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Some Galeomorph Sharks Express a Mammalian Microglia-Specific Protein in Radial Ependymoglia of the Telencephalon

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Keywords

Elasmobranch · Ependymoglia · Microglia · Ionized calcium-binding adapter molecule 1 · Iba1 · Allograft inflammatory factor 1 · AIF-1 · Astrocytes · Glial fibrillary acidic protein

Abstract

Ionized calcium-binding adapter molecule 1 (Iba1), also known as allograft inflammatory factor 1 (AIF-1), is a highly conserved cytoplasmic scaffold protein. Studies strongly suggest that Iba1 is associated with immune-like reactions in all Metazoa. In the mammalian brain, it is abundantly expressed in microglial cells and is used as a reliable marker for this cell type. The present study used multiple-label microscopy and Western blotting to examine Iba1 expression in the telencephalon of 2 galeomorph shark species, the swellshark (*Cephaloscyllium ventriosum*) and the horn shark (*Heterodontus francisci*), a member of an ancient extant order. In the swellshark, high Iba1 expression was found in radial ependymoglia cells, many of which also expressed glial fibrillary acidic protein. Iba1 expression was absent from most cells in the horn shark (with the possible exception of perivascular cells). The difference in Iba1 expression between the species was supported by protein analysis. These results suggest that radial ependymoglia of the elas-

mobranchs may be functionally related to mammalian microglia and that Iba1 expression has undergone evolutionary changes in this cartilaginous group.

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Introduction

Ionized calcium-binding adapter molecule 1 (Iba1), also known as allograft inflammatory factor 1 (AIF-1), is a cytoplasmic scaffold protein that was first isolated from peripheral tissues in the 1990s [Chen et al., 1997; Zhao et al., 2013]. This relatively small (17-kDa) protein has several PDZ interaction domains that mediate multiprotein complex assembly [Hung and Sheng, 2002; Zhao et al., 2013]. In the mammalian brain, Iba1 is abundantly expressed in “resting” microglia and is a reliable marker for these cells, distinguishing them from neurons and other glia [Ahmed et al., 2007; Wake et al., 2009; Bifari et al., 2017]. Among its known functions in microglia, Iba1 binds to fimbrin and cross-links actin molecules [Sasaki et al., 2001; Ohsawa et al., 2004] and also interacts with Pax6, a multifunctional protein involved in transcription regulation [Maurya and Mishra, 2017]. This protein has been structurally and functionally conserved among the Metazoa [Deininger et al., 2002; Drago et al., 2014]. It

has been found in a number of phyla, including marine sponges [Kruse et al., 1999], several mollusc groups [Hermann et al., 2005; De Zoysa et al., 2010; Zhang et al., 2011; Li et al., 2013; Wang et al., 2013; Zhang et al., 2013; Martin-Gomez et al., 2014], the medicinal leech (an annelid worm) [Drago et al., 2014], and echinoderms [Ovando et al., 2012; Ji et al., 2014]. The function of Iba1 is associated with tissue injury and immune challenge, including pathogen infection.

The conservation of Iba1 in evolution suggests that it may be present in the central nervous system (CNS) of the Elasmobranchii, a class of cartilaginous fishes that includes sharks, skates, and rays [Compagno et al., 2005; Ruggiero et al., 2015]. The information about the evolution of the CNS of this diverse group remains limited [Wullmann and Vernier, 2006; Lisney et al., 2008; Hofmann and Northcutt, 2012], but some conserved aspects of elasmobranch neuroanatomy are well understood, such as the structure of the serotonin (5-hydroxytryptamine, 5-HT) system [Stuesse et al., 1991; Stuesse et al., 1995; Carrera et al., 2008].

Cartilaginous fish brains contain several types of glia, including astrocyte-like cells [Kalman and Gould, 2001; Ari and Kalman, 2008a, b] and oligodendrocyte-like cells [Rotenstein et al., 2009]. Generally, these macroglial cell types are likely to be present in all vertebrates [Cuoghi and Mola, 2009]. Microglia-like cells have been described in the retina of the small-spotted catshark (*Scyliorhinus canicula*) [Bejarano-Escobar et al., 2013] and in the brains of bony fishes [Zupanc et al., 2003; Cuoghi and Mola, 2007; Li et al., 2016].

The predominant glial cells in many elasmobranchs are radial endymoglia (also known as tanocytes) with somata in the endymal layer (surrounding a ventricle) and long processes extending to the pia. Radial endymoglia can be present in all brain divisions of various elasmobranch species despite considerable variability in the abundance of astrocyte-like cells and overall glia composition [Kalman, 2002; Ari and Kalman, 2008a, b].

The present study investigated the expression of Iba1 in the telencephalon of 2 shark species in the superorder Galeomorphii, the swellshark (*Cephaloscyllium ventriosum*) (in the order Carcharhiniformes) and the horn shark (*Heterodontus francisci*) (in the phylogenetically ancient order Heterodontiformes). Both species are found along the Pacific coast from California to Mexico and may also occur in more southern regions [Compagno et al., 2005].

Materials and Methods

Animals

Specimens of adult swellsharks (*C. ventriosum*) and adult horn sharks (*H. francisci*) were obtained from the UCSB Parasitology Laboratory in 2015–2017, with a postmortem interval of 1–3 h (after terminal anesthesia with MS222). Six specimens of swellsharks (2 females, 3 males, 1 unidentified) and 4 specimens of horn sharks (3 females and 1 male) were studied.

The brains of the specimens were removed, rinsed in 0.1 M phosphate-buffered saline (PBS, pH 7.2) and either immersion fixed in 4% paraformaldehyde at 4°C overnight (for immunohistochemistry or Nissl staining) or frozen at –75°C (for Western blotting). The fixed brains were cryoprotected in 30% sucrose in 0.1 M phosphate buffer at 4°C for several days until they sank and then either sectioned immediately or transferred to a cryoprotectant solution (30% sucrose, 1% polyvinylpyrrolidone (PVP-40), and 30% ethylene glycol in PBS) at –20°C and sectioned later.

For comparisons with a mammalian brain, adult mice (adult C57BL/6J males; Jackson Laboratory) were used. They were deeply anesthetized with a mixture of ketamine (200 mg/kg) and xylazine (20 mg/kg), and their dissected brains were processed the same as shark brains (except they were not embedded). All procedures have been approved by the UCSB Institutional Animal Care and Use Committee.

Immunohistochemistry

Brains were embedded in 20% gelatin (type A), immersed in formalin with 20% sucrose for 3 h at room temperature, and sectioned coronally at 40 µm thickness on a freezing microtome. Sections were rinsed in PBS, blocked in 2% normal donkey serum (NDS) for 30 min, and double immunolabeled with rabbit anti-Iba1 IgG (1:500; #019-19741; Wako Chemicals USA) and either goat anti-glial fibrillary acidic protein (GFAP) IgG (1:500; #ab53554; abcam) or goat anti-5-HT IgG (1:500; #20079; ImmunoStar) with 2% NDS and 0.3% Triton X-100 for 2 days at 4°C. 5-HT immunohistochemistry was used to assess general tissue quality. Sections were rinsed several times in PBS for 30 min, incubated in Alexa Fluor 488-conjugated donkey anti-rabbit IgG (1:1,000; #A-21206; Thermo Fisher Scientific) and Cy3-conjugated donkey anti-goat IgG (1:200; #705-165-147; Jackson ImmunoResearch) with 2% NDS for 90 min, rinsed several times in PBS, mounted onto gelatin/chromium-subbed glass slides, allowed to air-dry, and coverslipped with ProLong Gold Antifade Mountant with DAPI (#P36931; Thermo Fisher Scientific). The specificity of the Iba1 antibody has been well established in the mouse brain [Wake et al., 2009; Bifari et al., 2017], and omission of the primary antibody abolished all fluorescence (with the exception of the vasculature in the case of the Iba1 and GFAP antibodies). Sections were imaged with a Zeiss AxioVision Z1 bright-field/epifluorescence microscope, and confocal z-stacks (30–60 optical sections, each 0.45 µm thick) were obtained with an Olympus FluoView FV1000S confocal microscope, using a ×60 objective (NA 1.4). Colocalization was detected with the “coloc” function implemented in Imaris 7 (Bitplane), based on a published algorithm [Costes et al., 2004].

Nissl Staining

Sections mounted on glass slides were rehydrated, stained with 0.25% thionine for 15 s, dehydrated in a graded series of ethanols,

differentiated in 1% glacial acetic acid in 95% ethanol for 4 min, further dehydrated in absolute ethanol, cleared in xylenes, and coverslipped with Permount.

Western Blotting

Brains were homogenized with a TissueRuptor (Qiagen) in Pierce RIPA buffer (#89900; Thermo Fisher Scientific) with the Halt protease inhibitor cocktail containing EDTA (#87786; Thermo Fisher Scientific) and incubated in the same buffer for 1 h on a shaker at 4°C. The lysates were centrifuged at 20,000 g for 5 min to remove unlysed tissue fragments and stored at -75°C. Their protein concentrations were measured with the Pierce PCA protein assay kit (#23227; Thermo Fisher Scientific), and proteins were separated by molecular weight with SDS-PAGE electrophoresis. The samples were mixed (1:1) with 2× Laemmli sample buffer (#161-0737; Bio-Rad) containing 5% 2-mercaptoethanol, boiled for 5 min (unless indicated otherwise), loaded onto a 15% Tris-HCl minigel (#161-1157; Bio-Rad) (an estimated 50 µg/lane) with Odyssey protein molecular weight markers (#928-40000; LI-COR), and electrophoresis was performed at 200 V for 30 min at room temperature. The proteins were transferred onto an Immobilon-FL PVDF membrane (#IPFL07810, 0.45 µm; Millipore,) for 1 h at 100 V at 4°C, and the transfer quality was assessed with Ponceau S solution. In the comparative analysis, the optical densities of bands were measured and plotted in Mathematica 11.1 (Wolfram Research, Inc.). The membranes were allowed to dry overnight at room temperature and immunoprobed. They were blocked in Odyssey blocking buffer (OBB; #927-40000; LI-COR) for 1 h, incubated in rabbit anti-Iba1 IgG (1:1,000; #016-20001; Wako Chemicals USA) and mouse anti-β-actin IgG (1:1,000; #926-42212; LI-COR) with 0.2% Tween 20 in OBB overnight at 4°C, rinsed 4 times (5 min each) in PBS with 0.1% Tween 20 (PBST), incubated in IRDye 800CW donkey anti-rabbit IgG (1:5,000; #925-32213; LI-COR) and IRDye 680RD donkey anti-mouse IgG (1:5,000; #926-68072; LI-COR) with 0.2% Tween 20 and 0.015% sodium dodecyl sulfate (SDS) in OBB for 1 h, rinsed 4 times (5 min each) in PBST, rinsed in PBS, and imaged with an Odyssey Fc Imaging System (LI-COR). To visualize GFAP bands, the mouse anti-β-actin IgG was replaced with goat anti-GFAP IgG (1:2,000; #ab53554; abcam) and the IRDye 680RD donkey anti-mouse IgG was replaced with IRDye 680RD donkey anti-goat IgG (1:5,000; #925-68074; LI-COR).

Results

The brains of the horn shark (Fig. 1a) and the swellshark (Fig. 1b) differ in size and in the relative extent of their subdivisions (Fig. 1c, d). The telencephala of both species are elaborated (type II) in that the majority of their neurons have migrated away from their original periventricular location (Fig. 2). This pattern is consistent with observations in other galeomorph sharks [Butler and Hodos, 2005].

In the adult rodent brain, Iba1 is a reliable marker for microglia [Wake et al., 2009], and GFAP is used as a marker for astroglia, even though not all astrocytes ex-

Fig. 1. Specimens of the horn shark (*Heterodontus francisci*) (a) and the swellshark (*Cephaloscyllium ventriosum*) (b). Both species can grow up to over 1 m in length [Compagno et al., 2005]. Brain specimens of the horn shark (c) and the swellshark (d). Tel, telencephalon. Scale bars, 10 cm (a, b) and 5 mm (c, d).

press GFAP [Zilles et al., 1991]. In order to assess the reliability of Iba1/GFAP immunolabeling in shark brains, the procedure was tested in the mouse telencephalon, where it produced robustly labeled microglia and astrocytes in the corresponding fluorophore channels (Fig. 3).

In the swellshark telencephalon, strong Iba1 immunoreactivity was found in radial endependymoglia cells (Fig. 4). Iba1 immunoreactivity was present in their periventricular somata and along their processes, including their distal segments at the brain surface (near the pia). Strong Iba1 immunoreactivity was also observed around putative blood vessels, probably due to perivascular endependymoglia processes. Iba1-positive fibers often traveled in fascicle-like groups, perhaps following blood vessels (Fig. 5).

Since radial endependymoglia express GFAP [Ari and Kalman, 2008a], the relationship between Iba1 and GFAP

Fig. 2. Coronal, Nissl-stained sections through the horn shark and swellshark telencephala. LV, lateral ventricle. Scale bar, 1 mm.

Fig. 3. Confocal image (flattened z-stack) of Iba1 (green) and GFAP (red) immunoreactivity in the mouse brain septum. The DAPI channel (blue) shows cell nuclei. **Inset** The asterisk indicates an astrocyte, and the arrow indicates a microglial cell. Scale bars, 30 μ m (main panels) and 10 μ m (inset).

Fig. 4. Epifluorescence images of Iba1 (green) and GFAP (red) immunoreactivity in the swellshark telencephalon. The DAPI channel (blue) shows cell nuclei. The asterisks indicate some blood vessels, and the arrows indicate some ependymoglial processes. LV, lateral ventricle. Scale bars, 200, 100, and 100 μ m (from left to right).

immunoreactivities was investigated with double-label immunohistochemistry. Epifluorescence microscopy revealed strong GFAP immunoreactivity in periventricular regions (the location of ependymoglial somata) and in some ependymoglial processes (Fig. 4). In addition, many free, astrocyte-like GFAP-positive cells were found throughout the telencephalon. When not obscured by dense ependymoglial processes, these cells were Iba1 neg-

ative. The overlap between Iba1 and GFAP signals was further analyzed with confocal microscopy by automatically detecting their colocalization in 3 dimensions (z-stacks) using a published algorithm [Costes et al., 2004; Chen et al., 2017]. The analysis showed that only a subset of Iba1-positive ependymoglial fibers was also GFAP positive (Fig. 5).

In the horn shark telencephalon, virtually all Iba1 and GFAP immunoreactivity was associated with vasculature, and only sparse Iba1-positive fibers were present near periventricular regions. Strong perivascular GFAP immunoreactivity has been reported in other cartilaginous species [Ari and Kalman, 2008a, b]. However, this immunoreactivity could not be reliably distinguished from nonspecific binding because similar immunostaining was observed in the swellshark telencephalon with omission of the primary antibodies (Fig. 6).

To investigate whether the striking difference between the swellshark and horn shark telencephala could be accounted for by uncontrolled differences between the quality of the 2 sets of specimens, brains were double immunostained for Iba1 and 5-HT (serotonin) (Fig. 7). Strong 5-HT immunoreactivity was found in both species that clearly visualized individual serotonergic fibers and their varicosities. In the same sections, the difference in Iba1 immunoreactivity was unambiguous. The potential difference between the tissue integrity of the swellshark and horn shark specimens was further assessed by quantifying the SDS-PAGE protein bands of tissue lysates (Fig. 8) as part of the Western blotting analysis described further. The band distributions were virtually indistinguishable between the 2 shark species. They were also very similar to the distributions of the mouse neocortex and cerebellum, dissected immediately after terminal anesthesia. As expected, the mouse spleen (a positive control) showed a distinctly different distribution. A single, strong β -actin band at the expected molecular weight (45 kDa) suggested that the proteins in all lysates were not degraded and migrated normally (Fig. 9).

Western blotting revealed major differences between the Iba1 bands of the swellshark and horn shark telencephala. These sets of bands were different from the Iba1 bands of the mouse brain (Fig. 9). This result was consistent with the expression of Iba1 in different cell types (radial ependymoglia, possible perivascular cells, and microglia, respectively). Iba1 is a 17-kDa protein that plays key roles in the formation of protein-protein complexes [Sasaki et al., 2001; Ohsawa et al., 2004; Maurya and

Mishra, 2017], and its function relies on interaction with PDZ domains present in many proteins [Hung and Sheng, 2002; Chang et al., 2011; Zhao et al., 2013]. The distinct but heavy bands of brain lysates could, therefore, be Iba1-linked protein complexes that did not completely dissociate under the used lysis and loading conditions. To investigate it further, a mouse spleen lysate was analyzed alongside brain lysates. The mammalian spleen is among the tissues that have the highest Iba1 expression (which also include the lungs and the bone marrow; The Human Protein Atlas, <http://www.proteinatlas.org>). The spleen produced a clear 17-kDa band under the same lysis and Western blotting conditions (Fig. 9). Notably, a heavy band similar to the heaviest band of the mouse brain (around 122 kDa) appeared in unboiled spleen samples, suggesting a protein complex (Fig. 10). Likewise, the heaviest band dominated in unboiled mouse brain samples, but another, lighter band (around 43 kDa) appeared in boiled samples (Fig. 10). The difference between 43 and 17 is 26 kDa, which closely approximates the molecular weight of some polypeptides comprising the mouse complement C1q complex (e.g., B chain [26.7 kDa] and C chain [26.0 kDa]). The C1q complex is expressed in the brain [Mosser et al., 2017; Presumey et al., 2017], and these polypeptides are predicted to interact with Iba1 (STRING [Protein-Protein Interaction Networks], <https://www.string-db.org>).

Since the 43-kDa band was close to the molecular weight of GFAP (around 50 kDa) and many Iba1-positive ependymoglia cells were also GFAP positive, potential antibody cross-reactivity was examined with double immunoprobings (Iba1/GFAP) in mouse tissues (Fig. 10). In brain samples, these 2 bands were close but distinctly different.

In summary, the available evidence suggests that Iba1 bands are specific and show various degrees of association of Iba1 with other proteins. Co-immunoprecipitation may provide more direct information about these Iba1 complexes, but immunoprecipitation is sensitive to purification conditions and is likely to pose nontrivial problems in elasmobranchs due to the limited information about their brain biochemistry.

Discussion

Studies in mammalian models have highlighted the importance of glial cells in shaping neural architecture and signaling [Stogsdill and Eroglu, 2016]. In particular, “resting” microglia have been recently shown to play key

Fig. 5. Confocal images (flattened z-stacks) of Iba1 (green) and GFAP (red) immunoreactivity in the swellshark telencephalon in a periventricular region (a) and in more superficial regions (b, c). The DAPI channel (blue) shows cell nuclei. Iba1/GFAP colocalization was detected automatically in 3 dimensions and is shown in yellow (bottom row). The arrows indicate some ependymoglia processes, and the gray arrowheads indicate some GFAP-positive but Iba1-negative astroglia. Scale bar, 30 μ m.

Fig. 6. Epifluorescence images of Iba1 (green) and GFAP (red) immunoreactivity in the horn shark telencephalon and epifluorescence images of the fluorescence signals in the swellshark telencephalon after omission of the anti-Iba1 and anti-GFAP antibodies. The DAPI channel (blue) shows cell nuclei. LV, lateral ventricle. Scale bars, 50, 100, and 100 μm (from left to right).

roles in the maintenance and plasticity of synapses in the healthy brain [Nimmerjahn et al., 2005; Wake et al., 2009; Hong et al., 2016; Mosser et al., 2017]. This adds an important functional dimension to neuroanatomical studies in cartilaginous fishes that have demonstrated consider-

able variability in the glial composition of different clades [Kalman and Gould, 2001; Kalman, 2002; Ari and Kalman, 2008a, b]. These differences include the expression of GFAP [Kalman, 2002] and regionally specific changes in the astroglial architecture between sharks and batoids

Fig. 7. Confocal images (flattened z-stacks) of Iba1 (green) and 5-HT (red) immunoreactivity in the horn shark and swellshark telencephala. LV, lateral ventricle. Scale bar, 50 μ m.

(skates and rays) [Ari and Kalman, 2008a]. The functional implications of these changes remain poorly understood.

It is generally agreed that all vertebrate brains contain the same fundamental types of glial cells, including radial ependymoglia (which are found only in restricted regions of adult mammalian brains) [Nacher et al., 1999; Cuoghi and Mola, 2007; Cuoghi and Mola, 2009]. This assumption is likely to be correct even though the classification of nonmammalian glia needs further refinement [Cuoghi and Mola, 2009] and the use of a single marker can be misleading [Ari and Kalman, 2008a]. A recently introduced, cell morphology-based approach may facilitate marker-independent analyses [Garcia-Cabezas et al., 2016].

In contrast to their structural similarity in different vertebrate groups, glial cells may have been a particularly plastic element in *functional* brain evolution. For example, no postlesion reactive gliosis has been found in elasmobranchs, and, more generally, it has been suggested that such gliosis may be confined to mammals and birds

despite the presence of astroglial cells in elasmobranchs [Kalman et al., 2013].

The present study found that some shark species express Iba1, a highly specific mammalian microglial marker, in radial ependymoglia. This protein may endow ependymoglia with new properties; for example, Iba1 has been associated with membrane ruffling and phagocytosis [Ohsawa et al., 2000; Kanazawa et al., 2002]. Little information is currently available about this potential function in ependymoglia. However, it has been shown that the radial ependymoglia of the Iberian wall lizard (*Podarcis hispanica*) can take up cell debris by lateral processes and transport it to the ventricular and pial poles of the cell, thus actively participating in phagocytosis [Nacher et al., 1999].

The absence of Iba1 immunoreactivity in the horn shark brain allows several explanations and requires further investigation. Neither immunohistochemistry nor Western blotting revealed differences in tissue integrity between the swellsharks and horn sharks. It is unlikely that the Iba1 antibodies used failed to detect Iba1 in the

Fig. 8. Distributions of proteins separated by molecular weight (MW) with SDS-PAGE. **a** The protein bands after transfer onto a membrane, visualized with Ponceau S (a general protein stain). Lanes 1 and 5 are standard ladders with MW shown on the left. CER, cerebellum; H, horn shark; NCX, neocortex; S, swellshark; Tel., telencephalon. **b** Quantitative analysis of the protein distribu-

tion in the mouse neocortex (black curve), the mouse cerebellum (gray curve), and the mouse spleen (red curve). **c** A quantitative analysis of the protein distribution in the swellshark telencephalon (blue curve) and the horn shark telencephalon (green curve). **d** The distribution curves in **b** and **c** plotted together (without the spleen). The y-axes represent relative optical densities.

horn shark because of changes in the amino acid sequence. Iba1 is a highly conserved protein [Deininger et al., 2002; Drago et al., 2014].

It is possible that the adult horn shark telencephalon lacks radial ependymoglia, as supported by GFAP immunohistochemistry. This finding is similar to observations in some skate brains that also show a virtual lack of ependymoglia in the telencephalon [Kalman, 2002; Ari and Kalman, 2008a]. However, it is possible that the horn shark contains astrocytes that were not detected by GFAP immunohistochemistry. In skates, GFAP-positive astrocytes have been found only in perivascular and perimeningeal locations, but glutamine synthetase immunohistochemistry revealed astrocytes throughout the

telencephalon [Ari and Kalman, 2008a]. Glutamine synthetase was not used in this study. Generally, the vertebrate patterns of glial evolution remain hypothetical and somewhat unpredictable. For example, GFAP immunoreactivity shows great regional variability in some elasmobranchs, as well as in birds and mammals [Kalman, 2002; Ari and Kalman, 2008a]. In contrast, teleost brains show strong GFAP immunoreactivity with predominating ependymoglia [Cuoghi and Mola, 2009]. An extensive study of the 3 elasmobranch superorders (Squalomorphii, Galeomorphii, and Batoidea) has found “no meaningful difference between the astroglial architectures of squalomorph and galeomorph sharks” [Ari and Kalman, 2008a]. Considering these findings, the lack of

Fig. 9. The Iba1 and β -actin bands in lysates of the mouse neocortex (NCX), cerebellum (CER), and spleen, and of the swellshark (S) and horn shark (H) telencephala. Two different swellshark and horn shark specimens are shown. The mouse brain produced a heavy band (around 122 kDa, **) and a lighter band (around 43 kDa, *), which suggest Iba1 complexes with other proteins. The spleen produced a single 17-kDa band (arrow), consistent with the molecular weight of a single Iba1 molecule. This band was also detectable in the mouse neocortex.

GFAP-positive ependymoglia in the horn shark telencephalon is not surprising. The horn shark represents the order of the bullhead sharks (Heterodontiformes) and is phylogenetically distant to the swellshark. Among some 500 known species of extant sharks, the bullhead sharks are one of the most ancient elasmobranch lineages that has been traced back to the Triassic period and is currently represented by only 9 species [Sato et al., 1983; Compagno et al., 2005; Naylor et al., 2012]. Interestingly, a mitochondrial analysis suggests that *H. fran-*

cisci may be the most ancient (extant) species of this group [Naylor et al., 2012]. This order has been used in studies of early vertebrate evolution, including their individual genes [Compagno et al., 2005; Komorowski et al., 2012].

It can be hypothesized, therefore, that Iba1 expression in radial ependymoglia emerged within the Elasmobranchii group and that Iba1 expression later shifted to microglial cells (the only Iba1-positive cells in the adult mammalian brain). However, it raises challenging ques-

Fig. 10. Control experiments in mouse tissues used to examine the specificity of Iba1 bands. In the neocortex (NCX), boiling of samples increased the intensity of the lighter (43 kDa) band, but sample dilution (from 1:1 to 1:8) had no detectable effect. In boiled samples, the spleen produced a single 17-kDa band (corresponding to the molecular weight of a single Iba1 molecule), but a heavy

band (similar to the heavy band in the brain) appeared in unboiled samples. The Iba1 and GFAP bands did not overlap. In the brain, the GFAP bands were consistent with the molecular weight of GFAP (around 50 kDa). The light GFAP band in the spleen may be due to partial proteolysis [Fields and Yen, 1985]. CER, cerebellum; RT, room temperature.

tions because mammalian microglia originate outside the CNS: its precursors invade the CNS from the yolk sac early in development [Mosser et al., 2017]. It is interesting to note that in the swellshark telencephalon radial endymoglia cells can express both GFAP and Iba1, but in the mammalian telencephalon these proteins are specifically expressed in astrocytes and microglia, respectively (GFAP-positive astrocytes are also present in elasmobranch brains). Little is known about how these expression similarities may be reflected in the function of the cells.

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Disclosure Statement

The author declares no conflict of interest.

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