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

Submitted 26 May 2014; accepted in final form 29 July 2014

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 (Faber 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 1988a; 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 brightfield 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-11052; 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 at 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 glued onto the slide with cyanoacrylate and sealed with nail polish. 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 moved through the spinal cord to create an incision. Then, the tip of a second tungsten needle, coated with crystals of tetramethylrhodamine-dextran (70,000 mol wt; D1818; Invitrogen), was quickly applied to the incision for ~10 s. Backfilled larvae were removed from the agarose 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% surose in PBSTx at room temperature for 3 h and processed for immunolabeling as described above.

Freeze-fracture replica immunogold labeling. Backfilling with tetramethylrhodamine-dextran was used to identify the position of the M-cells in the replica. Brains from zebrafish larvae 5–6 dpf were dissected, frozen, freeze-fractured, and replicated according to our detailed procedures (Pereda et al. 1994). A gold index grid was bonded to the frozen sample by using 3% Lexitan plastic dissolved in dichroloethane; the samples were thawed and “grid-mapped” by confocal microscopy, and cellular material was removed by gentle washing with SDS detergent. For freeze-fracture replica immunogold labeling (FRIL), residual connexin proteins adhering to the replica after SDS washing (Fujimoto 1995, 1997) were labeled with rabbit anti-Cx34.7-1 (JOB 2930-2), chicken anti-Cx34.7-CT (JOB), and mouse monoclonal antibody directed against Cx35 (MAB3043 and MAB3045; Chemicon) and counterlabeled with gold goat-conjugated anti-mouse (5- and 20-nm gold beads for Cx35; BBQ), anti-rabbit secondary antibodies (10- and 30-nm gold beads for Cx34.7; BBQ), and donkey anti-chicken antibodies (12-nm gold beads for Cx34.7; Jackson ImmunoResearch Laboratories). Replicas were examined using a JEOL 2000 EX-II transmission electron microscope operated at 100 kV.

Electrophysiology. Zebrafish 4–7 dpf were used for electrophysiology recordings. To expose the brain, the larvae were first paralyzed in a solution containing 10 μM t-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 HEPES, 0.1 EGTA, 2 MgCl2-6H2O, 4 Na2ATP, 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 an “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 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 PBSx5, 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 monoclonal 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 Eker; Xi et al. 2011), dat:EGFP zebrafish 4–5 dpf were backfilled with Texas red dextran (D-3328; Invitrogen). After fixation, the larvae were stained with mouse anti-Cx35 and rabbit recombinant monoclonal 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 teleost 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 (Bartelmex and Hoerr 1933). These endings can be unambiguously identified in the lateral dendrite of the goldfish M-cell by immunolabeling 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- to 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 octavolateralis 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 Thirumalisai 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 dpi (n = 11), 0.94 ± 0.04 in 5 dpi (n = 74), 1.06 ± 0.04 in 6 dpi (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)
containing 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), contained approximately 1,866 and 1,498 connexons, respectively. For their identification in the replica, the M-cells were retrogradely labeled by application of tetramethylrhodamine-dextran in the spinal cord (see Methods). As in adult goldfish (Rash et al. 2013), 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 Cx34.7 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

Fig. 3. Developmental profile and anatomic characteristics of CEs in larval zebrafish. A–C: labeling of afferents in the inner ear confirms that large oval areas labeled for Cx35 correspond to developing CEs. A: high-magnification laser-scanning confocal section showing labeling of auditory afferents (red) following application of 1,1,3,3′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) in the vicinity of the posterior macula of the ear. The M-cell lateral dendrite appears immunostained with anti-green fluorescent protein (anti-GFP) in a 4-dpf Tol-056 enhancer trap transgenic zebrafish larva (blue). B: labeling with Cx35 antibody (green); C: merge. D: cartoon illustrating the parameters used to characterize CEs in developing zebrafish as revealed by Cx35 labeling. E: number of CEs at 3-, 5-, and 9-dpf zebrafish (error bars here and the other graphs indicate SE). F: diameter of CEs at 4-, 5-, 6-, and 9-dpf zebrafish. G: ratio between the diameters of the Cx35-labeled oval areas (CEs) and lateral dendrite, or relative size (ratio between the average diameter of the CEs and the lateral dendrite), in 4-, 5-, 6-, and 9-dpf zebrafish.
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 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 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 their proximity to CEs.

Functional properties of synapses at larval zebrafish CEs. CEs in 4- to 9-dpf zebrafish are identifiable by labeling for gap-junction and scaffold proteins. On the other hand, the presence of these molecules does not necessarily imply that electrical synapses at these endings are functional. To explore the physiological properties of larval CEs, we performed whole cell recordings of the M-cell in 5- to 7-dpf zebrafish (Fig. 9A) 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).

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...
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 expres-
sion (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 (Con-
nors and Long 2004). Our labeling for Cx35 in CEs is consis-
tent 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 communi-
cation), 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 communica-
tion) 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 antibod-
ies. 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 neu-
ron, 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 pres-
ent 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 junc-
tion. 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 reso-
lution 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). E: 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 showing facilitation of the chemical component (recording represents the average of 10 individual traces).
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 measureable 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 response 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 anatomical 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 for other teleosts (Bartelmez and Hoerr 1933), suggesting that the number of these terminals gradually increases during development to reach the number observed in the adult. In summary, we show here that electrical synapses at CEs in 4- to 9-dpf zebrafish exhibit properties that are comparable with those of adult goldfish, suggesting they are at least partially responsible for the acquisition of auditory responses in the M-cell.

ACKNOWLEDGMENTS

We are deeply indebted to Florence Marlow for help and guidance. We also thank Yoichi Oda, Adam Miller, and John O’Brien for comments on the manuscript and Monica Zell for the goldfish images in Fig. 1.

GRANTS

This research was supported by National Institutes of Health Grants DC-03186, DC-011099, R21-NS-055726, and NS-0552827 to A. E. Pereda and S10-RR-05831, S10-RR-08329, NS-044395, and NS-044010 to J. E. Rash.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES


