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Central Nervous System

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A Periaxonal Net in the Zebrafish Central Nervous System.

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Abbreviated Title: oligodendrocyte extracellular matrix

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ABSTRACT

We produced a monoclonal antibody, named A20, which specifically recognizes a 35 kDa protein and stains myelinated axons in zebrafish brain. The A20 antigen is located at the outside of the myelin layer of large axons, and comprises a fine meshwork composed of thin unit fibers about 1-2 μm in length and about 100-200 nm in thickness. The unit fibers form pentagonal and hexagonal structures, which further polymerize into an envelope structure on the axons.

The A20 monoclonal antibody did not stain neuronal cell bodies nor synapses. Instead, the distribution of the A20 antigen was along axons, practically coincident with the distribution of myelin basic protein. The monoclonal antibody stained only axons in the CNS, and not the extracellular matrix surrounding Schwann cells (Martin 1994). These results suggest that this antigenic meshwork (which we call the periaxonal net) is synthesized by oligodendrocytes.

During the development of the zebrafish brain, the periaxonal net appeared after the formation of myelin on the axons. The periaxonal net developed first at the brain stem, then gradually appeared at the caudal end of the spinal cord. The thickness of the periaxonal net around the Mauthner axon changed during development. Although the thickness of the Mauthner axon continues to grow throughout life, the thickness of periaxonal net stopped growing at six months after fertilization.

INTRODUCTION

In addition to the major components of the nervous system, neurons and glia, there are non-cellular structural elements, collectively named the extracellular matrix (ECM), filling the intercellular space of the brain.

The perineuronal net (Blumcke 1995, Atoji 1997, Marco 1998) is one kind of brain-specific ECM. At first dismissed as an artifact of capricious staining techniques, this perineuronal net is now recognized as a genuine entity in neurocytology. It forms a layer at the surface of a neuronal cell body, but rarely does so on the surface of the axon. The chemical composition of the perineuronal net is not yet known, but it is thought to be composed of proteoglycans and associated proteins. The putative roles of these ECM molecules include the stabilization of synapses and the maintenance of cellular relationships in the adult brain (Hockfield 1983); the generation of a polyanionic, ion-buffering microenvironment (Bruckner 1993); and the prevention of extracellular-space occlusion by the hygroscopic proteoglycans (Carlson 1996).

The extracellular matrix of Schwann cells in the peripheral nervous system (PNS) is also well known. During peripheral nerve development, Schwann cells actively deposit ECM, composed of basal lamina sheets that surround individual axon-Schwann cell units and collagen fibrils. These ECM structures are formed from a diverse set of macromolecules, consisting of glycoproteins, collagens, and proteoglycans. The Schwann cell ECM acts as an organizer of peripheral nerve tissue and strongly influences Schwann cell adhesion, growth, and differentiation, and regulates axonal growth during development and

regeneration of motor and sensory neurons. In contrast, little has been reported regarding the corresponding structure in the central nervous system (CNS), the ECM of oligodendrocytes (Szuchet 2000).

In our laboratory, we have prepared a library of monoclonal antibodies specific to the proteins of the zebrafish brain (Miyamura 2001, Kawai 2001). Zebrafish (*Danio rerio*) is increasingly used as a model animal for neurobiology and embryology. In this paper, we report on one of the monoclonal antibodies in our library, A20, which recognizes a meshwork surrounding myelinated axons. We have named this meshwork the periaxonal net.

MATERIALS AND METHODS

Animals

Zebrafish (*Danio rerio*) were kept and raised according to standard conditions (Westerfield 1995, Tomizawa 2000a, b). Fertilization was carried out according to the conditions listed in “The zebrafish book, 3rd edition” (Westerfield 1995). When embryos were generated by natural spawning, they were raised at 28.5°C in a fresh-water tank; when they were generated by artificial spawning, they were raised first in fish water (Instant Ocean 60 mg/l) and later in the fresh-water tank. The age of the fish was counted in days after fertilization (Maeyama 2000).

Preparation of the membrane fraction from zebrafish brain

Membrane fraction was prepared from zebrafish brains according to our method for the preparation of a bovine brain membrane fraction (Nakayasu 1993, Tomizawa 2001a,b). The zebrafish were maintained at 28.5°C under a common light cycle (14 h light and 10 h dark; Westerfield 1995). Adult fish (about 5 or 6 months after fertilization) were anesthetized in ice water.

The skulls were removed from the heads in a ice box, and the isolated brains were homogenized at 0°C in a solution containing 0.25 M sucrose and the protease inhibitors, leupeptin and antipain (1 µg/ml). The homogenate was centrifuged at 640 x g for 2 min at 0°C to remove the nuclear fraction and any undestroyed cells. The supernatant was collected, and the precipitate was re-homogenized in the same buffer. The supernatants containing the membranes were combined and centrifuged at 10,000 x g for 10 min at 0°C. The precipitate (the crude membrane fraction) was washed twice with the same buffer, and was used for the immunization of animals for antibody production and for immunoblots.

Production of hybridomas

The membrane fraction from 20 fish was suspended in 0.75 ml of PBS (phosphate buffered saline; 10 mM sodium phosphate buffer, pH 7.4, containing 0.14 M NaCl) and emulsified with the same volume of Freund's complete adjuvant (Nakayasu 1993). The water-in-oil emulsion was injected into 3 male BALB/c mice (more than 5 weeks old) on day 1. The same amount of the antigen was emulsified with an incomplete adjuvant and injected into the same mice at day 15, day 29, and day 43. Four days after the final injection, spleen cells from

the mice were prepared and fused with P3U1 myeloma cells using polyethyleneglycol.

The hybridoma cells were grown in OPTI medium (Gibco) supplemented with 4% fetal calf serum (Sigma) according to the method given in 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 from the hybridoma cells, and cloned by a limited dilution method in 96 well plates (Falcon). Usually, conditioned medium, in which the myeloma cells had been grown for a week, was mixed with the same volume of fresh OPTI medium, because otherwise the hybridomas grew very slowly. The detection of a positive hybridoma clone was performed by a micro-scale immunoblot system (1 mm x 40 mm blot) using the zebrafish membrane fraction. These hybridomas were frozen in a liquid nitrogen case or allowed to grow for the preparation of ascitic fluid.

Preparation of ascitic fluid

About 0.5 ml of pristane (2,6,10,14-tetramethylpentadecane) was injected into male BALB/c mice at least 5 weeks before the injection of the hybridoma cells. Positive hybridoma cells (about 10^7 cells) were injected intraperitoneally. After 1-2 weeks, the ascitic fluid was collected and centrifuged at $10,000 \times g$ for 10 min to remove the pristane and cellular structures. The ascitic fluid was stored at -80°C until use, without any more purification of the antibody. The antibody used for the experiments in this report was designated A20. For the working solution, the ascitic fluid was mixed with an equal volume of glycerol and

stored at -30°C .

MBP primary antibody

For immunohistochemical studies rabbit polyclonal anti-human myelin basic protein (MBP) was obtained from YLEM and was used at a 2:3 dilution (Tomizawa 2000a).

Immunoblotting

SDS polyacrylamide gel electrophoresis (SDS-PAGE; 60 X 40 mm gels) and Western blotting were carried out by the methods of Nakayasu (1995). Following electrophoresis, a nitrocellulose filter of the same size as the gel was laid on the gel, and the protein bands were transferred onto the filter at 0.5 A for 30 min. The membrane filter was then blocked by shaking in a solution of 10 mM Tris-HCl (7.4), containing 0.1% Tween 20 (polyoxyethylene sorbitan monolaurate) and 0.14 M NaCl (TBS- Tween) in a 10 cm dish.

The monoclonal antibody (ascitic fluid) designated A20 was diluted with the same buffer at 1:50,000, and incubated with the filter at 4°C overnight. The filter was washed for four times with the same buffer, then incubated with alkaline phosphatase-conjugated secondary antibody (1:10,000-20,000 dilution in TBS-Tween buffer). After washing with the same buffer, the antigenic band was visualized using BCIP and NBT as alkaline phosphatase substrates (Sambrook 1989). For screening the monoclonal antibody, a small blot (1 mm x 40 mm) was incubated in a 24 well plate with 100 μl of the culture medium from the hybridoma cells.

Protein staining by colloidal gold

Colloidal gold particles (25 nm) were prepared by boiling 0.01% HAuCl_4 containing 0.04% sodium citrate- $2\text{H}_2\text{O}$ for 15 min. After cooling, polyethyleneglycol (0.03%) and Tween 20 (0.05%) were added to stabilize the gold particles (Nakayasu 1993). The pH of the solution was then adjusted to 3.0, and the colloidal gold suspension was kept at room temperature. The Western blot was blocked and washed twice with distilled water, and incubated with 10 ml of the colloidal gold solution overnight.

Immunohistochemistry

Immunohistochemistry was performed by the method of Westerfield (1995). Briefly, zebrafish heads were immersion-fixed in the skull with 0.1 M sodium phosphate buffer (pH 7.3) containing 4% freshly depolymerized paraformaldehyde and 4% sucrose at 4°C overnight (Westerfield 1995). The isolated brains were carefully removed from the skull and gently washed in 0.1 M sodium phosphate buffer (pH 7.3), containing 4% sucrose. The brains were sunk in a 30% sucrose solution overnight for cryo-protection, and stored in the same solution at 4°C until sectioning. The brains were embedded in OCT embedding compound (Tissue Tek) and cut into $15\ \mu\text{m}$ semi-thin sections on a cryostat.

The sections were put onto a glass slide coated with 1.5% gelatin and 0.15% $\text{CrK}(\text{SO}_4)_2 \cdot 2\text{H}_2\text{O}$, and dried at $55\text{--}60^\circ\text{C}$. The sections were treated with 0.3% H_2O_2 dissolved in absolute methanol for 15 min in order to inhibit

endogenous peroxidase activity, and washed well with PBS (pH 7.2) three times. Sections were then washed with PBS/Tween/DMSO (dimethylsulfoxide) solution (0.1 M sodium phosphate, pH 7.3; 0.2% Tween 20; and 1% DMSO) containing 0.1% TritonX-100, followed by a thorough washing with the same buffer without Triton X-100. Then non-specific protein binding sites were blocked with PBS/Tween/DMSO, containing 2% Tween 20, for 30 min.

The sections were incubated with the diluted ascites (1:10,000) or a commercially available rabbit polyclonal antibody against MBP (2:3) in PBS/Tween/DMSO at 4°C overnight. After washing with the same buffer three times, the sections were incubated with a peroxidase-conjugated goat anti-mouse IgG antibody (1:5000) or a peroxidase-conjugated anti-rabbit IgG antibody (1:500) in the same buffer at 4°C overnight, and washed well with PBS/Tween/DMSO and with 0.1 M sodium phosphate buffer (pH 7.3) for 10 min. The sections were pre-soaked with diaminobenzidine (DAB)-heavy metal staining solution (0.04% DAB, 0.1 M Tris-HCl (pH 7.4), 0.5% DMSO, and 0.3% $\text{Ni}(\text{NH}_4)_2(\text{SO}_4)_2$ for 5 min. To this solution, H_2O_2 was added at a final concentration of 0.003%. The enzymatic reaction was stopped by washing with 0.1M sodium phosphate buffer (pH 7.3), and then with distilled water, and the sections were dehydrated in a series of alcohols, cleared in xylene, and mounted with Permount. They were viewed with a Zeiss Axiophot.

Photographs were taken with a CCD camera (Photometrics KAF-1400) using V for Windows (Photometrics), arranged in Photoshop 6.0J (Adobe), and printed on a Pictography 3000 (Fujifilm).

Double immunofluorescence

For the double immunofluorescence study, the sections were dried, washed with PBS (pH 7.3), and washed with PBS/Tween/DMSO solution containing 0.1% TritonX-100. Then the sections were washed with the same buffer without Triton X-100, blocked, and incubated with a mixture of the diluted A20 (1:5,000) and MBP (2:3) in PBS/Tween/DMSO at 4°C overnight. After washing with the same buffer, the sections were incubated with a mixture of FITC-conjugated goat anti-mouse IgG antibody (1:100) and Cy3-conjugated anti-rabbit IgG antibody (1:200) for 2 hours at room temperature. After washing with the same buffer several times, they were mounted in Mowiol.

Decalcification

After fixation, the whole body of a zebrafish was washed several times in 0.1 M sodium phosphate buffer (pH 7.3) containing 4% sucrose. Then the tissue was soaked in 0.5 M EDTA (pH 7.3) and incubated for 1 week at room temperature. About 100 ml of the EDTA solution was used for one whole corpus and exchanged every 2 days. Following decalcification and washing with distilled water, the tissue was sunk in 30% sucrose solution overnight and stored in same solution until sectioning.

RESULTS

Production of the A20 monoclonal antibody

Seventy-seven hybridoma clones were immunoblot-positive, and they were cloned twice to yield stable hybridoma lines according to the limiting dilution-culture method. In the end, we obtained 25 positive hybridoma clones from this preparation. The pattern of the protein bands detected on the immunoblots did not change during screenings, indicating that these hybridomas were monoclones. Among the positive clones, an ascitic fluid produced by one hybridoma line (named A20) was used for further research.

Characterization of the A20 monoclonal antibody

As shown in Fig. 1, the A20 monoclonal antibody recognized a single protein band of 35 kDa in zebrafish brain (lane 3). No band was obtained when anti-mouse IgM was used as secondary antibody (lane 4), from which we deduced that the class of the A20 monoclonal antibody is IgG. The detected band was not a secondary-antibody artifact, because there was no similar band in the Western blot when the blots were incubated with anti-mouse IgG and IgM antibody, but without primary antibody (lane 5). As described in the following sections, the monoclonal anti-A20 antibody stained several areas in the zebrafish brain.

Distribution of the A20 antigen on the Mauthner axon

In coronal sections at the level of medulla oblongata, the white matter was preferentially stained (Fig. 2, A, C). In order to detect myelinated axons, alternating sections were stained with an anti-myelin basic protein (MBP) antibody (Fig. 2, B, D). Both antibodies stained the white matter of the medulla

and spinal cord (data not shown), and especially the Mauthner axon in the medial longitudinal fascicle (MLF). As shown in the enlarged pictures (Fig. 2, C, D), the pattern produced by both antibodies was doughnut-shaped, but the size of the doughnut formed by A20 staining (arrow) was larger than that formed by anti-MBP (arrowhead). This result suggests that the myelin layer might be surrounded by a layer of the A20 antigen on the Mauthner axon.

Double staining with these two antibodies (Fig. 3) confirmed the hypothesis mentioned above. The myelin layer (arrow) was structurally distinct from the A20 layer (arrowhead), and little myelin was found in the A20 layer. In the case of the Mauthner axon, these two layers were very thick and easy to see, but a similar relationship between the A20 and myelin layers was observed in the other MLF axons as well (small arrows in Fig. 3). These axons are smaller than the extremely large Mauthner axon, but they are also giant axons compared with ordinary axons outside the MLF. The same relationship between the A20 and myelin layers held true in the case of smaller myelinated axons, but it was very difficult to take pictures of these.

Sagittal sections (Fig. 4) and horizontal sections (data not shown) revealed more detail about the structure of the A20 antigen. There were many short areas of immunopositivity on the Mauthner axon and the other small axons. In some cases, the A20 layer was partially destroyed during the preparation of the sample, and we think the meshwork in Fig. 4 could be such a partially destroyed layer. Sometimes we observed a unit fiber of the A20 antigen, which was a very thin and short filament, about 100-200 nm thick and about 1-2 μm in length (Fig. 4, B). The unit fibers formed a pentagonal and hexagonal pattern, which fit

together to make a lattice, which in turn made up the A20 layer.

Localization of the A20 antigen along the optic nerve

This sort of reticular structure has been seen previously in the perineuronal net. We therefore tried to determine whether the antigen recognized by the A20 antibody was the same as the perineuronal net. We focused on the optic nerve, because the location of the perikarya and the nerve terminals are well known and the axons run in a single direction. The optic nerve fibers emerge from ganglion cells in the retina, and the axons are not myelinated inside the eyeball (Fig. 5, A,B). The fibers pass through the cribriform plate (large arrows in Fig. 5, A, B) after which they become myelinated nerves. MBP immunoreactivity appeared as soon as the optic nerve exited the eye (Fig. 5 B). The A20 antigen was also expressed after the nerve passed through the cribriform plate (Fig. 5 A).

Next, the distribution of the antigen was investigated in the optic tectum, where the fibers of the optic nerve synapse onto neurons of the tectum (Fig. 5, C, D). Optic nerve fibers from the retina enter the optic tectum through the optic tract. A major portion of the optic fibers runs through the second layer (open arrowhead in Fig. 5, C, D) and minor portion through the sixth layer (closed arrowhead), and then turns downward or upward, respectively. The A20 and MBP antibodies both stained the stratum opticum, but neither antigen was found in the deeper layers of the tectum (Fig. 5, F). Thus, the fibers were myelinated only in the stratum opticum, and the A20 antigen was also found only in this layer (Fig. 5, E).

These results indicate that the A20 antigen is not found on the perikarya of the optic nerve nor on its terminals. The A20 antigen was distributed only in the area where MBP, and by extension myelin, covered the axon as illustrated in Fig. 5.

Distribution of the A20 antigen on spinal nerves

Thus far we had found that the distributions of the A20 and MBP antigens were very similar. Therefore, it is likely that the A20 antigen is located on oligodendrocytes. Oligodendrocytes and Schwann cells provide the myelin layers on axons, but the former cells are found only in the CNS and the latter only in the PNS. We therefore asked whether the A20 antigen is present on the myelin layer of Schwann cells. In order to determine this, we immunostained both cranial and spinal nerves. Figures 6 B-D shows that the A20 antigen was located only inside of the spinal cord, and not in the spinal nerves. The staining was found at the dorsal root, but it disappeared in the dorsal root ganglia (asterisk in Fig. 6, C). In case of the ventral root, the A20 antigen was found at the ventral horn, but disappeared where the ventral nerve left the spinal cord (Fig. 6).

In the case of octaval nerve, there was also a clear boundary between the CNS and PNS. The A20 antibody stained areas inside the CNS heavily, but there was no staining in the PNS (Fig. 6, A). Similar results were obtained in cases of the fifth, seventh, and tenth cranial nerves (data not shown). These results indicate that the A20 antigen is expressed only on the myelin layers of oligodendrocytes, and not on those of Schwann cells.

Changes in the A20 antigen during development

We have described the tight relationship between the A20 antigen and the myelin layer of oligodendrocytes. If this is true, then it might be expected that the A20 antigen would be expressed only after the formation of the myelin layer on the axon. We therefore observed the expression of A20 during the development of the zebrafish brain. At seven days after fertilization, the distribution of the A20 antigen was very limited. It was found on the larval Mauthner axon and on some nerves in medulla oblongata (Fig. 7). Coronal sections from the same zebrafish revealed that the antigen was associated with the anterior part of Mauthner axon, but not the posterior part (Fig. 7, F). This result suggests that the A20 antigen first appears at the anterior part of the Mauthner axon, then gradually extends to the posterior section of the axon. In adult fish, the A20 antigen was distributed along the entire length of the Mauthner axon (data not shown). In contrast to the pattern of A20 at 7 days after fertilization (Fig. 8, A), MBP was already expressed along the entire length of the Mauthner axon at this timepoint (Fig. 8, B). At 3 weeks after fertilization, the A20 antigen had appeared on many axons in the larval brain and also at the caudal end of the Mauthner axon (Fig. 8, C, D).

We found that the thickness of the A20 layer gradually increased during development. Figure 9 shows the relationship between the thickness of the A20 layer and the size of the Mauthner axon (the diameter of the axon plus the myelin layer without the A20 layer). The size of the Mauthner axon and the whole length of the brain increased gradually up to 18 months, but the size of

A20 layer did not change after 6 months. The thickness of the A20 layer was measured using the monoclonal A20 antibody and the size of Mauthner axon was measured using the anti-MBP antibody. A two sample t-test was used to compare these two values.

DISCUSSION

The A20 monoclonal antibody recognizes a periaxonal net in the zebrafish brain

In this study, we prepared a hybridoma clone that produced a specific monoclonal antibody, A20, which recognized a protein of about 35 kDa in the zebrafish brain (Fig. 1).

The A20 antigen is present in a thick layer outside the myelin layer that surrounds the axons in the MLF. At first we presumed that the antigen was a component of the perineuronal net, which is known to surround neuronal cell bodies. However, the A20 antigen was not found at the perikaryon, but was located only on the outer layer of myelinated axons. Therefore, the A20 antigen is not a member of perineuronal net, but is a member of a new structural category, which we have named the periaxonal net.

The periaxonal net defined by A20 is also distinct from the network made by Schwann cells, because the A20 antigen was restricted to the CNS and was not seen in PNS, where Schwann cells are located. However, because the

extracellular matrix on Schwann cells is also expressed outside the myelin layer (Chernousov 2000 a and b), there could be some similarity between the two. The ECM of Schwann cells remains after the death of peripheral axons, and this is also true in the case of the periaxonal net. Work from our lab has shown that the A20 antigen and its typical structure are well maintained seven days after cutting the optic nerve in cultured zebrafish brain (Tomizawa 2001a). Therefore, the periaxonal net can exist independently of axons, similar to the Schwann cell ECM.

Although we have not yet directly compared these two ECM networks, it will be very important to do so in the near future. Our periaxonal net should be tested for the presence of common components of the ECM of Schwann cells, including heparin sulfate, laminins, and collagens. In general, the ECM of Schwann cells helps the regeneration of axons in the PNS. However, axons in the CNS are generally not thought to regenerate, so the function or the contribution of the periaxonal net to the axon could be considerably different from the Schwann cell ECM. However, recent reports by Becker (1997, 1998) indicated the regeneration of axon in CNS, if this is the case, the periaxonal net could be contributed the regeneration.

Possible functions of the periaxonal net

What is the function of A20 antigen in the brain? The antigen may possibly act as a road map during the generation of primary neurons, in which case the A20 antigen would interact with surface molecules on other cells. A periaxonal structure acting as a signal molecule has been reported in clusters of

Lazarillo-expressing cells of the grasshopper brain (Simon, 2000). However, not only is the structure of this net very different from that of the A20 antigen, but the expression of A20 begins after myelination (Fig.8), making a role in axonal pathfinding rather unlikely.

It is also possible that the A20 antigen acts to combine bundles of axons. The A20 antigen is found in axon bundles such as the optic nerve, but it is also found on thick axons that are completely separated from other axons, like the Mauthner axon (Fig. 3). Further research will be needed to evaluate A20's potential role in this regard.

Finally, A20 may be acting to regulate the microenvironment during development, since its thickness changes during the first 6 months of life. It could possibly generate a polyanionic and ion-buffering microenvironment. Since axons require a lot of extracellular sodium for their activity, if A20 is anionic and acts as an ion exchanger, it could modulate the ionic equivalence near axons, and therefore their level of depolarization. Hopefully, these questions will be resolved in the near future.

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FIGURE LEGENDS

Fig. 1. Characterization of the A20 antigen

Lane 1, molecular weight markers; lane 2, membrane proteins of the zebrafish brain. The proteins were electrophoresed, blotted, and stained with colloidal gold particles (brown). The blots were incubated with the A20 monoclonal antibody (1:100,000) then with anti-mouse IgG (1:20,000) (lane 3) or anti-mouse IgM (lane 4) as secondary antibodies. The arrowhead on the right indicates the position of the A20 antigen. The molecular weight of this antigen was approximately 35 kDa, and the class was IgG. As a control for this analysis, the blot was incubated with the two secondary antibodies but without the A20 antibody (lane 5).

Fig. 2. Distribution of the A20 antigen and MBP at the level of medulla

Coronal sections of zebrafish brain at the level of medulla were stained with an A20 monoclonal antibody (A, C) or an anti-MBP polyclonal antibody (B, D). (C) and (D) are higher magnifications of (A) and (B), respectively. The white matter was stained with both anti-A20 and anti-MBP antibodies. The staining of the gray matter was weaker. The Mauthner axon (MA, Alfei 1991) was heavily stained in a doughnut-shaped pattern in all sections. The doughnut-shaped staining of A20 in (C, arrow) is considerably larger than that of MBP in (D, arrowhead). Without primary antibody and with secondary peroxidase conjugated antibody, the sections were not stained at all. MLF, medial

longitudinal fascicle. Scale bar = 200 μm (A, B), 50 μm (C, D).

Fig. 3. Distribution of the A20 antigen and MBP on the Mauthner axon

A coronal section at the level of the medulla was double stained with the A20 antibody (A, green) and anti MBP antibody (B, red). (C) is a superimposition of (A) and (B). Arrows indicate the Mauthner axon. The A20 antigen (arrowhead) was localized outside of the myelin layer of Mauthner axon stained by the MBP antibody. Small arrows, the other axons running in MLF. Scale bar = 25 μm .

Fig. 4. The fine structure of the A20 antigen layer

(A) A sagittal section of zebrafish brain at the level of the Mauthner axon was stained with the A20 antibody. The meshwork on the Mauthner axon (m) and the other smaller axons in the MLF is visible. (B) Enlargement of meshwork. The reticular structure (about 1-2 μm in length and about 100-200 nm in thickness) can be seen. Scale bar = 50 μm (A), 2 μm (B).

Fig. 5. Distribution of A20 along the optic nerve

Sections at the level of the optic nerve were stained with the A20 monoclonal antibody (A, C, E) or the anti-MBP antibody (B, D, F). The optic nerve leaves the eye through the hole of cribriform plate (large arrow in A, B). Staining by both antibodies was observed only outside of the cribriform plate (arrowheads in A, B).

(C-F) Sagittal sections of optic tectum stained with the A20 antibody (C, E)

or anti-MBP antibody (D, F). (E, F) Higher magnification of the surface of optic tectum. Immunostaining was observed in the stratum opticum (second layer). Although the optic nerve fibers extend to the deeper layers (see illustration), neither antibody stained these deep fibers (E, F). The illustration shows the distribution of the myelin and A20 antigen on the neuron. The reticular structure covers only the axon and not the soma or the synapse. Open arrowhead in C-F, the second layer; close arrowhead in C, D, the sixth layer of the tectum. t, tectum; c, cerebellum. Scale bar = 150 μ m (A, B), 250 μ m (C, D), and 50 μ m (E, F).

Fig. 6. Localization of the A20 antigen in cranial and spinal nerves

A coronal section of zebrafish brain at the level of the octaval nerve was stained with the A20 antibody (A). The box in the illustration in left shows the octaval nerve. The immunoreactivity changed distinctively at the transition point between the CNS and PNS (arrow). The PNS was not stained.

A sagittal section of spinal cord was stained with the A20 antibody (B-D). (C, D) Higher magnifications of B. The staining was limited within the CNS to areas such as the spinal cord (s) and the basement of the dorsal root (open arrow in B and C). The ventral roots (closed arrow in B and D) and the dorsal root ganglia (asterisk) were not stained. Convex staining on the dorsal side was observed in the dorsal horn (C). However, no immunostaining was observed after the axons of the ventral horn passed out of the spinal cord (D). Arrowhead in B and C, melanocytes (not immunostaining). s, spinal cord; m, muscle. Scale bar = 250 μ m (A), 50 μ m (B), 100 μ m (C,D).

Fig. 7. Distribution of the A20 antigen in brain at 7 days after fertilization

Coronal section of a larva at seven days after fertilization (C-F). The black spots at the roof of the larva are melanocytes. A sagittal section of a zebrafish larva stained with A20 antibody at seven days after fertilization (A). Coronal sections of the larva were prepared at the planes indicated in this figure (C-F). (B) A sagittal section at 21 days after fertilization stained with the A20 monoclonal antibody. The numerous black spots on the surface of the larva are melanocytes (open arrowhead). Closed arrowhead indicates Mauthner axon. t, tectum; c, cerebellum, and f, facial lobe.

The A20 antigen first appeared on the rostral portion of the Mauthner axon (arrowheads in C-E). There was no staining at the caudal portion of the Mauthner axon (F) at this stage of the development. Scale bar = 250 μ m (A, B), 50 μ m (C-F).

Fig. 8. Distribution of the A20 antigen in the brain at 21 days after fertilization

A coronal section of caudal spinal cord in zebrafish larva at seven (A, B) and 21 days (C, D) after fertilization. The A20 antigen had not appeared at seven days (A), when MBP was already expressed on the Mauthner axon (B). At 21 days after fertilization, both antigens appeared on the Mauthner axon and on additional axons in the dorsal white matter. Scale bar = 50 μ m

Fig. 9. Development of periaxonal net during development

The thickness of the periaxonal net was measured at one month, 6 months, and about 18 months after fertilization (larva, adult, and old, respectively). The periaxonal net rapidly grew thicker during early development, but the thickness did not change between 6 and 18 months after fertilization. In contrast, the overall size of the brain and the thickness of the axon continued to increase during this period. Measurements were made in 23, 61, and 92 fish for the larva, adult, and old zebrafish Mauthner axons, respectively.