Published in final edited form as:

J Neurosci Methods. 2008 October 30; 175(1): 64–69. doi:10.1016/j.jneumeth.2008.08.009.

An immunochemical marker for goldfish Mauthner cells

Carmen E. Flores, Smaranda Ene, and Alberto E. Pereda*

Dominick P. Purpura Department of Neuroscience, Albert Einstein College of Medicine, Bronx, New York.

Abstract

The Mauthner cells are a pair of large reticulospinal neurons that mediate tail-flip escape responses in fish. Exploring the co-localization of scaffold and gap junction proteins at mixed (electrical and chemical) synapses we found that the use of a particular antibody against the scaffold protein Zonula Occludens 2 (ZO-2) resulted in labeling of these cells. We show here that this staining is restricted to the Mauthner cell and evenly distributed along its dendrites and axon, also prominent in small dendritic and axonal processes. Because the observed labeling is non-specific, we suggest that the antibody might recognize a soluble protein that is primarily expressed in the Mauthner cells. While the identity of this protein is presently unknown, the use of this antibody should facilitate the identification of the Mauthner cell and its fine processes during anatomical and immunohistochemical studies, which otherwise require intracellular injection of tracer molecules during electrophysiological recordings.

Keywords

Mauthner; Gap junction; Electrical synapses; ZO-1; ZO-2

1. Introduction

The Mauthner (M-) cells are a pair of reticulospinal neurons located in the medulla of teleost fish (Beccari, 1907). These uncommonly large cells are anatomically and physiologically identifiable and have historically constituted a valuable preparation for the study of cellular correlates of behavior and for the elucidation of basic mechanisms of synaptic transmission (for review see Korn and Faber, 2005). Their characteristic large myelinated axons, first noticed by Mauthner (Mauthner, 1859), cross the midline to descend the length of the spinal cord, issuing axon collaterals that massively activate cranial and spinal motor systems (Faber et al., 1989). Such an anatomical arrangement allows a single action potential in this cell to initiate an escape response by producing a tail flip.

From the anatomical point of view, the large size and distinctive morphology of the M-cell permits the imaging of long stretches of membrane in a single optical section, where it is possible to identify specific synaptic inputs and to examine the cellular and subcellular distribution of specific proteins. This is the case for the Large Myelinated Club endings (Club

*Corresponding author: Dominick P. Purpura Department of Neuroscience, Albert Einstein College of Medicine, 1300 Morris Park Ave., Bronx, NY 10461. Phone: (718) 430 3405. Fax: (718) 430 8821. E-mail address: apereda@aecom.yu.edu.
Dr. Greg A. Gerhardt, Co-Editor-in-Chief, *Journal of Neuroscience Methods*, University of Kentucky, Department of Anatomy and Neurobiology, 306 Davis Mills Building, Lexington, KY 40536

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endings) which due to their large size, characteristic myelination and dendritic localization, are the most recognizable synaptic input to the M-cells. This population of about 100 large afferents 5–15 μm in diameter, originates in the rostral portion of the saccular macula (Popper and Fay, 1998) and runs in the posterior branch of the VIIIth nerve of teleost fish to terminate as mixed, electrical and chemical, synaptic contacts on the distal portion of the lateral dendrite of the M-cell (Bartelmez, 1915; Bartelmez and Hoerr, 1933; Bodian, 1937). The unusual large size of these contacts makes them amenable for examining the presence and intraterminal distribution of specific proteins (Pereda et al., 2003; Flores et al., 2008). Because of the experimental accessibility and abundance of gap junctions, these contacts constitute a particularly valuable model for the study of the properties of electrical synapses in vertebrates (for review see Pereda et al., 2004).

While exploring the co-localization of scaffold proteins with connexin 35, the gap junction protein that mediates electrical transmission at Club endings (Pereda et al., 2003), we found that the use of a particular polyclonal Zonula Occludens 2 (ZO-2) antibody produced a Golgi-like staining of the M-cell. This labeling was non-specific and was restricted to this neuron, suggesting that the ZO-2 antibody is likely to recognize a protein that is primarily expressed in M-cells. While the identity of such a protein remains undetermined, the use of the antibody should constitute a valuable tool for the identification of the M-cell and its fine processes during anatomical and comparative studies.

2. Materials and Methods

2.1. Animals

Goldfish (*Carassius auratus*) of 10–12 cm in body length ($n=16$) were used for this study. The fish were kept in 150 liter tanks (maximum of 20 animals per tank) at temperatures of approximately 20 °C and exposed to a 12-h light/12-h dark cycle. On the day of experiment, animals were anesthetized using MS-222 (Ethyl m-aminobenzoate; 250 mg/l) in ice water and perfused intracardially with 1X saline phosphate buffer (PBS) at pH 7.4 for 10 minutes/80ml followed by a fixative containing 4% paraformaldehyde in 0.1 M phosphate buffer (PB) for 10–15 minutes/60ml at 4°C. Animal care, handling, and surgery were carried out according to U.S. government guidelines for the use of animals in research, and were approved by the Animal Welfare Committee at Albert Einstein College of Medicine.

2.2. Section preparation

After perfusion goldfish brains were removed and kept overnight at 4°C in 4% paraformaldehyde in phosphate buffer. Brains were sectioned (30–100 μm) using a TPI vibratome (Technical Products International, St. Louis, MO) in ice cold 0.1 M PB, and then collected in cold 1X PBS, pH 7.4. Sections containing both Mauthner cells, corresponding to the portion of the hindbrain located ventral to the cerebellum and delimited by the anterior and posterior part of the cerebellar peduncles were selected for further processing. These sections were either stored at 4°C in 1X PBS or rinsed at room temperature (RT) 3 times/10 minutes each with TBS (50 mM Tris-HCL+ 0.256 M NaCl, pH 7.4).

2.3. Immunohistochemistry

Selected sections were blocked and permeabilized for 45 minutes at RT in TBSTr (50 mM Tris-HCL+0.256 M NaCl+0.3–0.4% Triton X-100, pH 7.4) + 5–10% Normal Goat Serum (NGS). We first tested all the antibodies (Table 1) in single immunofluorescence experiments to determine the optimal working concentration and quality of the signal. For double immunofluorescence experiments, sections were incubated overnight at 4°C on a moving platform with polyclonal ZO-2 (711400) antibody and either monoclonal anti-connexin 35/36 (mCx35) or monoclonal ZO-1 (mZO-1) antibodies diluted in TBSTr + 5–10% NGS. Sections

were then rinsed four times for 10 minutes each, washed and incubated for 1–2 hr at RT on a moving platform with both Alexa Fluor 488- conjugated goat anti-mouse and Alexa Fluor 594- conjugated goat anti-rabbit secondary antibodies diluted in TBS + 0.1% Triton-X 100. Finally, sections were rinsed two to three times with TBS + 0.1% Triton-X 100 followed by one to two rinses with 50 mM TRIS-HCl, pH 7.4. The sections were mounted on glass microscope slides using a 0.2% n-propyl gallate-based antifading solution, to reduce photobleaching and covered with No.1 coverslips (Esco, Portsmouth, NH). They were left for 4–12 hours to dry at room temperature and then placed at 4°C. Control sections were incubated with secondary antibodies in the absence of primary antibodies and did not show meaningful labeling (not shown).

2.4. Confocal microscopy and Image processing

Slides were initially examined under transmitted light and epifluorescence in an upright Leica microscope and then imaged using an Olympus BX61WI confocal microscope with a motorized fixed stage using 20X air, 40Xapo/340 Water and 60X oil objective lenses. FLUOVIEW FV500 software was employed for data acquisition and analysis. Confocal double-immunofluorescence XY images were scanned in Z-axis intervals of 0.4–0.8 μ m for 3D reconstruction. Z-plane sections and Z-plane stacks from each image were employed for image analysis using Image J (NIH) software. A uniform threshold was applied through Image J to all images in all channels to reduce background. Some images were further processed using Adobe Photoshop (Adobe Systems, San Jose, Ca) and Canvas X (ACD Systems) for presentation purposes.

2.5. Immunoblotting

Small tissue samples containing both Mauthner cells obtained from goldfish hindbrain, corresponding to the portion defined above, were collected rapidly and stored at – 80°C until homogenization in IP buffer (20 mM TRIS-HCl, pH 8.0, 140 mM NaCl, 1% Triton X-100, 10% glycerol, 1 mM EGTA, 1.5 mM MgCl₂, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride (PMSF), 5 g/l of the protease inhibitors leupeptin, pepstatin A, and aprotinin) as described previously (Flores et al., 2008). Briefly, goldfish brain homogenates were sonicated at 4°C 3 times for 10 sec each, 35% duty cycle with a W-225R sonicator (Misonix, Inc), and centrifuged for 10 minutes at 20,000 $\times$ g and the supernatant was collected for western blotting. Protein was quantified by using the BCA™ Protein Assay Kit (Pierce). Samples were dissolved in reducing SDS sample buffer (containing 5–10% β -mercaptoethanol and 50–100 mM of 1,4-dithiothreitol) and resolved on 10% SDS-PAGE gels for immunoblots of ZO-1 and ZO-2. Proteins were transferred at 30 V overnight to nitrocellulose membranes (Schleicher and Schuell, Germany) in TRIS-glycine transfer buffer, pH 8.3, containing 0.5% sodium dodecylsulphate. In order to confirm transfer efficiency, membranes were stained with Ponceau-S (Sigma, USA). Blots were blocked for 1 hr at RT with 4% nonfat dry milk in TSTw (20 mM Tris-HCl, pH 7.4, 150 NaCl, 0.1% Tween 20), rinsed briefly in TSTw, and then incubated overnight at 4°C on a moving platform with mZO-1 and 71–1400 antibodies at 0.5 μ g/ml diluted in TSTw containing 1% nonfat dry milk. Membranes were washed 3 times/10 min each with TSTw, incubated for 1 hour with horseradish peroxidase-conjugated goat anti-mouse IgG or anti-rabbit IgG (Santa Cruz biotechnology, USA) diluted 1:5000, rinsed with TSTw 4 times/10 min and resolved by chemiluminescence ECL (Amersham Biosciences, USA).

3. Results

The large size of the M-cell and some of its inputs makes it ideal to study the subcellular distribution of specific proteins (Fig. 1A). Our recent work has shown that the gap junction protein connexin 35 (Cx35), the fish ortholog of the mammalian neuronal connexin 36 (Cx36) (Condorelli et al., 1998), co-localizes with the protein Zonula Occludens 1 (ZO-1) at Club endings and other mixed synaptic terminals on the M-cell and all throughout goldfish hindbrain

(Flores et al., 2008). ZO-1 is a scaffold protein member of the MAGUK family (Itoh et al., 1999), which has been shown to co-localize and interact with several connexins (Herve et al., 2004; Li et al., 2004; Flores et al., 2008). Consistent with this evidence, punctate labeling for ZO-1 (mZO-1 antibody) was detected at synaptic terminals across the soma and dendrites of the M-cell and, more diffusely, near its membrane and on this cell's axon cap (Fig. 1B), a specialized area that is essential for ephaptic interactions between the M-cell and inhibitory interneurons (Faber and Korn, 1978). ZO-1 labeling was found prominently at Club endings (Fig. 1A,C), which were unambiguously identified due to their large size and remote localization on the lateral dendrite of the M-cell (Pereda et al., 2003; Flores et al., 2008).

Because Zonula-Occludens 2 (ZO-2) was shown to co-localize with Cx36 in the inner plexiform layer of the retina (Ciolofoan et al., 2006), we asked if this protein could also co-localize with Cx35 at mixed synapses in M-cells. ZO-2 is a tight junction-related scaffold protein, and like ZO-1, also a member of the MAGUK family (Itoh et al., 1999). We found that, in contrast with the labeling pattern observed for ZO-1, the ZO-2 polyclonal antibody 71–1400 produced a Golgi-like staining of both M-cells in which most of their anatomical features could be recognized (Fig. 1A,B; see also Fig. 2). Detailed morphological analysis with the 71–1400 labeling revealed that the M-cells were uniformly stained by this antibody (Fig. 2). All portions of this cell appeared clearly identifiable, including the soma and its axon and the main dendrites (Fig. 2). Furthermore, very small secondary dendritic processes, including the small axon cap dendrites, were easily visualized (Fig. 2B,C, D). In some sections the staining with 71–1400 antibody appeared as not uniform, brighter at the initial segment and thin dendrites. This difference in labeling is likely to represent either a technical or imaging artifact and it was not consistently observed. This striking labeling of the M-cells was found in all the experiments and observed using two different batches of the 71–1400 antibody (50393525 and 299831).

The fact that the M-cell was uniformly labeled was inconsistent with the pattern expected for ZO-2 (which is functionally similar to ZO-1; Itoh et al., 1999) and suggested that the 71–1400 antibody was cross-reacting with a diffusible cytoplasmatic protein. Western blots obtained from goldfish brain showed that while mZO-1 recognized a band at the predicted molecular weight for this protein in teleost fish (Kiener et al., 2007; Flores et al., 2008), 71–1400 recognized two distinct bands which did not match the molecular weight predicted for ZO-2 (~169 kDa; Itoh et al., 1999). The lack of recognition could be ascribed to the degree of identity of mammalian ZO-2 with its teleost orthologs TJP 2.1 and TJP 2.2, which is of 53% and 48%, respectively (Kiener et al., 2007). Thus, this finding confirmed that the 71–1400 antibody non-specifically recognized proteins other than ZO-2 (Fig. 3). As a further indication of cross-reactivity, we found that this particular pattern of labeling was not observed with two other ZO-2 antibodies. While no labeling was observed with the monoclonal ZO-2 37–4700 antibody, a second polyclonal ZO-2 antibody (38–9100) yielded a pattern reminiscent of that observed for ZO-1 in the axon cap and near the M-cell membrane. In contrast with the ZO-1 antibodies (Flores et al., 2008) (Fig. 1 B,C), there was no punctate labeling observed at synaptic terminals across the somatodendritic membrane with the 38–9100 antibody, suggesting that ZO-2 does not co-localize with Cx35 in goldfish M-cells (not shown).

Interestingly, the 71–1400 labeling seems restricted to the M-cells, suggesting that this antibody recognizes a protein that is primarily expressed in these cells (Fig. 2A). An alternative interpretation would be that this molecule is present in other neurons but, because of its larger size, the M-cell could contain larger amounts of it, giving the appearance of being selectively stained. This does not seem to be the case because: 1) small dendritic processes appeared intensively labeled and 2) cell bodies corresponding to neighboring cells, which are significantly larger than those small processes, were not labeled by the 71–1400 antibody. This finding is illustrated in Fig. 4, where double-labeling with 71–1400 and a monoclonal Cx35 antibody (mCx35) was performed. The mCx35 was employed to label synaptic terminals at

both M-cells and neighboring vestibulospinal neurons (their synaptic inputs are also mixed and contain Cx35; Pereda et al, 2003) to reveal the cellular outlines. Consistent with the notion that 71–1400 labeling is specific for the M-cells, we did not observe 71–1400 labeling on the large vestibulospinal cell bodies (Fig. 4B,C,D), while smaller dendritic processes of the M-cell were intensively labeled (Fig. 4A,B).

4. Discussion

4.1. The 71–1400 antibody labels Mauthner cells

Our data indicates that the use of a particular ZO-2 antibody, 71–1400, results in labeling of both M-cells in goldfish. Previous studies showed that a monoclonal antibody raised against dissected goldfish M-cells was capable of recognizing this neuron (Triller et al., 1991). The antibody also labeled other large neurons of the nuclei reticularis, suggesting the expression of a common antigenic molecule in these functionally and/or ontogenetically related neurons (Triller et al., 1991). Labeling with this antibody was patchy and only observed in the soma, where electron microscopy showed it was associated with polyribosomes and the endoplasmic reticulum (Triller et al., 1991). In contrast with such restricted distribution, we show here that labeling with the 71–1400 antibody is uniformly distributed and restricted to the M-cells, suggesting it cross-reacts with a soluble protein that is, at least, primarily expressed in this pair of identifiable neurons. Such a conclusion is supported by the following findings: 1) the uniform distribution of the labeling, suggesting that it recognizes a soluble antigenic molecule, 2) western blots indicated that the antibody recognizes a protein/s different than ZO-2, and 3) the labeling in the goldfish hindbrain was, under our experimental conditions, restricted to the M-cells.

Further analysis is required to characterize the properties of the 71–1400 antibody in goldfish. The molecular and functional characteristics of the recognized molecule need yet to be determined with proteomic approaches. This antibody is directed against a synthetic peptide derived from the central portion of the ZO-2 protein, and its sequence specification is proprietary (Zymed Laboratories). Together with the identification of the antigenic molecule, future studies will be also necessary to establish the developmental profile and species-specificity of this antibody. While the phylogenetic distribution and anatomical similarity of the M-cell system suggest historical homology, it would be interesting to establish the prevalence of 71–1400 antibody labeling, and/or its recognized protein if identified in the future, amongst closely and distantly related teleost fish. This could potentially reveal evolutionary differences in biochemical and functional characteristics of the M-cell system across species of fish.

4.2. The use of the 71–1400 antibody as a cellular marker

Antibodies are popular tools used for both neuronal identification and to reveal their cellular anatomical features. For example, the monoclonal antibody A60 specifically recognizes the DNA-binding neuron-specific protein NeuN, and it is used as a diagnostic neuronal marker (Mullen et al., 1992; Wolf et al., 1996; Harada et al., 1999). In addition, although not cell-specific, the neurofilament antibody 3A10 has been used to reveal the anatomy of the M-cells in zebrafish (Lorent et al., 2001; Liu et al., 2003). The characteristics of the labeling with the 71–1400 antibody indicates that its use should facilitate the identification of the M-cell and its fine processes during morphological and immunochemical studies, as illustrated in Fig. 4B. Accordingly, the ability of 71–1400 to recognize the M-cell has been confirmed by others (T. Szabo and C. McCormick, personal communication), and labeling with this antibody allowed the identification and differentiation of its small axonal processes from those belonging to other neurons during detailed immunohistochemical studies (C. Grove, personal communication).

Because of the amenability for combining detailed anatomical and physiological analysis, the goldfish M-cells are considered a valuable preparation for the study of basic mechanisms of synaptic transmission in vertebrates (Faber and Korn, 1978; Korn and Faber, 2005). The labeling of the M-cells usually requires the need of intracellular injection of tracer molecules during electrophysiological recordings (Zottoli et al., 1987; Scott 1994; Pereda et al., 1995; Pereda et al., 2003; Weiss et al., 2006) which are time consuming and potentially harmful to the cell. Thus, the use of the 71–1400 antibody should constitute a useful tool not only as a marker of the M-cells but also to facilitate anatomical studies which require the identification of its fine cellular processes for immunochemical detection of specific synaptic proteins.

Acknowledgments

We thank T. Szabo, C. McCormick, C. Grove and S. Zottoli for useful discussions and critically reading the manuscript. Supported by NIH grants DC03186 and NS0552827 to A. Pereda.

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Figure 1. Double immunolabeling of the Mauthner Cell with monoclonal ZO-1 (mZO-1) and 71-1400 antibodies

(A) Schematic diagram of the Mauthner cell showing its soma, axon cap, and ventral and lateral dendrites. Auditory afferents terminating as Club endings are restricted to the distal portion of the lateral dendrite, and form mixed (electrical and chemical) synaptic contacts. (B) Laser scanning confocal projection of the M-cell soma using double immunolabeling with mZO-1 (green) and 71-1400 (red) antibodies. While mZO-1 antibody shows a characteristic punctate distribution in synaptic terminals and diffuse labeling of the axon cap (Flores et al., 2008), the 71-1400 antibody produces complete labeling of the Mauthner cell. Inset: high magnification of the boxed area illustrating unidentified synaptic terminals labeled for ZO-1. Right:

individual channel images obtained using mZO-1 (upper panel) and 71–1400 (lower panel) antibodies. (C) Double immunolabeling of the distal portion of the lateral dendrite. mZO-1 shows the characteristic punctate staining in Club endings (Flores et al., 2008). Inset: high magnification of the boxed area illustrating two Club endings exhibiting punctate staining with mZO-1. In contrast, labeling with the 71–1400 antibody reveals the Mauthner cell lateral dendrite and its bifurcation. Right: individual channel images obtained using mZO-1 (upper panel) and 71–1400 (lower panel) antibodies.

Figure 2. Labeling with the 71–1400 antibody reveals the detailed anatomy of the Mauthner cell
 Laser scanning confocal projection showing immunolabeling of the Mauthner cell using polyclonal 71–1400. (A) Low magnification image showing both Mauthner cells. The Mauthner cells bodies, dendrites and axons appear labeled with the 71–1400 antibody. There was no labeling of other cells. (B) High magnification of the boxed area in A, showing detailed morphological features of the Mauthner cell; the axon cap dendrites and other small dendritic processes can be clearly recognized. (C) Proximal portion of the lateral dendrite. Note the labeling of small dendritic processes. (D) Labeling of the ventral dendrite and its small dendritic processes with 71–1400.

Figure 3. Immunoblotting with monoclonal ZO-1 and 71-1400 antibodies

Lane 1: Immunoblot detection of ZO-1 from goldfish brain with mZO-1 antibody. The observed band is consistent with the estimated molecular mass for another teleost ZO-1 (~178 kDa; Flores et al., 2008). Lane 2: Immunoblot from goldfish brain using 71-1400 antibody. Three bands of relatively lower molecular mass were detected; none of which is consistent with the molecular mass of ZO-2 (~169 kDa).

Figure 4. Labeling with the 71–1400 antibody is restricted to the Mauthner cells

Laser scanning confocal projections of the Mauthner and vestibulospinal neurons using double immunolabeling with monoclonal mCx35 (green) and 71–1400 (red) antibodies. The Cx35 antibody was used to facilitate cellular identification. This connexin localizes to most excitatory inputs to Mauthner, vestibulospinal and other cells in goldfish hindbrain (Pereda et al., 2003). (A) Image corresponds to the proximal portion of the lateral dendrite, soma and axon cap, and illustrates the distribution of Cx35 to synaptic terminals. The use of 71–1400 resulted in labeling of the entire Mauthner cell. Inset: high magnification of the boxed area showing labeling of the small cap dendrites with 71–1400 antibody. (B) Double immunostaining with mCx35 and 71–1400 antibodies shows that 71–1400 labeling is restricted to the Mauthner Cells. Small dendritic processes corresponding to the bifurcation of the lateral dendrite of the Mauthner cell appeared labeled with the 71–1400 antibody. In contrast, no labeling was observed with this antibody in vestibulospinal neurons situated in the proximity of these small dendritic processes. Labeling with mCx35 was observed in synaptic contacts on both Mauthner and vestibulospinal cells (delineated by the dotted lines). Note that despite that their cell bodies are larger than the small dendritic processes of the Mauthner cell, no labeling with 71–1400 exist in vestibulospinal neurons. (C) A group of vestibulospinal neurons labeled with mCx35 but not with 71–1400 antibody. The DIC image of the area was superimposed to the fluorescent image to visualize the cells. (D) High magnification image of one of the neurons illustrated in C, showing punctate staining with Cx35 antibody but not for 71–1400 antibody.

Table 1

Antibodies and reagents

Target	Conjugate	Species	Dilution	Company	Lot
Connexin 35/36		Mouse	1:250–500	Chemicon MAB 3045	0511014892
Connexin 35/36		Mouse	1:250–500	Chemicon MAB 3045	0608037864
ZO-1		Mouse	1:250–500	Zymed 33–9100	60605689
ZO-2		Mouse	1:200–500	Zymed 37–4700	
Mouse IgG (H+L)	Alexa 488	Goat	1:250–500	invitrogen A11005	43241A
Mouse IgG (H+L)	Alexa 488	Goat	1:250–500	invitrogen A11005	52103A
ZO-2		Rabbit	1:500	Zymed 71–1400	50393525
ZO-2		Rabbit	1:500	Zymed 71–1400	299831
ZO-2 C-term		Rabbit	1:100–800	Zymed 38–9100	60203540R
Rabbit IgG (H+L)	Alexa 594	Goat	1:250–500	Invitrogen A11008	47101A
Rabbit IgG (H+L)	Alexa 594	Goat	1:250–500	Invitrogen A11008	4798A