Neuron, Volume 79

Supplemental Information

Molecular and Functional Asymmetry

at a Vertebrate Electrical Synapse

Table S1. Antibodies used and their patterns of connexin recognition

<table>
<thead>
<tr>
<th>Antibody and designation</th>
<th>Type</th>
<th>Epitope</th>
<th>Source and Reference</th>
<th>Connexin recognition****</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Cx34.7</td>
</tr>
<tr>
<td>Cx34.7 IL*</td>
<td>polyclonal</td>
<td>perch Cx34.7 intracellular loop</td>
<td>O'Brien, J. O'Brien et al., 2004</td>
<td>+</td>
</tr>
<tr>
<td>Cx36 Ab298*</td>
<td>polyclonal</td>
<td>mouse Cx36</td>
<td>Nagy, J.I. Rash et al., 2000</td>
<td>+</td>
</tr>
<tr>
<td>Cx36 39-4200**</td>
<td>monoclonal</td>
<td>mouse Cx36 CT *** perch Cx34.7 c-terminus</td>
<td>Life Technologies Li et al., 2004</td>
<td>+</td>
</tr>
<tr>
<td>Cx34.7 CT**</td>
<td>polyclonal</td>
<td>perch</td>
<td>O'Brien, J. O'Brien et al., 2004</td>
<td>+</td>
</tr>
<tr>
<td>Cx36 37-4600**</td>
<td>monoclonal</td>
<td>mouse Cx36 MID ***</td>
<td>Life Technologies Li et al., 2004</td>
<td>-</td>
</tr>
<tr>
<td>Cx36 36-4600**</td>
<td>polyclonal</td>
<td>mouse Cx36 CT ***</td>
<td>Life Technologies Li et al., 2004</td>
<td>nd</td>
</tr>
<tr>
<td>Cx36 51-6300**</td>
<td>polyclonal</td>
<td>mouse Cx36 MID ***</td>
<td>Life Technologies Li et al., 2004; Rash et al., 2000</td>
<td>-</td>
</tr>
</tbody>
</table>

* Antibodies used in current study.
** Additional antibodies tested and used as positive controls for connexin detection.
*** MID and CT refer to the mid and C-terminus regions, respectively, of the third cytoplasmic connexin domain.
**** Recognition of Cx34.7, Cx35 and Cx36 by the antibodies listed is indicated by “+” and lack of recognition is indicated by “-”. Not determined is indicated.
Figure S1
Figure S2
Figure S4
Figure S5
Figure S6
Figure S1. **Specificity of antibodies for Cx35 and Cx34.7.** Detectability of Cx34.7 and Cx35 by immunofluorescence labeling with antibodies against Cx34.7 and Cx36 in Cx34.7-transfected or Cx35-eYFP-transfected HeLa cells. Each row of images shows the same field in red and green channels, and in overlay (third column). Immunolabeling at cell-cell appositions is indicated by arrows. (A) Image showing detection of Cx34.7 with anti-Cx34.7 IL (A1) and co-detection of Cx34.7 by monoclonal anti-Cx36 Ab39-4200 (A2), as seen by yellow in overlay (A3). (B) Co-recognition of Cx34.7 by anti-Cx36 Ab298 (B1) and anti-Cx36 Ab39-4200 (B2) seen as yellow in overlay (B3). (C) Clusters of Cx35-eYFP expressing HeLa cells display intense eYFP green fluorescence (C1), but show lack of Cx35 detection with anti-Cx34.7 IL (C2, C3) antibody. (D) Recognition of Cx34.7 by anti-Cx36 39-4200 (D1) shown as positive control for transfection, and lack of Cx34.7 detection with anti-Cx36 Ab51-6300 (D2, D3). (E) Group of Cx35-eYFP cells with robust eYFP fluorescence (E1), showing labeling with anti-Cx36 Ab51-6300 (E2) seen as yellow in overlay (E3). Inset in E3 shows higher confocal magnification of connexin detection by Ab51-6300 at cell-cell gap junctional contacts.
Figure S2. Detectability of Cx34.7 and Cx35 by immunofluorescence with various antibodies against Cx34.7 and Cx36. To aid future studies of the two neuronal connexins in the M-cell/CE system, we tested the utility and specificity of an assortment of other anti-Cx34.7 and anti-Cx36 antibodies for their ability to detect Cx34.7 or Cx35 by immunofluorescence in HeLa cells transfected with either Cx34.7 or Cx35-eYFP. In cells transfected with Cx34.7, anti-Cx34.7 CT (A1) and Ab39-4200 (A2) were found to co-detect Cx34.7 (A3). In cells expressing Cx35-eYFP, anti-Cx34.7 CT was found not to cross-react with Cx35 (B1-3). While HeLa cells transfected with Cx34.7 showed intense labeling for Cx34.7 with anti-Cx34.7 IL (C1) and anti-Cx34.7 CT (D1), monoclonal anti-Cx36 Ab37-4600 did not recognize Cx34.7 in these same cultures (C2, C3 and D2 and D3, respectively). However, Ab37-4600 (E2, E3) and another anti-Cx36 (Ab36-4600) (F2, F3) labeled Cx35 in cells transfected with Cx35-eYFP (E1, F1). These results show that the anti-Cx34.7 antibodies selectively detect Cx34.7 but not Cx35, and that while several anti-Cx36 antibodies detect both Cx34.7 and Cx35, monoclonal Ab51-6300 and polyclonal Ab37-4600, which were generated against the same sequence in Cx36 with low homology to a corresponding sequence in Cx34.7, selectively detect Cx35.
Figure S3. **Electrical transmission at CEs is asymmetric.** A. Multiple (6) sequential CE recordings (at VIII\textsuperscript{th} N. root) with the same dendrite. CEs terminating in the same lateral dendrite were recorded in a sequential fashion while maintaining the dendritic recording electrode. B. Asymmetry of CCs in each direction for each CE. C. Differences in CC (\(\Delta G\)) and GJ conductance (\(\Delta CC\)) in each direction for each of the CEs.
Figure S4. Spike recorded at the site of depolarization in CE afferents regenerates in subsequent nodes and at the presynaptic terminal. (A) The amplitude of the CE spike evoked at the recording site in the VIIIth nerve root (Pulse spike, red trace) with long depolarizing (200 ms) pulses during simultaneous recordings is decreased by pulse depolarization and exhibits a pronounced AHP (asterisk), which is more evident at depolarized membrane potentials (depolarizing pulse). In contrast, the amplitude of the spike evoked at resting potential with extracellular VIIIth nerve stimulation (VIIIth N. spike, black trace) is of higher amplitude and lacks of a prominent AHP. Because the spike regenerates in subsequent nodes from resting potential, the amplitude of the spike evoked by extracellular stimulation is likely representative of that generated at the terminal. (B) The coupling potential recorded during simultaneous recordings is superimposed with both the presynaptic spike evoked with 200ms pulses and with the spike evoked by extracellular VIIIth nerve stimulation (traces are represented normalized). The coupling potential (as is the case for the spike evoked by VIIIth nerve stimulation) lacks of a detectable AHP, suggesting that is originated by a spike originated at resting potential in the presynaptic terminal. (C) Intracranial recordings in a CE terminal in response to current injection (using ether short or long depolarizing pulses) indicate that CE terminals generate action potentials.
**Figure S5.** The two components of the AD coupling voltage-dependence. Relationship between the antidromic coupling potential (AD Coup, ordinates) and the presynaptic membrane potential (Membrane potential, abscissa). The data (open circles) were fit with a function representing the sum of an exponential and a straight line \( [AD Coup = (A+k1*Vm) + B*e^{k2*Vm}] \) and the two calculated components are also plotted alone (k1, k2). The relationship observed after QX-314 is similar to the k1 component calculated early in the recording (k1 control).
**Figure S6. Modulation of asymmetric conductance-voltage relationship by intracellular free Mg$^{2+}$ in heterotypic GJs formed by Cx35 and Cx34.7.** (A) Fluorescence images of a pair of Rin cells expressing Cx35-EYFP (a) and Cx34.7-DsRed (b), exhibiting a heterotypic junctional plaque (c; arrow); corresponding bright field image (d). (B) Transjunctional voltage ($V_j$) protocol applied to measured steady-state junctional conductance ($g_j$) -$V_j$ dependence. (C) $V_j$ protocol applied to measured instantaneous $g_j$-$V_j$ dependence. (D-F), Instantaneous (black) and steady-state (grey) $g_j$-$V_j$ dependence measured in symmetric intracellular Mg$^{2+}$ conditions (D; see top diagram), and asymmetric conditions (E-F; see top diagram). (G) Summary of instantaneous $g_j$ rectification at $V_j$ equal -80 and + 80 mV for symmetric and asymmetric intracellular Mg$^{2+}$ conditions. Numbers of independent experiments are shown in the histogram bars. ** p < 0.001.
Immunohistochemistry. After anesthesia, goldfish brains were extracted by quick dissection and fixed immediately in freshly prepared 1% formaldehyde containing 0.2% picric acid. After allowing fixation for 1 h, brains were transferred into 30% sucrose solution and left for 18-20 h at 4°C. Sections 50 µm thick were cut in a cryostat maintained at -24 °C, collected on slides, allowed to dry for 2-3 h, and then rinsed in PBS. After blocking for 1 h in PBS containing 10% normal goat serum and 0.5% Triton X-100, the sections were incubated simultaneously with rabbit polyclonal anti-Cx34.7 IL antibody (see Supplemental Table 1) and mouse monoclonal anti-Cx35 (Chemicon MAB3043) antibody overnight at 4°C. Sections were then washed in 0.4% Triton in 1 x PBS, incubated with Alexa Fluor 488-conjugated goat anti-rabbit and/or Alexa Fluor 594-conjugated goat anti-mouse secondary antibodies for 1 h at room temperature, washed in 0.1-0.4% Triton in PBS, and coverslipped using n-propyl gallate based mounting media.

Confocal microscopy and image processing. Sections were imaged using an Olympus BX61WI confocal microscope with a mortised fixed stage using 20X air, 40X apo/340 water and 60X oil objective lenses. FLUOVIEW FV500 software was used for data acquisition. Confocal immunofluorescence XY images were scanned in Z-axis intervals of 0.2-0.8 µm for 3D reconstruction. Z-plane sections and Z-plane stacks from each image were employed for image analysis using Image J (NIH) and MetaMorph software (Universal Imaging, Downingtown, PA). For colocalization analysis of Cx35 and Cx34.7, images of individual CEs were background subtracted and thresholded to include signals approximately 2-3 fold greater than the scatter labeling at the dendrite. Regions of interest corresponding to individual CEs were identified in Metamorph using transmitted light images and Cx35 labeling. 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. Unlabeled areas within CEs were relatively small compared to labeled areas (see Fig. 1E). For presentation purposes, some images were processed using Adobe Photoshop (Adobe Systems, San Jose, CA) and Canvas X (ACD Systems).

**Antibodies.** The antibodies used are listed in Supplemental Table 1, their species of origin, designation, epitope recognition, source and characteristics of detection of either or both Cx34.7 and Cx35. Specificity of the anti-Cx36 antibodies has been confirmed using Cx36 KO mice (Li et al., 2004; Curti et al., 2012), and specificity of the anti-Cx34.7 antibodies has been reported (O’Brien et al., 2004). Antibodies were further tested for recognition of Cx34.7 vs Cx35 as described below.

**HeLa cell transfection and immunohistochemistry.** Clones for mammalian cell expression included Cx34.7-pcDNA (O’Brien et al., 2004), Cx36-pcDNA, and Cx35-EYFP. The Cx35-EYFP clone was made by PCR amplification of Cx35 from Cx35-pcDNA (O’Brien et al., 2004) with the primers CTAGAATTCTCCGGATGCTCAGAAATGG (forward) and GTGTGCCCGGACACATAAGCAGAGTCACTGG (reverse) and subcloning into EcoRI and SmaI sites of pEGFP-N1 (Clontech). The resulting clones were fully sequenced. EGFP was subsequently swapped for EYFP from pEYFP-C1 (Clontech Laboratories, Inc., Mountain View, CA) with AgeI and BsrGI.

HeLa cells (American Type Culture Collection, Manassas, VA) were grown in DMEM with 10% fetal bovine serum and transiently transfected with Cx34.7-pcDNA3, Cx35-eYFP and Cx36-pcDNA plasmids using Lipofectamine 2000 reagent (Life Technologies, Grand Island, NY), as previously described (Li et al., 2008, O’Brien et al., 1998). Following a 24 h transfection period, transfected cells were taken for analysis. For immunolabeling of Cx34.7 or Cx35-eYFP
transfected HeLa cells grown on glass coverslips, cells were fixed with ice-cold 1% formaldehyde in PBS for 8 min, washed in PBS, and incubated overnight at 4°C simultaneously with rabbit anti-Cx34.7 and mouse anti-Cx36 or with anti-GFP antibodies, or with mouse anti-Cx36 and rabbit anti-Cx36 in 50 mM Tris-HCl, pH 7.4, containing 1.5% sodium chloride (TBS) with 0.3% Triton-X-100 (TBSTr) containing normal donkey serum. Cultures were washed in TBSTr for 30 min and incubated simultaneously for 1 h at room temperature with DyLight 549-conjugated goat anti-rabbit IgG (Thermo Fisher Scientific, Inc., Waltham, MA) diluted 1:1000 and Alexa Fluor 488-conjugated goat anti-mouse IgG (Invitrogen) diluted 1:1000. Cultures were then washed for 30 min in TBSTr and placed on slides with anti-fade medium.

**Freeze-Fracture Replica Immunogold Labeling (FRIL).** A combination of physiological and anatomical techniques was used to identify the VIIIth nerve terminals on the M-cell (*Carassius auratus*). After formaldehyde fixation, as above, three specimens were processed by single-replica FRIL (Rash and Yasumura, 1999; Pereda et al., 2003), and one additional specimen by double-replica SDS-FRL [sodium dodecyl sulfate-digested fracture replica labeling (Tanaka et al., 2005; Masugi-Tokita et al., 2007), which we designate as DR-FRIL. Slices containing the lateral dendrite of M-cells that were injected with Lucifer Yellow during intracellular *in vivo* recordings were freeze-fractured and coated with 1.5 nm of platinum and 20 nm of carbon (Figs. 3G and 4D). For single-replica samples, a gold "index" grid (aka "Finder"® grid, aka "Gilder Grids TEM finder grid" Grantham, UK) was bonded to the coated surfaces using Lexan plastic (polycarbonate plastic) dissolved in dichloroethane; the samples were thawed and "grid-mapped" by confocal microscopy, with which the location of the M cell lateral dendrite was determined; then, cellular material was removed from the tissue (i.e., the side opposite the grid) by gentle washing with 2.5% SDS detergent for 28 h at 49°C. Residual connexin proteins adhering to the replica after SDS washing were labeled as follows: Replica #811 (Figs. 2 and 3)
was labeled for 1 h with rabbit anti-Cx34.7 IL and mouse anti-Cx35 (EMD Millipore, Billerica, MA, MAB3043), then counter-labeled for 13 h with gold-conjugated goat anti-rabbit secondary antibodies from Chemicon (10-nm), Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA) (12-nm), BBI Solutions (Cardiff, UK) (10-nm) plus gold-conjugated goat anti-mouse secondary antibodies from Jackson ImmunoResearch (6-nm and 18-nm) and Chemicon (20-nm). Replica #812 was double-labeled for 1 h with rabbit polyclonal antibody against Cx36/Cx34.7/Cx35 (Cx36 Ab298; Pereda et al., 2003; made against an 18-amino acid sequence in human Cx36 beginning at amino acid #298, which is almost identical to corresponding sequences in Cx34.7 and Cx35) (O’Brien et al, 1998) and mouse anti-NMDAR1 (BD Biosciences, San Jose, CA), then counter labeled for 13 h with the same goat anti-rabbit and goat anti-mouse gold-conjugated secondary antibodies as used for sample #811.

Double-replica FRIL. A 150 µm-thick slice of goldfish hindbrain containing a Lucifer Yellow-injected M-cell was cryoprotected with 30% glycerol, mounted between two 4.6-mm gold sample holders, and frozen by plunging into a mixture of propane-ethane at its freezing point (−196°C). The sample was fractured at −105°C in a prototype JFD-2 freeze-etch machine equipped with a turbopump but lacking a liquid-nitrogen-cooled shroud, previously described as necessary for minimizing water vapor “contamination” (Rash et al., 1992). The opened double-replica “sandwich” was coated with 3-5 nm of carbon, 1.5 nm of platinum, and ca. 20 nm of carbon. In combination with slight water vapor deposition described above, this pre-coat of carbon [which is used to increase labeling efficiency (Fujimoto, 1995; Schlörmann et al., 2007) has the disadvantage that it partially obliterates E-face pits, making it somewhat more difficult to resolve GJ E-face pits (Fig. 2D,E). After replication, the matching replica complements were cleaned at 80°C for 29 h using 2.5% SDS in 15 mM Tris-buffered saline, pH 8.3.
For the double-replicas DRD#1 “top” and “bottom”, both matching replica complements were washed for 1 h in “labeling-blocking buffer” (Dinchuk et al., 1987), then labeled simultaneously for 4 h at 22°C with rabbit polyclonal antibody against Cx34.7 (Cx34.7IL) and mouse monoclonal antibody directed against Cx35 (Chemicon MAB3045), rinsed, then counter-labeled for 4 h at 22°C with 5-nm goat anti-rabbit IgG and 10-nm goat anti-mouse IgG (both from BBI). Complementary replicas obtained using a hinged double-replica device are mirror images in depth and angle of contour (i.e., hills opposite valleys). To facilitate precise matching of image details, including matching of connexon E-face pits with P-face intramembrane particles (IMPs) in apposed hemiplaques (Chalcroft and Bullivant, 1970), one of the complementary replicas was inverted on the specimen support, and the two matching samples were placed in the electron microscope in matching orientations.

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 about 250-300 µm from the M-cell soma; both current clamp and single-electrode voltage clamp (SEVC; sampling frequency from 14-21 kHz; Axoclamp 2; Molecular Devices) techniques were employed (electrode solution 5 M KAc; 4-12 MΩ). Individual VIIIth nerve afferents were penetrated either at the posterior VIIIth root during simultaneous recordings with the M-cell’s lateral dendrite (Fig. 4A), or less often, intracranially close to the dendrite (Fig. 4C) (electrode solution 2.5 M KCl; 35-45 MΩ). The “spike height method” was used to balance the voltage drop produced by the resistance of the electrode (Frank and Fourtes, 1956). Responses were quantified after averaging sets of 12 or more consecutive traces. Student’s t test was used to assess statistical significance. Errors are presented as standard error of the mean (SEM). Because the AD coupling is amplified by a persistent sodium current present in CEs, we corrected its amplitude using a prediction based on previous results in which we measured the reduction in amplitude of the AD coupling.
produced by Na⁺ channels blockers (Curti and Pereda, 2004).

**Estimate of junctional resistance.** Junctional resistance in each direction was estimated following Devor and Yarom (Devor and Yarom, 2002):

\[ R_{ortho} = \frac{R_{Mcell} \times R_{CE} \times [CC_{ortho} \times CC_{anti} \times (1 - CC_{ortho}) - (1 - CC_{ortho})]}{CC_{ortho} \times (R_{Mcell} \times CC_{anti} - R_{CE})} \]

\[ R_{anti} = \frac{R_{CE} \times R_{ortho} \times (1 - CC_{anti})}{R_{ortho} \times CC_{anti} - R_{CE} \times CC_{anti} \times (1 - CC_{ortho})} \]

Where \( R_{ortho} \) and \( R_{anti} \) are the values of the junctional resistance in the orthodromic (from CEs to M-cell) and antidromic (from M-cell to CEs) directions, respectively. As defined, \( R_{CE} \) and \( R_{Mcell} \) are the input resistance of the CEs and M-cell respectively, and \( CC_{ortho} \) and \( CC_{anti} \) the coupling coefficient in the orthodromic and antidromic directions respectively, estimated using the corresponding presynaptic action potentials. These estimates of the junctional 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. Extracellular solution contained (in mM): 140 NaCl, 4 KCl, 2 CaCl₂, 1 MgCl₂, 2 CsCl, 1 BaCl₂, 5 glucose, 2 pyruvate, 5 HEPES (pH 7.4). Recording pipettes (3-5 MΩ) were filled with pipette solution containing (in mM): 140 KCl, 10 NaAsp, 1 MgCl₂, 0.26 CaCl₂, 2 EGTA, 5 HEPES (pH 7.2). To adjust the concentration of intracellular free Mg²⁺ we used pipette solutions containing different concentrations of MgCl₂ and EDTA, and the standard web-based Maxchelator software (www.stanford.edu/~cpatton/webmaxcS.htm) to calculate free ionic concentrations. We
examined junctional conductance \( (g_j) \) and \( g_j \) dependence on transjunctional voltage \( (V_j) \) using dual whole-cell voltage clamp. Each cell was voltage clamped with a patch clamp amplifier (EPC-8; HEKA). \( V_j \)s were generated by stepping the voltage in on cell, while keeping constant voltage in the other cell. Junctional current \( (I_j) \) was measured as the negative of the current change measured in the cell where voltage was kept constant. The \( g_j \) was obtained from the equation \( g_j = I_j/V_j \). Data acquisition and analysis was performed with a custom-made software (Trexl er et al., 1999).

SUPPLEMENTAL REFERENCES


