Electrical synaptic transmission in developing zebrafish: properties and molecular composition of gap junctions at a central auditory synapse

Cong Yao, Kimberly G. Vanderpool, Matthew Delfiner, Vanessa Eddy, Alexander G. Lucaci, Carolina Soto-Riveros, Thomas Yasumura, John E. Rash, and Alberto E. Pereda

1Dominick P. Purpura Department of Neuroscience, Albert Einstein College of Medicine, Bronx, New York; 2Department of Biomedical Sciences, Colorado State University, Fort Collins, Colorado; and 3Program in Molecular, Cellular and Integrative Neurosciences, Colorado State University, Fort Collins, Colorado

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Because of its experimental accessibility, developing zebrafish has become an increasingly popular organism for not only the study of genetic and developmental aspects of the vertebrate nervous system (Roussigné et al. 2012; Stuckenholz et al. 2005; Ulrich et al. 2011), but also the understanding of diverse neural processes at the systems level (Ahrens et al. 2012; Beck et al. 2004; Bianco et al. 2012; Burgess and Granato 2007; McLean et al. 2007). Consequently, a good understanding of the properties of various forms of synaptic communication operating between larval zebrafish neurons is required to appreciate the network mechanisms underlying these processes. Whereas chemical excitatory (Patten and Ali 2007, 2009; Patten et al. 2010; Wen and Brehm 2005; Wen et al. 2013) and inhibitory (Legendre and Korn 1994, 1995; Legendre 1999) transmissions have been the focus of several studies, significantly less is known regarding the properties and molecular composition of electrical synapses in developing zebrafish. Moreover, a better understanding of the mechanisms underlying electrical transmission in developing zebrafish will contribute to a better characterization of neural processes that heavily rely on this modality of transmission, including retinal mechanisms (Li et al. 2012; Morris et al. 2008; Wei et al. 2012; Wong et al. 2004) and motor behaviors (Ahrens et al. 2012; Koyama et al. 2011; McLean and Fetcho 2009; Saint-Amant and Drapeau 2001).

Developing zebrafish offer a convenient model to study the development of the auditory system (Kohashi and Oda 2008; Nicolson 2005; Tanimoto et al. 2009). Auditory “mixed” (electrical and chemical) synapses terminating on the teleost Mauthner cell (M-cell), known as “large myelinated club endings” or simply club endings (CEs), constitute a valuable model for the study of vertebrate electrical transmission, as they are amenable to correlation of their ultrastructural and biochemical features with their in vivo physiological properties (Pereda et al. 2004). The M-cell network, centered on the properties and spinal connectivity of these colossal reticulospinal neurons, mediates auditory-evoked tail-flip escape responses in teleost fish (Fabre and Pereda 2011). A wealth of data support the primary role of CEs in these behaviors (Curti and Pereda 2010; Mu et al. 2012). Recent evidence (Tanimoto et al. 2009) indicates that the functional maturation of the inner ear after formation of the auditory pathway is a critical process in the acquisition of auditory inputs by second-order neurons located in the central nervous system (CNS) such as the M-cell (Tanimoto et al. 2009). However, the developmental properties of auditory synapses themselves on the M-cell remain unknown.

To gain insight into the maturation and properties of electrical synapses in developing zebrafish, we took advantage of our knowledge of the properties (Lin and Faber 1988; Pereda et al. 2004) and molecular determinants (Flores et al. 2008; Pereda et al. 2003; Rash et al. 2013) of electrical transmission at CEs in adult goldfish. Here, we show that, parallel to the development of auditory responses in the M-cell of larval zebrafish (Tanimoto et al. 2009), CEs become identifiable by immunohistochemistry after day 3 postfertilization (dpf) to reach a number of ~6/M-cell between 4 and 9 dpf. As in goldfish CEs, gap junctions at these terminals are formed by homologs of the mammalian connexin 36 (Cx36; Condorelli et al. 2000), Cx35, and Cx34.7 (O’Brien et al. 1998) and contain the connexin-associated protein zonula occludens-1 (ZO-1; Flores et al. 2008), suggesting that gap-junction channels are supported by a similar molecular scaffold. We also demonstrate using electrophysiological approaches that these mixed synapses exhibit functional properties that are comparable with those of adult goldfish, indicating that a relatively small number of audi-
tory terminals (~6 vs. ~85 in adult goldfish; Furukawa and Ishii 1967) is sufficient to support escape responses (Tanimoto et al. 2009) at these early developmental stages.

**METHODS**

Zebrafish husbandry and mutants. AB zebrafish were maintained under a 13:11-h light-dark cycle at 28°C using standard laboratory practices (Westerfield 2000). Zebrafish embryos were obtained by natural mating and were staged according to number of dpf and confirmed according to standard morphological criteria (Kimmel et al. 1995). All procedures were performed with approval from the Institutional Animal Care and Use Committee of Albert Einstein College of Medicine.

**Immunohistochemistry and confocal imaging.** Zebrafish at 2–9 dpf were fixed in either 4% formaldehyde made from freshly depolymerized paraformaldehyde (PFA) or 2% TCA at 4°C overnight or at room temperature for 3 h, dehydrated into methanol using a graded series (20, 40, 60, 80, and 100% methanol), and stored overnight or longer at −20°C. Before staining, fixed larvae were rehydrated into PBSTx (PBS with 0.5% Triton X-100) through a graded methanol series (100, 80, 60, 40, 20, and 0% methanol) and washed in PBSTx three times for 5, 10, and 15 min, respectively. Larvae were then individually transferred onto a glass slide for dissection. The brains were carefully removed using tweezers and sharp tungsten needles under a bright-field microscope. In some larvae, the forebrain, the midbrain, and the cerebellum were also removed to reduce the thickness of the sample. The isolated brains were collected and washed in PBSTx, incubated at room temperature in blocking solution (10% newborn goat serum in PBSTx) for at least 1 h, and then transferred into blocking solution containing the primary antibodies at the following dilutions: Cx35 monoclonal antibody (MAB3043; EMD Millipore), 1:300; ZO-1 monoclonal antibody (cat. no. 33-9100; Invitrogen), 1:200; and ZO-1 rabbit polyclonal antibody (cat. no. 61-7300; Invitrogen), 1:200. The following day, brains were washed in PBSTx five times (5, 10, 15, and 30 min). After the washes, the brains were transferred into the blocking solution containing secondary antibodies at 1:200 dilutions. Secondary antibodies included: Cy5-tagged goat-anti-mouse IgG (A-10524; Invitrogen), Cy2-tagged goat-anti-rabbit IgG (cat. no. 111-225-003; Jackson ImmunoResearch Laboratories), Alexa Fluor 488-tagged goat-anti-mouse IgG (A11001; Invitrogen), and Alexa Fluor 594-tagged goat-anti-rabbit IgG (A11012; Invitrogen). The brains were incubated in the secondary antibody solution in the dark at room temperature for 4 h or 4°C overnight and handled in the dark. Then, the brains were washed three times for 5, 10, and 15 min, transferred to methanol using a graded series (20, 40, 60, 80, and 100%), and stored in methanol until imaging (usually the same day of experiment). To mount the brains on a slide, each brain was transferred onto a slide in the dark. Brains were mounted on a slide with a drop of BBA (33% benzyl benzoate and 67% benzyl alcohol) and covered with a coverslip, which was quickly applied to the incision for ~10 s. Backfilled larvae were removed from the agarsore 5 min after this procedure and allowed to recover in egg water at 28°C for 3–4 h. Larvae were then fixed in 4% formaldehyde with 1% sucrose in PBSTx at room temperature for 3 h and processed for immunolabeling as described above.

**Freeze-fracture replica immunogold labeling.** Backfilling with tetramethylrhodamine-dextran (70,000 mol wt; D1818; Invitrogen), was quickly applied to the incision for ~10 s. Backfilled larvae were removed from the agarsore 5 min after this procedure and allowed to recover in egg water at 28°C for 3–4 h. Larvae were then fixed in 4% formaldehyde with 1% sucrose in PBSTx at room temperature for 3 h and processed for immunolabeling as described above.

**Electrophysiology.** Zebrafish 4–7 dpf were used for electrophysiological recordings. To expose the brain, the larvae were first paralyzed in a solution containing 10 μM d-tubocurarine (Sigma) in a solution consisting of (in mM): 134 NaCl, 2.9 KCl, 2.1 CaCl₂, 1.2 MgCl₂, 10 HEPES glucose, pH 7.8, 274 mosM (Koyama et al. 2011). Larvae were placed on their backs and held with pins in a Sylgard-coated petri dish, and the brain stem was accessed ventrally following the procedure described in Koyama et al. (2011). The dissected larvae were transferred to the recording setup and maintained with the same external solution. M-cells were identified using far-red differential interference contrast (DIC) under ×40 water-immersion objective and recorded using a whole cell recording configuration under current-clamp. The electrode (5 MΩ) internal solution consisted of (in mM): 105 CH₃O₃SK, 10 HEPS, 0.1 EGTA, 2 MgCl₂·6H₂O, 4 Na₃ATP, 0.4 Tris-GTP, 10 K₂-phosphocreatine, 23 mannitol, adjusted to pH 7.2, 274 mosM (Koyama et al. 2011). The junction potential was estimated to be ~12 mV. Resting potentials ranged from 60 to 72 mV; M-cell action potential ranged from 73 to 80 mV; the input resistance of the M-cell ranged from 5 to 7 MΩ. Bipolar stimulation of the ear was obtained using a “theta” (septated) glass pipette filled with external solution. This pipette was positioned under visual guidance in the posterior macula in the ear, near the hair cells. In vivo intradendritic recordings (250–350 μm from the M-cell soma) of mixed synaptic responses in adult goldfish were done using the experimental approaches that were described previously (Smith and Pereda 2003). Population synaptic responses were obtained by stimulating the posterior branch of the VIIIth nerve. Single terminal synaptic responses were obtained by recording from individual VIIIth nerve afferents in the optic terminals (~6 vs. ~85 in adult goldfish; Furukawa and Ishii 1967) is sufficient to support escape responses (Tanimoto et al. 2009) at these early developmental stages.
the posterior VIIIth nerve outside of the brain while simultaneously recording the lateral dendrite of the M-cell.

Labeling of auditory afferents. 4-dpf Tol-056 enhancer trap transgenic zebrafish [where M-cells are tagged with green fluorescent protein (GFP); kindly provided by Dr. Michael Granato] were fixed overnight at 4°C in 4% PFA with 4% sucrose. Following washing with PBSTx, the fish were mounted on 3% agar and covered with 1.5% low-melting-point agarose. A glass micropipette containing 1.0% 1,1′-dioctadecyl-3,3′,3′,3′-tetramethylindocarbocyanine perchlorate (DiI; D282; Invitrogen) in chloroform was inserted into the ear directly dorsal to the posterior otolith, and DiI was applied by pressure (40 psi during 12 ms) using a Picospritzer (Picospritzer III; Parker Automation). Following this procedure, larvae were removed from the agar plate and placed at room temperature in 1× PBS overnight. Brains were then removed, stained with mouse anti-Cx35 and rabbit recombinant monocular anti-GFP antibodies (G10362; Invitrogen) at 1:300 dilutions, and finally imaged using the procedures described above.

Labeling of dopaminergic fibers. The relative position of dopaminergic fibers to the M-cells was revealed using the Tg(dat:EGFP) transgenic zebrafish expressing green fluorescent protein in dopaminergic neurons of the ventral diencephalon (kindly provided by Dr. Mark Ekker; Xi et al. 2011). dat:EGFP zebrafish 4–5 dpf were backfilled with Texas red dextran (D-332; Invitrogen). After fixation, the larvae were stained with mouse anti-Cx35 and rabbit recombinant monocular anti-GFP antibodies (G10362; Invitrogen) at 1:300 dilution.

RESULTS

Identification of CEs in developing zebrafish. CEs are terminations of auditory afferents originating in the telencephalic sacculus (Fig. 1A). They comprise a relatively homogeneous morphological group of terminals that are tightly clustered on the distal portion of the lateral dendrite of the M-cell (Bartelmez and Hoerr 1933). These endings can be unambiguously identified in the lateral dendrite of the goldfish M-cell by immunolabelling with anti-Cx35 (Fig. 1B; Flores et al. 2008; Pereda et al. 2003), as the approximately 60–100 gap-junction plaques contained at each of these unusually large contacts are homogenously distributed throughout the whole appositional surface (Fig. 1, B and C). Thus we first determined that CEs in developing zebrafish could also be identified by Cx35 labeling. For this purpose, reticulospinal neurons including the M-cell and MiD2cm and MiD3cm cells of 4–8 dpf zebrafish were backfilled by spinal application of tetramethylrhodamine-dextran, and the intact brains were labeled with monoclonal Cx35 antibody (Fig. 2A). As illustrated in Fig. 2, immunofluorescence was observed along the distal portion of the M-cell lateral dendrite at large ovoid areas that, because of their relative size, ovoid shape, and location, were presumed to correspond to CEs. As in adult goldfish, Cx35-labeled ovoid areas were defined by multiple fluorescent patches (Fig. 2, B–D). To confirm that they correspond to auditory afferents, we performed DiI applications in the vicinity of the posterior macula of the ear (thought to give rise to the saccular macula; Nicolson 2005), which resulted in the labeling of axons that terminated on these large ovoid areas (Fig. 3, A–C). Other, much smaller Cx35 immunoreactive puncta were found in more proximal areas of the lateral dendrite and soma (Fig. 2B), as observed in goldfish (Flores et al. 2008; Pereda et al. 2003), which probably correspond to other octovalateralis inputs to the M-cell (Szabo et al. 2007). This labeling is also consistent with recent reports indicating the presence of Cx35 in the M-cell dendrite of developing zebrafish (Jabeen and Thirumalai 2013). Finally, numerous immunoreactive puncta also were observed along the hindbrain, in particular at areas corresponding to the developing ear (Fig. 2B).

Developmental profile of CEs. The ability to identify CEs with Cx35 labeling provided us with the opportunity to examine the developmental appearance of these contacts. Zebrafish at 3–9 dpf were examined with anti-Cx35, and their diameter, number, and relative size (estimated as the diameter of the CE divided by the diameter of the lateral dendrite of the M-cell) were quantified (Fig. 3D). Although fewer and smaller in diameter than those of adult goldfish, these large Cx35-labeled areas became identifiable at day 3, reaching a stable number of ~6/M-cell between days 4 and 9; the average number of CEs was 6.2 ± 0.68 SE (n = 10 dendrites) in 5 dpf and 6.02 ± 0.41 (n = 4 dendrites) in 9-dpf zebrafish (P = 0.40, 1-tailed t-test comparing 5 vs. 9 dpf; Fig. 3E). Their diameter and relative size were also found to be relatively stable between days 4 and 9 (Fig. 3, F and G). The average CE diameter was 1.90 μm ± 0.15 in 4-dpf larvae (n = 11), 1.81 ± 0.04 in 5-dpf larvae (n =
74), 1.77 ± 0.08 in 6-dpf larvae (n = 13), and 1.90 ± 0.06 in 9-dpf larvae (n = 24; Fig. 3F); these measurements were not significantly different from each other (P > 0.05). The CE relative size at different ages was also not significantly different (P > 0.05), averaging 1.01 ± 0.06 in 4 dpf (n = 11), 0.94 ± 0.04 in 5 dpf (n = 74), 1.06 ± 0.04 in 6 dpf (n = 12), and 1.01 ± 0.06 in 9-dpf zebrafish (n = 24).

We next compared these parameters with those of adult goldfish (7–12 cm long) and adult zebrafish (3–4 cm long; Fig. 4, A–C). The average diameter of these Cx35-labeled areas was 6.46 μm ± 0.22 in adult goldfish (n = 45), 4.5 μm ± 0.23 in adult zebrafish (n = 21), and 1.83 μm ± 0.04 in 4- to 9-dpf zebrafish (n = 98; Fig. 4E). The relative size was 0.27 in adult goldfish, 0.27 in adult zebrafish, and 0.97 in 4- to 9-dpf zebrafish (Fig. 4F). The small number of CEs identified in larval zebrafish markedly contrasted with those observed in adult goldfish (Furukawa and Ishii 1967) and other teleosts (Bartelmez and Hoerr 1933; Bodian 1937), which are estimated to be ~85/M-cell (Fig. 4D), a number that is also likely to apply to adult zebrafish (see DISCUSSION). Thus, from the comparative point of view, CEs in 4- to 9-dpf larval zebrafish are fewer in number, but their relative size is larger (Fig. 4G), suggesting that a number of ~6 terminals/M-cell is sufficient to support their function during these developmental stages.

Number and molecular composition of gap junctions in CEs of larval zebrafish. We then determined the number of gap junctions and gap-junction channels that were present at a single larval zebrafish CE. It has been established that the number of Cx35 puncta at single goldfish CEs (Fig. 1B) correlates with the number of gap junctions present at these terminals (Flores et al. 2008). To improve visualization of these puncta at single larval zebrafish CEs, we performed deconvolution analysis of confocal images (Fig. 5, A and B; AutoQuant software). The number of manually counted puncta averaged 17.72 ± 0.99 (SE; n = 22). A second, automatic deconvolution analysis (Volocity software) of the same set of images yielded a similar number, averaging 17.82 ± 2.12 puncta (see METHODS for details), suggesting that 4- to 6-dpf zebrafish CEs contain on average ~18 gap-junction plaques (Fig. 5C). This suggestion was further supported by ultrastructural analysis with FRIL of two probable, partially reconstructed CEs. For their identification in the replica, the M-cells were retrogradely labeled by application of tetramethylrhodamine-dextran in the spinal cord (see METHODS). Because of their unusually large size (~1.3- to 1.9-μm diameter and 1.5- to 3-μm² area of apposition) and presence of multiple (>10) gap junctions/axon terminal and based on the presence of multiple large synapses on the same neuron as well as the anatomic location of these cells in hindbrain, these mixed (electrical and chemical) synapses are identified as CEs. CEs are the only axon terminals of that size that we found labeled with Cx35 under confocal microscopy in this area of the larval zebrafish hindbrain (Fig. 2), and their distinctively larger size is consistent with previous ultrastructural findings (Kimmel et al. 1981). The size of the gap-junction plaques was highly variable (0.001-0.239 μm²), and 2 CEs examined by FRIL (Fig. 6D)

Fig. 2. Labeling for Cx35 in M-cells of larval zebrafish. A: low-magnification laser-scanning confocal projection showing the staining pattern of monoclonal Cx35 antibody (green; MAB3043; EMD Millipore) in the hindbrain of a 4-days-postfertilization (dpf) zebrafish larva. Reticulospinal neurons (Mauthner, MiD2cm, and MiD3cm) were backfilled by spinal application of tetramethylrhodamine-dextran (Backfill-TRho; red). B: high-magnification image of the M-cell showing punctate staining for Cx35 on the cell body and lateral dendrite. Note the larger Cx35-labeled oval areas located at the distal portion of the lateral dendrite (arrows inside dotted box), which because of their relative size and location, are reminiscent of Cx35-labeled CEs in goldfish. Insert: high magnification of the boxed area (*) showing Cx35-labeled CEs. C: high-magnification confocal projection showing punctate labeling for Cx35 at CEs on the M-cell on the bifurcation of the lateral dendrite (6 dpf). D: confocal projection showing punctate labeling for Cx35 at CEs in an 8-dpf zebrafish larva. Note the similarity of Cx35 labeling with that observed in adult goldfish (Fig. 1, B and C).
contained approximately 1,866 and 1,498 connexons, respectively. Because in both cases the fracture plane was diverted from the CE plasma membrane into the M-cell cytoplasm at an area of perpendicular membrane apposition (Fig. 6, from the CE plasma membrane into the M-cell cytoplasm at an area of perpendicular membrane apposition (Fig. 6, from the CE plasma membrane into the M-cell cytoplasm at an area of perpendicular membrane apposition (Fig. 6, from the CE plasma membrane into the M-cell cytoplasm at an area of perpendicular membrane apposition (Fig. 6, from the CE plasma membrane into the M-cell cytoplasm at an area of perpendicular membrane apposition (Fig. 6, from the CE plasma membrane into the M-cell cytoplasm at an area of perpendicular membrane apposition (Fig. 6, from the CE plasma membrane into the M-cell cytoplasm at an area of perpendicular membrane apposition (Fig. 6, from the CE plasma membrane into the M-cell cytoplasm at an area of perpendicular membrane apposition (Fig. 6, from the CE plasma membrane into the M-cell cytoplasm at an area of perpendicular membrane apposition (Fig. 6), and, 22 of the 25 gap junctions found in postsynaptic hemiplaques in these 2 adjacent CEs were labeled almost exclusively for Cx34.7 (113 of 116 gold beads with 3 beads/2.5% of intermediate diameter (25 nm), reflecting either 2.5% of gold beads labeling for Cx35 or, more likely, the unavoidable but minor variability in sizes of gold beads (4.8% coefficient of variation; product data sheet; BBInternational)]. The other 7 unlabeled gap junctions were small (4–85 connexons) and therefore may have gone unlabeled due to the moderate labeling efficiency (1 gold bead/30 connexons) and the stochastic nature of immunogold labeling or represent evidence for a few neuronal gap junctions composed of another as yet unidentified connexin other than Cx34.7 and Cx35. (Four additional potential gap junctions were not positively identified because they were not shadowed with platinum but coated only with carbon. Combined with steep viewing angle of the carbon coat, the connexons were not clearly resolvable. Thus, because the size of those probable gap junctions and their number of connexons could not be determined accurately, the data from these 4 possible gap junctions were not included in the calculations above.) Combined with our confocal immunocytochemistry showing strong colocalization of both Cx35 and Cx34.7 at CE/M-cell synapses, our FRIL demonstration of essentially pure Cx34.7 in M-cell hemiplaques suggests that gap-junction channels in larval zebrafish, as in adult goldfish (Rash et al. 2013), are heterotypical, with Cx35 primarily or exclusively presynaptic and Cx34.7 primarily or exclusively postsynaptic. In support, gold beads for Cx35 (5- and 20-nm gold beads without 10-, 12-, or 30-nm gold beads for Cx34.7) were found in three presynaptic hemiplaques of unidentified neuronal gap junctions located >50 μm from the M-cell, demonstrating the efficacy of the Cx35 antibodies in this replica. Gold beads for Cx34.7 but not for Cx35 were detected in hemiplaques of an additional seven unidentified neuronal gap junctions that were >50 μm from the M-cell (Fig. 6) and, because of the location and sizes, presumably were on neighboring vestibulospinal or reticulospinal neurons. Finally, one gap junction contained gold beads for both Cx35 and Cx34.7, suggesting that, in contrast to CEs, the observed asymmetry in the distribution of connexins might not apply to all neuronal gap junctions at this developmental stage. Overall, at 5 dpf, both Cx34.7 and Cx35 occur abundantly at CE/M-cell and other neuronal gap junctions, with Cx34.7 found by FRIL only in the postsynaptic hemiplaques and Cx35 found almost exclusively in presynaptic hemiplaques.

ZO-1 is a member of the MAGUK family of proteins that was reported to interact with several connexins (Hervé et al. 2004), including Cx36 (Li et al. 2004). Recent work indicates that ZO-1 also colocalizes and interacts with Cx35 in goldfish CEs (Flores et al. 2008). Because of its extensive colocalization with Cx35 and direct interaction with the COOH terminus of Cx36-like connexins, ZO-1 is expected to play an important structural and functional role in CEs (Flores et al. 2008, 2010). Therefore, we asked whether ZO-1 is also present in CEs of larval zebrafish. In double-labeling experiments with anti-Cx35 and anti-ZO-1 antibodies, we found that these proteins...
colocalize in larval zebrafish CEs (Fig. 7A). The pattern and extent of colocalization between these two proteins were similar to those observed in adult goldfish CEs (Fig. 7B). Thus the results suggest that gap-junction channels in larval zebrafish CEs are formed by the fish homologs of Cx36 found in adult goldfish and supported by a similar molecular scaffold.

Proximity to regulatory elements. Electrical (and chemical) synaptic transmission in CEs has been shown to be under the regulation of dopamine (Cachope et al. 2007; Pereda et al. 1992, 1994), which is released from varicosities located at dopaminergic fibers in the vicinity of CEs (Cachope et al. 2007; Pereda et al. 1992). Recent data indicate that auditory responses to the M-cell in larval zebrafish are also under the regulatory control of dopamine (Mu et al. 2012). Therefore, we investigated the presence of dopaminergic fibers near the M-cell lateral dendrite and their proximity to CEs. In Tg(dat:EGFP) transgenic zebrafish expressing green fluorescent protein in dopaminergic neurons of the ventral diencephalon (Xi et al. 2011), fixed and labeled with anti-GFP antibodies, we observed the presence of dopaminergic fibers near the M-cell lateral dendrite and expressed here as percentage, was 27.29% for adult goldfish, 26.56% for adult zebrafish, and 95.04% for larval zebrafish. G: the 3-axis diagram summarizes the data combining number, diameter, and comparative size (size/dendrite) of CEs. Note that, although less numerous, larval CEs are proportionally larger. *The number of CEs in adult zebrafish was assumed to be 85, the same number observed in adult goldfish and other teleosts (see DISCUSSION).

while monitoring the responses evoked by a bipolar stimulating electrode placed in the vicinity of the hair cells in the posterior macula (Fig. 9B). Stimulation of the dendritic end of auditory afferents to the M-cell (for which the central endings could be identified by DIC optics on the lateral dendrite; Fig. 9C) evoked a low-threshold synaptic response composed of a brief and large early component followed by a delayed and longer-lasting component (Fig. 9D). These responses were indistinguishable from the mixed (electrical and chemical) synaptic response observed in adult goldfish in response to stimulation of CEs in the posterior branch of the VIIIth nerve (Fig. 9E), where the first component was shown to represent the electrical coupling produced by presynaptic action potentials in CEs and the second the release of neurotransmitter from these terminals (Furshpan 1964; Lin and Faber 1988b). Stimulation with higher strength evoked additional delayed responses, likely representing the activation of higher threshold and lower conducting afferents to the M-cell (data not shown). Remarkably, the time of arrival of the electrical component to the M-cell was similar in both adult goldfish and larval zebrafish ( ~200 μs; compare Fig. 8, E and F) and was usually “riding” on the stimulus artifact of the ear stimulation. The short delay cannot be ascribable to the relatively shorter distance between the ear and the M-cell in larval zebrafish, as stimulation of other afferent inputs by simply reversing the polarity of the stimulating electrode evoked synaptic responses with considerable longer synaptic delays (Fig. 8F), indicating that axons in larval zebrafish are capable of conducting action potentials at different speeds and that CEs are relatively fast conducting.

The two components of the mixed synaptic response also could be observed in isolation (Fig. 9G); whereas the first component was always present, the second component, representing the probabilistic release of neurotransmitter, could be absent from...
trial to trial and was facilitated by paired pulses, a stimulation protocol known to facilitate transmitter release in adult goldfish CEs (Fig. 9, G and H; Curti et al. 2008; Lin and Faber 1988a,b). Spontaneous synaptic responses, usually lacking chemical transmission, were observed during whole cell recordings (Fig. 10A). These responses were reminiscent of unitary CE responses obtained in adult goldfish during paired pre- and postsynaptic recordings (compare Fig. 10, B and C) and had similar amplitude (0.50 mV ± 0.10 SD, n = 65) to that observed by “minimal stimulation” (all-or-none response obtained with minimal stimulation strength) obtained by extracellular stimulation of the ear (0.56 mV ± 0.01 SD, n = 9; Fig. 10, D and E), indicating that they represent the natural activation of single terminals.

CEs become identifiable by Cx35 labeling at day 3 to reach a stable number of ~6 CEs/M-cell between days 4 and 9; the gradual increase in number suggests that not all CEs are at the same developmental and functional stage. Stimulation of the ear evoked graded responses that usually had six clear discernible steps (Fig. 10D). Increasing the stimulus strength did not further enhance the early response but rather resulted in the activation of components with longer synaptic delays (data not shown). This evoked “maximal” response was of 2.99 mV ± 0.65 SD, n = 9. Because the unitary responses are ~0.5 mV (spontaneous and evoked minimal; see above), it indicates, confirming the graded responses (Fig. 10D), that the maximal response represents the activation of six terminals, each contributing ~0.5 mV (Fig. 10E). In summary, both components of the mixed synaptic response are present in CEs of larval zebrafish, indicating that gap junctions at these terminals are functional at these developmental stages. Furthermore, all CEs are functional and seem to provide timing information (arrival time to the M-cell) comparable with those in adult goldfish.

**DISCUSSION**

The teleost CEs: a unique model to study vertebrate electrical transmission. Since their description by Bartelmez (Bartelmez 1915; Bartelmez and Hoerr 1933), CEs have provided an invaluable window for the study of vertebrate synaptic transmission. In his classic paper describing these terminals, Bodian (1937) provided conclusive evidence for the absence of direct protoplasmic continuity between the pre- and postsynaptic elements. By applying electron microscopy to goldfish CEs, Robertson et al. (1963) provided early evidence of the structures we know today as gap junctions. Parallel electrophysiological studies by Furshpan (1964) in CEs provided unequivocal evidence for the presence of electrical transmission in vertebrates. More recently, it was found that gap junctions at CEs contain teleost homologs of Cx36 (Pereda et al. 2003; Rash et al. 2013) and, like their chemical counterparts, electrical synapses at CEs undergo activity-dependent potentiation (Cachope et al. 2007; Pereda and Faber 1996; Pereda et al. 2004; Yang et al. 1990). Investigations on CEs unmasked the intimate functional relationship between glutamatergic and electrical transmission that are responsible for triggering activity-dependent changes (Cachope et al. 2007; Pereda et al. 2004; Smith and Pereda 2003), which recently also were shown to occur at thalamic inhibitory interneurons (Landisman and Connors 2005) and in the inferior olive (Mathy et al. 2014; Turecek et al. 2014). We show here that these CE terminals also can be identified in larval zebrafish and, moreover, their molecular composition and physiological properties are similar to those of adult goldfish. The present characterization will allow this unique model synapse to be used for new levels of analysis that combine electrophysiology, in vivo imaging, and genetic manipulations.

**Properties of electrical transmission in larval zebrafish.** In contrast to chemical transmission (Legendre and Korn 1994, 1995; Legendre 1999; Patten and Ali 2007, 2009; Patten et al. 2010; Wen and Brehm 2005), less is known regarding the properties of electrical transmission in developing zebrafish. We report here the molecular composition and functional properties of electrical synapses in developing CEs. We found that contacts between CEs and the M-cells in larval zebrafish each contain ~18 gap junctions, representing in total between 2,000 and 3,000 channels/CE. Interestingly, assuming that values of presynaptic action potentials and input resistance are similar to those measured in goldfish, the average amplitude of the recorded unitary electrical synaptic responses, together with measurements of M-cell input resistance, allowed tentative calculations of gap-junctional conductance, which suggested that only 2.5% of channels present at each larval zebrafish CE are functional [see Rash et al. (2013) for rationale and methods]. Although this highly speculative estimate needs to be confirmed, such a small fraction of functional channels would be consistent with that estimated for adult goldfish CEs (1–4%; Flores et al. 2012; Lin and Faber 1988b). Gap junctions at larval zebrafish contain two homologs of the mammalian Cx36, Cx35 and Cx34.7, which were also identified by immunochemical means at gap junctions between CEs and the M-cell in adult goldfish (Rash et al. 2013). Because of its
widespread brain distribution and preferred neuronal expression (Belluardo et al. 1999), Cx36 could be considered the main synaptic connexin in mammals and was reported to mediate electrical transmission in most CNS structures (Connors and Long 2004). Our labeling for Cx35 in CEs is consistent with recent reports indicating the presence of this connexin in zebrafish brain (Carlisle and Ribera 2014; Jabeen and Thirumalai 2013). As in adult goldfish, we found that Cx34.7 labeling was restricted to postsynaptic hemiplaques, at least by 5 dpf, suggesting that gap junctions between CEs and the M-cells in larval zebrafish are also made by the apposition of hemicannels, most of which are formed by two homologs of the mammalian Cx36 (Rash et al. 2013). Interestingly, several isoforms of these connexins have been recently identified in zebrafish. Given the presence of several Cx35 (Cx35.1 and Cx35.5) and Cx34 (Cx34.1 and Cx34.7) genes/proteins in zebrafish (Adam Miller and John O’Brien, personal communication), it is unclear whether the antibodies used in this study recognize two or more orthologs. However, our previous work (Rash et al. 2013) has found that the antibody used against Cx35 does not recognize Cx34.7, whereas the antibody against Cx34.7 does not recognize Cx35. Additionally, based on the epitopes for which these antibodies were raised, we expect that the Cx35 antibody will not recognize either of the Cx34 proteins and the Cx34.7 antibody will not recognize either of the Cx35 proteins. These channels seem to be supported by a similar molecular scaffold, as we detected the presence of the scaffolding protein ZO-1 at larval zebrafish CEs. ZO-1 is a member of the MAGUK family of proteins that, because of its extensive colocalization and interaction with Cx36-like con-

Fig. 6. CEs in larval zebrafish contain teleost homologs of mammalian Cx36. A–C: Cx34.7 (likely recognizing zebrafish Cx34.7; John O’Brien, personal communication) and Cx35 labeling colocalize at CEs. Double-immunolabeling for polyclonal Cx34.7 (JOB 2930-2 IL; red) and monoclonal Cx35 antibody (green; MAB3043; EMD Millipore) antibodies at CEs in a 5-dpf zebrafish (the lateral dendrite appears outlined by the dotted lines). C: merge showing colocalization of the antibodies. D: stereoscopic pair (left and right) and higher-magnification nonstereoscopic FRIL image (E) showing immunogold labeling for Cx34.7 (10-, 12-, and 30-nm gold beads) but not for Cx35 (5- and 20-nm gold beads) on several gap junctions of a large reticulospinal neuron, presumably a CE/M-cell synapse (see METHODS for antibodies used). Because of the removal of presynaptic connexins in this view, only M-cell connexins are present for labeling, as detailed in Rash et al. (2013). Note that a replicated debris fragment fell onto the CE and overlies a portion of 1 large Cx34.7-labeled gap junction. Boxed area in D enlarged as E. Circles in E = gap junctions labeled by Cx34.7; rectangles = small unlabeled gap junctions. Calibration bars in FRIL images (D and E) are 0.4 μm, corresponding to the limit of resolution of light microscopy in the red wavelength, as used for immunofluorescence of Cx34.7 (A and C).
nexins, was suggested to play an important structural and functional role in goldfish CEs (Flores et al. 2008). Thus our results indicate that gap junctions at larval zebrafish and adult goldfish CEs share a similar molecular composition and have similar functional properties.

Both components of the mixed synaptic response were observed at CEs of larval zebrafish. Mixed synaptic responses in the M-cell evoked by electrical stimulation of CEs were indistinguishable from the mixed synaptic responses observed in adult goldfish, where the first component was established to correspond to the electrical coupling potential of a presynaptically occurring action potential (Lin and Faber 1988a), and the delayed and longer-lasting component results from the release of glutamate from these terminals (Pereda et al. 2004; Wolszon et al. 1997). Although glutamatergic transmission was not the emphasis of this study, it appeared to exhibit kinetics and release properties (paired-pulse facilitation) that were consistent with those observed in adult goldfish (Wolszon et al. 1997). The electrical and chemical components of the mixed unitary synaptic responses were also indistinguishable from goldfish responses, indicating that gap junctions (and glutamatergic synapses) at these terminals are fully functional at the developmental stages under study.

The presence of large and abundant gap junctions at CEs guarantees fast synaptic transmission needed for an escape behavior. Nevertheless, whereas CEs are thought to provide critical timing information, the sensory input required to trigger an escape response is likely multimodal and requires not only auditory, but also vestibular and lateral line information (Casagrand et al. 1999; Mirjany and Faber 2011). In addition, gap junctions support a mechanism of lateral excitation in which the depolarization produced by some active CE afferents spreads back to neighboring, inactive CE terminals to increase their excitability (Curti and Pereda 2004; Pereda et al. 1995). This mechanism is thought to promote the coordinated activity of this population of afferents, increasing the probability of initiating an escape response. Lateral excitation is supported by the rectification properties of gap junctions. Because rectification relies on the existence of heterotypical Cx35/Cx34.7 channels (Rash et al. 2013), lateral excitation is also likely to operate in larval zebrafish, whose gap-junction channels are similarly constructed.

Fig. 7. Colocalization of Cx35 and zonula occludens-1 (ZO-1) at CEs in developing zebrafish. A: laser-scanning confocal projection of double-immunolabeling with polyclonal ZO-1 antibody (Zymed Ab 61-7300; red) and monoclonal Cx35 antibody (green; MAB3043; EMD Millipore) in a 5-dpf zebrafish. The M-cell (blue) was backfilled by spinal application of tetramethylrhodamine. Inset: higher magnification of the boxed area of the M-cell lateral dendrite (asterisk) showing high colocalization of Cx35 and ZO-1 labeling in 2 CEs (arrowheads). B: colocalization of Cx35. Colocalization of Cx35 (B1) and ZO-1 (B2) in a 5-dpf zebrafish.

Fig. 8. Presence of dopaminergic fibers in the vicinity of CEs. A: laser-scanning confocal image showing the presence of dopaminergic fibers in the vicinity of the lateral dendrite of M-cell revealed with an anti-GFP antibody (red) in a 4-dpf Tg(dat:EGFP) larval transgenic zebrafish expressing green fluorescent protein in dopaminergic neurons of the ventral diencephalon. B: high-magnification image showing an area of the M-cell lateral dendrite with double-immunolabeling for Cx35 and GFP in a 4-dpf Tg(dat:EGFP) larval zebrafish. The image illustrates the close proximity of dopamine-containing varicose fibers (red) to Cx35-labeled CEs (green). The M-cell (blue) was in both cases backfilled by spinal application of Texas red dextran.
Thus our results indicate that these developing synapses have properties that are comparable with those of adult teleosts. Consistent with this conclusion, a recent report (Mu et al. 2012) indicates that CEs in larval zebrafish, as those in adult goldfish (Cachope et al. 2007; Pereda et al. 1992, 1994), exhibit dopamine-dependent potentiation of the mixed synaptic response. This finding is consistent with the proximity of dopaminergic fibers to CEs that we describe here, which exhibit a pattern similar to that observed in adult goldfish (Cachope et al. 2007; Pereda et al. 1992).

Altogether, the data suggest that electrical synapses in developing zebrafish have properties similar to those observed in adult zebrafish.

Fig. 9. Mixed synaptic transmission in larval zebrafish. A–C: differential interference contrast (DIC) images of a 6-dpf zebrafish M-cell obtained using a ventral dissection approach. A: whole cell recording electrode in the M-cell. B: stimulating electrode (bipolar theta glass electrode) positioned in the posterior macula in the ear, near the hair cells. Note the otolith (asterisk), appearing bright inside of the ear. C: high magnification of the lateral dendrite of the M-cell showing fibers, which likely correspond to CEs, terminating in the distal part of the lateral dendrite. The glare in the bottom left corner (ear) produced by the brightness of the otolith, which is at a different focal plane, illustrates the proximity of the lateral dendrite to the ear. D: bipolar stimulation in the ear in a 6-dpf larva near the dendritic terminations of CEs evokes a mixed synaptic response in the M-cell, composed of a fast (short-delay) brief early component that is followed by a delayed, longer-lasting component of smaller amplitude (recording represents the average of 20 individual traces). The 1st component (electrical) is produced by the electrotonic propagation of presynaptic action potentials followed by a delayed glutamatergic response (chemical). F: top: bipolar stimulation in the ear evokes a mixed synaptic response in the lateral dendrite of the M-cell (single trace). Note the short delay between the stimulus artifact (arrowhead) and the electrical component of the synaptic response similar to that of the mixed synaptic response in adult goldfish (E). Bottom: reversing the polarity of the bipolar stimulating electrode (Rev. polarity) evokes synaptic responses in the M-cell with longer delays, produced presumably by stimulation of other adjacent afferent fibers having slower conduction velocities (single trace). G: paired-pulse stimulation in the ear. The 1st stimulus evokes a response consisting of only electrical transmission, whereas the 2nd stimulus additionally evokes facilitated transmitter release (single trace). H: paired-pulse stimulation of the ear showing facilitation of the chemical component (recording represents the average of 10 individual traces).

Fig. 10. Electrical synapses are functional at all CEs. A: spontaneous responses recorded in the M-cell of 6-dpf zebrafish larvae. Spontaneous and likely electrical synaptic potentials (empty circles) appear intermixed with longer-lasting spontaneous chemical (likely of inhibitory nature) synaptic potentials (filled circles), which were reversed by increased intracellular Cl− (Korn et al. 1987). Spontaneous electrical responses are reminiscent of unitary responses in adult goldfish (Lin and Faber 1988a; Smith and Pereda 2003). B: spontaneous responses boxed in 1 and 2 are illustrated superimposed. The presence of a chemical component in the response boxed in 2 confirms that brief responses correspond to electrically mediated unitary responses. C: superimposed traces represent single unitary responses obtained in adult goldfish evoked by presynaptic spikes during simultaneous recordings of a CE afferent and the M-cell lateral dendrite. D: graded evoked responses obtained in the M-cell of a larval zebrafish. Six amplitude steps can be identified following graded electrical stimulation of the ear. Stimulation with larger current does not increase the early, short-latency response and recruits components with longer delays (data not shown). E: histogram summarizing the average amplitudes of spontaneous electrical responses (Spont.; 0.50 ± 0.10 SD, n = 65) vs. minimal (0.56 ± 0.09 SD, n = 9) and maximal (2.99 ± 0.56 SD, n = 9) electrical synaptic response evoked by stimulation of the ear. PSP, postsynaptic potential.

Thus our results indicate that these developing synapses have properties that are comparable with those of adult teleosts. Consistent with this conclusion, a recent report (Mu et al. 2012) indicates that CEs in larval zebrafish, as those in adult goldfish (Cachope et al. 2007; Pereda et al. 1992, 1994), exhibit dopamine-dependent potentiation of the mixed synaptic response. This finding is consistent with the proximity of dopaminergic fibers to CEs that we describe here, which exhibit a pattern similar to that observed in adult goldfish (Cachope et al. 2007; Pereda et al. 1992). Altogether, the data suggest that electrical synapses in developing zebrafish have properties similar to those observed in adult zebrafish.
CEs and the development of auditory responses in the M-cell. Developing zebrafish have recently become a convenient organism for the study of the development of the auditory system (Nicolson 2005). We describe here the properties of a central auditory synapse in larval zebrafish, whose participation is thought to be critical for an essential escape behavior. Because of its experimental accessibility, the development of auditory responses in the M-cell has provided a model to correlate the development of the external ear and primary auditory afferents with an easily measurable animal behavior (Kohashi and Oda 2008; Kohashi et al. 2012; Tanimoto et al. 2009). The developmental profile of CEs (as detected by Cx35 labeling) is consistent with the acquisition of auditory responses by the M-cell (Tanimoto et al. 2009), and the switch to auditory evoked escape responses by the M-cell system occurs at ~75 h postfertilization (Kohashi et al. 2012). Our results are also consistent with previous ultrastructural data (Kimmel et al. 1981) demonstrating at these developmental stages the presence of large gap-junction-containing single synaptic contacts in the distal portion of the M-cell lateral dendrite, on which myelinated axons originating in the ear form synapses.

The mixed synaptic response evoked by CEs in larval zebrafish is indistinguishable from that of adult goldfish, where both seem to provide similar timing information (arrival time) to the M-cell, suggesting that their anatomic and physiological properties are scaled to produce the same function despite the striking size differences between larval zebrafish and adult goldfish. Interestingly, the degree of myelination reported by Kimmel et al. (1981) seems to be much less than that observed in adult goldfish CEs (Robertson et al. 1963). Remarkably, our results indicate that only a small number of terminals are required to support escape responses during these early developmental stages: only ~6/M-cell in 4- to 9-dpf larval zebrafish vs. ~85/M-cell in adult goldfish (Furukawa and Ishii 1967) with similar large numbers likely in adult zebrafish. That is, although we were not able to estimate rigorously the number of CEs in adult zebrafish, their number and density seem consistent with those observed in other teleosts such as bullhead (Bodian 1937) and goldfish (Furukawa and Ishii 1967). The presence of a higher number of CEs in older larvae was reported in other teleosts (Bartelmez and Hoerr 1933), suggesting that the presence of a higher number of CEs in older larvae was reported (Bodian 1937) and goldfish (Furukawa and Ishii 1967). The variability of distribution of Ca2+calmodulin-dependent kinase II at mixed synapses on the Mauthner cell: interactions between membrane and synaptic properties. J Physiol (Paris) 104: 203–214, 2010.


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