Calretinin-Like Immunoreactivity in Mormyrid and Gymnarchid Electrosensory and Electromotor Systems

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ABSTRACT

Calretinin-like immunoreactivity was examined in the electrosensory and electromotor systems of the two families of mormyriform electric fish. Mormyrid fish showed the strongest immunoreactivity in the knollenorgan electroreceptor pathway, in the nucleus of the electrosensory lateral line lobe (ELL) and the big cells of the nucleus exterolateralis pars anterior. Mormyromast and ampullary zones of the ELL showed calretinin-like immunoreactivity in the ganglion, granule, and intermediate cell and fiber layers. Mormyromast zones additionally showed labeling of apical dendrites and commissural cells, but the ampullary zone did not. In the electromotor system, two nuclei in the corollary discharge pathway showed labeling: in the paratrigeminal command-associated nucleus and the juxtalobar nucleus.

Gymnarchus niloticus (Gymnarchidae) showed strongest calretinin-like immunoreactivity in part of the phase-coding pathway, in S-type electroreceptor afferents. Zones of the ELL not receiving phase-coder input had weak labeling. The electromotor system showed labeling in the lateral relay nucleus and less strongly in the medullary relay nucleus, but none in the pacemaker.

The concentration of calcium-binding proteins in mormyrid and gymnarchid time-coding electrosensory pathways is consistent with the hypothesis that they play a role in preserving temporal information across synapses. Cell types that encode temporal characteristics of stimuli in precise spike times have high levels of calcium-binding proteins, but cells that re-code temporal information into presence or magnitude of activity have low levels.

Some cell types in the electromotor pathways and early in the time-coding electrosensory pathways do not follow this hypothesis, and therefore preserve temporal information using a mechanism independent of calcium-binding proteins. In particular, electromotor systems may use extensive electrotonic coupling within nuclei to ensure precise timing.

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Indexing terms: temporal coding; electric fish; recoding; calcium-binding protein
calcium-binding proteins than pathways in the same sensory modality that analyze other stimulus features, such as amplitude (Carr, 1986). Calcium-binding proteins have been found in time-coding auditory pathways of birds and mammals (Rogers, 1987; Takahashi et al., 1987; Zettel et al., 1991; Réosibois and Rogers, 1992; Vater and Braun, 1994) and electro sensory pathways of South American gymnotiform fish (Maler et al., 1984; Losier and Matsubara, 1990). African mormyromiform electric fish have independently evolved electro sensory systems that specialize in the analysis of temporal information, and therefore we describe their immunoreactivity for the calcium-binding protein, calretinin, too Obtain further support for the involvement of calretinin in time-coding pathways.

Calretinin is a 28 kDa protein belonging to the 6-EF hand subfamily of calcium-binding proteins (Baimbridge et al., 1992; Andressen et al., 1993; Seto-Ohshima, 1994). Several calcium-binding proteins, including calretinin, calbindin, parvalbumin, neurocalcin, and hippocalcin, are expressed mainly in the central nervous system (CNS) and diffuse throughout entire neurons, making them useful histological markers (Seto-Ohshima, 1994). The specific importance to time-coding pathways of intracellular calcium binding is not clearly understood. Faithful transmission of spike times through chemical synapses may require short integration times and therefore short synaptic potentials, so that presynaptic calcium must be brought back down to baseline levels quickly. Likewise, calcium buildup could activate calcium-dependent channels that could distort repetitive signals by adaptation. Preservation of finetemporal information may require larger synapses onto large somata, with large pre- and post-synaptic currents that could cause high calcium loads that are potentially toxic. For example, chick magnocellular neurons have AMPA (a-amino-3-hydroxy-5-methyl-4-isoxazolepropionate) glutamate receptors with unusually high calcium permeability (Otis et al., 1995). The high activity of these receptors probably induces a high calcium load. However, the question of whether calcium-binding proteins confer resistance to excitotoxicity is unresolved (Baimbridge et al., 1992; Andressen et al., 1993).

The anatomical and physiological specializations to preserve temporal information in precise spike times are no longer necessary once fine temporal discriminations have been made and the information is re-coded into another format, such as presence or magnitude of responses, or a computational map. In barn owls and Eigenmannia, calcium-binding proteins are strongly present in low-order nuclei of time-coding pathways, and are absent in high-order nuclei (Maler et al., 1984; Carr, 1986; Takahashi et al., 1987; Losier and Matsubara, 1990).

Motor systems may also need to preserve fine temporal information to co-ordinate muscle fibers or muscle groups. For electric organs to generate a strong current during the discharge, the electrocytes have to be activated simultaneously (Bennett, 1968). If calcium-binding proteins help in temporal fidelity, then they might also be concentrated in the electromotor system. High immunoreactivity to calbindin was found in part of the electromotor system. High immunoreactivity to calbindin was found in part of the electromotor system. Several calcium-binding proteins, including calbindin, parvalbumin, neurocalcin, and hippocalcin, are expressed mainly in the central nervous system (CNS) and diffuse throughout entire neurons, making them useful histological markers (Seto-Ohshima, 1994). The specific importance to time-coding pathways of intracellular calcium binding is not clearly understood. Faithful transmission of spike times through chemical synapses may require short integration times and therefore short synaptic potentials, so that presynaptic calcium must be brought back down to baseline levels quickly. Likewise, calcium buildup could activate calcium-dependent channels that could distort repetitive signals by adaptation. Preservation of finetemporal information may require larger synapses onto large somata, with large pre- and post-synaptic currents that could cause high calcium loads that are potentially toxic. For example, chick magnocellular neurons have AMPA (a-amino-3-hydroxy-5-methyl-4-isoxazolepropionate) glutamate receptors with unusually high calcium permeability (Otis et al., 1995). The high activity of these receptors probably induces a high calcium load. However, the question of whether calcium-binding proteins confer resistance to excitotoxicity is unresolved (Baimbridge et al., 1992; Andressen et al., 1993).

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1984). Furthermore, mormyrids have a corollary discharge pathway, whereby the motor command to generate a discharge travels not just to the electric organ, but also to primary electroeysensory areas to prepare them for reafferent sensory information (see Fig. 2C; Bell et al., 1983; Bell, 1989). If the precise temporal relationship between the actual and the expected discharge must be maintained, then calcium-binding proteins might be concentrated in the corollary discharge pathway.

MATERIALS AND METHODS

For this study, we used nine mormyrid species (four Brionomyrus brachyistius, three B. niger, and two Gnathonemus petersii, all ~10–12 cm, non-breeding individuals) and three juveniles (11–13 cm) of Gymnarchus niloticus. Our protocol was approved by the Institutional Animal Care and Use Committee at Cornell University (number 85-1-95). Fish were anesthetized by immersion in MS-222 (500–1000 mg/l) and then perfused through the heart with saline (0.9% NaCl) followed by 4% buffered paraformaldehyde. We removed the brains and post-fixed them for 1–2 days in 4% buffered paraformaldehyde.

Brains to be cut on the Vibratome (Pelco 101, Redding, CA) were embedded in 4% agar just prior to sectioning and were cut at 50 µm. Agar-embedded sections were more difficult to handle throughout the histological procedure, so in later experiments we cut brains as gelatin-embedded frozen sections. Brains were cleaned of meninges, post-fixed for a second time in 4% buffered paraformaldehyde 10% sucrose for 1 day, embedded in 10% 100 bloom gelatin-10% sucrose, and post-fixed for another 1–3 days. We cut the brains on a sliding microtome (American Optical, Buffalo, NY) at 50 µm.

Sections were washed three times in phosphate-buffered saline (PBS; 0.02 M phosphate buffer, pH 7.6, 0.9% NaCl), blocked in 5% normal goat serum (Vector Laboratories, Burlingame, CA) in PBS with 0.3–1% Triton X-100 (PBST; Aldrich Chemical Co., Milwaukee, WI), and incubated in anti-calretinin primary antibody (Swiss Antibodies, Bellinzona, Switzerland, lot 25392) with 1% normal goat serum in PBST for 2 days. Dilutions ranged from 1:3,000 to 1:14,000, all of which produced quite satisfactory results. A Triton X-100 concentration of 1% and a 30 minute wash in methanol before blocking were necessary to facilitate antibody penetration into highly myelinated regions, such as fiber tracts.

Sections were then washed in PBS three times and incubated for 2 hours in secondary antibody (biotinylated goat anti-rabbit; Vector Laboratories, Burlingame, CA) at a dilution of 1:300 with 2% normal goat serum in PBST. Sections were washed three times in PBS, incubated in avidin-biotin complex (ABC) solution (Vector Laboratories) for 2 hours, washed two times in PBS, and two times in 0.1 M, pH 7.2, tris buffer (TB). Sections were soaked for 15 minutes in a solution of 10 mg DAB (Sigma Chemical Co., St. Louis, MO) in 50 ml TB. We added 30 µl 3% H2O2 and let the reaction continue for 15 minutes. Finally, we washed the reaction four times in TB, and then mounted, dehydrated, and coverslipped them under Permount or DepeX.

To control for non-specific 2° antibody binding, representative sections from one Gymnarchus and one B. brachyistius were treated as above, except that they were not incubated in primary antibody. None of these showed any immunoreactivity whatsoever. From most series, we also took every fourth or fifth section to generate a Nissl series using neutral red or cresyl violet.

The primary antibody used in this study is a polyclonal antibody raised in rabbits against recombinant human calretinin produced in E. coli (Schwaller et al., 1993). To verify antibody specificity in the species used here, we deeply anesthetized one animal of each species in 0.1% MS-222 and removed their brains. We homogenized each brain in lysis buffer (20 mM Tris, pH 7.2, 2 mM EDTA, 25 µg/ml leupeptin, 2.5 µg/ml aprotenin, 10 µg/ml pepstatin, and 1 mM pefabloc), assessed the total protein (protein assay kit, Bio-Rad, Hercules, CA), and ran 30 µg of protein on a 12% SDS gel. We transferred the gel to a polyscreen PVDF membrane (Dupont-NEN, Boston, MA), blocked the membrane in 4.5% nonfat dry milk, and incubated it with the calretinin antibody at a dilution of 1:10,000 overnight. We visualized the 1° antibody by incubating the membrane with horseradish peroxidase (HRP)-conjugated mouse anti-rabbit for 1 hour, soaking it for 1 minute in DuPont Chemiluminescence kit, and exposing it to X-ray film for 10–30 seconds. When three species showed two bands (Fig. 1A), we performed another Western blot to compare one species (Gymnarchus) against 250 ng pure calretinin and calbindin protein (Sigma Chemical Co., St. Louis, MO; 1:1,000).

Anatomical figures were transferred to a Kodak photo CD, and gels were scanned on an Epson ES-1200C scanner. We made minor brightness and contrast adjustments and applied the unsharp mask filter to the digitized images using Adobe Photoshop 3.0. Images were assembled in Macromedia Freehand 5.0.

RESULTS

Antibody specificity and staining

We verified the specificity of the calretinin antibody used in this study by Western blot of all the species described in
anatomical detail below (Fig. 1A). In all species, the antibody recognized a band at 30 kDa (close to the reported molecular weight of 29 kDa) (Rogers, 1987; Schwall et al., 1993), but a faint band at 28 kDa was visible for Gymnarchus niloticus, Brienomyrus niger, and Gnathonemus petersii.

Because we were concerned about cross-reactivity with calbindin, we performed a Western blot of Gymnarchus with pure calretinin protein and calbindin protein, stained with the calretinin antibody, and we stained a duplicate membrane with a monoclonal calbindin antibody (Fig. 1B). We found that both bands on the fish matched bands in the pure calretinin protein lane (compare Fig. 1B, lanes 1 and 2), suggesting that both bands are calretinin. However, in contrast to the results of Schwall et al. (1993), the calretinin antibody did recognize pure calbindin protein (Fig. 1B, lane 3), and this band was at a different molecular weight (∼29 kDa) from either of the calretinin bands observed in Gymnarchus (Fig. 1B, lane 1). Also, we found that the monoclonal calbindin antibody recognized a faint 29 kDa band in Gymnarchus (Fig. 1B, lane 4), which aligned with the pure calbindin protein (Fig. 1B, lane 5), but which was distinct from either of the calretinin bands (Fig. 1B, lane 2). This result is unexpected because some studies have reported that calbindin is not present in teleosts (Parmentier et al., 1987; Schwall et al., 1993 Arevalo et al., 1995). We could not see any bands at 29 kDa labeled by the calretinin antibody in the fish samples (Fig. 1A, all lanes, and Fig. 1B, lane 1), so we conclude that cross-reactivity is low and most of the staining reported below is due to calretinin.

Histological sections generally showed well-differentiated staining. No within-species variation was seen in any of the cell types described here, although there were differences between species as described below.

**Mormyrid electrosensory areas**

The four types of electroreceptors in mormyrid fish have different temporal sensitivities. Since they follow distinct pathways in the CNS (Figs. 2A, 3; Szabo and Fessard, 1974; Bell, 1989), they can be analyzed individually for their calretinin-like immunoreactivity.

The knollenorgan electroreceptor encodes the temporal structure of brief stimuli (<1 ms) with precise spike latencies; that is, it is a time coder (Szabo et al., 1979; Amagai et al., 1993). The knollenorgan primary afferents run in the posterior and anterior lateral line nerves (pPLL, nPLL), which show weak calretinin-like immunoreactivity (Fig. 5). This weak labeling is not a general feature of cranial nerves, for example the statoacoustic (VIII) nerve was darkly labeled (Fig. 5). Knollenorgan primary afferents project to the nucleus of the ELL (NELL; Fig. 2A; Zipser and Bennett, 1976; Szabo et al., 1983; Bell et al., 1989). The NELL shows heavy calretinin-like immunoreactivity in Brienomyrus brachyistius and B. niger (Fig. 4). Adendritic round somata (10–15 µm diameter) are dark as are the initial segments of axons. The axons project into the lateral lemniscus (II), which has scattered, labeled fibers in these two species (Figs. 6–8). In Gnathonemus petersii by contrast, although labeling in the rest of the ELL is identical, the NELL and lateral lemniscus are unlabeled. Therefore, the labeled axons in the lateral lemniscus of B. niger and B. brachyistius probably arise solely from NELL somata.

The NELL projects bilaterally to the midbrain nucleus extrolateralis pars anterior (ELa), and ends on big and small cell types (Fig. 2A; Mugnaini and Maler, 1987a; Amagai, 1993; Amagai et al., 1993). The ELa receives its only input from the NELL. The ELa is very darkly labeled (Fig. 8). In Brienomyrus brachyistius, the labeling is due only to fibers from the NELL (Fig. 8B). However, in B. niger, round somata of diameter 10–15 µm are also strongly labeled. The ELa contains only two cell types (Mugnaini and Maler, 1987a), and these labeled cells are judged by their size to be the ELa big cells (also called “interstitial cells”). Their characteristic cup-shaped terminals around the unlabeled small cells are also labeled (diameter ∼5–10 µm; Fig. 8C). In Gnathonemus petersii, the ELa shows strong labeling in ELa big cell somata and a dense network of fibers, which probably arise from the big cells because the NELL in G. petersii was unlabeled.

ELa small cells project to the ELp, and there immunoreactivity also varies across species. In B. brachyistius, labeling in the ELp is weak (Fig. 7), although there appears the suggestion of a gradient of labeling of a few irregularly shaped cells (6–8 µm diameter), from darkest at the lateral edge to lightest at the medial edge (Fig. 7B). In the ELp of G. petersii, a large number of round (6–10 µm diameter) and elongate (∼5 × 10 µm) cells are lightly to darkly labeled, with one or two processes visible above background. In B. niger, no ELP cells are labeled.

Mormyromast electroreceptors are used in active electrolocation and detect the fish’s own electric organ discharge (EOD) as it is affected by objects near the fish (Szabo and Hagiwara, 1967). The mormyromast type A, which projects to the medial zone (MZ) of the ELL cortex, responds to impedance-induced EOD amplitude changes, whereas the mormyromast type B, which projects to the dorsolateral zone (DLZ), is more sensitive to capacitance-induced phase-shifts in the waveform (Fig. 2A; von der Emde and Bleckmann, 1992). Both mormyromast types encode stimuli by using spike latency and number of spikes (Bell, 1990), and therefore spike latencies and timing differences between types A and B encode electrical characteristics of objects near the fish.

Ganglion (GA), granule (GR), and intermediate cell and fiber (ICF) layers in mormyromast zones show strong calretinin-like immunoreactivity in all three mormyrid species (Fig. 4). Most of the labeling is small round and oval cells (diameter 5–7 µm). The pleomorphic (P) layer shows fibers running radially through it. We also labeled large round cells (diameter 10–15 µm) in the ICF, which are probably the commissural cells that connect left and right halves of the ELL (Fig. 4; Bell et al., 1981). Additionally, apical dendrites in the molecular layer (ML) are labeled (Fig. 4), which are difficult to ascribe to a particular cell type. We saw no output cell types labeled.

Ampullary receptors encode the amplitudes of low-frequency electrical stimuli by spike rate and have high spontaneous activity (Bell and Russell, 1978). Therefore, the importance of precise spike times is expected to be comparatively low. Ampullary primary afferents terminate in the ventrolateral zone (VLZ). Like the mormyromast zones, many small round cells are labeled in the GA, GR, and ICF layers (Fig. 4). However, unlike the mormyromast zones, we see no label either in apical dendrites in the ML or in commissural cells in the ICF layer.

All three zones of the ELL cortex project to the preeminent nucleus (PE) and the lateral toral nucleus (L; Fig.
Fig. 2. Electromotor and electrosensory pathways in mormyrid and gymnarchid. The mormyrid electromotor pathway includes a corollary discharge pathway that affects electrosensory areas. Items in boldface were found, in this study, to show strong calretinin-like immunoreactivity. Dotted lines indicate time-coding pathways.
2A). In the L of all three mormyrid species, there is a varied population of round (5–12 µm diameter) to elongate (5–7 × 11–18 µm) cells, scattered at low density throughout the nucleus. They appear to be mainly multipolar, but background labeling precludes detailed description of different cell types. The PE shows labeling only in about 30 oval multipolar cells (~11 × 18 µm) along the medial border for B. brachyistius and G. petersii (Fig. 6). They have thin, moderately branched processes that extend out more than 100 µm along the medial border of the PE and laterally into the PE. Large cells in this position have been described as projecting bilaterally to the L (Finger et al., 1981) and to the ELL (Bell et al., 1981), but double labeling is necessary to confirm this identification. These cells were not labeled in B. niger.

To summarize, we found the strongest calretinin-like immunoreactivity in the time-coding knollenorgan pathway, in NELL cells and ELa big cells. All three zones of the ELL cortex showed strong labeling in certain layers, but only the mormyromast zones, which may have greater dependence on temporal information than the ampullary zone, also show labeling in apical dendrites in the molecular layer and in the commissural cells.

**Mormyrid electromotor areas**

Mormyrids have irregular, pulse-type EODs. The command nucleus (COM) initiates the discharge (Grant et al., 1986). It projects to the medullary relay nucleus (MR) both directly and indirectly through the bulbar command-associated nucleus (BCA; Fig. 2C; Bell et al., 1983). The electromotor pathway does not show labeling. The COM has densely labeled fibers running through it, but no visible cell bodies (Fig. 4C). The BCA is unlabeled, and the medullary relay nucleus (MR) is only lightly labeled (Fig. 4C).

Five nuclei have been described so far in the corollary discharge pathway from the BCA to the electrosensory areas (Fig. 2C; Bell et al., 1983, 1995; Bell, 1989; Bell and von der Emde, 1995). The paratrigeminal command-associated nucleus (PCA) is very darkly labeled with somata from round (17–20 µm diameter) to oval (~12 × 20 µm), embedded in very dense fibers (Fig. 6B). The juxtalobar nucleus (JL) shows ~13 × 20 µm oval somata and dense fibers (Fig. 5B). No label above background is found in the medullary command-associated nucleus, the sublemniscal nucleus, or the juxta-lemniscal nucleus. Just these two nuclei in the corollary discharge pathway, and none in the direct electromotor pathway, show strong calretinin-like immunoreactivity.

**Mormyrid auditory areas**

The statoacoustic (VIII) cranial nerve showed strong calretinin-like immunoreactivity (Fig. 5B), but because it contains afferents from several end organs (the sacculus, the utriculus, the lagena, and the semi-circular canals; Bell, 1981a), it is not possible to tell from which end organ they originated. Two nuclei that receive input from the statoacoustic nerve were conspicuously labeled (Fig. 5C). The anterior nucleus, which receives input from saccular and lagener affrents (Bell, 1981a), showed strong labeling in round somata of 13–16 µm diameter (this nucleus is also called the dorsal zone of the descending octaval nucleus, or d2D; McCormick, 1992; Crawford, 1997a,b). The somata appear adendritic. The nucleus octavius, which receives input from all octavolateral organs except electroreceptors, showed strong labeling in 6–9 µm diameter round somata. Background labeling in octavius obscured any processes.

The medial octavolateral nucleus projects to the toral nucleus medialis dorsalis (MD; Bell, 1981b; Haugé-Carré, 1983), and responses to auditory stimuli have been recorded there (Crawford, 1993; Crawford, 1997a,b). The soma appear adendritic. The nucleus octavius, which receives input from all octavolateral organs except electroreceptors, showed strong labeling in 6–9 µm diameter round somata. Background labeling in octavius obscured any processes.

**Gymnarchid electrosensory areas**

Similarly to the mormyrid, the three types of electroreceptors in Gymnarchus have different temporal sensitivities and follow distinct pathways in the CNS (Szabo and Fessard, 1974; Bullock et al., 1975; Kawasaki and Guo, 1996), so we could analyze their calretinin-like immunoreactivity individually (Fig. 2B).
S-afferents encode the temporal characteristics of \(~400\) Hz sine-wave stimuli with precisely phase-locked spikes, that is, they are time-coders (Bullock et al., 1975; Szabo et al., 1992; Kawasaki and Guo, 1996). S-afferents run in the nALL and nPLL, which both show strong calretinin-like immunoreactivity (Figs. 9, 10). The thick axons of S-afferents label the most darkly, although other fiber types appear to label moderately. The S-afferents project to the medial zone (MZ) of the ELL, both directly and indirectly, through a group of giant cells that lie in the deep fiber layer (DF) within the afferent projection (Fig. 2B; Bass and Hopkins, 1982; Kawasaki and Guo, 1996). The giant cells have similar physiology to S-type afferents (Kawasaki and Guo, 1996). Giant cell somata show no calretinin-like immunoreactivity, but are visible as \(~50\) µm spherical holes within the fiber tract to the MZ (Fig. 10B). Dark staining of the large S-afferent axons obscures the small terminals that S-afferents make against the giant cells. The commissure that contains collateral axons from the giant cells is unlabeled (Figs. 9A, 10A).

Giant cells and S-afferents both terminate in the MZ (Szabo et al., 1992, 1993; Kawasaki and Guo, 1996). The MZ shows strong labeling of fibers that end at the inner cell layer (IC; Fig. 9B). These terminals are probably from S-afferents, since the giant cells are elsewhere entirely unlabeled (Fig. 10B) and we could not see the calyceal synapses of giant cells (Szabo et al., 1993; Kawasaki and Guo, 1996). Cell bodies in the IC or the outer cell layer (OC) of the MZ do not label for calretinin. The MZ shows moderate undifferentiated staining, and the OC is pale. The plexiform layer has tangentially running fibers.

Output cells in the MZ, which are sensitive to phase differences between different parts of the body, project up the lateral lemniscus into the torus semicircularis (TS; Kawasaki and Guo, 1996). No fibers in the lateral lemniscus show calretinin-like immunoreactivity (Figs. 11, 12), so the output cells from all zones of the ELL probably do not contain calretinin. Unlike Mormyrids, no parts of the torus semicircularis (TS) in Gymnarchus have particularly high levels of calretinin-like immunoreactivity, other than scattered cells. These cells are extremely varied in soma shape, from round (5–6 µm diameter) to elongate (\(\sim 5\times 10\) µm). Golgi studies of Gymnarchus are lacking, so a more detailed identification is not possible. Part of the lateral nucleus is distinctly unlabeled (Fig. 12). The PE also has a diverse set of scattered labeled cells, from round (5–10 µm diameter) to elongate (\(\sim 5\times 10\) µm; Fig. 11).

O-afferents encode the amplitude characteristics of EOD stimuli by the probability of firing on a given EOD cycle and are not as tightly phase-locked as S-afferents (Bullock et al., 1975; Kawasaki and Guo, 1996). O-afferents project
to the dorsal zone of the ELL (DZ; Szabo et al., 1992). Nowhere in the DZ is the staining as dark as the S-afferents, but there are distinct patches of more or less intense background staining (Fig. 10A). The darker patches have more frequent multipolar cell bodies labeled (8–10 µm diameter). The background staining in the different

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**Fig. 5.** Section at the level of the entry of the anterior lateral line nerve (nALL) of Brienomyrus brachyistius (see Fig. 3A). **A:** Overview. **B:** Close-up of boxed area in A showing the nALL and the juxtalobar nucleus (JL). **C:** Section just anterior to A, in the approximate location of the boxed area in the medial octavolateral nucleus (MO). Both nucleus anterior (ant) and nucleus octavius (oct) contain darkly labeled cell bodies. Ventral to the nucleus anterior are darkly stained fibers in the medial longitudinal fasciculus (mlf) surrounding the two large Mauthner axons. Scale bars = 500 µm in A, 100 µm in B, 50 µm in C.
patches is too undifferentiated to determine if it arises from cells inside or outside the DZ. Inner and outer divisions of the DZ do not stain differentially.

Ampullary afferents encode low-frequency electrical stimuli and have high spontaneous activity (Kawasaki and Guo, 1996). The precise timing of spikes is therefore expected to be comparatively unimportant. Ampullary afferents project to the ventral zone (VZ) of the ELL. Calretinin-like immunoreactivity in the VZ is faint, except in the thin ampullary afferent fibers (Fig. 9).

In summary, we found the strongest calretinin-like immunoreactivity in the phase-coding S-afferents. The giant cells, which are physiologically similar to S-afferents, were unlabeled. The O-afferent and ampullary zones of the ELL, which are not precise time-coders, were comparatively weakly labeled.

Gymnarchid electromotor areas

Gymnarchus has a regular, wave-type EOD. Gymnarchus has a linear chain of electromotor nuclei, starting with the pacemaker nucleus (PN) relayed through the lateral relay nucleus (LRN) and the medial relay nucleus (MR), ending with the electromotorneurons in the spinal cord (Fig. 2D; Szabo, 1961; Kawasaki, 1994). Gymnarchus has no corollary discharge system.
The PN is unlabeled, but the LRN is very darkly labeled. Round somata (diameter 18–24 µm) are dark, as are the axons as they make their way to the MR (Fig. 9C). MR somata are round (~50 µm diameter) and are moderately labeled. No other motor areas have been identified.

**DISCUSSION**

In mormyriform electrosensory systems, the cell types most devoted to preserving temporal information in precise spike latencies stain most prominently for calretinin-like immunoreactivity. The mormyrid knollenorgan pathway is characterized by labeling in the NELL (for Brienomyrus niger and B. brachyistius) and in the EL a big cells (for Gnathonemus petersii and B. niger). In the gymnarchid phase-coding system, S-type primary afferents are strongly labeled. This is consistent with the hypothesis that calcium-binding proteins are involved in the preservation of timing information across synapses.

It remains to be determined in what ways time-coding systems differentially rely on calcium buffering, but many scenarios are possible. In time-coding systems, variation in spike times need only be small to affect behavior, from tens of microseconds in the barn owl (Carr and Konishi,
1990) to hundreds of nanoseconds in Eigenmannia (Carr et al., 1986a). With repetitive firing, calcium buildup could activate calcium-dependent conductances and change the input resistance presynaptically or postsynaptically, thereby modifying the timing of transmitter release or spike initiation. Indeed, calcium buffers injected into cells can modify synaptic activity (Adler et al., 1991). Additionally, time-coding systems make heavy use of electrotonic synapses which can be blocked by calcium (Spray and Burt, 1990). In general, calcium-binding proteins could be protecting the cells in time-coding pathways from spatial or temporal spread of calcium. We speculate that their expression in a small subset of cells in cortex, hippocampus, or cerebellum (Andressen et al., 1993) could indicate a particular reliance of those cells on spike timing. Indeed, the importance of precise spike times in cortex may be underappreciated (Mainen and Sejnowski, 1995).

There are cell types in the time-coding pathways studied here that are unlabeled, even though they show similar spiking patterns. In the mormyrid, NELL cells and ELa big cells, which are strongly labeled, relay spikes from primary knollenorgan afferents, which are only faintly labeled. Giant cells in Gymnarchus follow one-to-one with their S-afferent inputs (Kawasaki and Guo, 1996), yet they are entirely unlabeled. Assuming that the primary role of calcium binding proteins in time-coding systems is to ensure fidelity of precise spike times across synapses, the lack of labeling in these cell types suggests that they use other mechanisms of synaptic transmission that are insen-

Fig. 8. Section at the level of nucleus externolateralis pars anterior (ELa) of Brienomyrus brachystius (see Fig. 3A). A: Overview. B: Close-up of boxed area in A. Fibers from NELL are heavily labeled, but no cell bodies are visible. C: ELa of B. niger. Big cell bodies are visible in the ELa, as are their cup-shaped terminals around unlabeled small cells (arrows). Scale bars = 500 in A µm, 50 µm in B, C.
sitive to calcium buildup. The most likely alternative mechanism is electrotonic synapses. Indeed, both the synapse from S-afferents to giant cells and the synapse from knollenorgan afferents to NELL cells are electrotonic (Szabo and Ravaille, 1976; Mugnaini and Maler, 1987b; Szabo et al., 1993). On the other hand, the synapse from NELL cells to ELa big cells is mixed electrotonic and chemical (Mugnaini and Maler, 1987a), and both those cell types contain calcium-binding proteins.

Parts of the cortex of the mormyrid ELL that have greater reliance on spike arrival times also show more calretinin-like immunoreactivity. Mormyromast afferents code stimulus intensity by first spike latency (Szabo and Hagiwara, 1967; Bell, 1990; Hall et al., 1995), and behaviorally fish are able to make fine temporal discriminations (von der Emde and Zelick, 1995). Commissural cells and apical dendrites of an undetermined cell type show calretinin-like immunoreactivity in the mormyromast zones. The ampullary zone shows no labeling in these cell types, which agrees with the ampullary afferents not carrying precise timing information. The role of calcium-binding proteins in the apical dendrites of cells in the mormyromast zones is unclear, but it is unlikely to be due to calcium spikes in the dendrite, because apical dendrites in the

Fig. 9. Section at the level of the ELL of Gymnarchus niloticus (see Fig. 3B). A: Overview. Scattered cell bodies are visible in dorsal zone (DZ) and ventral zone (VZ). B: Close-up of boxed area of the medial zone (MZ) in A. Fibers in MZ are heavily labeled, but no cell bodies in the inner or outer cell layers (IC, OC) are labeled. Some fibers are visible running tangentially in the plexiform layer (P). C: Close-up of boxed area in ventral part of A, showing the motor circuit. The pacemaker nucleus (PN) is unlabeled, but the lateral relay nucleus (LRN, small arrow) stains heavily, and the medial relay nucleus (MR, small arrow) stains moderately. Note the labeled LRN fibers projecting to MR (large arrows). Scale bars = 500 µm in A, 100 µm in B, 200 µm in C.
ampullary zone also show spikes (Bell et al., 1993), and because in the gymnotiform Apteronotus leptorhynchus, apical dendritic spikes are carried by TTX-sensitive sodium channels (Turner et al., 1994).

In Gymnarchus, we found only light calretinin-like immunoreactivity in all zones of the ELL. Most MZ labeling is due to S-afferent fibers, and we saw no cell bodies in the MZ. The VZ and DZ do show some labeled cell...
bodies, but since these areas are completely unexplored physiologically, it is difficult to make functional conclusions. We suggest that the patches of calretinin-like immunoreactivity in the DZ indicate some sort of functional subdivisions, particularly as they appear to coincide with "dips" in the height of the dorsal cell layer visible in Nissl stain (Bass and Hopkins, 1982).

**Calretinin and transformation of temporal information**

Within time-coding pathways, low-order nuclei in the sequence tend to have high concentrations of calcium-binding proteins, but the expression stops as the pathway progresses through some high-order cell type. The change of expression tends to coincide with the point at which temporal information ceases to be represented as precisely phase-locked spikes, and is transformed, or re-coded, such that presence of activity itself in a subset of cells becomes the representation of temporal information. The subset of cells may be further organized into a computational map. Thus in the gymnotiform phase-coding pathway, high immunoreactivity to calbindin was found in T-afferents, spherical cells of the electrosensory lateral line lobe (ELL), and giant cells in layer VI, but not in the small cells of layer VI, where phase-locked spikes from different parts of the body are re-coded to phase difference information (Maler et al., 1984; Heiligenberg and Rose, 1985; Carr et al., 1986b; Losier and Matsubara, 1990). Similarly, in the barn owl time-coding pathway, high immunoreactivity to calbindin antibodies was found in auditory afferents,

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Fig. 12. Section at the level of the torus semicircularis (TS) of Gymnarchus niloticus (see Fig. 3B). A: Overview. B: Close-up of boxed area in A. All midbrain nuclei have scattered labeled cells, except in a conspicuously unlabeled central region of the lateral nucleus (asterisk). Scale bar = 500 µm in A, 200 µm in B.
nucleus magnocellularis neurons, and nucleus laminaris neurons, where time-of-arrival of sounds at the two ears is re-coded into a place map of interaural delay, but not in the postsynaptic targets of nucleus laminaris (Carr, 1986; Takahashi et al., 1987; Carr and Konishi, 1990). The immunohistochemical border between presence and absence of calcium-binding proteins correlates with the functional border between representing fine temporal information in spike arrival times and re-coding it into a computational map.

As this study shows, in some mormyrids, NELL cells (Fig. 4) and ELa big cells (Fig. 8) had calretinin-like immunoreactivity, but the ELa small cells did not. The small cells have been hypothesized to re-code temporal information because they are targets of an excitatory direct input from the NELL and a presumably inhibitory indirect input through the ELa big cells (Mugnaini and Maler, 1987a; Amagai, 1993). Small cells also lack many of the anatomical specializations that correlate with preservation of fine temporal information: they have small somata, thin axons, less heavy myelination, and small synapses (Mugnaini and Maler, 1987a; Friedman, 1995). Lack of calretinin-like immunoreactivity reinforces the hypothesis that ELa small cells re-code temporal information.

A parallel case appears in the mormyrid auditory system. Statoacoustic nerve afferents (Fig. 5B), cells in the hindbrain medial octavolateral nucleus (Fig. 5C), and fibers in the mediodorsal-most part of the midbrain MD (Fig. 7) showed calretinin-like immunoreactivity, but somata in the MD did not. On the basis of calretinin-like immunoreactivity, therefore, this pathway may process temporal characteristics of auditory signals, in which re-coding occurs in MD. In fact, in a related species of mormyrid, Pollimyrus, this part of MD has been found to have complex response properties to auditory stimuli, such as responding to particular temporal characteristics (inter-click interval; Crawford, 1993, 1997a).

The relationship between calretinin-like immunoreactivity and re-coding is less clear in Gymnarchus; however. Kawasaki and Guo (1996) showed that giant cells have the same phase-locked spike pattern as S-type afferents, but the giant cells do not label for calretinin (Fig. 10). In this system, re-coding is performed by cells in the inner cell layer in the medial zone, which responds to phase differences between distant parts of the body, by using a computational algorithm similar to that of the small cells of Eigenmannia (Kawasaki, 1993). Giant cells may not have high levels of calretinin because they contain a different calcium binding protein, because they accumulate less calcium, or because it affects them less, for unknown reasons.

**Electromotor systems**

Electromotor systems also require preservation of timing information to ensure that the electrocytes are activated simultaneously. In Gymnarchus, the LRN has high calretinin-like immunoreactivity (Fig. 9), and the MR is also somewhat labeled, which is consistent with the hypothesis that calretinin helps in the preservation of temporal information from the PN through the LRN to the MR. However, the electromotor system of mormyrids does not show labeling in the COM, BCA, or MR. The electromotor system of mormyrids may use mechanisms to preserve temporal information that do not require calcium-binding proteins. In particular, cells within the COM and the MR in mormyrids are strongly coupled electrotonically, and the synapse between the COM and the MR is mixed chemical and electrotonic (Bennett et al., 1967; Elekes and Szabo, 1985, 1986; Grant et al., 1986). Since all the neurons in an electromotor nucleus should be active simultaneously, electrotonic coupling can increase fidelity without degrading utility. Interestingly, in the gymnotiform Eigenmannia, MR cells are not electrotonically coupled with each other (Elekes and Szabo, 1981), and they are heavily calbindin immunoreactive (Maler et al., 1984).

Unlike motor systems, sensory systems cannot increase temporal fidelity by electrotonically coupling units within one level of a sensory pathway, because this mechanism would degrade spatial resolution. Sensory systems are therefore constrained to optimize synapses to preserve temporal information across them. Sensory systems do use convergence, but this is primarily effective for reducing temporal variation among peripheral receptors, whose sensitivity makes them prone to noise (Carr et al., 1986a; Carr, 1993). Convergence sacrifices spatial resolution for sensitivity. Indeed, reducing jitter through convergence relies on temporal fidelity of synapses so that no additional temporal noise is introduced at the synapse (Amagai, 1993; Carr and Amagai, 1996).

Mormyrids show significant labeling in only two nuclei of the corollary discharge pathway. The PCA (Fig. 6B) and the JL (Fig. 5B) both show staining, but no intervening nuclei show staining. The unlabeled nuclei may preserve temporal information with a mechanism that does not require calretinin, such as using electrotonic coupling, or they may not have the same requirement for temporal fidelity. The ultrastructure of the corollary discharge pathway has not been described. It is interesting to note that the two nuclei that do show calretinin-like immunoreactivity project out of the corollary discharge pathway. One might speculate that the synaptic specializations the rest of the pathway uses to ensure temporal fidelity may not be available at this juncture.

**Species differences in calretinin expression**

We found species differences in calretinin-like immunoreactivity in NELL cells, ELa big cells (Fig. 8) and cells in E.Lp. Species differences in calcium-binding proteins are common (Baimbridge et al., 1992). Losier and Matsubara (1990) found that calbindin immunoreactivity in the gymnotiform electrosonorous time-coding pathway correlated with EOD discharge rate. Apteronotus (500–1,000 Hz) and Eigenmannia (250–500 Hz) both had strong reactivity, but the low-frequency Sternopygus (50–150 Hz) had none. This is consistent with the hypothesis that calcium-binding proteins keep intracellular calcium low, since time-coding units in Apteronotus and Eigenmannia fire more frequently than in Sternopygus, and therefore could be accumulating higher calcium loads. In the mormyrids studied here, however, EOD characteristics are very similar. All three species tested have similar EOD duration (~0.5 ms), peak power content (3.5–5 kHz), and interpulse intervals.

Part of the variation in expression could be that other calcium-binding proteins are expressed in place of calretinin in calretinin-negative areas. Assessing the expression of different calcium-binding proteins has historically had trouble due to antibody specificity problems. Antibodies to calbindin have recognized more than one protein, such as....
calbindin (Rogers, 1987) or perhaps a second "form" of calbindin (Parmentier et al. 1987). So, for example, the calbindin reactivity in the barn owl described by Takahashi et al. (1987) was probably due to cross-reactivity with calretinin (Rogers, 1987). Likewise, calbindin reactivity in Eigenmannia reported by Maler et al. (1984) and Losier and Matsubara (1990) overlaps with reactivity to the calretinin antibody used in this study (Cawong, personal communication). As we show here (Fig. 1), this antibody has similar problems, although the cross-reactivity is low. Despite reports that antibodies against calretinin or calbindin recognize the same single band in Western blots of teleosts (Parmentier et al., 1987; Schwaller et al., 1993), we found evidence of both calretinin and calbindin in Gymnarchus niloticus. The expression of further calcium-binding proteins in mormyrid and gymnarchid fish will require more study.

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LITERATURE CITED


