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REGULAR ARTICLE

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Zonal distribution of Purkinje cells in the zebrafish cerebellum: analysis by means of a specific monoclonal antibody

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Abstract We have isolated a monoclonal antibody that recognizes a 42-kDa protein from adult zebrafish brain. The antibody stains the typical drop-shaped perikaryon of Purkinje cells and their dendrites. The cerebellum of teleosts has complex features. It is composed of three parts; the valvula cerebelli (Va), the corpus cerebelli (CCe), and the crista cerebellaris (CC). In higher vertebrates, the molecular layer is always found as the most outer layer of the cerebellum, but in teleosts, some of the granular cells are located on the surface of the Va. In higher vertebrates, the boundary between the granular and molecular layers always contains Purkinje cells, but this does not occur in teleosts. The Purkinje cells are found only in a part of the boundary in Va. We have found that the layer containing Purkinje cells forms a continuous zone in the cerebellum in the zebrafish. The complex structure of the cerebellum is more easily understood with the aid of the concept of a "Purkinje zone". The Purkinje zone starts at the caudal end of Val (lateral division of Va), turns at the edge of Va toward Vam (medial division of Va), connects to CCe, and ends at the bottom of CCe. The dendrites are found only on one side of the zone. The dendrites of the Purkinje cells in Vam are planar and are packed regularly, similar to those of higher vertebrates. However, the dendrites in Val and the posterior part of CCe are not planar and are irregularly packed.

Keywords Purkinje cells · Dendrites · Cerebellum · Immunohistochemistry · Zebrafish, Danio rerio (Teleostei)

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Introduction

The cerebellum in lower vertebrates is different from that in mammals (Sarnat and Netsky 1974; Pouwels 1978). It is not found in amphioxus but is detectable in the most primitive vertebrates, such as myxinoids (Sarnat and Netsky 1974). In these animals, the cerebellum is mainly lateral and vestibular and distributed along the fourth ventricle. Basically, it is composed of three parts; the valvula cerebelli (Va), the corpus cerebelli (CCe), and the crista cerebellaris (CC; Alonso et al. 1992; Finger 1983; Maeyama and Nakayasu 2000; Meek and Nieuwenhuys 1991). The CC, which receives inputs from the vestibular apparatus, is believed to be the most primitive part of the cerebellum (Finger 1983).

The CCe is homologous with the vermis of higher vertebrates, and the Va is thought to be related to the flocculi (Sarnat and Netsky 1974). Usually, the Va penetrates into the midbrain but does not connect to structures there, such as the tectum.

In higher vertebrates, the molecular layer is located at the surface of the cerebellum and, beneath this, is the granular cellular layer. Large drop-shaped Purkinje neurons are present between the two layers. The tightly packed Purkinje neurons are found throughout the boundary.

These basic features of the cerebellum are not present in teleosts. Some of the granular cells are located on the surface of the Va (Wullimann et al. 1996) and CCe, and the boundary between the molecular layer and the granular layer is very complex. Therefore, the relationship between the two could be different from that in higher vertebrates.

We have isolated a monoclonal antibody that recognized Purkinje-like cells in zebrafish. This antibody has revealed the presence of a zonal distribution of the Purkinje-like cells. Although the zone is curved and twisted in a complex manner throughout the cerebellum, the distribution of the Purkinje-like cells is restricted to within the zone and their dendrites extend vertically from this zone. The dendrites are located only on one

side of the zone, and there is no extension into the other side, where the granular cells were found. The identification of the Purkinje zone should enable a better understanding of the complex structure of the zebrafish cerebellum.

Materials and methods

Preparation of synaptic membrane fraction from zebrafish brain

Synaptic membranes from zebrafish brain were prepared according to the method for the preparation of bovine brain synaptic membrane fractions (Tomizawa et al. 2000a, 2000b). Zebrafish were maintained at 28.5°C under an established cycle of 14 h light and 10 h dark (Westerfield 1995). Adult fish (about 5 or 6 months after fertilization) were chilled in ice water in which they entered into a state of suspended animation or were anesthetized according to Westerfield (1995). This treatment was approved by our animal care committee.

The skulls were 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 640 *g* for 2 min at 0°C to remove the nuclear fraction and intact cells, and the supernatant was collected. The pellets were homogenized once more in the same buffer. The combined supernatant containing synaptic membranes was then centrifuged at 10,000 *g* for 10 min at 0°C. The pellet (crude synaptic membrane fraction) was washed twice with the same buffer. This fraction was used for the preparation of monoclonal antibodies and in immunoblot studies (Nakayasu and Berezney 1991; Nakayasu 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 Freund's complete adjuvant (Nakayasu et al. 1993). The water in oil emulsion was injected into three male Balb/c mice (5 weeks old) at day one. The same amount of antigen was emulsified with an incomplete adjuvant and injected into the same mice at days 15, 29, and 43. Four days after the final injection, spleen cells were prepared and fused with P3U1 myeloma cells by using polyethylene glycol (Mortillaro et al. 1996).

Hybridoma cells were grown in OPTI medium (Gibco) supplemented with 4% fetal calf serum (Sigma) according to 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, and the hybridoma cells were cloned by a limited dilution method in 96-well plates (Falcon). Usually, a conditioned medium, in which the myeloma cells had been grown for a week, was mixed with the same volume of fresh OPTI medium, otherwise the hybridomas grew very slowly. The detection of positive hybridoma clones was performed with a micro-scale immunoblot system by using the zebrafish synaptic membrane fraction (4 cm × 1.5 mm blot). We isolated 25 positive clones from one immunized mouse. These hybridomas were stored under liquid nitrogen or used for the preparation of ascitic fluid (Nakayasu et al. 1993).

Preparation of ascitic fluid

About 0.5 ml pristane (2,6,10,14-tetramethylpentadecane) was injected into male Balb/c mice at least 5 weeks before the injection of hybridoma cells. Positive hybridoma cells (about 10^7 cells) were injected intraperitoneally. After 12 weeks, the ascitic fluid was collected and centrifuged at 10,000 *g* for 10 min to remove pristane and other cellular structures. Ascitic fluid was stored at –80°C until use without further purification of the antibody. For working solutions, the ascitic fluid was mixed with an equal volume of glycerol and stored at –30°C.

Immunoblotting

SDS polyacrylamide gel (60×40 mm) electrophoresis (SDS-PAGE) and Western blotting were carried out following 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 with 10 mM TRIS-HCl (pH 7.4), containing 0.1% Tween 20 (polyoxyethylene sorbitan monolaurate) and 0.14 M NaCl (TBS-Tween) in a 10-cm dish on a shaker.

The monoclonal antibody (ascitic fluid) was diluted with this buffer at 1:500,000 and incubated with the filter at 4°C overnight. The filter was washed four times with the same buffer and then incubated with an alkaline-phosphatase-conjugated secondary antibody (1:20,000 dilution in TBS-Tween buffer). After being washed with the same buffer, the antigenic band was visualized by using bromochloroindoxyl phosphate and nitroblue tetrazolium as alkaline phosphatase substrates (Nakayasu et al. 1993). In screening for monoclonal antibodies, small blots (1.5×40 mm) were incubated in 24-well plates with 100 µl of the culture medium from the 96-well plate.

Protein staining by colloidal gold

Colloidal gold particles (25 nm) were prepared by boiling 0.01% $HAuCl_4$ containing 0.04% sodium citrate.$2H_2O$ at 100°C 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 keep at room temperature. The Western blot was blocked, washed twice with distilled water, and then incubated with 10 ml colloidal gold solution overnight.

Immunohistochemistry

Immunohistochemical studies were performed as described by Maeyama and Nakayasu (2000). Briefly, a zebrafish head was fixed with the skull bone in 50% ethanol containing 4% freshly depolymerized paraformaldehyde at 4°C overnight, washed with 50% ethanol for 15 min, 30% ethanol for 10 min, 5% ethanol for 15 min, and then with PBS for 15 min. The brain was carefully removed from the skull, gently washed in 0.1 M sodium phosphate buffer (pH 7.3) containing 4% sucrose, and placed into a 30% sucrose solution until it sank. The brain was stored in a 30% sucrose solution containing 30% OCT embedding compound (Tissue Tek) at 4°C for 30 min. The brain was embedded in OCT embedding compound, frozen on a cryostat, and cut into sections of about 20 µm in thickness.

The sections were placed onto glass slides coated with 1.5% gelatin and 0.15% $CrK(SO_4)_2.2H_2O$, soaked with PBS (pH 7.3), and dried at 55–60°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 then washed well with PBS (pH 7.3) three times. This was followed by washing first with a PBS/Tween/DMSO (dimethylsulfoxide) solution (PBS pH 7.3, 0.2% Tween 20, and 1% DMSO) containing 0.1% Triton X-100 and then with the same buffer without Triton X-100. 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 ascitic fluid (1:5000) in PBS/Tween/DMSO at 4°C overnight. After being washed with this buffer three times, sections were incubated with peroxidase-conjugated goat anti-mouse IgG antibody (1/2000) in

the same buffer at 4°C overnight and then washed well with PBS/Tween/DMSO and with 0.1 M sodium phosphate buffer (pH 7.3) for 10 min. The slices were pre-soaked with diaminobenzidine (DAB) in heavy metal staining solution (0.1 M TRIS-HCl, pH 7.4), containing 0.04% DAB, 0.5% DMSO, and 0.45% $Ni(NH_4)_2(SO_4)_2$ for 5 min. H_2O_2 was added to this solution to give a final concentration of 0.003%. The enzymatic reaction was stopped by washes in 0.1 M sodium phosphate buffer (pH 7.3) and then in distilled water. The slices were dehydrated in series of alcohols, cleared in xylene, mounted with Permount, and viewed with a Zeiss Axiophot. Photographs were taken with a charge-coupled device camera (Photometrics KAF-1400) by using V for Windows (Photometrics), arranged by Photoshop (Adobe, Version 6), and printed on a Pictrography 3000 (Fujifilm).

Results

Characterization of M1 monoclonal antibody

We isolated a hybridoma clone that produced a monoclonal antibody that we named M1. After isolation of the hybridoma, recloning in 96-well plates was carried out twice to ensure that the hybridoma was a single clone. Immunoblot analyses (Fig. 1) showed that the monoclonal antibody recognized only one protein band of about 42 kDa in size on Western blots prepared from proteins from adult zebrafish brain. The nature of the antigen is unknown. The monoclonal antibody belongs to the IgG class (Fig. 1, lane 4), and no band was seen when anti-mouse IgM secondary antibody was used instead of anti-IgG antibody (Fig. 1, lane 3). There was no detectable band on the Western blots without primary monoclonal antibody (Fig. 1, lane 5).

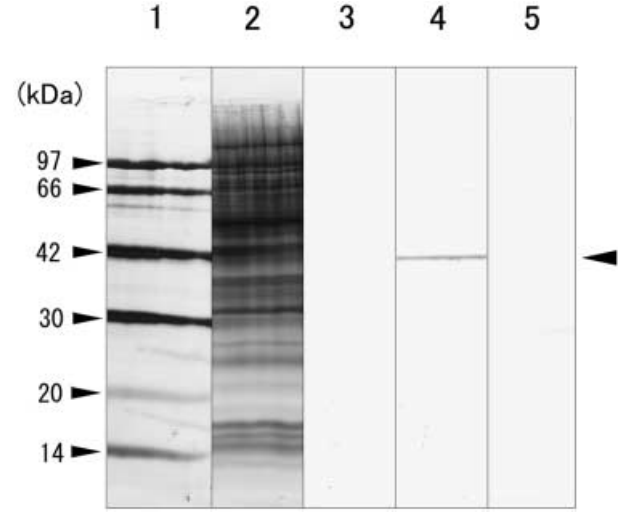


Fig. 1 Immunoblot analysis with M1 monoclonal antibody. *Lane 1* Molecular weight markers, *lane 2* membrane proteins of zebrafish brain. The proteins were electrophoresed, blotted, and stained with colloidal gold. The blots were incubated with M1 monoclonal antibody and then with anti-mouse IgM (*lane 3*) or anti-mouse IgG (*lane 4*) as secondary antibodies (*arrowhead right* position of the M1 antigen, 42 kDa). As a control for this analysis, the blot was incubated without the M1 antibody and then with the two secondary antibodies (*lane 5*)

Monoclonal antibody stains Purkinje-like cells in zebrafish brain

Thin sections of zebrafish brain were stained with the M1 monoclonal antibody. As shown in Fig. 2, the molecular layer of the cerebellum was heavily stained, as were large neurons between the molecular layer and granule cell layer. However, the granule cells were completely free of staining. Other areas in the brain, such as the forebrain, midbrain, medulla, and spinal cord were not stained or very weakly stained. This antibody also stained the surface of the tectum, but the reason for this is not yet clear.

The antibody M1 bound to cells that were very similar to Purkinje neurons (Fig. 3). The stained cells were large and drop-like. In addition, the large cells were stained with anti-calbindin D28 K antibody used for the identification of Purkinje neurons in higher vertebrates. The cells were not stained with anti-GFAP antibody, a marker for astrocytes/Bergmann glia in the cerebellum (Tomizawa et al. 2000b). Numerous dendrites extended from the perikarya to reach the surface of the molecular layer. Besides Purkinje neurons, the cerebellum also contained stellate cells, Golgi cells, basket cells, and glial-

Fig. 2 Distribution of M1 antigen in a sagittal section. The schematic diagrams show the outline of the zebrafish brain. The *solid line* in the *insert* indicates the location of the sagittal section. *Bar* 750 µm, 500 µm in *insert*

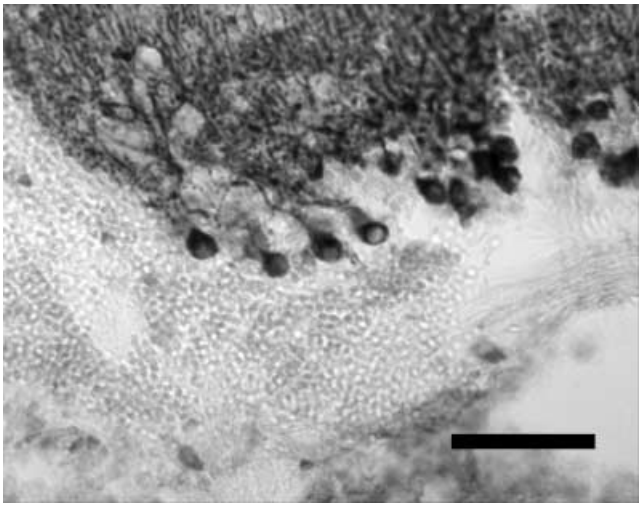


Fig. 3 Higher magnification of antibody staining. Drop-shaped Purkinje-like cells and their dendrites are heavily stained, but cerebellar granule cells are not stained. *Bar* 50 µm

like Bergmann cells or astrocytes. The stained cells were very similar to the typical Purkinje neurons but not to the other cell types.

The sizes and shapes of the stained perikarya were almost the same throughout the cerebellum. These neurons had thick branched dendrites. Counter-staining with toluidine blue indicated that these large neurons were very common in the cerebellum and that all of these cells were stained by the monoclonal antibody. The monoclonal antibody also stained similar cells in the cerebellum of goldfish and medaka fish (*Oryzias latipes*) but failed to stain mouse brain (data not shown).

Direction of dendrites

In higher vertebrates, the dendrites of Purkinje neurons extend toward the surface of the cerebellum; however, in teleosts, this does not appear to occur. Figure 4 shows the dendrites of the Purkinje-like cells in several area of the cerebellum. In Val (Fig. 4A, B), the dendrites extended from the surface of the cerebellum (top) to the inside (bottom). In Vam (Fig. 4C, D), the dendrites extend very regularly, from the inside of the cerebellum (bottom) to the surface (top). The dendrites of a Purkinje neuron in higher vertebrates are planar; they grow only in a two-dimensional plane.

The Purkinje-like cells in zebrafish seemed to be different. In Vam, the arrangement of the dendrites was similar to that of higher vertebrates (Fig. 4C, D). They were tree-shaped with many branches and extended widely when examined in sagittal section (Fig. 4D), but not in coronal section (Fig. 4C) where they appeared to have a regular stripe-shape. Therefore, it is possible that the planar dendrites in Vam were regularly arranged only in a two-dimensional plane, as in higher vertebrates. On the other hand, the dendrites in Val and in caudal CCe looked highly irregular in sagittal and coronal or horizontal sections (Fig. 4A, B, E, F). They did not show the regular stripe-shape.

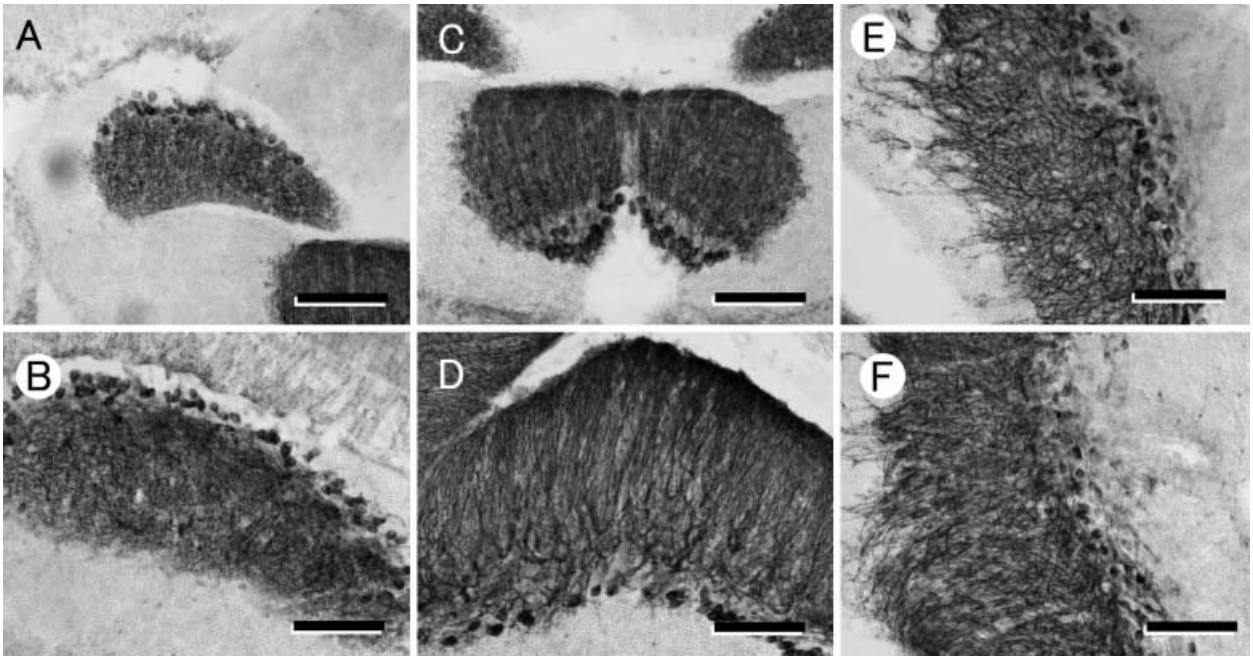
In Vam, the perikarya were arranged in a single or, at the most, double layer. However, in Val and caudal CCe, there were three or more layers of perikarya (Fig. 4A, B, E, and F).

Purkinje-like cells form a complex curved neuronal zone

Large stained neurons were present between the molecular and granular cell layers. However, there was a boundary area, free from such large cells. Using continuous sections, we surveyed the distribution of Purkinje-like cells. We found that the neurons were distributed in a zone that was curved and twisted in a complex manner. For example, the neurons were present at the top of Val but not at the bottom. On the other hand, the granular cells were localized at the top, lateral sides, and bottom of Val.

At the rostral edge of Val, the dendrites of the Purkinje-like cells seemed to extend backward, whereas in Vam, the dendrites extended from the bottom to the top. The cell bodies were located at the bottom of Vam. These finding suggested that there was a continuous zone of the large neurons. It started at the caudal end of Val and proceeded to the rostral end of Val. The zone turned and twisted then proceeded to Vam (Fig. 5). The

Fig. 4A–F Dendritic morphology of Purkinje cells. **A** Lateral part of the valvula cerebelli in coronal section. **B** Lateral part of the valvula cerebelli in sagittal section. **C** Medial part of the valvula cerebelli in coronal section. **D** Medial part of the valvula cerebelli in sagittal section. **E** Caudal region of corpus cerebelli in sagittal section. **F** Caudal region of corpus cerebelli in horizontal section. *Bar* 100 μm



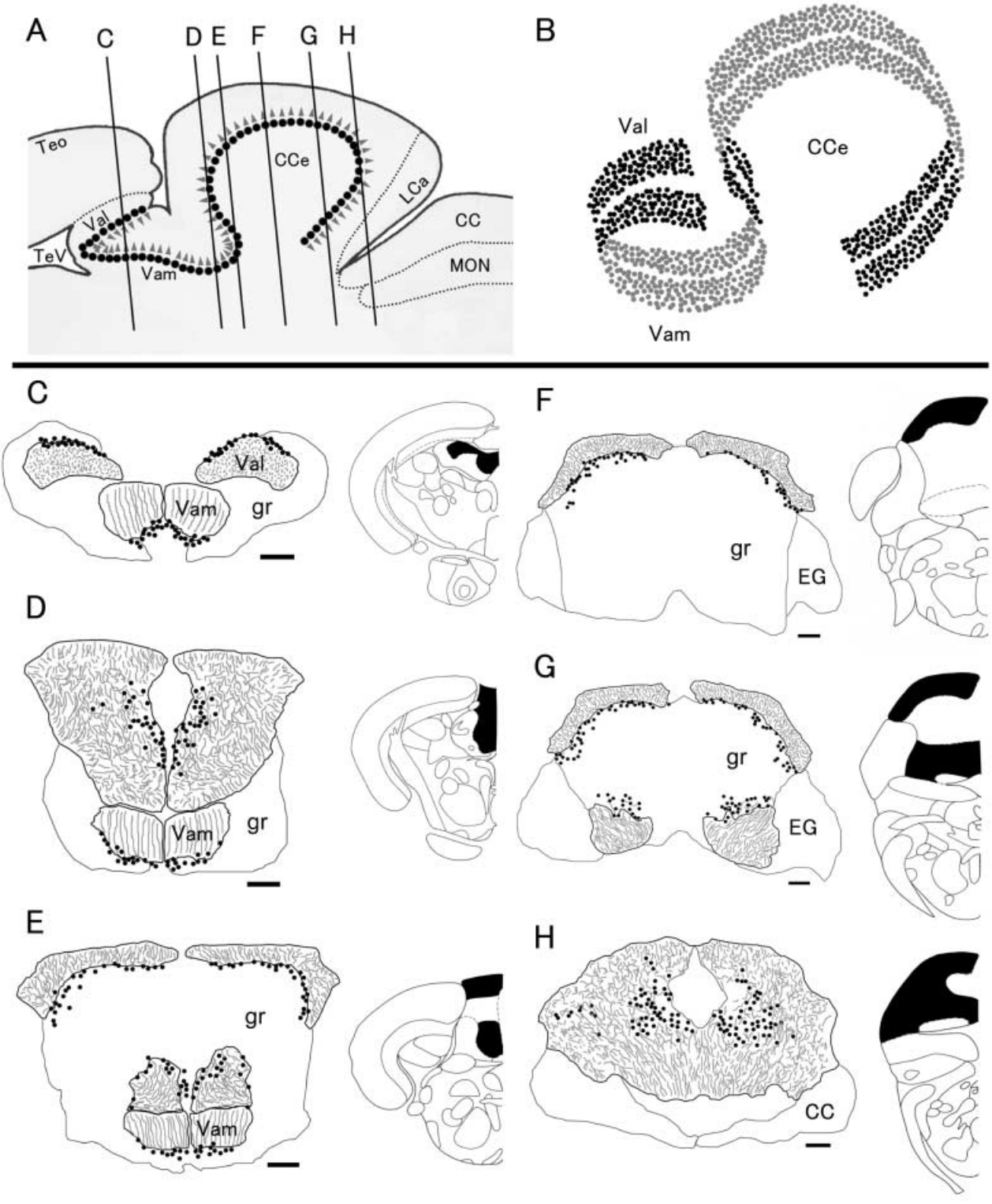


Fig. 5A–H Zone of Purkinje cells. **A, B** Illustration of zone of Purkinje cells. *Solid lines* (*C–H*) Each coronal section level in **C–H** (*gray triangles* Purkinje dendrites, *large black or gray closed circles* cell bodies of the Purkinje cells). **C–F** Illustrations of stained coronal sections of the cerebellum (*large closed circles* cell bodies of the Purkinje cells, *gray lines* dendrites, *dark area right* molecular layer in the cerebellum). The area indicated by *small gray dots* is the granular cell layer (*CC* crista cerebelli, *CCe* corpus cerebelli, *EG* eminentia granularis, *gr* granule cell layer, *LCa* lobus caudalis cerebelli, *MON* medial octavolateralis nucleus, *TeO* tectum opticum, *TeV* tectal ventricle, *Val* lateral division of the valvula cerebelli, *Vam* medial division of the valvula cerebelli). *Bar* 100 µm

cellular zone continued to the rostral end of CCe, then to the top of CCe, and finally extended to the bottom of CCe. Overall, it made an "Ω" shape.

It is interesting that the dendrites were found only on one side of the zone, even where the zone was curved or twisted. The granular neurons were always found the other side of the zone. The dendrites seemed to extend vertically away from the zone. There were no Purkinje-like cells in the boundary between the molecular layer and the granular cell layer that was not part of the zone.

Discussion

Monoclonal antibody M1 recognizes Purkinje neurons

The monoclonal antibody M1 recognized Purkinje-like cells in adult zebrafish cerebellum. These cells were located between the granular cell layer and the molecular layer, were large and drop-shaped, and had numerous dendrites that extended into the molecular layer. The dendrites branched many times.

These features are typical of Purkinje cells and also are very similar to those of higher vertebrates. Double staining with the M1 monoclonal antibody and the anti-calbindin D28 K antibody (Rodriguez-Moldes et al. 1990) has revealed both antigens are co-located in the Purkinje-like cells (data not shown). Therefore, it is highly likely that the antibody recognizes Purkinje cells.

Nevertheless, it is possible that the stained cells are Bergmann glia, which also have long processes that extend into the molecular layer (Krah and Meller 1999). However, the processes of these glia usually do not branch many times. The precursor of this glial cell type is generally thought to be a radial glia, and the granular cells located on the surface of the cerebellum migrate by using the processes of the radial glia (Tomizawa et al. 2000b). In addition, the number of stained cells is too large for them to be Bergmann glia. Anti-GFAP antibody, which decorates Bergmann glia and radial glia, does not stain the Purkinje-like cells in the layer. There also are eurydendroid cells in the Purkinje cell layer. These large cells are output neurons for the cerebellum, as teleosts do not have the deep nuclei that are present in higher vertebrates. It is also possible that the antibody stains these neurons. However, there are thought to be fewer eurydendroid cells than Purkinje cells. Indeed, the monoclonal antibody stains almost all of the large cells in the boundary, and, therefore, the cells must be Purkinje cells.

Porteros et al. (1998) have reported that their antibody (anti-parvalbumin antibody) also recognizes Purkinje cells and that they have observed eurydendroid cells surrounded by immunopositive Purkinje's synapses. In the Purkinje cell layer, we have observed similar cells, which are larger than Purkinje cells. These immunonegative cells could be eurydendroid neurons. Several calcium-binding proteins, such as parvalbumin and calretinin (Celio et al. 1988; Porteros et al. 1997, 1998), are known to be good markers for Purkinje cells. Therefore, the target protein for our monoclonal antibody could be a member of the large family of these proteins; however, there are no reports of any of these proteins being 42 kDa in size.

Our monoclonal antibody M1 stains almost all large neurons in the boundary layer. Brochu et al. (1990) have reported that anti-zebrin II antibody recognizes Purkinje cells in teleosts, but their antibody stains only a subgroup of Purkinje cells. Fortin et al. (1998) have also suggested that there is a difference of contents of several calcium-binding proteins in Purkinje cells. Almost all Purkinje cells in monkey cerebellum seem to be immunopositive for calbindin, but some subpopulations of the Purkinje cells are immunonegative for calretinin or parvalbumin. In zebrafish, the dendrites are planar in Vam but arbor-shaped in the caudal CCe. Therefore, it is possible that there are several subtypes of Purkinje cells resulting from the compartmentalization of the cerebellum (Boegman et al. 1988; Herrup and Kuemerle 1997).

Connection to crista cerebellaris

It is still uncertain whether the CC belongs to the cerebellum. It is clear that the axons of granule cells in the cerebellum extend into the CC (Sarnat and Netsky 1974). Crest cells are present in the CC and have a similar shape to cerebellar Purkinje cells (Diaz-Regueira and Anadon 1995). The dendrites of crest cells form synapses with axons from cerebellar granule cells. It should be noted that the crest cells also directly receive information from the 7th and 8th cranial nerves, which carry lateral and vestibular signals (O'Marra and McCormick 1999; Northcutt et al. 2000). The crest cells are located under the CC, but this area is usually thought to be part of the medulla and not the cerebellum.

Therefore, we thought it would be interesting to determine whether our monoclonal antibody stains the crest cells. The monoclonal does indeed stain these cells (data not shown), but the staining is weak compared with that of Purkinje cells in Va and CCe. In addition, the stained crest cells appear to be slightly smaller than the Purkinje cells, and the crest cells are distributed more irregularly than the Purkinje cells. It is interesting to note that the line of stained crest cells seems to continue to the zone of Purkinje cells in the caudal CCe. Therefore, it is possible that the crest cells are also a subgroup of Purkinje cells.

Zone of Purkinje cells

The Purkinje cells have a zonal distribution in the cerebellum. It starts at the caudal Val and ends at the caudal CCe (or CC). In Val and Vam, the width of the zone is narrow, and the two-dimensional density of the Purkinje cells is high. On the other hand, in the dorsal CCe, the width of the zone is much greater, and the density is rela-

tively low. The dendrites extend vertically from the tangential plane of the zone. Therefore, this is the fundamental axis of the cerebellum. The organization of the cerebellum of teleosts is very complex; however, this idea makes it easier to understand. The basic features of the teleost's cerebellum are the same as those of higher vertebrates, not only in CCe, but also in Val and Vam. In the latter areas, the narrow Purkinje zone is bent and twisted. This structure places the granular cells on the surface of the Val and causes the Purkinje-free boundary to be localized between the granular cell layer and the molecular layer.

It should be noted that the Purkinje belt in the zebrafish brain is divided into right and left belts by the central line. Purkinje cells are extremely rare in the central line. Both right and left zones are closely contacted in the Vam but are separated in the Val and caudal CCe. We do not know the reason for the presence of the central void line, but it might be related to the neurogenesis in the adult zebrafish (Maeyama and Nakayasu 2000). In lower vertebrates, neurogenesis continues even in adult. The sites of neurogenesis in the zebrafish cerebellum are located at the central line.

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