

Novel Tools and Methods

Sources of Calcium at Connexin 36 Gap Junctions in the Retina

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Abstract

Synaptic plasticity is a fundamental feature of the CNS that controls the magnitude of signal transmission between communicating cells. Many electrical synapses exhibit substantial plasticity that modulates the degree of coupling within groups of neurons, alters the fidelity of signal transmission, or even reconfigures functional circuits. In several known examples, such plasticity depends on calcium and is associated with neuronal activity. Calcium-driven signaling is known to promote potentiation of electrical synapses in fish Mauthner cells, mammalian retinal All amacrine cells, and inferior olive neurons, and to promote depression in thalamic reticular neurons. To measure local calcium dynamics *in situ*, we developed a transgenic mouse expressing a GCaMP calcium biosensor fused to Connexin 36 (Cx36) at electrical synapses. We examined the sources of calcium for activity-dependent plasticity in retina slices using confocal or Super-Resolution Radial Fluctuations imaging. More than half of Cx36-GCaMP gap junctions responded to puffs of glutamate with transient increases in fluorescence. The responses were strongly dependent on NMDA receptors, in keeping with known activity-dependent signaling in some amacrine cells. We also found that some responses depended on the activity of voltage-gated calcium channels, representing a previously unrecognized source of calcium to control retinal electrical synaptic plasticity. The high prevalence of calcium signals at electrical synapses in response to glutamate application indicates that a large fraction of electrical synapses has the potential to be regulated by neuronal activity. This provides a means to tune circuit connectivity dynamically based on local activity.

Key words: calcium; Connexin 36; electrical synapse; retina; SRRF

Significance Statement

Electrical synapse plasticity is controlled at the level of the individual synapse, and several mechanisms of plasticity depend on the presence of calcium. Tools available to researchers to study calcium dynamics generally provide a spatially diffuse view, without single-synapse resolution. We have developed a transgenic mouse that reports calcium dynamics at individual Cx36 electrical synapses, enabling investigators to study calcium microdomain dynamics in circuits of interest. Preliminary studies in retina reveal that calcium microdomains are dynamic at a large fraction of electrical synapses, demonstrating extensive potential for activity-dependent plasticity. Such plasticity is likely to be the norm for electrical synapses rather than an exception limited to a few studied circuits.

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Introduction

All mature neurons operate in networks functionally connected to other neurons by synapses. Chemical and electrical synapses generally have different functions within these networks. Although there are numerous important exceptions, chemical synapses generally form inhibitory or excitatory connections between neurons of different type, whereas electrical synapses form connections between cells of the same type. In this way, electrical synapses play important roles in establishing networks of neurons.

Electrical synaptic plasticity is a central element of many adaptive processes that optimize circuit function under changing conditions (Haas et al., 2016). Sensory systems must encode information over an enormous range of signal intensities. In the retina, the intensity of incident light may range over 10 orders of magnitude during the day, but encoding of retinal output in spike rates of retinal ganglion cells spans only about two orders of magnitude. Adaptation at many levels contributes to the signal compression that makes this coding possible. Electrical synapse plasticity contributes to both large-scale switching from high-sensitivity rod pathways to lower-sensitivity cone pathways (Ribelayga and O'Brien, 2017), as well as to more temporary adaptation to contrast (Shi et al., 2020). Motor control systems also depend on dynamic tuning of electrically coupled circuits in the inferior olive nucleus and the cerebellum to correct errors in movement timing (Schweighofer et al., 2013). In a similar manner, the thalamic reticular nucleus is thought to focus attention on certain streams of sensory information through dynamic adjustment of electrically coupled networks (Haas and Landisman, 2011; Coulon and Landisman, 2017). Such changes in coupling could be a general mechanism to regulate high-order processes in the brain (Pernelle et al., 2018).

In several known examples, electrical synapse plasticity depends on calcium and is associated with neuronal activity. Calcium-driven signaling promotes potentiation of electrical synapses in fish Mauthner cells (Pereda et al., 1998; Smith and Pereda, 2003), a motor neuron that controls the escape swimming reflex. Similar potentiation by

a calcium signal has been observed in mammalian retinal All amacrine cells (Kothmann et al., 2012) and inferior olive neurons (Turecek et al., 2014). In contrast, calcium signaling promotes depression in thalamic reticular neurons (Haas and Landisman, 2011; Sevetson et al., 2017). Calcium signaling has also been found to promote activity-dependent potentiation of innexin-based electrical synapses in a motor circuit in the leech (Welzel and Schuster, 2018), revealing that this type of signaling is both ancient and conserved through a broad phylogenetic range of the animal kingdom. These observations reveal that sensory, motor, and cognitive functions can all be modified by changes in electrical synapses instigated by calcium signaling.

Because of the widespread influence of calcium signaling on electrical synapse function, it would be useful to have the ability to study these calcium signals in real time at electrical synapses. To make this possible, we developed a transgenic mouse expressing a GCaMP calcium biosensor fused to Connexin 36 (Cx36) at electrical synapses, which enables the measurement of local calcium dynamics *in situ*. This study presents validation of this model as well as a survey of the sources of calcium for activity-dependent plasticity in retina slices. We find that a large fraction of Cx36-GCaMP gap junctions experience transient increases in local calcium concentration when stimulated with glutamate and that the responses depend both on NMDA receptors and voltage-gated calcium channels. This reveals that a dynamic calcium microenvironment at electrical synapses is widespread in the retina, providing a substrate for activity-dependent plasticity.

Materials and Methods

Development of the Cx36-GCaMP transgene

We customized the pBluescript-based vector pl-Scel (a gift from Jochen Wittbrodt; Thermes et al., 2002) by cutting the vector with SacI and NotI and inserting a linker comprising the 5' phosphorylated oligonucleotides ACCTCGAGGACTT AAGA and GGCCTCTTAAGTCCTCGAGGTGC. This added restriction sites AclI and AflII to assist with our specific cloning requirements while removing the parent restriction sites. The new vector was termed pBS-ISAA. Cx36-GCaMP (plasmid #123604, Addgene; Moore et al., 2020) was cut with BamHI and AflII to excise a fragment containing the GCaMP3-tagged Cx36 exon 2 and the SV40 polyadenylation sequence of the plasmid; this fragment was cloned into pBS-ISAA. A 5.3 kb fragment of the Cx36 (*Gjd2*) gene containing intron 1, exon 1, and 4 kb of upstream sequence was amplified from mouse genomic bac RP23-230H3 (BACPAC) with primers CTTGATATCGAATTCTC CAGTCAGGGAACGTGTAGC and ACCACAGTCAACAG GATCCTGAACAGAGGAAGAGG using Phusion DNA polymerase (New England Biolabs). The amplified fragment was cloned into the Cx36 exon2-GCaMP clone in pBS-ISAA cut with BamHI and EcoRI using Cold Fusion Cloning Kit (System Biosciences), recreating the natural exon 1-intron 1-exon 2 structure of Cx36.

To test for proper splicing and gap junction formation of the construct, we cut the full-length Cx36 promoter-Cx36-GCaMP clone with EcoRV and SmaI, removing most of the

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promoter fragment, and resealed the plasmid. We then cut the entire insert out with *AbsI* and transferred it to pCAGEN (plasmid #11160, Addgene; Matsuda and Cepko, 2004). Transfection of this construct into HeLa cells (catalog# CCL-2, ATCC; RRID:CVCL_0030) resulted in expression of GCaMP-tagged Cx36 that formed gap junctions between cells, verifying that the construct was spliced and trafficked properly (data not shown).

Development of Cx36-GCaMP transgenic mice

All experimental procedures described in this study comply with the U.S. Public Health Service policy on humane care and use of laboratory animals and the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and were approved by the Institutional Animal Care and Use Committees under protocols HSC-AWC-16-0063 at the University of Texas Health Science Center at Houston and ASP 1220 at the National Institute of Neurologic Disorders and Stroke. Mice were used in this study and maintained on a daily 12 h light/dark cycle. The full-length Cx36 promoter-Cx36-GCaMP clone was linearized with *I-SceI* and sent to the transgenic mouse core facility at the University of Texas Health Science Center at Houston for blastocyst injections in C57BL/6 background, embryo implantation, and rearing filial (F0) pups. The F0 founder animals were crossed with C57BL/6J mice (Jackson Laboratory; RRID:IMSR_JAX:000664) and offspring screened for the transgene by PCR of tail DNA with primers CAGGACTCAGGACAGTGACTCTGCCTATG and GCGGCGGTACGAACTCC. Several lines of mice were propagated from founders that displayed germline transmission of the transgene. Both female and male mice were used for the experiments described in this report.

Immunofluorescence staining and confocal microscopy

Cryostat sections of retina, brain, and pancreas from Cx36-GCaMP mice were used for the assessment of protein expression levels and localization. Mice were anesthetized with isoflurane and killed by cervical dislocation. For frozen sections of retina or pancreas, freshly dissected tissues were fixed with 4% formaldehyde (Electron Microscopy Sciences) in 0.1 M PB, pH 7.5, for 30 min and then washed with PBS. Fixed tissues were cryoprotected overnight with 30% sucrose in 0.1 M PB, embedded in optimum cutting temperature compound (Sakura Finetek), and sectioned vertically with a cryostat. For frozen sections of brain, anesthetized mice were arterially perfused with PBS, followed by 2% formaldehyde in PBS for 20 min. Whole brains were then dissected out and further immersion fixed in 2% formaldehyde in 0.1 M PB for 5 h. Fixed brains were cryoprotected, embedded, and sectioned as for retina and pancreas. The cryostat sections of retina and brain were blocked in 10% donkey serum (Jackson ImmunoResearch) in PBS and 0.3% Triton X-100 (PBST) for 1 h and incubated with primary antibodies diluted in 5% donkey serum in PBST overnight at room temperature (RT). For cryostat sections of pancreas, an additional antigen retrieval step was included. Sections were incubated in 10 mM sodium citrate, pH 6.0, at 100°C for 1 h, cooled to RT in the same buffer for an additional 1 h, and washed with PBS. Slides were then blocked and probed as for other

tissues. Antibodies used include mouse anti-Cx35/36 (catalog #MAB3045 Millipore-Sigma; RRID:AB_94632), goat anti-Cx36 (catalog #sc-14904, Santa Cruz Biotechnology; RRID:AB_2111311), rabbit anti-insulin (catalog #sc-9168, Santa Cruz Biotechnology; RRID:AB_2126540), and Alexa Fluor 488-conjugated rabbit anti-GFP (catalog #A-21311, Thermo Fisher Scientific; RRID:AB_221477). Secondary antibodies were made in donkeys and sourced from Jackson ImmunoResearch. Sections were coverslipped with Vectashield mounting medium with DAPI (Vector Laboratories) before imaging. Samples were imaged on a Zeiss LSM 780 confocal microscope with 63×/1.4 NA or 40×/1.4 NA oil objectives. For localizing and quantifying the expression levels of intrinsic GCaMP, or immunostains of Cx36 or GFP, three consecutive confocal slices with a total thickness of 0.6 μm in the middle of the inner plexiform layer (IPL) were selectively imaged and flattened with a maximum intensity projection using Zeiss Zen 2.3 software.

Immunofluorescence image processing and data quantification

Images for display in figures were enhanced for contrast and brightness with Adobe Photoshop (RRID:SCR_014199) or Fiji software (RRID:SCR_002285; Schindelin et al., 2012). Image quantification was performed using Fiji. Regions of interest (ROIs) selection was made through thresholding on the fluorescence channels of Cx36 and anti-GFP immunolabeling with a standard 15% threshold. The selections of detected binary objects were further modified by the editor function *Wellspring* to isolate individual gap junctions and limited to objects larger than 1 pixel and smaller than 50 pixels. The coexpression of Cx36 and GCaMP was assessed by measuring the fluorescence intensity of the nonselected channel in each ROI. Positive colocalization was scored if the measured intensity exceeded a threshold greater than the summed level of the background average plus two times the background SD in that channel.

Solutions

Eight puffing and three bathing solutions were used for detecting the transient puffing responses during live imaging. All puffing and bathing solutions were made in Ames medium (Thermo Fisher Scientific) and complemented with none, 20 μM (R)-CPP (3-((R)-2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid; Tocris Bioscience), 100 μM picrotoxin (Tocris Bioscience), 20 μM Nifedipine (Tocris Bioscience), or 100 μM CdCl₂. Puffing solutions were completed by adding either glutamic acid at 1 mM or NMDA at 10 μM along with 1 mM glycine (Sigma-Aldrich) or AMPA at 100 μM. In addition, all bathing and puffing solutions were supplemented with 10 μM L-AP4 (L-(+)-2-amino-4-phosphonobutyric acid; Tocris Bioscience) to suppress light-driven On pathway responses.

Retina dissection and slicing for transient puffing responses

Mice were anesthetized with isoflurane and killed by cervical dislocation. After eyeball enucleation, the retina was dissected in oxygenated Ames medium and mounted

photoreceptor side up on black nitrocellulose filter paper (EMD Millipore). The retina was sliced with a homemade micrometer-controlled positioning stage and slicing blades (Feather Double Edge) into 150 μm slices. The retina and slices were maintained submerged in oxygenated Ames medium throughout the procedure. Retina slices were mounted in perfusion chambers (Warner Instruments) and perfused continuously with oxygenated Ames medium using a peristaltic pump (Instech Laboratories) at 2 ml/min.

Borosilicate glass puffing pipettes were fabricated with a tip width of 20 μm and a taper of 3 mm with a Flaming–Brown pipette puller (Sutter Instrument). Pipettes were loaded with puffing solutions and localized close to retinal IPL within the field of view. Puff solutions were administered 10 s after the start of image acquisition at the injection pressure of 0.2 psi for 2 s using a Pico-Injector (Harvard Apparatus).

Live imaging and image processing

Retina slices were imaged live with visible light excitation and L-AP4 in the bath to suppress excitatory On pathway responses. Preliminary experiments were performed with a Zeiss LSM510 confocal microscope using a 40 $\times$ /1.0 NA water immersion objective. Subsequent experiments were performed in an upright Olympus BX51-WI conventional light microscope. Slices were viewed with 4 $\times$ dry or 40 $\times$ /0.8 NA water immersion objectives. GCaMP fluorescence was excited with a Xenon lamp (Sutter Instrument) with a 470BP40 filter and detected through a 525BP50 filter. Images were captured with an ANDOR iXon Life 887 EMCCD camera (Oxford Instruments) using Micro-Manager 1.4 software (Edelstein et al., 2014). The acquisition of Super-Resolution Radial Fluctuations (SRRF) images (Gustafsson et al., 2016; Lee et al., 2018) was performed with an electron-multiplying gain of 200 and an exposure time of 10 ms, 30 frames per image, producing a time resolution of ~ 1 s per SRRF image.

The 16-bit SRRF image sequences of live retina slices were processed through Fiji and MATLAB R2018a (MathWorks; RRID:SCR_001622) software to identify local fluorescence intensity peaks labeled as ROIs and to capture ROI intensity through time. ROIs were initially identified manually in maximum intensity projections of the image sequence through time, which permitted identification of Cx36-GCaMP gap junction plaques despite fluctuations of fluorescence intensity and also captured any small movements of the slice during the acquisition. Fluorescence intensity within each selected ROI was extracted from each image using MATLAB. Subsequent analyses used R software (R Project for Statistical Computing, www.r-project.org; RRID:SCR_001905). For each ROI, the baseline decay in fluorescence intensity (F_0) with time was estimated with the whole SRRF image stack excluding images acquired during the puff and the subsequent 20 s responding window. By fitting the baseline with an exponential decay model (Eq. 1), the difference (ΔF_t) between the measured fluorescence levels and the baseline ($F_{0,t}$) was then divided by the corresponding baseline fluorescence level to calculate response at

each time point ($\Delta F/F_0$). Response area under the curve for each ROI was calculated as the sum of response values from the puff through the end of the acquisition (Eq. 2) as follows:

$$Y = (Y_0 - \text{plateau}) * e^{-X/\lambda} + \text{plateau}, \quad (1)$$

where Y_0 denotes the initial intensity of GCaMP fluorescence, $1/\lambda$ denotes the rate of decay, and plateau denotes the late residual intensity of decaying GCaMP fluorescence; and

$$\text{AUC} = \sum (\delta F_t / F_{0,t}). \quad (2)$$

Positive GCaMP responses to different puffing conditions were defined by at least three points of measurements in a 20 s postpuff responding time window (between the 11th and the 30th s of live imaging) exhibiting ΔF larger than two times the SD of measurements acquired outside the responding window used to calculate the baseline. The fraction of gap junctions responding was calculated as a population measure within each slice experiment as the number of individual gap junction plaques with a positive response, as defined above, divided by the total number of gap junction plaques analyzed in that experiment.

Statistical analyses

Statistical comparisons were performed using estimation statistics (Ho et al., 2019) using Web-based tools accessible at <https://www.estimationstats.com/#/>. Additional traditional statistical analyses were performed using Prism software (GraphPad; RRID:SCR_002798).

Results

Development of transgenic mice to monitor electrical-synapse-localized calcium signaling

Calcium signaling has been demonstrated to instigate profound changes in coupling of Cx36-containing electrical synapses (Pereda et al., 1998; Kothmann et al., 2012; Turecek et al., 2014; Sevetson et al., 2017), but to date it has not been possible to monitor changes in the calcium microenvironment surrounding electrical synapses. This presents challenges in understanding how neuronal activity may influence functional connectivity of electrically coupled networks. To overcome this problem, we used the gap junction-tethered calcium biosensor Connexin36-GCaMP (Cx36-GCaMP; Moore et al., 2020) to develop transgenic mice with electrical synapses tagged with the calcium biosensor. Cx36-GCaMP contains GCaMP3 (Tian et al., 2009) fused to the C terminus of mouse Cx36 (Fig. 1A). This fusion construct reports the local calcium microenvironment around gap junctions and retains both channel function and functional plasticity (Moore et al., 2020). We developed a transgene construct containing ~ 5 kb of the mouse Cx36 (*gjd2*) gene, including ~ 4 kb of the upstream regulatory region, exon 1 and intron 1, with exon 2 derived from Cx36-GCaMP (Fig. 1B). This construct was used to develop transgenic mice (see above, Materials and Methods),

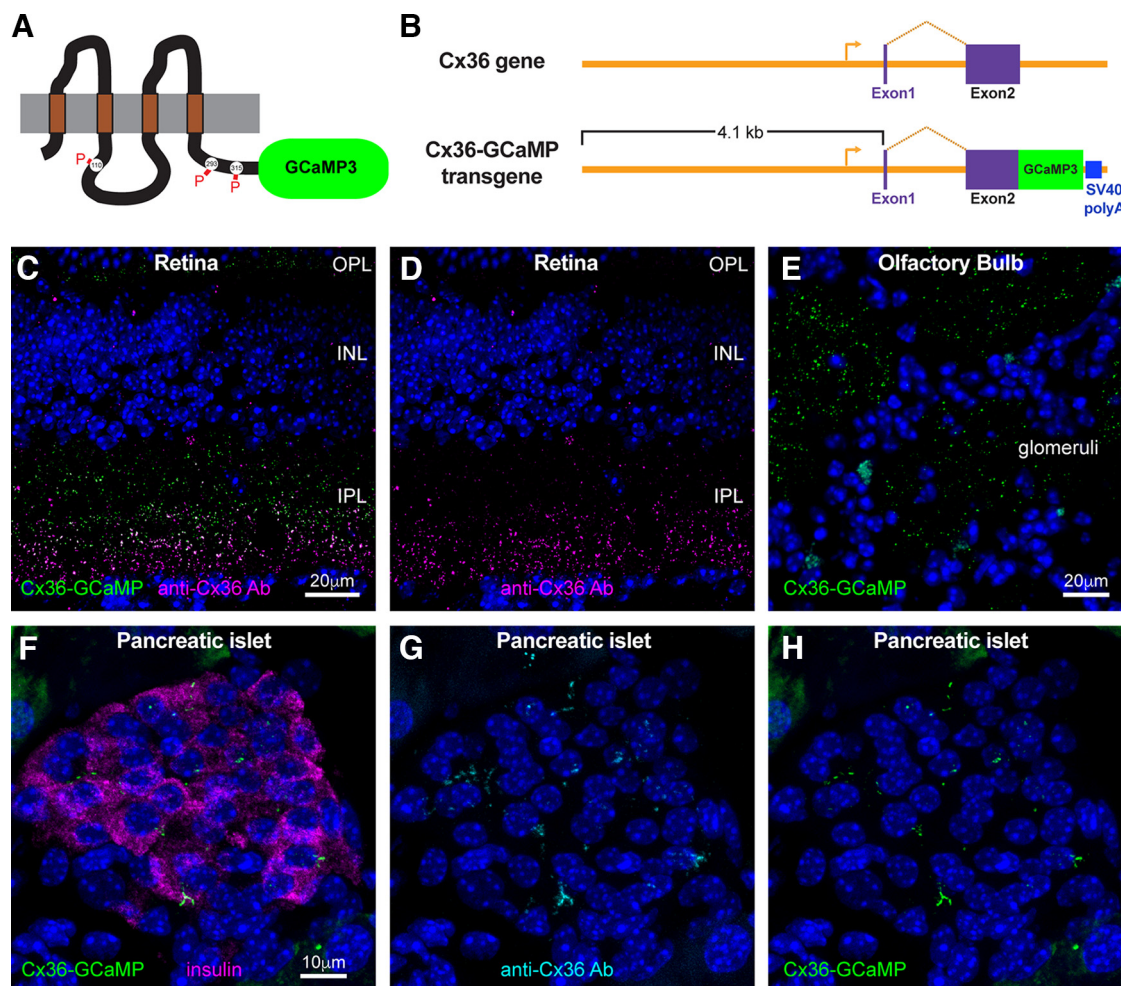


Figure 1. Cx36-GCaMP transgenic mouse. **A**, Cx36-GCaMP consists of mouse Cx36 with GCaMP3 fused to the C terminal. Regulatory phosphorylation sites of Cx36 are shown. Cx36-GCaMP expressed in cultured cells has been shown to display normal functional regulation by protein kinases (Moore et al., 2020). **B**, The Cx36-GCaMP transgene consists of 4.1 kb of genomic sequence upstream of mouse Cx36 exon 1, exon 1, intron 1, and exon 2 and SV40 polyadenylation signal derived from the Cx36-GCaMP expression plasmid. The endogenous transcription initiation site is indicated by the right arrow, and transcript splicing is indicated by the chevron between exons 1 and 2. **C**, **D**, Cx36-GCaMP expression in retina of transgenic mice. Cx36-GCaMP labeled with an anti-GFP antibody is green; Cx36 immunolabeling is magenta. Cx36-GCaMP is expressed in punctate spots in both IPL and OPL, where Cx36 is normally expressed. INL, Inner nuclear layer. Scale bars: 20 μ m. **E**, Cx36-GCaMP expression in olfactory bulb glomeruli labeled with anti-GFP antibody. Scale bar: 20 μ m. **F–H**, Expression of Cx36-GCaMP in the pancreas. Cx36-GCaMP (green) is found between islet cells in the pancreas that contain insulin (**F**, magenta). Labeling with an anti-Cx36 antibody (**G**, cyan) is nearly identical to labeling of Cx36-GCaMP with an anti-GFP antibody (**H**, green). Scale bars: 10 μ m.

and F1 offspring were screened for uniformity of expression in the retina. Two lines with consistent expression, G01 and G43, have been propagated by incrossing. Data from G01 are shown in this communication.

Figure 1C shows expression of Cx36-GCaMP in vertical sections of mouse retina with signal enhanced with an anti-GFP antibody (green). Cx36-GCaMP was expressed in punctate clusters in the retinal IPL and outer plexiform layers (OPL), a distribution typical of Cx36 expression in gap junctions in the retina (Feigenspan et al., 2001; Mills et al., 2001). Immunostaining with anti-Cx36 antibody revealed precisely overlapping distribution of the two labels through most of the inner plexiform layer (Fig. 1C,D). A narrow band of Cx36 gap junctions in the lower edge of the inner plexiform layer failed to contain Cx36-GCaMP,

suggesting that at least one cell type did not express the transgene. Ninety-six $\pm$ 3% of Cx36-GCaMP puncta colocalized with anti-Cx36 immunostaining, whereas 75 $\pm$ 10% of Cx36 puncta colocalized with Cx36-GCaMP (n = 8 animals).

Cx36-GCaMP was also found in a punctate distribution in other brain regions known to express Cx36. As an example, Figure 1E shows labeling for Cx36-GCaMP in olfactory bulb glomeruli, where Cx36 expression is enriched (Belluardo et al., 2000; Degen et al., 2004), and Cx36 supports lateral excitation and spike synchrony of mitral cells (Christie et al., 2005; Christie and Westbrook, 2006). We also examined expression of Cx36 in the pancreas, where Cx36 is expressed in endocrine beta cells and is required for synchrony of activity and efficient secretion of insulin

(Calabrese et al., 2001; Serre-Beinier et al., 2009). Figure 1*F* shows that Cx36-GCaMP is found between insulin-containing beta cells of pancreatic islets. Furthermore, Cx36-GCaMP labeling overlaps completely with labeling for Cx36 (Fig. 1*G,H*), which includes both endogenous Cx36 and transgenic Cx36-GCaMP.

In mammalian retina, the largest Cx36 gap junctions are found predominantly in the All amacrine (amacrine type 2) cells (Feigenspan et al., 2001; Mills et al., 2001) and are located in the lowest sublaminae of the IPL. As much as 98% of Cx36 is localized to All amacrine cells in this lowest layer (Mills et al., 2001). This is the area where Cx36-GCaMP expression is missing from some large gap junctions (Fig. 1*C*), suggesting that All amacrine cells may not express the transgene in this transgenic line. Some Cx36-GCaMP expression was detected in the lowest layer (Fig. 1*C*). All amacrine cells make gap junctions both with each other and with several classes of cone On bipolar cells (Kolb and Nelson, 1983; Anderson et al., 2011; Sigulinsky et al., 2020), and trafficking to these two classes of gap junctions is dependent on different factors (Meyer et al., 2014). One potential explanation for the Cx36-GCaMP signal in the lower IPL is that it is expressed in some cone On bipolar cells and localized to gap junctions with All amacrine cells. This hypothesis will require further investigation to verify.

The calcium microenvironment is dynamic at electrical synapses

All amacrine cell gap junctions display profound activity-dependent potentiation driven by activation of NMDA receptors and subsequent activation of calcium/calmodulin-dependent protein kinase II (CaMKII; Kothmann et al., 2012). This signaling pathway appears to require calcium influx, but the existence of a glutamate-driven calcium signal at electrical synapses has not been demonstrated. To examine this signaling pathway, we imaged slice preparations from Cx36-GCaMP mouse retina presented with puffs of glutamate; 1 mM glycine was present in both superfusion and puff solutions to enable NMDA receptor activation by glutamate. We used two types of apparatus for these experiments, a confocal microscope using scanning laser excitation (Fig. 2*Ai–Aiii*) and a widefield microscope using SRRF imaging (Gustafsson et al., 2016; Lee et al., 2018; Fig. 2*Bi–Biii*; see above, Materials and Methods). Both systems use visible light excitation, so retinal On pathway light responses were suppressed by the presence of L-AP4 in perfusion and puff solutions throughout the experiments. Figure 2, *Ai* and *Bi*, show maximum intensity projections through time of a 20 s confocal sequence and a 45 s SRRF sequence, respectively, centered on the retinal inner plexiform layer where amacrine cell, ganglion cell, and some bipolar cell gap junctions are located. Individual frames are shown 2 s before a 2 s glutamate puff (Fig. 2*Aii,Bii*) and 2 s after the puff (Fig. 2*Aiii,Biii*). Individual Cx36-GCaMP gap junctions can be followed through time in these experiments (Movie 1).

Figure 2*C* shows raw fluorescence intensity of three individual Cx36-GCaMP gap junctions from the sequence in Figure 2*B* (arrowheads). A single exponential baseline

was fit to each individual spot to represent F_0 , as shown by the gray lines in Figure 2*C*. In each spot, an increase in fluorescence time locked to the application of the glutamate puff (black bar) was detected. This was superimposed on a declining baseline that was at least partially because of a tissue-wide increase in fluorescence caused by the onset of the imaging light and is calculated into the F_0 baseline. In the experiment in Figure 2*B* 15 of 15 measured spots showed positive responses (at least three time points 2 SD above the baseline); 18 of 34 measured spots showed positive responses in the experiment in Figure 2*A*. These spots showing positive responses are interpreted to be gap junctions that experienced a transient increase in local calcium concentration in response to the glutamate puff. Spots lacking a response are interpreted to be gap junctions that did not encounter a change in local calcium concentration above the noise level of the measurement time locked to the puff stimulus. Figure 2*D* shows the average glutamate puff response $\pm$ 1 SD of the 15 measured Cx36-GCaMP spots from the experiment in Figure 2*B*. Calcium rose rapidly at the gap junctions immediately following the glutamate puff and declined steadily as the puff washed out. Control experiments using puffs of Lucifer yellow dye revealed persistence of the puff solution was similar to the duration of Cx36-GCaMP responses (Fig. 2*E,F*), suggesting that the duration of Cx36-GCaMP calcium responses in these experiments depended on the washout of glutamate from the tissue slice. It is unclear to what extent Cx36-GCaMP responses may be shaped by cellular factors such as the kinetics of ion channels or transporters. These experiments indicate that a large fraction of electrical synapses expressing Cx36 GCaMP experience dynamic increases in calcium on glutamate stimulation.

Calcium transients come from multiple sources

Direct modulation of gap junction coupling by activity and glutamate has been shown to depend on NMDA receptors in the All amacrine cells (Kothmann et al., 2012). To examine the potential sources of glutamate-induced calcium signals at the broader sample of Cx36-GCaMP gap junctions in our experiments, we performed a series of pharmacological interventions. Puffs of glutamate produced responses of variable amplitude in different individual gap junctions (Fig. 3*A*, left) and between slices. This suggests that individual gap junctions within the slice encounter quantitatively different changes in local calcium concentration, and some reveal no detectable change. Note that because of the wide-field imaging paradigm, some background tissue fluorescence is incorporated into the F_0 baseline used to calculate $\Delta F/F_0$. So, to the extent that scattered light from positive responses is incorporated into F_0 , the fraction subtracted from positive spot responses may affect the response amplitude measured for small gap junctions more than that measured for larger ones exposed to comparable calcium changes. Glutamate puffs produced a population-wide average time-integrated response of $0.436 \pm 0.312 (\Delta F/F_0) \cdot \text{sec}$ ($n = 7$ slices from 7 animals), with $77 \pm 28\%$ ($n = 7$) of gap junctions showing a positive response (Fig. 3*K,L*). Inhibition of NMDA receptors

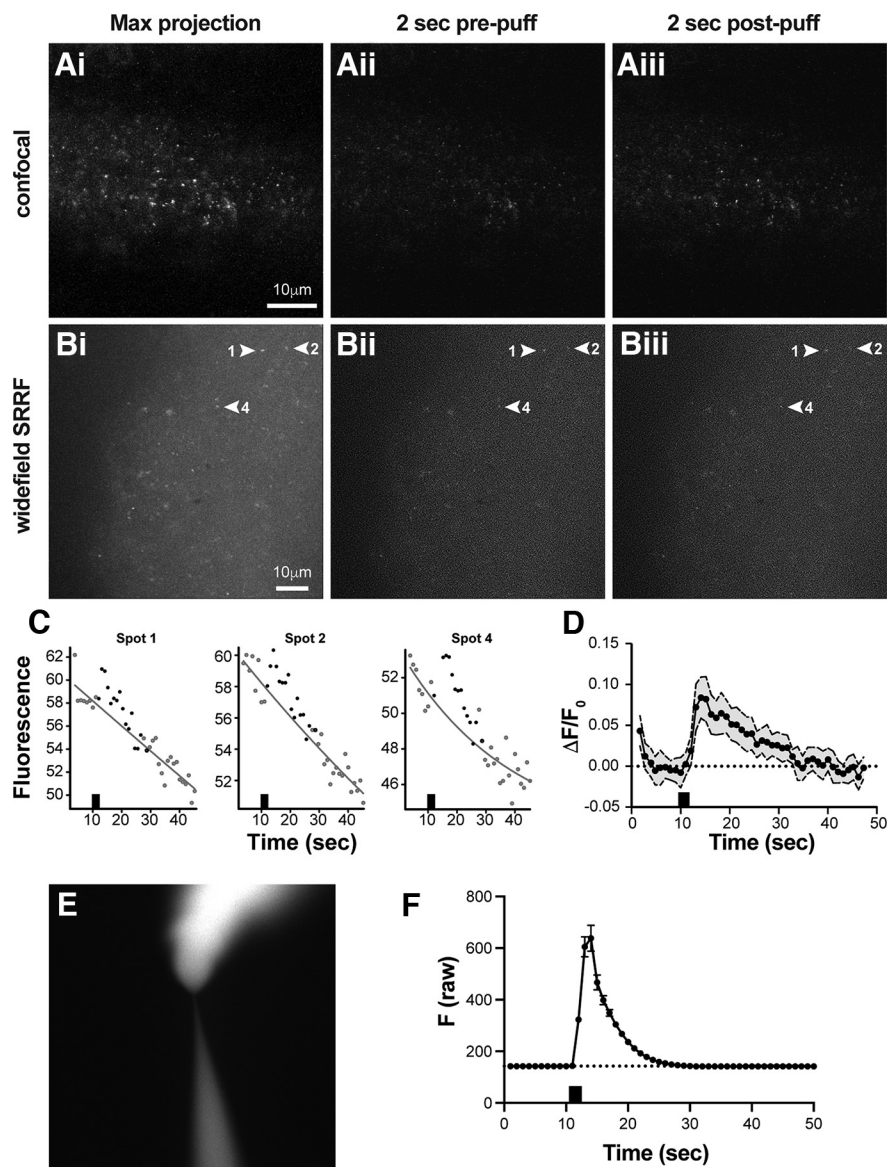
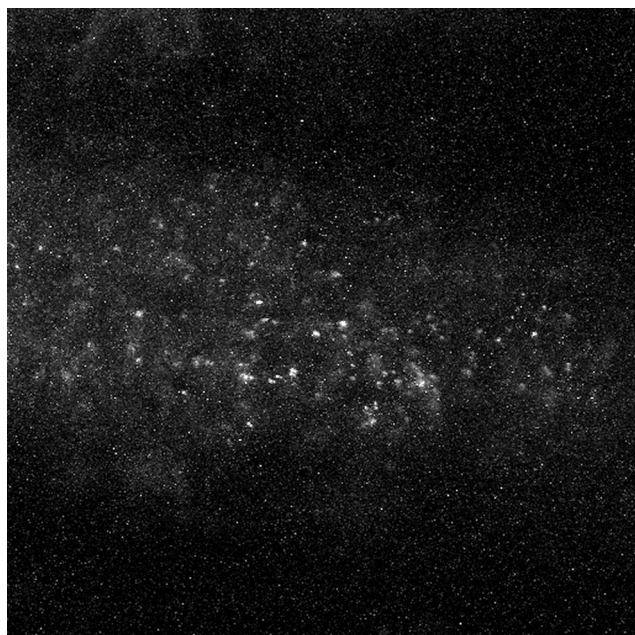


Figure 2. Glutamate puff responses of Cx36-GCaMP in retina slices. **Ai-iii**, Confocal imaging of a retina slice preparation centered on the inner plexiform layer presented with a 1 s puff of 750 μ M glutamate (**Ai**). Maximum intensity projection across the full 20 s time course (**Aii**). Single image 2 s before glutamate puff (**Aiii**). Single image 2 s after glutamate puff. **Bi-iii**, Widefield SRRF imaging of retina slice preparation centered on the inner plexiform layer presented with a 2 s puff of 1 mM glutamate (**Bi**). Maximum intensity projection across the full 45 s time course (**Bii**). Single image 2 s before glutamate puff (**Biii**). Single image 2 s after glutamate puff. Scale bars in **A**, **B**: 10 μ m. **C**, Raw fluorescence intensity sequences of three example gap junctions from the sequence in **Bi** (arrowheads). The glutamate puff is indicated by the black box on the time axis. Black spots following the puff are data points within the designated responding window used to assess a positive response. Gray lines are the F_0 baselines fit to the intensity data outside the responding window (gray data points). **D**, Mean responses of 15 gap junctions from the sequence in **Bi-iii** expressed as $\Delta F/F_0$. Error bars are ± 1 SD. The glutamate puff is shown by the black box on the time axis. **E**, **F**, Time course of fluorescence of a Lucifer yellow dye puff in the recording chamber under the superfusion conditions of experiments shown in this study. **E**, Image of a puffing pipette and dye plume at the mouth of the pipette. **F**, Mean (symbols) ± 1 SD of raw fluorescence intensities of 12 spots within the plume at a greater distance from the pipette over the course of one puff; 2 s puff is indicated by the black box on the x-axis.

with CPP largely blocked responses of some gap junctions, whereas other gap junctions retained some response (Fig. 3A, right), indicating that some of the gap junctions imaged required NMDA receptors for the local calcium rise, although not all did. Figure 3B shows individual spot responses, and Figure 3, C and L, shows the fraction of spots showing a positive response in three

slices from three animals in which both control glutamate puff and glutamate and CPP conditions were tested. The median difference of the response area was -0.514 ($\Delta F/F_0$) $\bullet$ sec, and the p value of the two-sided permutation t test of 5000 bootstrap samples of the data was 0.0. This indicates that NMDA receptors were required for a large fraction of transient calcium signals



Movie 1. Cx36-GCaMP mouse retina slice recording. Response of a slice to a 2 s puff of 1 mM glutamate begun at 4 s; 20 s total record time. [View online]

in response to glutamate puffs detected at Cx36-GCaMP gap junctions in the inner plexiform layer.

Suppression of glutamate-driven calcium signals at Cx36 gap junctions by an NMDA receptor antagonist was the expected result. Nonsynaptic NMDA receptors very closely associated with Cx36 gap junctions have been shown to contribute significantly to the signaling that drives CaMKII phosphorylation of Cx36 in All amacrine cells (Kothmann et al., 2012). However, as described above, it is possible that Cx36-GCaMP is not expressed in All amacrine cells, and it is known that Cx36 is expressed by additional cell types in the inner retina, including retinal ganglion cells and other amacrine cells (Hidaka et al., 2002; Schubert et al., 2005; Hoshi and Mills, 2009; Pan et al., 2010; Kirkby and Feller, 2013; Harrison et al., 2021). Furthermore, most ganglion cells and some amacrine cells use NMDA receptors in post-synaptic response to glutamatergic bipolar cells (Massey and Miller, 1990; Marc, 1999; Gustafson et al., 2007). In cells with synaptic NMDA receptor responses, depolarization may activate voltage-gated calcium channels, providing another potential source of calcium at Cx36 gap junctions. To examine whether voltage-gated calcium channels were involved, we treated retina slices with CdCl₂ to block calcium channels nonselectively. Figure 3D shows individual spot responses from one slice to a puff of glutamate without or with 100 μ M CdCl₂. CdCl₂ reduced the amplitude of most spot responses. Figure 3E shows individual spot responses, and Figure 3F shows the fraction of spots showing a positive response in three slices from three animals in which both control glutamate puff and glutamate plus CdCl₂ conditions were tested. The median difference of the response area was $-0.211 (\Delta F/F_0) \bullet \text{sec}$, and the *p* value of the two-sided permutation *t* test of 5000 bootstrap samples of the data was 0.0002. Population average data from four slices are shown in Figure 3, K and L.

These results suggest that voltage-gated calcium channels contributed to the calcium responses of the population of electrical synapses in these slices. However, it has been reported that CdCl₂ directly inhibits NMDA receptors (Legendre and Westbrook, 1990; Tu et al., 2016), which confounds this interpretation. As a more focused test of the involvement of voltage-gated calcium channels, we used NMDA puffs to stimulate NMDA receptors more specifically. This should activate both synaptic NMDA receptor populations, which may depend on voltage-gated calcium channels to generate calcium transients at distant gap junctions, and nonsynaptic NMDA receptor populations that may not if they are close enough to Cx36 gap junctions to elicit a calcium signal via direct calcium influx through the NMDA receptor channel. Figure 3G shows that in two slices from two animals, NMDA puffs in the presence of CdCl₂ tended to elicit smaller responses than control NMDA puffs. The median difference of the time-integrated response area was $-0.184 (\Delta F/F_0) \bullet \text{sec}$, and the *p* value of the two-sided permutation *t* test of 5000 bootstrap samples of the data was 0.0016. There was a variable effect on the fraction of gap junctions showing a positive response to NMDA puffs on treatment with CdCl₂ (Fig. 3H). These modest effects of CdCl₂ on response amplitude and fraction of gap junctions displaying a response indicate that CdCl₂ did not fully inhibit NMDA receptors at the concentration applied. The much larger effect on glutamate-stimulated responses suggest that voltage-gated calcium channels supplemented the transient calcium increase at Cx36 gap junctions when all types of glutamate receptors were activated. Finally, we examined the effects of the selective L-type calcium channel blocker nifedipine (20 μ M) in experiments with puffs of 100 μ M AMPA to activate AMPA receptors without substantial contributions from NMDA receptors. In this paradigm, nifedipine tended to reduce the amplitude of the population average response (Fig. 3K,L), again suggesting a contribution of voltage-gated calcium channels to the response.

As a final test, we also evaluated the effects of blocking ionotropic GABA receptors, which are likely to be activated in the glutamate puff paradigm because of glutamate excitation of amacrine cells and synaptic GABA release in the IPL. Figure 3I shows that application of picrotoxin had no effect on the time-integrated response area [median difference 0.12 ($\Delta F/F_0$) $\bullet \text{sec}$; *p* value of the two-sided permutation *t* test of 5000 bootstrap samples of the data was 0.224], and Figure 3J shows that there was no consistent effect on the fraction of gap junctions showing a positive response in two slices from two animals. Thus, GABA receptor activation appeared to have little influence on calcium transients at Cx36-GCaMP gap junctions at the population level.

Discussion

Activity-dependent tuning of electrical coupling

Electrical synapses are abundant in the retina, being found in all classes of retinal neuron and being subject to extensive plasticity (Ribelayga and O'Brien, 2017; O'Brien

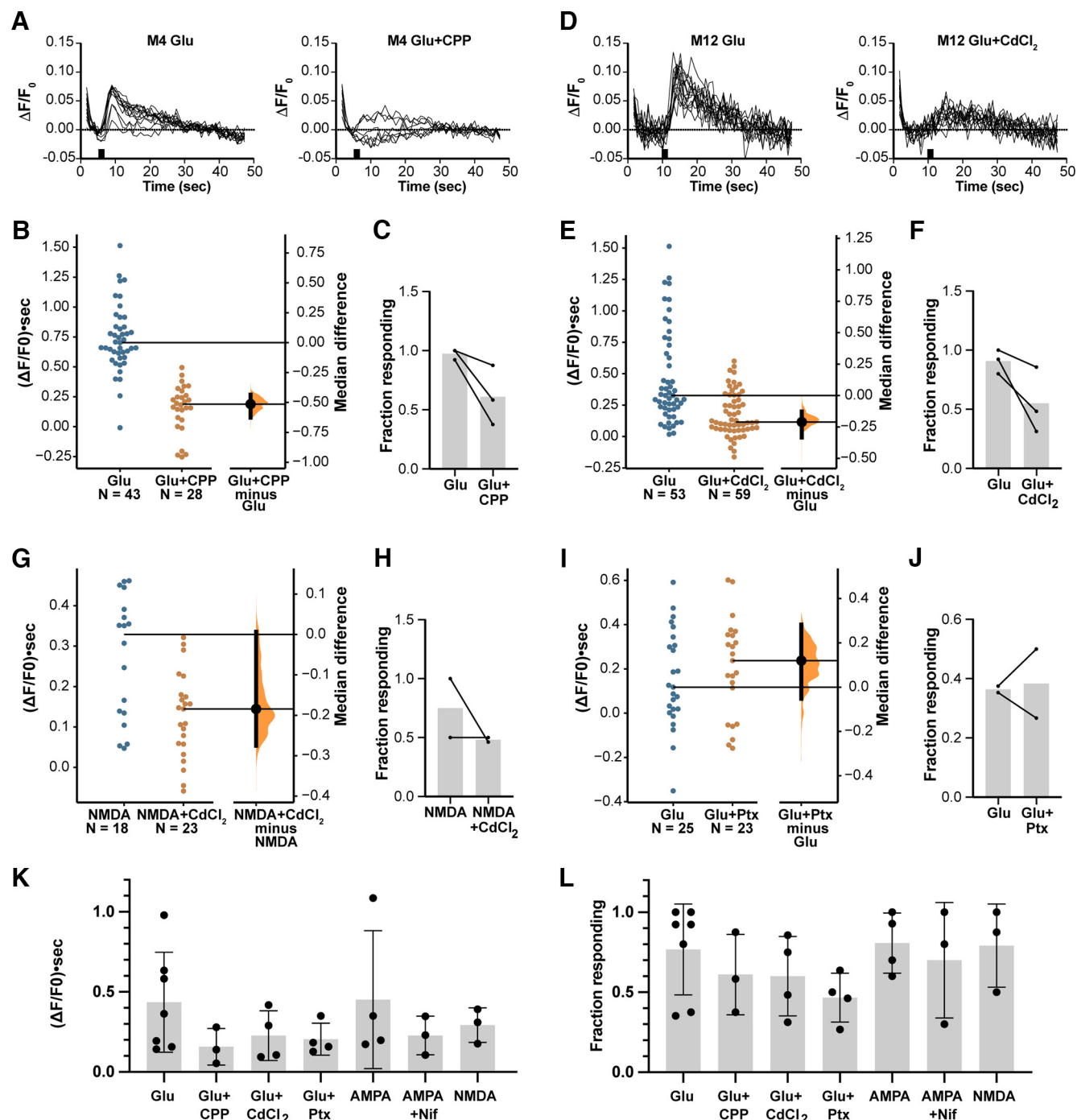


Figure 3. Pharmacological analysis of sources of calcium at Cx36-GCaMP electrical synapses. **A**, Left, Individual responses to a 2 s glutamate puff (black bar) of 13 Cx36-GCaMP spots. There is some variation in amplitude, with some spots failing to respond. Right, Individual responses of eight Cx36-GCaMP spots in the same slice with 10 μM CPP in the bath and puff solutions. Very few spots showed responses, but some persisted. **B**, Gardner-Altman estimation plot of pooled individual spot response amplitudes from three slices recorded in control and CPP conditions. Median responses are indicated by the black lines. Right, The frequency distribution of the median treatment, control difference in 5000 bootstrap replicates; 95% confidence intervals of the median difference are indicated by the black lines. **C**, Fraction of Cx36-GCaMP spots showing a positive response in the three slices shown in **B**. **D**, Individual responses to a glutamate puff of 15 spots in control (left) or solutions containing 100 μM CdCl₂ (right) in the same slice. **E**, Gardner-Altman estimation plot of pooled individual spot response amplitudes from three slices recorded in control and CdCl₂ conditions. **F**, Fraction of Cx36-GCaMP spots showing a positive response in the three slices shown in **E**. **G**, Gardner-Altman estimation plot of pooled individual spot response amplitudes to puffs of 10 μM NMDA from two slices recorded either in control solution or with 100 μM CdCl₂ in the perfusion and puff solutions. **H**, Fraction of Cx36-GCaMP spots showing a positive response in the two slices shown in **G**. **I**, Gardner-Altman estimation plot of pooled individual spot response amplitudes to puffs of glutamate from two slices recorded either in control solution or with 100 μM CdCl₂ in the perfusion and puff solutions. **J**, Fraction of Cx36-GCaMP spots showing a positive response in the two slices shown in **I**. **K**, Bar graph showing the fraction of Cx36-GCaMP spots showing a positive response to puffs of glutamate, glutamate + CPP, glutamate + CdCl₂, glutamate + Ptx, AMPA, AMPA + Nif, and NMDA. **L**, Bar graph showing the fraction of Cx36-GCaMP spots showing a positive response to puffs of glutamate, glutamate + CPP, glutamate + CdCl₂, glutamate + Ptx, AMPA, AMPA + Nif, and NMDA.

continued

two slices recorded either in control solution or with 100 μM Picrotoxin in the perfusion and puff solutions. **J**, Fraction of Cx36-GCaMP spots showing a positive response in the two slices shown in **I**. **K**, Population average responses to puffs of 1 mM glutamate, 100 μM AMPA, or 10 μM NMDA. Response amplitudes are shown in **K** and fraction of gap junctions responding in **L**. Each point represents average responses from Cx36-GCaMP spots in one slice; bars indicate the mean, and error bars indicate 1 SD. A total of 16 slices from 12 animals is included.

and Bloomfield, 2018). Activity-dependent plasticity is a subset of the types of plasticity observed at electrical synapses. In retinal All amacrine cells, light-dependent and bipolar cell activity-dependent signaling drives a more than threefold increase in coupling, as measured by the diffusion coefficient for Neurobiotin tracer diffusion through the All amacrine cell network (Kothmann et al., 2012). This signaling is initiated by spillover glutamate-activating nonsynaptic NMDA receptors, with activation of CaMKII and phosphorylation of Cx36 (Kothmann et al., 2012; Fig. 4, left). In the present study, we find that NMDA-receptor-dependent calcium transients are widespread at Cx36 gap junctions in the retina. However, direct calcium flux through NMDA receptors is not the only source of calcium that may induce Cx36 plasticity in the retinal inner plexiform layer. Additional signaling driven by glutamate-dependent activation of voltage-gated calcium channels (Fig. 4, right) contributes to calcium transients that we can detect at Cx36-GCaMP gap junctions. Although not unexpected, the presence of calcium fluctuations at retinal electrical synapses derived from voltage-gated calcium channels has not been formally demonstrated. This expands the repertoire of signaling mechanisms that have potential to regulate electrical synapse plasticity.

The most striking observation of this study is that >90% of gap junctions containing Cx36-GCaMP experience transient increases in calcium on glutamate puff stimulation. These gap junctions have been selected essentially randomly from the entire population of Cx36 gap junctions in the retinal inner plexiform layer that harbor Cx36-GCaMP. With the caveat that All amacrine cells may not express Cx36-GCaMP, this observation is all the

more striking, suggesting that a dynamic calcium micro-environment around electrical synapses is commonplace in retinal neurons.

Studies of phosphorylation of Cx36 in the retina demonstrate that gap junctions within one micron of each other on the same dendrite can be in very different phosphorylation states and, hence, different states of functional plasticity (Kothmann et al., 2009). This emphasizes the fact that functional plasticity of each individual Cx36 gap junction plaque is highly dependent on local cellular signaling. Most studies of calcium dynamics in neurons record signals in the cell soma, often at a great distance from electrical synapses. Although such changes may be correlated with calcium changes in the vicinity of electrical synapses, it has not previously been possible to assess whether that is the case. Cx36-GCaMP allows the direct investigation of calcium signals relevant to the functional regulation of Cx36 electrical synapses.

Cx36 is intrinsically capable of plasticity over an order of magnitude dynamic range (O'Brien, 2019). Outside the retina, long-term potentiation of electrical synapses is driven by calcium influx through synaptic NMDA receptors in Mauthner cell mixed synapses (Pereda and Faber, 1996; Haas et al., 2016) and through nonsynaptic NMDA receptors in inferior olive neurons (Turecek et al., 2014). Curiously, synaptic activation of NMDA receptors in inferior olive neurons has the opposing action to trigger electrical synaptic depression (Mathy et al., 2014). This depression is similar to the effects of activity in thalamic reticular neurons (Landisman and Connors, 2005; Haas et al., 2011). In the latter, two distinct pathways cause electrical synaptic depression; a calcium-independent pathway depends on

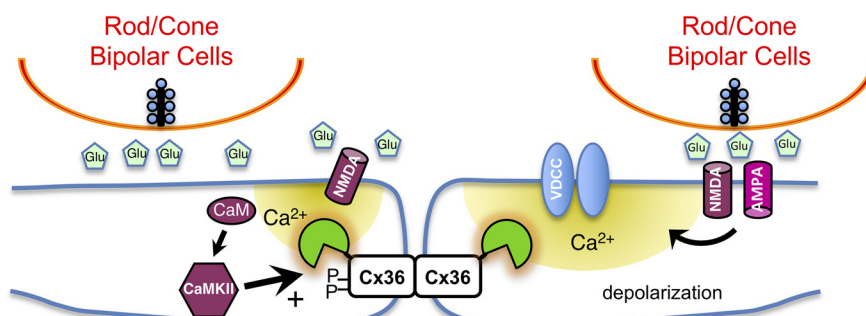


Figure 4. Schematic model of sources of calcium available for electrical synapse plasticity. Left, A Ca^{2+} –CaMKII signaling pathway has been well documented to enhance Cx36 coupling by promoting Cx36 phosphorylation; an opposing mechanism activating calcineurin and dephosphorylating Cx36 has also been proposed. Extracellular sources of calcium triggered by glutamatergic synaptic activity are shown, including direct influx through NMDA receptors (left) and indirect influx through voltage-gated calcium channels triggered by membrane depolarization (right). Direct calcium influx may involve nonsynaptic NMDA receptors as shown, or may involve synaptic NMDA or calcium-permeable AMPA receptors in close proximity to electrical synapses. Indirect signaling may involve synapses at any distance from the electrical synapse and may also occur in excitatory neurons shown as presynaptic in this diagram.

presynaptic glutamate activation of metabotropic glutamate receptors, whereas the own activity of the neuron itself drives a calcium-dependent pathway that involves voltage-gated calcium channels and recruitment of intracellular store calcium (Sevetson et al., 2017). An activity- and calcium-dependent potentiation of electrical synapses can also be elicited in the same neurons under specific conditions that limit calcium influx, indicating that calcium drives bidirectional plasticity dependent on the specific type of activity and level of calcium experienced (Fricker et al., 2021). The similarities and differences in signaling in these example networks demonstrate that the signaling pathways that control electrical synapse plasticity are circuit specific and very sensitive to the magnitude of the calcium signal experienced locally at the electrical synapse.

Retinal ganglion cells adapt in a matter of seconds to both average image contrast and to the local distribution of contrast features (Smirnakis et al., 1997). Our experiments in the Cx36-GCaMP mouse demonstrate that a large fraction of electrical synapses experience a dynamic calcium microenvironment, providing the molecular underpinnings to contribute to adaptation on a seconds time scale. This mouse will be a useful tool to examine the plasticity within specific circuits when paired with strategies to label specific cell types.

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