proteins and suggest that this protein may play quite distinct roles in brain development and maintenance.



**Keywords:** zebrafish, extracellular matrix, monoclonal antibody, brain-specific ECM

# INTRODUCTION

The extracellular matrix (ECM) is involved in various aspects of brain development (Venstrom and Reichardt, 1993), including nerve regeneration (Masuda-Nakagawa and Nicholls, 1991; Bignami et al., 1991; Tona et al., 1993), migration of neuronal cells, axonal outgrowth (Kafitz and Greer, 1998; Pires-Neto et al., 1999), and cell-to-cell signaling (Arber et al., 1995). About 20% of the weight of the living brain consists of extracellular water associated with the brain ECM that fills the empty spaces between cells (Rutka et al., 1988). ECMs are generally composed of many proteins and saccharides, and in the brain the predominant component is thought to be a glycosaminoglycan. This molecule consists of a very long hyaluronan backbone composed of two sugars, glucuronate and N-acetylglucosamine, that associate through proteinaceous linkers with core proteins carrying chondroitin sulfate or keratan sulfate chains. These core proteins - aggrecan, versican, neurocan, brevican - all belong to the lectican family and can be assembled with tenascin into matrices (Asher, 1991; Ruoslahti, 1996). Collagen and erastin are not common in the brain. The functions of the lectican matrices are not yet understood. Many studies have, however, shown that ECM components are heterogeneously distributed in the brain, that expression of particular ECM components varies between the different brain regions (Brückner et al., 1996; Hobohm et al., 1998), and that specific neuronal or glial cells may be responsible for the synthesis of certain ECM proteins in these regions (Soussi-Yanicostas et al., 1996). Murakami et al. (1999) have also proposed a model that describes the way perineuronal sulfated proteoglycans associate with the neuronal cell surface**(Author: it is not clear what is meant by the underlined sentence, perhaps you should describe the hypothesis of Murakami in more detail if it is important)**.

Much of the brain-specific ECM thus far identified is perineuronal, being distributed at the surface of the neuronal cells. These ECM comprise the “glycocortex”**(Author: please check underlined sentences for accuracy)**. Little, however, is known about those ECM components that evenly fill the empty spaces between the brain cells. In the present study, we describe a 53 kDa protein that is expressed in the zebrafish brain. We have characterized the distribution of this protein in the zebrafish body and show that it is an ECM component that is evenly distributed in the interneuronal spaces of the adult zebrafish brain. Its possible functions are discussed.

# MATERIALS AND METHODS

***Preparation of the synaptic membrane fraction from zebrafish brain***

Synaptic membranes from zebrafish brain were prepared by the method used to prepare bovine brain synaptic membrane fractions (Tomizawa, 2000a and 2000b). Zebrafish were maintained at 28.5$^{o}$C under a 14h light and 10h dark cycle (Westerfield, 1995). Before use, adult fish (about 5 or 6

mo. after fertilization) were chilled in ice water, where they entered into a state of suspended animation.

The skulls were then removed from the heads on ice and the isolated brains were homogenized at 0°C in 0.25 M sucrose solution with the protease inhibitors, leupeptin and antipain (1 μg/ml). The homogenate was centrifuged at 2,000 rpm for 2 min at 0°C to remove the nuclear fraction and intact cells, and the supernatant was collected. The pellets were again homogenized in the same buffer, recentrifuged and the second batch of supernatant obtained**(Author: please check underlined words contributed by the editor for accuracy)**. The two supernatants containing the synaptic membranes were combined and then centrifuged at 12,000 rpm for 10 min at 0°C. The pellet containing the crude synaptic membrane fraction) was washed twice with the same buffer and used for the preparation of monoclonal antibodies and in immunoblot studies (Nakayasu, 1991 and 1995).

***Production of hybridomas***

The synaptic membrane fraction from 20 fish was suspended in 0.75 ml of PBS (phosphate buffered saline; 10 mM sodium phosphate buffer, pH 7.3, containing 0.14 M NaCl) and emulsified with the same volume of complete Freund's adjuvant (Nakayasu, 1993). The emulsion was injected into three male Balb/C mice (more than five weeks old) at day one. The same mice were given booster shots consisting of the same amount of antigen emulsified with incomplete Freund's adjuvant on days 15, 29, and 43. Four days after the last injection, spleen cells were prepared and fused with P3U1 myeloma cells using polyethylene glycol (Mortillaro, 1996).

Hybridoma cells were grown in OPTI medium (Gibco) supplemented with 4% fetal calf serum (Sigma) as recommended by the Gibco manual. They were selected in HAT medium (OPTI medium containing 4% fetal calf serum, 5 mM hypoxanthine, 0.02 mM aminopterin and 0.8 mM thymidine) for more than 10 days to remove surviving myeloma cells, after which the hybridoma cells were cloned by limiting dilution in 96-well plates (Falcon). The cells had to be weaned off HAT by mixing the conditioned medium in which the myeloma cells had been grown for a week with the same volume of fresh OPTI medium, otherwise the hybridomas grew very slowly**(Author: please check underlined words for accuracy)**. Detection of positive hybridoma clones was performed by using a micro-scale immunoblot system using the zebrafish synaptic membrane fraction (4 cm x 1.5 mm blot) (see below). We isolated 25 positive clones from one immunized mouse and stored them in liquid nitrogen or used them to prepare ascites (Nakayasu, 1993).

***Preparation of ascitic fluid***

Male Balb/c mice were injected with 0.5ml of pristane (2,6,10,14-tetramethylpentadecane) at least five weeks before the intraperitoneal injection of about $10^7$ hybridoma cells. After 12 weeks, the ascitic fluid was collected, centrifuged at 10,000 x g for 10 min to remove pristane and cellular structures, and stored without further purification of the antibody at –80 °C until use. For working solutions, the ascites was mixed with an equal volume of glycerol and stored at –30°C.

***Immunoblot***

SDS polyacrylamide gel (60 x 40 mm) electrophoresis (SDS-PAGE) and western blotting were carried out according to the methods of Nakayasu (1995). A nitrocellulose filter of the same size was overlaid on the gel and the protein bands were transferred by electroblotting onto the filter at 0.5 A for 30 min. The membrane was blocked in a 10 cm dish on a shaker with 10 mM Tris-HCl (pH 7.4) containing 0.1% Tween 20 (polyoxyethylene sorbitan monolaurate) and 0.14 M NaCl (TBS-Tween).

The monoclonal antibody (ascitic fluid) was diluted with the same buffer at 1:500,000 and incubated overnight at 4$^{o}$C with the filter. The filter was washed four times with TBS-Tween buffer and then incubated with an alkaline phosphatase-conjugated secondary antibody (1:20,000 dilution in TBS-Tween buffer). After washing with TBS-Tween buffer, the antigenic band was visualized using BCIP and NBT as alkaline phosphatase substrates (Nakayasu, 1993). To screen for monoclonal antibodies, small blots (1.5 x 40mm) were incubated in 24 well plates with 100 μl of the culture medium from each well of the 96-well plate**(Author: please check that underlined words are correct)**.

***Protein staining by colloidal gold***

Colloidal gold particles (25 nm) were prepared by boiling 0.01% $HAuCl_4$ containing 0.04% sodium citrate-2$H_2O$ for 15 min (Nakayasu, 1995). After cooling, polyethyleneglycol (0.03%) and Tween 20 (0.05%) were added to stabilize the gold particles. The pH of the solution was then adjusted to 3.0 and the colloidal gold suspension was kept at room temperature. Western blots were blocked and washed twice with distilled water prior to overnight incubation with 10 ml of the colloidal gold solution.

***Immunohistochemistry***

Immunohistochemical studies were performed as previously described (Maeyama, 2000). Briefly, a zebrafish head was fixed with the skull bone at 4$^{o}$C overnight in 4% freshly depolymerized paraformaldehyde. The brain was then carefully removed from the skull and gently washed in 0.1 M sodium phosphate buffer (pH 7.3) containing 4% sucrose, after which it was placed into a 30% sucrose solution until it sank. The brain was then incubated at 4$^{o}$C for 30 min in a 30% sucrose solution containing 30% OCT embedding compound (Tissue Tek) before being embedded in OCT embedding compound, frozen on a cryostat, and cut into 20 μm thick sections. The sections were placed onto glass slides coated with 1.5% gelatin and 0.15% $CrK(SO_4)_2$-2$H_2O$, soaked with PBS (pH 7.3) and dried at 55-60$^{o}$C. The sections were treated for 20 min with 0.3% $H_2O_2$ in PBS (pH 7.3) in order to inhibit endogenous peroxidase activity and then washed well three times with PBS (pH 7.3). This was followed by washing first with PBS/Tween/DMSO (dimethylsulfoxide) solution (PBS, pH 7.3, 0.2% Tween 20 and 1% DMSO) containing 0.1% TritonX-100 and then with the same buffer without Triton X-100. Non-specific protein binding sites were then blocked for 30 min with PBS/Tween/DMSO containing 2% Tween 20. The sections were incubated at 4 $^{o}$C overnight with the ascites diluted (1:5000) in PBS/Tween/DMSO. After washing with this buffer three times, sections were incubated at 4$^{o}$C overnight with peroxidase-conjugated goat anti-mouse IgG antibody diluted (1/5000) in the same

buffer and then washed well for 10 min with PBS/Tween/DMSO containing 0.1 M $PO_4$ (pH 7.3). The slices were pre-soaked for 5 min with diaminobenzidine (DAB)-heavy metal staining solution (0.1 M Tris-HCl (pH 7.4) containing 0.04% DAB, 0.5% DMSO and 0.45% $Ni(NH_4)_2(SO_4)_2$. To this solution $H_2O_2$ was added to yield a final concentration of 0.003%. The enzymatic reaction was stopped by washing first with 0.1 M sodium phosphate buffer (pH 7.3) and then with distilled water. The slices were dehydrated in series of alcohol treatments, cleared in xylene, mounted with Permount and viewed with a Zeiss Axiophot.

Photographs were taken with a CCD camera (Photometrics KAF-1400) using V for Windows (Photometrics), arranged by Photoshop (Adobe, Version 5) and printed by Pictrography 3000 (Fujifilm).

# RESULTS

***Isolation and characterization of monoclonal antibody K-54***

A hybridoma clone producing a monoclonal antibody denoted as K-54 was isolated and twice recloned in 96-well plates to ensure its monoclonality. Immunoblot analyses (Fig. 1) showed that K-54 recognizes only a 53 kDa protein from the adult zebrafish brain. K-54 is an IgG antibody (Fig. 1, lane 4) as no band was seen when anti-IgM secondary antibody was used instead of anti-IgG antibody (Fig. 1, lane 3). If K-54 was not used, bands were not detected in Western blotting (Fig. 1, lane 5).

The band seen in the western blots was sharp. This lack of diffusion indicates that the antigenic protein recognized by K-54 is not an ECM core protein such as neurocan or phosphacan that carry large amounts of sulfated glycosaminoglycans. The molecular weight of the antigen also does not match the molecular weights of any previously identified ECM core or linker proteins.

***Distribution of the antigen recognized by K-54 in zebrafish***

Our preliminary experiments indicated that the antigen is found only in the brain of the zebrafish. To confirm this, sections from various tissues of the adult zebrafish were incubated with K-54. Indeed, the antibody stained the brain but not the other tissues such as skeleton, muscle, or dermis (Fig. 2). The cartilaginous bone of young larva or the hard bone of the adult fish were also not recognized by K-54. Such skeletal tissues are rich in ECM, with cartilaginous bone being composed of chondroitin sulfates and other proteoglycans and adult bone consisting of a collagenous matrix. Basal laminas found around the muscle cells or between epidermis and dermis were also not stained by K-54 (Fig. 2). This was confirmed by using K-54 in immunoblots of proteins prepared from muscle (Fig. 3, lane 1-2) or dermis (Fig. 3, lane 3-4). Thus, the antigen recognized by K-54 is brain-specific and not present in the ordinary ECM found in bone, muscle and dermis.

***Distribution of the antigen recognized by K-54 in the brain***

The distribution of the K-54 antigen in the brain (Fig. 4A) was assessed relative to the location of neuronal cells (Fig. 4C). Neuronal and glial cell bodies were Nissl stained using propidium iodide (PI). This substance preferentially binds to ribosomes, as do other Nissl dyes like toluidine blue. The K-54 antigen was found to localize in the extracellular spaces around these cells, with the densities at the cell surface and in the intercellular spaces being equivalent. This pattern of distribution is quite different from that of proteins in the perineuronal matrix or glycocortex of the neurons, as these antigens are found only at the neuronal cell surface.

The areas occupied by cell bodies were immunonegative and looked like void volume (Fig. 4A). Also observed were immunonegative spaces lacking cells that could be dead neurons or blood vessels. Unlike many other antibodies that recognize ECM antigens, K-54 did not stain the surface of the blood vessel, arachnoid and pia mater. These characteristics of K-54 staining, as depicted by the photographs in Fig. 4 of the coronal section of the hypothalamus, were also noted for other areas of the brain.

***Distribution of the antigen recognized by K-54 in specific domains of the adult zebrafish brain***

While the K-54 antigen appears to be an ECM-like protein found only in the brain, it is not ubiquitously expressed throughout the brain. Figure 5 shows it to be limited to the telencephalon, the optic tectum, the tectobulbar tract, the interpenduncular nucleus, a part of hypothalamus, the outer layer of cerebellum, and the ventral rim of medulla. Neural cell bodies were not stained at any of these locations. The overall distribution of the antigen is shown schematically in Fig. 6. Of the brain areas where the K-54 antigen was found, very strong staining of the cerebellum, the tectum, the ventricular rim of the medulla, and the hypothalamus were observed (Fig. 7). The fish cerebellum (which differs markedly from that of higher vertebrates, being composed of curved and bent molecular and granular cell layers) appears to express the K-54 antigen only in the molecular layer, not in the granular cell layer (Fig. 7, A-D). The molecular layer is a well-known structure that contains numerous non-myelinated parallel fibers emanating from granular cells.

In the hypothalamus, the antibody did not stain the areas of the corpus mamilare (CM) and periventricular hypothalamus that were crowded with many small neurons (Fig. 7 E). It did, however, strongly stain the empty area in the hypothalamus that consists of a little perikaryon and many non-myelinated thin axons.

In the tectum, the antibody stained its surface (stratum marginale) (Fig. 7 F). The structure of the stratum marginale is similar to the molecular layer of the cerebellum as it contains non-myelinated axons derived from granular cells in the torus longitudinalis located just beneath the tectum. The other layer in the deep tectum also stained weakly. However, the spherical neurons found at the bottom of the tectum were completely immunonegative.

## DISCUSSION

***Characterization of a brain-specific ECM protein***

Using the monoclonal antibody K-54 that was raised against the synaptic membrane fraction of the zebrafish brain, we have identified an apparently novel component of the brain ECM. This antigen appears to be found only in the brain and, more specifically, only in the intercellular spaces of the brain, unlike other known ECM components.

Commonly occurring ECM components, namely the collagens and proteoglycans, are found in cartilage and basal lamina. The K-54 antibody did not, however, stain these structures, nor did the ECM in the cartilaginous bone of fish larvae or the adult bone contain the K-54 antigen. Only fibrous materials found outside the neurons were stained. These observations are not altogether unexpected, as Ruoslahti (1996) has previously commented on the fact that common matrix proteins found outside the brain appear to be largely absent from the adult brain.

A notable observation regarding the K-54 antigen is that its distribution in the brain is not pericellular and covering the neuronal cell surface. This contrasts with many of the other ECM components previously detected in the adult brain, as these are usually found to be located near the cell surface but rarely in the intracellular spaces. These latter ECM components mostly consist of hyaluronate chains that are anchored on the surface of the nerve cells and associated with core proteins attached by link proteins (Murakami, 1999). The thickness of these pericellular layers is usually less than the diameter of a nerve cell. Thus, the ECM component detected by the K-54 antibody is considerably different in its pattern of distribution from the typical pericellular glycocortex ECM found in the brain.

In higher vertebrates, ECM protein expression is very active in the young brain, and the relative amount of matrix components is also high compared to the adult brain. However, as the individual grows and matures, the brain gradually loses much of this matrix by proteolysis. It has been previously observed that the overall structure of brain ECM in zebrafish resembles that of the very young brain of higher vertebrates (Ruoslahti, 1996). This may be related to the fact that the fish brain continues to grow after sexual maturation (Maeyama 2000). Even in adulthood the teleost brain contains stem cells that divide continuously right up until the death of the individual. These characteristics are not observed in the higher vertebrates and it is thus believed that the brain ECM of the adult teleost may resemble that found in the young brains of higher vertebrates. We found that the K-54 antibody stained young as well as adult zebrafish brains, although the pattern and density of staining differed somewhat (data not shown)**(Author: please check underlined sentence for accuracy)**.

Expression of the K-54 antigen does not depend upon scar formation, the presence of a tumor, or regeneration of the brain. In higher vertebrates, the ECM produced rapidly during these events. In teleosts, however, the antigen is found in normal adults.**(Author: it is not clear what is meant by this paragraph. Do you wish to emphasize the point that the teleost brain resembles the young brain of higher vertebrates? Please clarify)**

The staining pattern of the K-54 antibody resembles that of lectins like Concanavalin A and Wheat germ agglutinin as these lectins also stain the fibrous network distributed throughout the intercellular

spaces (data not shown). However, unlike the K-54 antibody, these lectins stained not only brain ECM but also ECM outside the brain. In addition, unlike the K-54 antibody (discussed below), they did not stain in a domain-specific manner within the brain.

***Domain-specific distribution of the K-54 antigen within the brain***

Several areas of the brain, namely the cerebellum, tectum, medulla rim and hypothalamus, were stained particularly intensely by the K-54 antibody. There is no obvious structural feature that is common between these areas but it appears that the antibody preferentially stains those areas that contain few nerve cells. The antibody also appeared to particularly stain non-myelinated fibers like those found in the molecular layer in the cerebellum and the outermost layer in the tectum. Crowded areas, like cerebellar granular cell layer, the spherical cell layer in deep tectum or the mamilary body in the hypothalamus, were in contrast poorly recognized.

Considerable tracts of extracellular space in the adult zebrafish brain were clearly not occupied with the K-54 ECM. It is probable that these spaces are filled with other, probably more common brain-specific ECM components. These as yet unidentified ECM components may serve the function of maintaining the structure of the brain, such that the unoccupied areas of the brain are not crushed or collapsed by its own weight. Given the relatively limited distribution of the K-54 antigen, however, it may be that this ECM component may serve a quite different function to that of more common ECM components. Perhaps the K-54 antigen supports the elongation or maintenance of non-myelinated axons. It may also be that this ECM maintains the empty areas in the brain so that space is available for neuronal migration. Supporting this notion are our observations of the very young teleost brain (about 1 week after fertilization), wherein the K-54 antigen was found in the neuron-less periphery of the neural tube (data not shown). At this stage, neurons have not yet migrated to the periphery, although as the brain develops, the neuronal cells gradually migrate to the immunopositive areas. The K-54 antigen may play a role in guiding or supporting such neuronal migration.

Rettig et al. (1988) have previously reported the existence of the NEC1 antigen, which is expressed in the rostral portions of the human brain. Like K-54, their antibody stained the ECM that fill the empty intercellular spaces in specific domains of the brain. The domains being stained appeared to change rapidly during development. It is thus possible that the K-54 antigen is a teleost homolog of NEC1, and we are currently screening our library of monoclonal antibodies to further examine this possibility.**(Author: please check the last sentence of this paragraph. It was not clear what you wished to say with the original sentence. In addition, you did not explicitly state in the orginal sentences of this paragraph that the NEC1 antigen may be equivalent to the K-54 antigen, although this is implied)**

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## REFERENCES

Arber, S., Caroni, P. (1995) Thrombospondin-4, an extracellular matrix protein expressed in the developing and adult nervous system promotes neurite outgrowth. *J. Cell Biol.* **131** 1083-1094.

Asher, R., Perides, G., Vanderhaeghen, J.-J., Bignami, A. (1991) Extracellular matrix of central nervous system white matter: demonstration of an hyaluronate-protein complex. *J. Neurosci. Res.* **28** 410-421.

Bignami, A., Asher, R., Perides, G. (1991) Brain extracellular matrix and nerve regeneration. In: *Plasticity and regeneration of the nervous system* (Timiras, P. S. ed.), pp197-206. New York: Plenum Press.

Brükner, G., Hätig, W., Kacza, J., Seeger, J., Welt, K., Brauer, K. (1996) Extracellular matrix organization in various regions of rat brain grey matter. *J. Neurocytol.* **25** 333-346.

Hobohm, C., Hätig, W., Brauer, K., Brükner, G. (1998) Low expression of extracellular matrix components in rat brain stem regions containing modulatory aminergic neurons. *J. Chem. Neuroanat.* **15** 135-142.

Kafitz, K. W., Greer, C. A. (1998) Differential Expression of Extracellular matrix and cell adhesion molecules in the olfactory nerve and glomerular layers of adult rats. *J. Neurobiol.* **34** 271-282.

Maeyama, K., Nakayasu, H. (2000) Postembryonic neurogenesis in zebrafish (*Danio rerio*) brain: presence of two different systems. *Zoolog. Sci.* **17** 285-294.

Masuda-Nakagawa, L. M., Nicholls, J. G. (1991) Extracellular matrix molecules in development and regeneration of the leech CNS. *Phil. Trans. R. Soc. Lond. B* **331** 323-335.

McComb R. D., Bigner, D. D. (1985) Immunolocalization of monoclonal antibody-defined extracellular matrix antigens in human brain tumors. *J. Neuro-Oncol.* **3** 181-186.

Mortillaro, M. J., Blencowe, B. J., Wei, X., Nakayasu, H., Du, L., Warren, S. L., Sharp, P., Brezney, R. (1996) A hyperphosphorylated form of the large subunit of RNA polymerase II is associated with splicing complexes and the nuclear matrix. *Proc. Natl. Acad. Sci. USA.* **93** 8253-8257.

Murakami, T., Ohtsuka, A., Su, W.-D., Taguchi, T., Oohashi, T., Murakami, T., Abe, K., Ninomiya, Y. (1999) The extracellular matrix in the mouse brain: its reactions to endo-alpha-N-acetylgalactosaminidase and certain other enzymes. *Arch. Histol. Cytol.* **62** 273-281.

Nakayasu, H. (1995) Crystal violet as an indicator dye for nonequilibrium pH gradient electrophoresis (NEpHGE). *Anal. Biochem.* **229** 259-262.

Nakayasu, H., Berezney, R. (1991) Nuclear matrins: Identification of the major nuclear matrix proteins. *Proc. Natl. Acad. Sci. USA.* **88** 10312-10316.

Nakayasu, H., Nishikawa, M., Mizutani, H., Kimura, H., Kuriyama, K. (1993) Immunoaffinity purification and characterization of γ- aminobutyric acid (GABA)B receptor for bovine cerebral cortex. *J. Biol. Chem.* **268** 8658-8664.

Pires-Neto, M. A., Braga-de-Souza, S., Lent, R. (1999) Extracellular matrix molecules play diverse roles in the growth and guidance of central nervous system axons. *Brazilian J. Med. Biol. Res.* **32** 633-638.

Rettig, W. J., Chesa, P. G., Beresford, H. R., Melamed, M. R., Old, L. J. (1988) Definition of an extracellular matrix protein in rostral portions of the human central nervous system. *Brain Res.* **438** 315-322.

Ruoslahti, E. (1996) Brain extracellular matrix. *Glycobiol.* **6** 489-492.

Rutka, J. T., Apodaca, G., Stern, R., Rosenblum, M. (1988) The extracellular matrix of the central and peripheral nervous systems: structure and function. *J. Neurosurg.* **69** 155-170.

Soussi-Yanicostas, N., Hardelin, J.-P., del Mar Arroyo-Jimenez, M., Ardouin, O., Legouis, R., Levilliers, J., Traincard, F., Betton, J.-M., Caban, L., Petit, C. (1996) Initial characterization of anosmin-1, a putative extracellular matrix protein synthesized by definite neuronal cell populations in the central nervous system. *J. Cell Sci.* **109** 1749-1757.

Tomizawa, K., Inoue, Y., Doi, S., Nakayasu, H. (2000a) A monoclonal antibody stains

oligodendrocytes and Schwann cells in zebrafish (*Danio rerio*). *Anat. Embryol.* **201** 399-406.

Tomizawa, K., Inoue, Y., Nakayasu, H. (2000b) A monoclonal antibody stains radial glia in the adult zebrafish. *J. Neurocytol.* **29** 119-128.

Tona, A., Perides, G., Rahemtulla, F., Dahl, D. (1993) Extracellular matrix in regenerating rat sciatic nerve: a comparative study on the localization of laminin, hyaluronic acid, and chondroitin sulfate proteoglycans, including versican. *J. Histochem. Cytochem.* **41** 593-599.

Venstrom, K. A., Reichardt, L. F. (1993) Extracellular matrix 2: role of extracellular matrix molecules and their receptors in the nervous system. *FASEB J.* **7** 996-1003.

Westerfield, M. (1995) Histological methods. In: *The Zebrafish Book, 3.* (Westerfield, M. ed.), pp 8.1-8.12. Oregon: University of Oregon Press.

Wilson, M.T., Snow, D.M. (2000) Chondroitin sulfate proteoglycan expression pattern in hippocampal development: potential regulation of axon tract formation. *J. Comp. Neurol*, **424** 532-546.

## FIGURE LEGENDS

***Fig. 1 Immunoblot analysis of the K-54 monoclonal antibody.***

Lane 1, molecular weight markers; lanes 2-5, membrane proteins of zebrafish brain that were electrophoresed and blotted. The proteins were visualized with colloidal gold (lane 2) or incubated with K-54 followed by anti-mouse IgG (lane 3) or anti-mouse IgM (lane 4) as secondary antibodies. The arrowhead on the right indicates the position of the antigen recognized by K-54 (53 kDa). As a control, the blot was incubated without the monoclonal antibody and then with the two secondary antibodies (lane 5).

***Fig. 2 Distribution of K-54 antigen throughout the zebrafish body.***

Coronal sections of the whole zebrafish body (about 72 hours after fertilization) were prepared and stained with the monoclonal antibody K-54. Only the central nervous system was stained. Section at the level of the ear. b, bone (cartilage or skeleton); c, CNS; d, dermis; p, pharynx; m, muscle; arrow head, chromatophore; *, immunoreactive area. Scale bar = 100 μm.

***Fig. 3 Presence of K-54 antigen in zebrafish muscle and dermis.***

Muscle (lanes 1-2) and dermis (lanes 3-4) were isolated from adult zebrafish and the proteins were electrophoresed and blotted. The membranes were treated as described in the legend for Fig. 1. Lanes 1 and 3, whole protein staining with colloidal gold particles; lanes 2 and 4, immunblots using monoclonal antibody K-54 and alkaline phosphatase-conjugated secondary antibody.

***Fig. 4 Staining of intercellular spaces of the brain by the K-54 antibody.***

The coronal section at the level of hypothalamus was stained with the monoclonal antibody (FITC, green; A and B), then with propidium iodide (PI, red) to identify the cell bodies (B and C) . Scale bar = 50 μm.

***Fig. 5 Distribution of K-54 antigen within different areas of the brain.***

Coronal sections from adult zebrafish were stained with the monoclonal antibody K-54. (A) Coronal section at the level of the tectum; (B) cerebellum. CCe, corpus cerebelli; TeO, tectum opticum; Vam, medial division of valvula cerebelli; TTB, tractus tectobulbaris; Nln, nucleus interpeduncularis; IL, inferior lobe; LCa, lobus caudalis cerebelli. Scale bar = 200 μm.

***Fig. 6 Schematic diagram of K-54 antibody staining of the teleost brain.***

The distribution of the protein recognized by K-54 as revealed by experiments shown in Figure 6. Dark areas, strongly immunopositive areas; gray areas, weakly immunopositive areas. PGZ, periventricular gray zone of optic tectum; Val, lateral division of valvula cerebelli; IRF, inferior reticular formation; DH, dorsal horn; VH, ventral horn.

***Fig. 7 Distribution of K-54 antigen in the cerebellum, hypothalamus and tectum.***

K-54 was used to stain the cerebellum (A-D), the hypothalamus (E) and the tectum (F). In the cerebellum, the antigen is found in the molecular (CCe, LCa, Vam, Val) but not the granular layer. In the hypothalamus, the antibody stained a strongly diffuse nucleus (DIL) and a central nucleus where few neuronal cells were found but not the periventricular hypothalamus (HD) nor corpus mamillare (CM). In the tectum, the antibody stained the surface layer strongly, and also, more weakly, the internal part of the tectum. Scale bar = 200 μm.