Molecular and Functional Asymmetry at a Vertebrate Electrical Synapse

John E. Rash, 1 Sebastian Curti, 2, 3 Kimberly G. Vanderpool, 1 Naomi Kamasawa, 4 Srikant Nannapaneni, 2 Nicolas Palacios-Prado, 2 Carmen E. Flores, 2 Thomas Yasumura, 1 John O’Brien, 5 Bruce D. Lynn, 6 Feliksas F. Bukauskas, 2 James I. Nagy, 6 and Alberto E. Pereda 2, *

1 Department of Biomedical Sciences, Colorado State University, Fort Collins, CO 80523, USA
2 Dominick P. Purpura Department of Neuroscience, Albert Einstein College of Medicine, Bronx, NY 10461, USA
3 Laboratorio de Neurofisiología Celular, Departamento de Fisiología, Facultad de Medicina, Universidad de la República, Montevideo 11800, Uruguay
4 Max Planck Florida Institute, Jupiter, FL 33458, USA
5 University of Texas Health Science Center, Houston, TX 77030, USA
6 Department of Physiology, University of Manitoba, Winnipeg, MB R3E 0J9, Canada

*Correspondence: alberto.pereda@einstein.yu.edu

http://dx.doi.org/10.1016/j.neuron.2013.06.037

SUMMARY

Electrical synapses are abundant in the vertebrate brain, but their functional and molecular complexities are still poorly understood. We report here that electrical synapses between auditory afferents and goldfish Mauthner cells are constructed by apposition of hemichannels formed by two homologs of mammalian connexin 36 (Cx36) and that, while Cx35 is restricted to presynaptic hemiplaques, Cx34.7 is restricted to postsynaptic hemiplaques, forming heterotypic junctions. This molecular asymmetry is associated with rectification of electrical transmission that may act to promote cooperativity between auditory afferents. Our data suggest that, in similarity to pre- and postsynaptic sites at chemical synapses, one side in electrical synapses should not necessarily be considered the mirror image of the other. While asymmetry based on the presence of two Cx36 homologs is restricted to teleost fish, it might also be based on differences in postranslational modifications of individual connexins or in the complement of gap junction-associated proteins.

INTRODUCTION

While the physiological importance of electrical synaptic transmission in cold-blooded vertebrates has long been established (Bennett, 1977), progress over the last decade has also revealed the widespread distribution of electrical synapses, and this modality of synaptic transmission was reported to underlie important functional processes in diverse regions of the mammalian CNS (Connors and Long, 2004). Consequently, electrical transmission is now considered an essential form of interneuronal communication that, together with chemical transmission, dynamically distributes the processing of information within neural networks. In contrast to detailed knowledge of the mechanisms underlying chemical transmission, far less is known about how the molecular architecture or the potentially diverse biophysical properties of electrical synapses encountered in physiologically disparate neural systems govern their function or impact on characteristics of electrical transmission in those systems.

Electrical synaptic transmission is mediated by clusters of intercellular channels that are assembled as gap junctions (GJs). Each intercellular channel is formed by the docking of two hexameric connexin hemichannels (or connexons), which are individually contributed by each of the adjoining cells, forming molecular pathways for the direct transfer of signaling molecules and for the spread of electrical currents between cells. As a result, electrical synapses are often perceived as symmetrical structures, at which pre- and postsynaptic sites are viewed as the mirror image of each other. Connexons are formed by proteins called connexins that are the products of a multigene family that is unique to chordates (Cruciani and Mikalsen, 2007). Because of its widespread expression in neurons, connexin 36 (Cx36) is considered the main “synaptic” connexin in mammals. In contrast to other connexins, such as some found in glia (Yum et al., 2007; Orthmann-Murphy et al., 2007), all pairing configurations tested so far indicate that Cx36 forms only “homotypic” intercellular channels (Teubner et al., 2000; Li et al., 2004), where connexons composed of Cx36 pair only with apposing Cx36-containing connexons. Notably, the number of neuronal connexins is higher in teleost fishes, which, as a result of a genome duplication (Voll, 2005), have more than one homolog gene for most mammalian connexins (Eastman et al., 2006). This raised the possibility of more complex configurations of neuronal connexin coupling in teleost fish evolving in response to functional demands.

Because of their experimental access, auditory “mixed” (electrical and chemical) synapses on the teleost Mauthner cell (M-cell) (a reticulospinal neuron involved in tail-flip escape responses; Faber and Pereda, 2011), known as “large myelinated club endings” (CEs), constitute a valuable model for studying vertebrate electrical transmission (Pereda et al., 2011). Consequently, electrical transmission is now considered an essential form of interneuronal communication that, together with chemical transmission, dynamically distributes the processing of information within neural networks. In contrast to detailed knowledge of the mechanisms underlying chemical transmission, far less is known about how the molecular architecture or the potentially diverse biophysical properties of electrical synapses encountered in physiologically disparate neural systems govern their function or impact on characteristics of electrical transmission in those systems.

Electrical synaptic transmission is mediated by clusters of intercellular channels that are assembled as gap junctions (GJs). Each intercellular channel is formed by the docking of two hexameric connexin hemichannels (or connexons), which are individually contributed by each of the adjoining cells, forming molecular pathways for the direct transfer of signaling molecules and for the spread of electrical currents between cells. As a result, electrical synapses are often perceived as symmetrical structures, at which pre- and postsynaptic sites are viewed as the mirror image of each other. Connexons are formed by proteins called connexins that are the products of a multigene family that is unique to chordates (Cruciani and Mikalsen, 2007). Because of its widespread expression in neurons, connexin 36 (Cx36) is considered the main “synaptic” connexin in mammals. In contrast to other connexins, such as some found in glia (Yum et al., 2007; Orthmann-Murphy et al., 2007), all pairing configurations tested so far indicate that Cx36 forms only “homotypic” intercellular channels (Teubner et al., 2000; Li et al., 2004), where connexons composed of Cx36 pair only with apposing Cx36-containing connexons. Notably, the number of neuronal connexins is higher in teleost fishes, which, as a result of a genome duplication (Voll, 2005), have more than one homolog gene for most mammalian connexins (Eastman et al., 2006). This raised the possibility of more complex configurations of neuronal connexin coupling in teleost fish evolving in response to functional demands.

Because of their experimental access, auditory “mixed” (electrical and chemical) synapses on the teleost Mauthner cell (M-cell) (a reticulospinal neuron involved in tail-flip escape responses; Faber and Pereda, 2011), known as “large myelinated club endings” (CEs), constitute a valuable model for studying vertebrate electrical transmission (Pereda et al., 2011).
We previously reported that connexin 35 (Cx35) (O’Brien et al., 1998), a fish homolog of mammalian neuronal Cx36 (Condorelli et al., 1998), is present at CEs (Pereda et al., 2003). Given the genome duplication that occurred in teleost fish, we investigated the presence of an additional Cx36 homolog at these terminals. Using immunofluorescence and ultrastructural approaches, we show that a second homolog of Cx36, connexin 34.7 (Cx34.7) (O’Brien et al., 1998), is also present at CEs. Strikingly, matched double-replica freeze-fracture immunogold labeling revealed that Cx35 is restricted to presynaptic CE hemiplaques, whereas Cx34.7 is restricted to postsynaptic M-cell hemiplaques. Asymmetry in the molecular composition of adjoining connexons was proposed to allow electrical rectification at some GJs (Barrio et al., 1991; Phelan et al., 2008). Consistent with this notion, our estimates of GJ resistance in each direction revealed a near 4-fold difference in conductance, favoring the spread of postsynaptic membrane responses to presynaptic endings, which, by acting as a mechanism of lateral excitation (Pereda et al., 1995; Curti and Pereda, 2004), would facilitate the fish’s escape. Thus, molecular asymmetries in neuronal gap junctions can underlie complex functional properties and suggest that the apposed sides of electrical synapses are not necessarily the mirror images of each other.

RESULTS

Cx35 and Cx34.7 Colocalize in Club Endings

The large size and distinctive morphology of the M-cell (Figure 1A) allows the imaging of long stretches of membrane in a single optical section. Because of their unusually large size, CEs can be unequivocally identified on the distal portion of the M-cell lateral dendrite using Cx35 labeling (Figures 1B and 1C; Pereda et al., 2003; Flores et al., 2008). To determine whether other teleost homologs of Cx36 are present at CEs, we investigated whether Cx35 colocalizes with Cx34.7 at CEs by performing double immunofluorescence labeling using an anti-Cx35 antibody (Chemicon MAB3043) and an anti-Cx34.7 intracellular loop (IL) antibody (see below; Experimental Procedures; Table S1 available online). Both Cx35 and Cx34.7 antibodies showed intense punctate staining and colocalization at contacts between CEs and M-cells (Asymmetry in the molecular composition of adjoining connexons was proposed to allow electrical rectification at some GJs (Barrio et al., 1991; Phelan et al., 2008). Consistent with this notion, our estimates of GJ resistance in each direction revealed a near 4-fold difference in conductance, favoring the spread of postsynaptic membrane responses to presynaptic endings, which, by acting as a mechanism of lateral excitation (Pereda et al., 1995; Curti and Pereda, 2004), would facilitate the fish’s escape. Thus, molecular asymmetries in neuronal gap junctions can underlie complex functional properties and suggest that the apposed sides of electrical synapses are not necessarily the mirror images of each other.

RESULTS

Cx35 and Cx34.7 Colocalize in Club Endings

The large size and distinctive morphology of the M-cell (Figure 1A) allows the imaging of long stretches of membrane in a single optical section. Because of their unusually large size, CEs can be unequivocally identified on the distal portion of the M-cell lateral dendrite using Cx35 labeling (Figures 1B and 1C; Pereda et al., 2003; Flores et al., 2008). To determine whether other teleost homologs of Cx36 are present at CEs, we investigated whether Cx35 colocalizes with Cx34.7 at CEs by performing double immunofluorescence labeling using an anti-Cx35 antibody (Chemicon MAB3043) and an anti-Cx34.7 intracellular loop (IL) antibody (see below; Experimental Procedures; Table S1 available online). Both Cx35 and Cx34.7 antibodies showed intense punctate staining and colocalization at contacts between CEs and M-cells (Figures 1B–1D). We previously showed that the number of anti-Cx35 fluorescent puncta at individual CEs (Figures 1E–1G) was consistent with ultrastructural demonstration of 63–243 closely spaced GJ plaques at these terminals (Tuttle et al., 1986), suggesting that each punctum represents an individual plaque (Flores et al., 2008). Accordingly, confocal line-scan imaging illustrated the high degree of colocalization of Cx35 and Cx34.7. (H) Plot represents the ratio of superposition of Cx35 to Cx34.7 labeling at individual CEs (converted to percentage) against the ratio of Cx34.7 to Cx35 (n = 30). See also Figures S1 and S2.

Figure 1. Presence of Two Homologs of Cx36 at CEs
(A) Diagram of the M-cell. Auditory afferents terminate as CEs in the distal portion of the lateral dendrite, forming mixed (electrical plus chemical) synaptic contacts (dashed box).
(B–D) Laser scanning confocal projection of the distal portion of the lateral dendrite. Double immunolabeling with a monoclonal Cx35 antibody (C, red) and a polyclonal Cx34.7 (IL) antibody (D, green) shows a high degree of colocalization at individual CEs, shown by red/green overlay (B, asterisks).
(E–G) High magnification of an individual CE showing intense punctate labeling for Cx35 (F, red) and Cx34.7 (G, green) and high colocalization (E).
(H) Confocal line-scan imaging (blue line in inset) illustrates the high degree of colocalization of Cx35 and Cx34.7.
(I) Plot represents the ratio of superposition of Cx35 to Cx34.7 labeling at individual CEs (converted to percentage) against the ratio of Cx34.7 to Cx35 (n = 30).
See also Figures S1 and S2.
Ultrastructural Analysis Reveals that Cx35 and Cx34.7 Are Differentially Segregated to Pre- versus Postsynaptic Sides

To confirm that Cx35 and Cx34.7 colocalize at individual GJ plaques, we performed conventional freeze-fracture replica immunogold labeling (FRIL), which allows broad expanses of tissues to be examined and facilitates unambiguous assignment of specific connexin labeling to GJ hemiplaques in either of two apposed cells (see Supplemental Experimental Procedures). Four replicas of goldfish hindbrain contained CE synapses on identified M-cells. The CE terminals were identified on confocal grid-mapped M-cells that had been injected with Lucifer yellow during in vivo recordings prior to tissue fixation as well as in one set of matched double replicas prepared by SDS-FRIL (see Supplemental Experimental Procedures). Samples were either single-labeled with anti-Cx36 Ab298, which binds to both Cx34.7 and Cx35 (see below), or double-labeled for Cx35 and Cx34.7 IL. In a double-labeled replica of a positively identified M-cell, labeling for Cx35 was found directly associated with GJ plaques in presynaptic membranes of CEs (n = 20 GJs). In contrast, labeling for Cx34.7 was only on identified M-cell postsynaptic membranes (n = 53 GJs). Consistent with this distribution, anti-Cx36 Ab298, which recognizes both Cx35 and Cx34.7 (see next section and Table S1), was found to label both pre- and postsynaptic membranes (data not shown, but see data in Pereda et al., 2003). Such differential distribution to pre- versus postsynaptic membranes was investigated further by double-immunolabeling for Cx35 and Cx34.7 using matched double-replica FRIL (DR-FRIL). Initially, a sample prepared for DR-FRIL was fractured and major portions of both matching complements were retrieved and labeled. In one of the two M-cell complements, more than 400 labeled GJs were found; 367 were viewed toward the M-cell side of the junction (Figures 2A–2D), all of which were labeled for Cx34.7 and none for Cx35; and 79 were viewed from the M-cell side of the synapse toward the CE (Figure 2E), all of which were labeled for Cx35 and none for Cx34.7. A diagram of that same cell is indicated in Figures 2F and 2G, illustrating the two primary views seen in Figures 2D and 2E.

To further investigate this apparent GJ connexin asymmetry, analysis was performed on matching complements of individual M-cell/CE GJs in these same samples. However, because of damage to one of the matching replicas, only about 30 M-cell/CE GJs could be matched in the two complementary replicas (Figure 3). Of those 30 matching complements, 100% had labeling for Cx35 (10 nm gold beads) within the CE plasma membrane, without labeling for Cx34.7 IL, and 100% had labeling for Cx34.7 IL (5 nm gold beads) within the postsynaptic M-cell plasma membrane, with no labeling for Cx35. Thus, whether examined in single replicas or in matched complementary double replicas of the same GJ hemiplaques, Cx35 was restricted to the CE side of GJs (presynaptic hemiplaques) and Cx34.7 was present only in the M-cell side of GJs (postsynaptic hemiplaques), unambiguously demonstrating that GJ channels between CEs and the M-cell dendrite are heterotypic.

Specificity of Anti-Connexin Antibodies

Because of substantial amino acid sequence identity of Cx35 and Cx34.7, the specificity of the antibodies used here is critical for the accurate identification of these two connexin homologs. Our previous studies on connexins at CEs focused largely on Cx35 at these synapses, using either anti-Cx35 antibodies or anti-Cx36 antibodies that were shown to recognize Cx35. In the present study, HeLa cells transfected with Cx34.7 or Cx35 were used to confirm the quality and specificity of a set of anti-Cx34.7 antibodies and to establish which of the previously utilized as well as currently available anti-Cx36 antibodies either do or do not cross-react with Cx34.7 or Cx35 (Table S1).

HeLa cells were found to readily express Cx34.7 upon transfection, and robust immunofluorescence detection of this connexin both intracellularly and at plasma membrane contacts was obtained with anti-Cx34.7 IL (Figure S1A1). The same culture labeled with anti-Cx36 Ab39-4200 showed codetection and subcellular colocalization of labeling (Figures S1A2 and S1A3), indicating Ab39-4200 recognition of Cx34.7 and therefore serving as a positive control for Cx34.7 expression. The anti-Cx36 Ab298 previously shown in our earlier study to recognize Cx35 (Pereda et al., 2003) also recognized Cx34.7 (Figure S1B1) and produced labeling that corresponded with labeling produced by Ab39-4200 (Figures S1B2 and S1B3). We next tested immunofluorescence detectability of Cx35 with anti-Cx34.7 IL in HeLa cells transfected with Cx35-enhanced yellow fluorescent protein (eYFP). Clusters of HeLa cells with high transfection efficiency displayed intense intracellular eYFP fluorescence as well as detection of Cx35-eYFP at cell-cell contacts (Figures S1C1 and S1E1). In these cultures, Cx35 was not recognized by anti-Cx34.7 IL (Figures S1C2 and S1C3), indicating specificity of this antibody for Cx34.7. In contrast, while Cx34.7-transfected cells showed robust labeling of Cx34.7 with anti-Cx36 Ab39-4200 (Figure S1D1), polyclonal anti-Cx36 Ab51-6300 did not cross-react with Cx34.7 in this same culture (Figures S1D2 and S1D3) but showed robust detection of Cx35 (Figures S1E1 and S1E3). See Figure S2 for additional antibodies tested. In addition, we previously established that anti-Cx35 (Chemicon MAB3043) antibody does not crossreact with Cx34.7 (Pereda et al., 2003).

Electrical Transmission between CEs and the M-cell Is Asymmetric

Heterotypic GJ channels have been associated with asymmetry of electrical transmission (Barrio et al., 1991; Phelan et al., 2008). While simultaneously recording a single CE afferent at the VIIIth nerve root and the M-cell lateral dendrite (Figure 4A), we found a dramatic difference between orthodromic and antidromic coupling coefficients (CCs), calculated using the M-cell and CE action potentials and their respective coupling potentials (CC = coupling/action potential). The CCs averaged 0.009 ± 0.001 (SEM) in the orthodromic direction and 0.083 ± 0.009 (SEM) in the antidromic direction (p < 0.0005; n = 36). The ~9-fold disparity indicates that electrical transmission is stronger in the antidromic direction. This difference is observed in the simultaneous recording illustrated in Figure 4A and is more clearly observed in the experiment of Figure S3A, where multiple CEs terminating in the same lateral dendrite were recorded sequentially while maintaining the dendritic recording electrode. There was a dramatic difference for CCs in the antidromic direction at each CE (Figure S3B), indicating that the functional asymmetry
represents a general property of CEs likely operating under physiological conditions, as it was observed using physiological signals, such as action potentials.

**Electrical Synapses between CEs and the M-cell Rectify**

The strength of electrical transmission (amplitude of the coupling potential) does not solely depend on the conductance of the GJ channels but also on the passive properties determined by the resistance (and capacitance under some conditions) of the coupled neurons. The relatively smaller size of CEs indicates that their input resistance is likely higher than that of the M-cell dendrite, thus contributing to the asymmetry between orthodromic and antidromic CCs. To evaluate the contribution of heterotypic GJ channels to asymmetric electrical transmission, we investigated possible asymmetries in GJ resistance between CEs and the M-cell. Rectification refers to the propensity of some electrical synapses to display differential resistance to current flow in one versus the other direction across the junction between two coupled cells (Furshpan and Potter, 1959). While properties of junctional conductance (inverse of resistance) are...
Molecular Asymmetry and Electrical Rectification

generally examined with simultaneous recordings from two cells under voltage clamp configuration (Barrio et al., 1991), this approach in our case would require simultaneous in vivo intraterminal and intradendritic recording, which is feasible (Pereda et al., 2003) but not sufficiently stable for analysis of rectification. Moreover, the resistance of the presynaptic electrode and geometrical characteristic of the afferents make it impractical to use the voltage clamp configuration to directly determine junctional resistance. Therefore, we followed an established indirect approach (Bennett, 1966; Devor and Yarom, 2002), where junctional resistance can be estimated from measurements of CCs in each direction (from pre- to post and vice versa), in combination with measurements of the input resistances of the coupled cells (see Experimental Procedures). While this approach might also be challenging in some cell types (GJs are dendrodendritic in most mammalian neurons), the M-cell and the CEs offer several unusual anatomical and physiological characteristics that make it possible to estimate these parameters in vivo: (1) CE afferents terminate with a single contact and are tightly segregated to the distal portion of the lateral dendrite of the M-cell; (2) the M-cell lateral dendrite as well as both the axons and terminals of CEs are accessible for intracellular recordings; and (3) the M-cell and the CEs have comparable and unusually fast membrane time constants, estimated to be 400 μs in the M-cell (Fukami et al., 1965) and 200 μs in CEs (Curti et al., 2008), which allow the use of physiological signals, such as action potentials, for measurements of CCs. Due to spatial considerations, measurements of CCs during simultaneous recordings of CE afferents in the VIIIth nerve root and the M-cell dendrite are useful to expose asymmetry of electrical transmission (Figures 4A and S3B) but not accurate enough for estimating GJ conductance (see below). To overcome this problem, we calculated average values of CCs for the population of afferents, using values obtained under various experimental arrangements that maximize their accuracy (see below).

The "population CC" in the orthodromic direction (CE to M-cell) for a number of CEs was estimated as the ratio between the average amplitude of the electrical component (or coupling potential) of the unitary postsynaptic potential and the average amplitude of the presynaptic spike (CC, postsynaptic coupling potential/presynaptic spike; Figure 4A). The orthodromic coupling potential (recorded during paired recordings with intradendritic recordings in the terminal field of CEs) averaged 0.73 ± 0.04 mV SEM (n = 76). (Because the strength of electrical synapses between individual CEs varies dramatically [Smith and Pereda, 2003], it was not possible to assign differences in the amplitude of individual coupling potentials to their relative position within the dendritic field and therefore correct for potential electrotonic attenuation. Thus, although potentially slightly underestimated, we believe the average amplitude of orthodromic coupling potentials represents the most appropriate value to use for calculating the CC in the orthodromic direction.)

During simultaneous recordings, the amplitude of the presynaptic spike evoked at the recording site with long (200 ms) depolarizing pulses does not represent the spike that ultimately generates coupling, as the spike recorded at the site of depolarization regenerates in subsequent nodes and, finally, at the presynaptic terminal (see Figure S4). More importantly, its...
amplitude is affected by the pulse depolarization. Therefore, for values of presynaptic spike amplitude, we used short depolarizing pulses, at which spikes initiate from resting potential and are likely representative of those normally occurring at the contact, averaging 87.6 ± 0.9 mV SEM (n = 203). These measurements yielded an orthodromic CC of 0.008. The input resistance of the M-cell lateral dendrite was directly measured under single-electrode voltage-clamp configuration during intradendritic recordings (see Experimental Procedures) and found to be, on average, 1.32 ± 0.3 MΩ SEM (n = 9; Figure 5B).

The population antidromic CC (M-cell to CE) was calculated as the ratio between the amplitude of the antidromic (AD) coupling potential (the coupling of the antidromic spike of the M-cell in the CE) and the amplitude of the antidromic M-cell spike (AD spike). Because the AD coupling potential is greatly reduced by electrotonic attenuation when recorded at the VIIIth nerve root during simultaneous recordings, we estimated its average value by performing intraterminal recordings in the vicinity of the M-cell lateral dendrite. This recording position allows measurement of the true amplitude of the AD coupling without the effect of attenuation by electrotonic axonal propagation (Figure 4C, bottom right). The coupling averaged 5.07 ± 0.31 mV SEM (n = 24) but was subsequently corrected to 1.85 ± 0.11 mV SEM to take account for the amplification of the AD coupling produced by a persistent sodium current (INaP), which is present in these afferents. (The correction was based on a predicted amplification of 63.6% of the average AD coupling amplitude from previous correlations of percent INaP amplification versus AD coupling amplitude at resting potential; see Experimental Procedures; Curti and Pereda, 2004.)

We next considered the AD spike amplitude that is, on average, most representative of that “seen” by the population recorded in the dendrite (Figure 5C; CC, AD coupling potential/AD spike). Because the AD coupling potential is greatly reduced by electrotonic attenuation when recorded at the VIIIth nerve root during simultaneous recordings, we estimated its average value by performing intraterminal recordings in the vicinity of the M-cell lateral dendrite. This recording position allows measurement of the true amplitude of the AD coupling without the effect of attenuation by electrotonic axonal propagation (Figure 4C, bottom right). The coupling averaged 5.07 ± 0.31 mV SEM (n = 24) but was subsequently corrected to 1.85 ± 0.11 mV SEM to take account for the amplification of the AD coupling produced by a persistent sodium current (INaP), which is present in these afferents. (The correction was based on a predicted amplification of 63.6% of the average AD coupling amplitude from previous correlations of percent INaP amplification versus AD coupling amplitude at resting potential; see Experimental Procedures; Curti and Pereda, 2004.)
of CEs. We reasoned that the amplitude of the AD spike at the center of the terminal field of CEs in the lateral dendrite would yield a good approximation. Because the amplitude of the M-cell AD spike decays along the lateral dendrite (the M-cell spike is generated at the axon initial segment and neither the soma nor dendrite have active properties; Furshpan and Furukawa, 1962) and because the precise location of the electrode in the dendrite cannot be controlled, this AD spike amplitude varies between experiments (10–20 mV). Therefore, to estimate the amplitude of the AD spike at the center of the terminal field of CEs, where most CEs terminate (Lin et al., 1983), we performed multiple sequential recordings along the M-cell dendrite (Figure 4D). Initial extracellular recordings were made in the M-cell axon cap, which served as a “spatial calibration” marker, as the distinctive field amplitude denotes the proximity of the electrode to the initial segment (Furshpan and Furukawa, 1962). The electrode was then moved at regularly spaced intervals along the lateral dendrite for multiple recordings, during and after which no changes were observed in the electrical properties of the M-cell (Figure 4E). The amplitude of the AD spike decayed

Figure 5. Voltage Dependence of Electrical Transmission
(A) The AD coupling is voltage dependent.
(B) Superimposed traces show the AD coupling potential recorded at resting potential (–73 mV) and near the threshold of the cell (–64 mV).
(C) Relationship between the antidromic coupling potential (AD coup, ordinates) and the presynaptic membrane potential (membrane potential, abscissa). The dramatic increase in AD coupling with depolarization was blocked by QX-314 (Curti and Pereda, 2004), revealing a second voltage-dependent mechanism. See also Figure S5.
(D) Only the QX-314-resistant mechanism is observed at the end of a 200 ms pulse.
(E) Superimposed traces show the AD coupling potential recorded at resting potential (–73 mV) near the threshold of the cell (–66 mV) and at –80 mV.
(F) Traces are illustrated scaled.
(G) Amplitude of AD coupling (obtained at the end of the 200 ms pulse, red circles) and the afferent’s input resistance (blue boxes) versus membrane potential in a representative experiment. Changes in amplitude of AD coup (K1) occur in the absence of changes in the afferent axon’s input resistance.
(H) Summary for ten experiments (red dots are AD coupling; blue squares are input resistance).
INa+P present at presynaptic terminals that acts to amplify the amplitude of the junctions themselves but rather the activation of an electrical coupling upon depolarization does not represent a property of GJs (Giaume and Korn, 1987). We therefore hypothesized that the QX-314-insensitive voltage-dependent component could correspond to either (1) a voltage-dependent behavior of GJ channels or (2) a voltage-dependent behavior of the cell’s membrane resistance, which could proportionally modify the amplitude of the coupling potential. To distinguish between these two possibilities, we measured both the amplitude of the AD coupling potential and the CE’s input resistance under different membrane potentials at the end of a 250 ms pulse, where active conductances do not contribute to coupling amplification. As illustrated in Figures S5 (single experiment) and 5H (n = 10), changes in amplitude of the AD coupling potential were independent of the CE’s input resistance, which remained constant through the full range of membrane potentials. As is the case with other rectifying electrical synapses (Giaume and Korn, 1984), we found a difference between the resting potentials of the coupled cells. The values averaged −71.7 ± 0.32 mV SEM (n = 203) for CEs, where −74 mV was the most hyperpolarized value, and −78.7 ± 2.5 mV SEM (n = 95; p < 0.01) for the M-cell, where −85 mV was the most hyperpolarized value, suggesting the existence of a transjunctional voltage of ~10 mV, on top of which electrical signals operate. Thus, we conclude that electrical synapses at CEs exhibit voltage-dependence, where depolarization of the presynaptic terminal enhances retrograde electrical communication. By virtue of their electrical directionality and voltage-dependence, heterotypic GJs in CEs act synergistically with the presynaptic QX-314-sensitive component.

### Table 1. Values Used for Estimates of Gap Junctional Resistance

<table>
<thead>
<tr>
<th></th>
<th>Presynaptic Spike</th>
<th>Postsynaptic Coupling</th>
<th>Population Coupling Coefficient</th>
<th>Postsynaptic Cell Input Resistance</th>
<th>Junctional Resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Orthodromic (CE to M-cell)</td>
<td>87.6 ± 0.9 mV, n = 203</td>
<td>0.73 ± 0.04 mV, n = 76</td>
<td>0.008</td>
<td>1.32 ± 0.3 MΩ, n = 9</td>
<td>168.3 MΩ</td>
</tr>
<tr>
<td>Antidromic (M-cell to CE)</td>
<td>10.6 mV</td>
<td>1.85 ± 0.11 mV, n = 24</td>
<td>0.175</td>
<td>8.05 ± 0.74 MΩ, n = 20</td>
<td>39.8 MΩ</td>
</tr>
</tbody>
</table>

Mean ± SEM.

The experiments were performed under different experimental conditions for calculating accurate antidromic CCs (Curti and Pereda, 2004). Blockade of the INa+P reveals a second, less prominent, voltage-dependent component that is symmetrical relative to resting membrane potential. This second voltage-dependent component can also be observed in the absence of TTX and QX-314 at the end of a long (250 ms) depolarizing pulse (Figure 5D) when the above-mentioned conductances are no longer active, further indicating the existence of two different voltage-dependent mechanisms (Curti and Pereda, 2004). Both components can also be isolated by curve fitting (Figure S5). The QX-314-insensitive voltage-dependent behavior had a slope of 0.094, equivalent to a change in AD coupling amplitude of 3.81% per mV of membrane potential change, which is symmetrical from resting potential, and unlike the INa+P component, it does not modify the time to peak (Figure 5E) nor the kinetics of the coupling potential (Figure 5F). We hypothesized that the QX-314-insensitive voltage-dependent component could correspond to either (1) a voltage-dependent behavior of GJ channels or (2) a voltage-dependent behavior of the cell’s membrane resistance, which could proportionally modify the amplitude of the coupling potential. To distinguish between these two possibilities, we measured both the amplitude of the AD coupling potential and the CE’s input resistance under different membrane potentials at the end of a 250 ms pulse, where active conductances do not contribute to coupling amplification. As illustrated in Figures S5 (single experiment) and 5H (n = 10), changes in amplitude of the AD coupling potential were independent of the CE’s input resistance, which remained constant through the full range of membrane potentials. As is the case with other rectifying electrical synapses (Giaume and Korn, 1984), we found a difference between the resting potentials of the coupled cells. The values averaged −71.7 ± 0.32 mV SEM (n = 203) for CEs, where −74 mV was the most hyperpolarized value, and −78.7 ± 2.5 mV SEM (n = 95; p < 0.01) for the M-cell, where −85 mV was the most hyperpolarized value, suggesting the existence of a transjunctional voltage of ~10 mV, on top of which electrical signals operate. Thus, we conclude that electrical synapses at CEs exhibit voltage-dependence, where depolarization of the presynaptic terminal enhances retrograde electrical communication. By virtue of their electrical directionality and voltage-dependence, heterotypic GJs in CEs act synergistically with the presynaptic QX-314-sensitive component.
Figure 6. Rectification Promotes Lateral Excitation
Cartoon illustrates the lateral excitation of CE afferents on the M-cell lateral dendrite and the contribution of electrical rectification to this phenomenon. Dendritic synaptic potentials (yellow) evoked by suprathreshold electrical stimulation of VIIIth nerve afferents (orange labeled CEs) spread to neighboring subthreshold terminals (yellow labeled CEs). Electrical rectification favors the retrograde transmission of dendritic signals toward CEs in a higher resistance pathway (>Rn), counteracting the leak of currents toward the soma following a pathway of low resistance (<Rn). Because of its voltage-dependent properties, electrical transmission acts as a coincidence detector, facilitating the recruitment of CEs that are already depolarized, such as during the invasion of an incoming action potential, whose depolarization travels ahead several nodes (bottom left CE; note that the arrow is bigger and the yellow in the CE more intense, denoting the increase in coupling produced by presynaptic depolarization).

Heterotypic Channels Formed by Two Teleost Homologs of Cx36 Mediate Electrical Transmission at CEs
We have previously reported the presence of Cx35 at CEs and suggested that intercellular channels were likely homotypic but specifically noted the possible presence of other connexins at these junctions (Pereda et al., 2003). We report here the presence of Cx34.7, a second teleost homolog of Cx36 (O’Brien et al., 1998), at CE/M-cell contacts. Our earlier results are nevertheless consistent with the detailed characterization of antibodies we report here, which indicates that some of the Cx36 antibodies previously used (i.e., Ab298) recognize both Cx35 and Cx34.7, therefore labeling both pre- and postsynaptic hemiplaques. Members of the connexin protein family can be permissive or nonpermissive for forming functional intercellular channels with each other. Heterotypic channels are especially prominent among glial cells (Rash, 2010) and are found in various tissues (Elenes et al., 2001), where they provide diversity for intercellular communication (Rackauskas et al., 2007, Palacios-Prado and Bukauskas, 2009). Heterotypic junctions at CEs are somewhat unconventional, in that they are formed by two teleost homologs of a connexin that is normally not permissive for forming intercellular channels with any other connexins. In tests of the capacity of Cx36 to form channels with ten other connexin family members, Cx36 was permissive for channel formation only with itself (Teubner et al., 2000). The limited amino acid sequence difference between Cx34.7 and Cx35 appears not to have caused sufficient structural changes to render these connexins incompatible, and indeed, our data show that adult CE/M-cell GJs gap junctions are formed exclusively from heterotypic coupling of these two connexins.

Heterotypic GJs between CEs and the M-cell Rectify
Although the experimental access did not allow us to perform a detailed biophysical analysis, our data indicate that these rectifying junctions are associated with voltage-dependent properties having kinetics similar to those at the classic crayfish rectifying synapse (Furshpan and Potter, 1959; Giaume and Korn, 1984). (These results contrast with a previous report suggesting that electrical synapses at CEs do not rectify [Lin and Faber, 1988].) The discrepancy with our estimates mainly arises from differences in the values of AD coupling and dendritic input resistance used for the calculations of junctional resistance that
were critical for revealing the asymmetry.) Heterotypic channels formed by recombinant Cx32 and Cx26 exhibit rectification properties (Barrio et al., 1991; Rubin et al., 1992; Bukauskas et al., 1995) that are reminiscent of those observed at rectifying synapses in crayfish (Furshpan and Potter, 1959) and hatchetfish (Auerbach and Bennett, 1969), indicating that molecular asymmetry between hemichannels might constitute a principal determinant of electrical rectification. Supporting Furshpan and Potter’s hypothesis that junctional membranes behave as a diode (electrical rectifier) rather than a simple electrical resistor (Furshpan and Potter, 1959), biophysical modeling combined with genetic analysis of heterotypic Cx32/Cx26 channels (Oh et al., 1999) suggested that instantaneous rectification of electrical coupling observed at these channels can be explained by the separation of fixed positive (p) and negative (n) charges across the junctional membrane, which results from the pairing of hemichannels with opposite charges at their channel, leading to the formation of a diode or “p-n junction,” which was shown to be capable of generating steep current-voltage relations (Oh et al., 1999). We show here that, in addition to molecular asymmetries, cytosolic-soluble cell-specific factors (such as Mg$^{2+}$) can contribute substantially to the generation of rectification in electrical synapses (Figure S6). Furthermore, although both Cx34.7 and Cx35 sides were sensitive to changes in [Mg$^{2+}$], they were differentially affected, indicating that molecular differences might contribute to a differential sensitivity of each hemichannel to soluble factors to enhance electrical rectification. While Mg$^{2+}$ is unlikely to be the factor creating rectification under physiological conditions at CE/M-cell synapses, as yet undetermined channel interacting cytosolic soluble factors (including intracellular polyamines; Shore et al., 2001; Musa and Veenstra, 2003; Musa et al., 2004) may induce electrical rectification, either because their concentrations are different on each side of the junction (coupling in the M-cell occurs between two different cell types and their intracellular milieu could be different) and/or by preferentially interacting with hemichannels of one side of the heterotypic junction. Finally, asymmetry could be also generated by differences in posttranslational modifications of the apposing hemichannels, such as connexin phosphorylation, which may contribute to rectifying properties by altering surface charge or conformation of the proteins (Alev et al., 2008; O’Brien et al., 1998).

Rectification Promotes Bidirectionality of Electrical Communication

Although closely associated with early evidence for electrical transmission (Furshpan and Potter, 1959), electrical rectification is an underestimated property of electrical synapses. Notably, rectification is generally associated with unidirectionality of electrical communication. Our results clearly separate the two notions (rectification and directionality), as rectification in this case acts to promote bidirectionality of electrical communication, which otherwise is challenged by the geometrical characteristics and electrical properties of the M-cell and CEs. We suggest that rectification, as in the M-cell, could also underlie bidirectional communication between neuronal processes of dissimilar size elsewhere, compensating for potentially challenging electrical and geometrical conditions for the spread of currents.

The M-cell network mediates auditory-evoked tail-flip escape responses in teleost fish, and much data support CEs as having a primary role in generating these responses (Faber and Pereda, 2011). Because electrical synapses at CEs are bidirectional, signals originating in the M-cell dendrite can influence CE excitability (Pereda et al., 1995). We propose that retrograde transmission is relevant functionally based on the following: (1) it allows CEs to be electrically coupled to each other through the lateral dendrite of the M-cell (Figure 6; Pereda et al., 1995); (2) this coupling serves as a mechanism for “lateral excitation” (Pereda et al., 1995; Herberholz et al., 2002; DeVries et al., 2002); (3) lateral excitation promotes the coordinated activity of a population of CEs; and (4) the coordinated activity likely increases efficacy of auditory input for the initiation of an escape response. These events are likely enhanced by electrical rectification, which favors the spread of currents from the M-cell lateral dendrite toward the presynaptic CEs. That is, because dendritic currents would encounter lower resistance to spread across these junctions than those generated presynaptically, electrical rectification favors the retrograde transmission of dendritic signals, counteracting the leak of currents toward the soma, following a pathway of low resistance (Figure 6). Moreover, given that coupling increases with presynaptic depolarization, the voltage dependence of electrical coupling we describe here acts as a “coincidence detector,” promoting the recruitment of CEs that are already depolarized, such as during the invasion of an incoming action potential, whose depolarization (because of cable properties) travels several nodes ahead without reaching threshold (Figure 6). Thus, although differences in input resistance significantly contribute to the asymmetry of electrical transmission between these cells, rectification plays a critical functional role by directing currents toward the presynaptic endings.

Are Hemiplaques in Electrical Synapses the Mirror Images of Each Other?

A generalized perception is that each side in most GJ plaques represents the mirror image of the other, as its formation requires the symmetric arrangement of hemichannels. This view, however, is changing with the recognition that connexins associate with a variety of proteins, resulting in the formation of macromolecular complexes (Hervé et al., 2012). Furthermore, electrical synapses have been shown to be dynamic structures, where connexins actively turnover (Flores et al., 2012) and exhibit activity-dependent regulation of their coupling strength (Yang et al., 1990; Pereda and Faber, 1996; Landsisman and Connors, 2005; Cachope et al., 2007; Haas et al., 2011). These properties suggest that each side in a GJ plaque must be supported by a scaffold structure, similar to postsynaptic densities at chemical synapses (Kennedy, 2000). While the detailed composition of this scaffold is largely unknown, several molecules interact with Cx36 (Li et al., 2004, 2009; Burr et al., 2005; Ciolfano et al., 2006; Alev et al., 2008) and its teleost homologs (Flores et al., 2008, 2010). Thus, molecular diversity in electrical synapses might not only be endowed by the connexins present but also potentially by differences in the ensemble of scaffold and regulatory molecules associated with each side of the gap junctions that form these synapses, which could be an additional
means of creating molecular asymmetry, impacting on the functional properties of these channels.

**EXPERIMENTAL PROCEDURES**

For full methodological details see the Supplemental Experimental Procedures.

**Immunohistochemistry**

Goldfish brains were fixed and sections were incubated simultaneously with rabbit polyclonal anti-Cx34.7 IL antibody (see Table S1) and mouse monoclonal anti-Cx35 (Chemicon MAB3043) antibody, incubated with Alexa Fluor 488-conjugated goat anti-rabbit and/or Alexa Fluor 594-conjugated goat anti-mouse secondary antibodies, and coverslipped using n-propyl gallate-based mounting media.

**Confocal Microscopy and Image Processing**

Sections were imaged using an Olympus BX61WI confocal microscope. Image analysis was performed using Image J (National Institutes of Health [NIH]) and MetaMorph software. Colocalization of Cx35 and Cx34.7 was measured as the percentage of the area labeled for Cx35 that was also labeled for Cx34.7 and the converse.

**Antibodies**

The antibodies used are listed in Table S1 along with their species of origin, designation (epitope recognition, source), and characteristics of detection of either or both Cx34.7 and Cx35.

**Freeze-Fracture Replica Immunogold Labeling**

Specimens were processed by single-replica FRIL and one additional specimen by double-replica SDS-FRIL (sodium dodecyl sulfate-digested fracture replica labeling, which we designate as DR-FRIL). For single-replica samples, a gold “index” grid (aka “Finder” grid) was bonded to the coated surfaces using Lexan plastic (polycarbonate plastic) dissolved in dichloroethane; the samples were thawed and “grid-mapped” by confocal microscopy, and covered with n-propyl gallate-based mounting media.

**Electrophysiology**

Surgical and recording techniques were similar to those described previously (Smith and Pereda, 2003; Curti and Pereda, 2004). Intracellular recordings were obtained in vivo from the lateral dendrite; both current clamp and sin-

**Estimate of Functional Resistance**

Functional resistance in each direction was estimated following Devor and Yarom (2002). These estimates of the functional resistance assume a simple two neuron model with passive membrane properties coupled directly by a single junction.

**In Vitro Electrophysiological Measurements**

Experiments were performed on Rin cells expressing Cx35 or Cx34.7 tagged with eYFP or DsRed, respectively. To adjust the concentration of intracellular free Mg2+, we used pipette solutions containing different concentrations of MgCl2 and EDTA and the web-based Maxchelator software (http://www.stanford.edu/~cpattan/webmaxc5.html) to calculate free ion concentrations.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures, six figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.neuron.2013.06.037.

**ACKNOWLEDGMENTS**

We thank Michael V.L. Bennett and Peter Sterling for their comments on the manuscript. This research was supported by National Institutes of Health grants S10RR025831, S10RR038329, NS044395, and NS044010 (to J.E.R.), EY012857 (to J.O.), R01NS072238 (to F.F.B.), Canadian Institute for Health Research (to J.J.N.), and by NIH grants DC03186, DC011099, R21NS055726, and NS0552827 (to A.E.P.).

Accepted: June 13, 2013
Published: September 4, 2013

**REFERENCES**


Neuron 79, 957–969, September 4, 2013 ©2013 Elsevier Inc. 967


