Amacrine Cells Forming Gap Junctions With Intrinsically Photosensitive Retinal Ganglion Cells: ipRGC Types, Neuromodulator Contents, and Connexin Isoform

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Received: September 14, 2020
Accepted: November 30, 2020
Published: January 7, 2021

Citation: Harrison KR, Chervenak AP, Resnick SM, Reifler AN, Wong KY. Amacrine cells forming gap junctions with intrinsically photosensitive retinal ganglion cells: ipRGC types, neuromodulator contents, and connexin isoform. Invest Ophthalmol Vis Sci. 2021;62(1):10. https://doi.org/10.1167/iovs.62.1.10

PURPOSE. Intrinsically photosensitive retinal ganglion cells (ipRGCs) signal not only centrally to non-image-forming visual centers of the brain but also intraretinally to amacrine interneurons through gap junction electrical coupling, potentially modulating image-forming retinal processing. We aimed to determine (1) which ipRGC types couple with amacrine cells, (2) the neuromodulator contents of ipRGC-coupled amacrine cells, and (3) whether connexin36 (Cx36) contributes to ipRGC-amacrine coupling.

METHODS. Gap junction-permeable Neurobiotin tracer was injected into green fluorescent protein (GFP)–labeled ipRGCs in Opn4Cre+/EYFP; Z/EG mice to stain coupled amacrine cells, and immunohistochemistry was performed to reveal the neuromodulator contents of the Neurobiotin-stained amacrine cells. We also created Opn4Cre+/EYFP; Cx36fllox/flox; Z/EG mice to knock out Cx36 in GFP-labeled ipRGCs and looked for changes in the number of ipRGC-coupled amacrine cells.

RESULTS. Seventy-three percent of ipRGCs, including all six types (M1–M6), were tracer-coupled with amacrine somas 5.7 to 16.5 μm in diameter but not with ganglion cells. Ninety-two percent of the ipRGC-coupled somas were in the ganglion cell layer and the rest in the inner nuclear layer. Some ipRGC-coupled amacrine cells were found to accumulate serotonin or to contain nitric oxide synthase or neuropeptide Y. Knocking out Cx36 in M2 and M4 dramatically reduced the number of coupled somas.

CONCLUSIONS. Heterologous gap junction coupling with amacrine cells is widespread across mouse ipRGC types. ipRGC-coupled amacrine cells probably comprise multiple morphologic types and use multiple neuromodulators, suggesting that gap junctional ipRGC-to-amacrine signaling likely exerts diverse modulatory effects on retinal physiology. ipRGC-amacrine coupling is mediated partly, but not solely, by Cx36.

Keywords: melanopsin, ipRGC, amacrine cells, gap junctions, neuromodulators
cells are not dopaminergic.12,20 What neuromodulators do about M5 and M6 ipRGCs? (2) ipRGC-coupled amacrine light phase.

An hour dark cycle, with experiments conducted during the sexes were used. Animals were housed in a 12-hour light/12-hour dark cycle. All mice were 3 to 10 months old, and both sexes were used. Animals were housed in a 12-hour light/12-hour dark cycle, with experiments conducted during the sexes were used.

Table. Primers for Genotyping

| Gene     | Primer Name | Sequence            |
|----------|-------------|---------------------|
| GFP      | Z/EG for    | CCC CTG CTG TCC ATT CCT TA |
| Z/EG rev |             | GGG AGG AGG GGC TGG |
| Cre      | Cre for     | CTG CTT CAG GTC GCT TAG |
| Cre rev  |             | GCT TGT CTG GCT GCT CAG |
| Opn4     | Opn4 for    | GGT TGG GAT GGA GGA GAG |
| Opn4 rev |             | GCC TCG TCG TCT GAG |
| Cx36     | U1/Cx36 for | AAG AAG TCG TGC TGC TTC |
|          | D1/Cx36 rev1| GCC TGC TGC TGC TTC |
|          | D2/Cx36 rev2| AAG AAG TCG TGC TGC TTC |

(1) Müller et al.,11 saw tracer coupling between amacrine cells and M1–M3 ipRGCs, and amacrine cells also tracercouple with ON α cells,15,16 which are M4 ipRGCs.17–19

How about M5 and M6 ipRGCs? (2) ipRGC-coupled amacrine cells are not dopaminergic.12,20 What neuromodulators do they contain? (3) Cx36 mediates heterologous gap junction coupling between amacrine cells and many types of mouse ganglion cells.21,22 Does ipRGC-amacrine coupling involve Cx36?

Materials and Methods

Mouse Lines

All procedures were approved by the University of Michigan Institutional Animal Care and Use Committee. This study used two mouse lines. In Opn4Cre/+; Z/EG mice, Cre recombinase expressed under the melanopsin (Opn4) promoter induces Cre-dependent green fluorescent protein (GFP) expression in ipRGCs.17 In Opn4Cre/+; Cx36flox/+, Z/EG mice, which were created by mating Cx36 flox mice23 with Opn4Cre/+; Z/EG mice over two generations, melanopsin-driven Cre induces Cx36 knockout as well as GFP expression in ipRGCs. We had previously used this Opn4Cre/+ line to knock out the NRI subunit of N-methyl-D-aspartate (NMDA) receptors in ipRGCs.24

The Table lists the genotyping primers. All mice were 3 to 10 months old, and both sexes were used. Animals were housed in a 12-hour light/12-hour dark cycle, with experiments conducted during the light phase.

Tracer Injection and Immunohistochemistry

After overnight dark adaptation, a mouse was euthanized by CO2 and cervical dislocation under dim red light. Both eyes were enucleated and hemisected in room temperature Ames’s medium (MilliporeSigma, St. Louis, MO, USA) gassed with 95% O2 5% CO2. Each retina was isolated and cut into three to four pieces, which were kept in darkness for up to 7 hours before being used for tracer injection. A piece was flattened ganglion cell side up on a superfusion chamber, stabilized by a weighted harp, and superfused by 32°C Ames at 2 ml/min. The GCL was visualized through infrared transillumination under an Eclipse E600FN microscope (Nikon, Melville, NY, USA) and GFP+ somas identified using FITC epifluorescence. A randomly selected GFP+ soma was impaled with a glass microelectrode (100- to 150-Ω tip resistance) containing 1 M KCl, 4% Neurobiotin (Vector Laboratories, Burlingame, CA, USA), and 0.1% Lucifer Yellow. A MultiClamp 700B amplifier (Molecular Devices, San Jose, CA, USA) was used to generate ~1 to ~3-nA pulses to iontophorese Lucifer Yellow until this dye’s fluorescence appeared in the soma. The FITC stimulus was then extinguished and the retina kept in darkness. Membrane resistance was estimated using Clampex software (Molecular Devices), and pulse polarity was switched to positive to iontophorese Neurobiotin for 15 minutes. Pulse amplitude was 3 nA for cells with <300-Ω membrane resistances, 1.5 to 2.5 nA for 300 to 600 Ω, and 1 nA for >600 Ω, so that all ipRGCs experienced comparable voltage changes. Due to technical difficulty, we did not inject displaced ipRGCs, which constitute 6% to 10% of all ipRGCs.25,26

After injecting three ipRGCs in a retinal piece, it was fixed in 4% paraformaldehyde for 15 minutes, washed in PBS three times, and incubated for 2 hours at room temperature in primary block (10% normal donkey serum and 2% Triton X-100 in PBS) and then for 5 days at 4°C in primary block plus Alexa Fluor 568 streptavidin (1:250; Thermo Fisher, Waltham, MA, USA). After four PBS rinses, the retina was incubated overnight at 4°C in secondary block (5% normal donkey serum and 0.5% Triton X-100 in PBS) plus Alexa Fluor 568 streptavidin (1:250). In some experiments, the 5-day incubation also included one of these primary antibodies: rabbit anti-RNA binding protein with multiple splicing (RBPMs; 1:500; PhosphoSolutions, Aurora, CO, USA; catalog no. 1830-RBPMs), mouse anti–brain nitric oxide synthase (bNOS; 1:400; MilliporeSigma, catalog no. N2280), rabbit anti–neuropeptide Y (NPY; 1:1000; Cell Signaling Technology, Danvers, MA, USA; catalog no. 11976), rabbit anti-serotonin (1:250; Immunostar, Hudson, WI, USA; catalog no. 20080), and sheep anti–vasoactive intestinal peptide (VIP; 1:250; MilliporeSigma, catalog no. AB1581). To identify serotonin-accumulating amacrine cells, Neurobiotin-injected retinas were incubated in 2 μM serotonin hydrochloride (Tocris, Minneapolis, MN, USA) for 15 minutes before paraformaldehyde fixation.24 To visualize the primary antibodies, the secondary block included these secondary antibodies at 1:250: donkey anti-rabbit FITC, donkey anti-mouse Cy3, or donkey anti-sheep FITC (all from Jackson Immuno Research, West Grove, PA, USA).

Afterward, the retinas were rinsed nine times in PBS, flattened ganglion cell side up on glass slides, mounted using Vectashield (Vector Laboratories), and imaged using an SP5 confocal microscope (Leica, Buffalo Grove, IL, USA) at 0.5-μm steps from the vitreal surface through ∼20% of the inner nuclear layer (INL). Neurobiotin filled each ipRGC’s dendrites, enabling classifying the ipRGC as one of the six types based on dendritic stratification and morphology.27–29 For many ipRGCs, Neurobiotin also filled nearby somas. To distinguish the somas from nonselective streptavidin staining, we counted only round staining within or near the injected ipRGC’s dendritic field. Soma diameter was measured along the longest axis. Statistical comparisons used the Mann-Whitney U test, with P values <0.05 indicating significant differences.

Some retinas were stained with mouse anti-connexin 36 (1:250; Thermo Fisher, catalog no. 37-4600) and donkey anti-mouse FITC, without Neurobiotin injection. The entire thickness of these retinas was imaged confocally, and the z-stack was rotated 90° to show the side view of the imaged volume.
RESULTS

ipRGC Types With Coupled Amacrine Cells

To ascertain whether all ipRGC types form gap junctions, we injected the gap junction–permeable tracer Neurobiotin into 183 Opn4Cre+/Z/EG ipRGCs. Tracer-coupled somas were observed for all six ipRGC types, including 9 of 19 M1 cells (47%), 31 of 39 M2 cells (79%), 32 of 45 M3 cells (71%), 24 of 30 M4 cells (80%), 19 of 26 M5 cells (73%), and 20 of 24 M6 cells (83%). As shown in the exemplary data in Figure 1A, some of the coupled somas appeared to be in contact with the injected ipRGCs’ dendrites, potentially suggesting dendrosomatic gap junctions, whereas others were outside the ipRGCs’ dendritic fields, which probably connected with the ipRGCs via dendrodendritic gap junctions or an intermediary cell. To learn whether any of the somas were ganglion cells, 110 somas (98 in the GCL and 12 in the INL) coupled to 19 ipRGCs were tested with the RBPMS antibody, which labels all and only ganglion cells,30 and none were stained (Fig. 1B). Since Neurobiotin
ipRGC-Coupled Amacrine Cells

FIGURE 2. ipRGCs were tracer-coupled with a wide range of soma sizes. (A) Frequency distribution of the soma diameters of all the couple cells tracer-coupled to all injected cells of every ipRGC type. (B) The average numbers of small, medium, and large somas that coupled with each M1–M6 ipRGC, including uncoupled ipRGCs.

FIGURE 3. Some ipRGC-coupled amacrine cells had somas in the INL. (A) An M2 ipRGC that was tracer-coupled with three INL somas (arrowheads) and eight GCL somas (arrows). The rectangles mark the two regions that have been rotated 90° in the bottom panels to show their side views. (B) Frequency distribution of all GCL versus INL somas tracer-coupled to all injected M1–M6 ipRGCs.

in ganglion cells does not diffuse into glia,22,31 we inferred that the ipRGC-coupled somas were amacrine cells. Figure 1C shows the population-averaged number of amacrine cells coupled to each M1–M6 ipRGC, including ipRGCs lacking coupled somas.

The somas coupled to each ipRGC type spanned a wide diameter range: M1, 7.3–15.4 μm; M2, 6.0–16.2 μm; M3, 5.8–16.2 μm; M4, 6.8–16.5 μm; M5, 7.1–16.0 μm; and M6, 5.7–14.7 μm. Figure 2A shows frequency histograms plotting the diameter distribution of all the somas coupled to all injected cells of every ipRGC type. In Figure 2B, we have binned the ipRGC-coupled somas into three diameter ranges22 and plotted the population-averaged numbers of small, medium, and large somas coupled to each M1–M6 ipRGC to illustrate that different ipRGC types coupled with somewhat different proportions of the soma size groups (e.g., M2 and M6 coupled almost exclusively with medium somas, while M4 coupled with a higher proportion of large somas).

Of the 824 tracer-coupled somas, 759 (92.1%) were in the GCL and hence displaced amacrine cells, while the rest were conventionally placed in the INL. Figure 3A shows an M2 ipRGC that was tracer-coupled with 8 GCL somas (arrows)
and 3 INL somas (arrowheads). Figure 3B shows that, collectively, M3 ipRGCs coupled with the most INL somas, whereas M4 coupled exclusively with GCL somas.

**Neuromodulator Contents of ipRGC-Coupled Amacrine Cells**

ipRGC-coupled amacrine somas vary considerably in size (Fig. 2A), suggesting they comprise multiple types. To learn whether they contain multiple neuromodulators, we tested each of 33 coupled ipRGCs with one of four antibodies. Specifically, we tested anti-bNOS on 4 M3 ipRGCs, which were coupled to a total of 35 GCL somas and 1 INL soma; anti-NPY on 1 M2, 2 M3, 1 M4, 1 M5, and 1 M6, coupled to 37 GCL and 4 INL somas; anti-serotonin on 3 M2, 3 M3, 3 M4, 2 M5, and 2 M6, coupled to 61 GCL and 12 INL somas; and anti-VIP on 2 M2, 2 M3, 1 M4, 3 M5, and 2 M6, coupled to 60 GCL and 5 INL somas. Results showed that each of 3 M3 ipRGCs coupled to 1 bNOS-immunopositive GCL soma (Fig. 4A); 1 M3 and 1 M4 ipRGCs coupled to 2 and 1 NPY-immunopositive GCL somas, respectively (Fig. 4B); and each of 1 M2, 2 M3, and 1 M4 ipRGCs coupled to 1 serotonin-immunopositive GCL soma (Fig. 4C). None of the ipRGC-coupled amacrine cells tested with anti-VIP were stained (Fig. 4D).

**Connexin Isoform Mediating ipRGC-Amacrine Coupling**

To test the hypothesis that ipRGC-amacrine coupling involves Cx36, we created Opn4Cre+/; Cx36floxflox; Z/EG mice to knock out Cx36 in GFP-labeled, Cre-expressing ipRGCs. Cx36 immunostaining in both plexiform layers remained robust, confirming nonglobal Cx36 knockout (Fig. 5A). We injected Neurobiotin into 55 M1–M6 cells (i.e., M4).15,16 For M1–M3, Müllerm et al.11 reported behavior of coupling. In pilot tests, we injected Neurobiotin for various durations and found that while 15-minute injections stained more somas than 5-minute injections, injecting for >15 minutes did not stain any more somas, so our 15-minute injection protocol likely stained all coupled somas. Even if it did not, both mouse lines would presumably be affected more or less equally, so all observed control versus knockout differences should remain valid. Another caveat is that the low percentage of coupled M1 cells could have been due to the difficulty of injecting their relatively small somas.18,25,32 and M1 cells indeed seemed less well filled than M2–M6 (Figs. 1A, 5B). Nevertheless, our finding that M1 cells couple with about half as many somas as M2 and M3 agrees with Müller et al.11

While Müller et al.11 saw ipRGC-coupled somas only in the GCL, we found some in the INL. Considering that they injected Neurobiotin via 120- to 145-MΩ microelectrodes for 3 minutes whereas we injected using similar electrodes but...
**FIGURE 5.** ipRGC-amacrine coupling is mediated in part by Cx36. (A) Cx36 immunostaining confirms nonglobal Cx36 knockout in Opn4Cre<sup>C105/105</sup>; Cx36<sup>flx/flx</sup>; Z/EG retinas. A1: Cx36 immunostaining was imaged confocally in whole-mount retinas, and the z-stacks were rotated 90° to show these orthogonal views of Opn4Cre<sup>C105/105</sup>; Cx36<sup>+/+</sup>; Z/EG (left) and Opn4Cre<sup>C105/105</sup>; Cx36<sup>flx/flx</sup>; Z/EG (right) retinas. A2: Representative whole-mount images at focal planes within the outer (top) and inner (bottom) plexiform layers. (B) Neurobiotin staining patterns of six representative Opn4Cre<sup>C105/105</sup>; Cx36<sup>flx/flx</sup>; Z/EG ipRGCs. Arrowheads mark Neurobiotin-filled somas within the M3 ipRGC's dendritic field. (C) Population-averaged numbers of somas coupled to each Opn4Cre<sup>C105/105</sup>; Cx36<sup>flx/flx</sup>; Z/EG ipRGC of every type, including uncoupled ipRGCs (black columns). The number above each column is the number of ipRGCs analyzed for that ipRGC type. The Opn4Cre<sup>C105/105</sup>; Z/EG control data (gray columns) have been replotted from Figure 1C. ***P < 0.001.
for 15 minutes, it is conceivable that the INL somas require longer injection to get labeled. We previously presented preliminary evidence that some displaced amacrine cells receive gap junctional ipRGC input indirectly, by way of other amacrine cells that are directly ipRGC coupled. It seems plausible that the INL somas stained in the present study likewise coupled indirectly with ipRGCs and thus could only be stained by prolonged tracer injection. Primate ipRGCs have also been shown to couple with both INL and GCL somas.

Müller et al. detected GABA immunoreactivity in all ipRGC-coupled somas and concluded that ipRGCs couple only with amacrine cells, since practically all displaced amacrine cells are GABAergic. But some ganglion cells contain GABA, and primate ipRGCs appear to couple with other ganglion cells in addition to amacrine cells, so some of the ipRGC-coupled somas in mice could potentially be ganglion cells. We ruled this out by showing that none of those somas contained RBPMS, a reliable ganglion cell marker.

Our previous rat study detected three morphologic types of ipRGC-coupled amacrine cells, all generating tonic ON photoresponses: two types stratified in the innermost sublamina ("S5") of the inner plexiform layer, and a third type bistratified in S5 and the outermost sublamina, S1. In the present study, the wide size range of ipRGC-coupled somas suggests that mice likewise possess multiple morphologic types of ipRGC-coupled amacrine cells. Immunohistochemistry revealed further diversity: some ipRGC-coupled cells use NPY or nitric oxide as neuromodulators, and some accumulate serotonin. (Although mammalian amacrine cells do not synthesize serotonin, some can accumulate it, which is probably secreted from centrifugal fibers. A consideration of the known properties of NPY-containing, bNOS-containing, and serotonin-accumulating mouse amacrine cells suggests potential additional morphologic and physiologic diversity of ipRGC-coupled amacrine cells. Specifically, NPY-containing displaced amacrine cells stratify mainly in S4; NOS-containing amacrine cells stratify in the middle of the inner plexiform layer and generate ON-OFF photoresponses, and serotonin-accumulating amacrine cells stratify in S1 and S3. We previously found ipRGC-coupled amacrine cells by searching specifically for amacrine cells with tonic ON photoresponses. Thus, we could have missed ipRGC-coupled cells exhibiting other photoresponses (e.g., ON-OFF), a possibility reinforced by the present immunohistochemical data. The actual diversity could be even greater because mouse displaced amacrine cells contain many neuromodulators we did not probe for (e.g., adrenomedullin, corticotropin-releasing hormone, encephalin, somatostatin, and over a dozen others). We ruled out VIP and can also eliminate of those somas contained RBPMS, a reliable ganglion cell marker.

To learn whether Cx36 contributes to ipRGC-amacrine coupling, we tested whether its elimination would reduce such coupling. Since many neurons presynaptic to ganglion cells contain Cx36, a panretinal knockout would disrupt neural signaling extensively and could cause widespread developmental alterations. Thus, we created Opn4Cre+/−; Cx36flox/flox; Z/EG mice to knock out Cx36 only in melanopsin-expressing cells and confirmed that Cx36 expression in both plexiform layers largely remained. A few rods and cones in Opn4Cre+/−; Cx36flox/flox; Z/EG mice express Cre, so we presumably also eliminated these cells' Cx36 and hence coupling. But since only a few photoreceptors were uncoupled, any impact on inner retinal development was likely minimal, and indeed ipRGC morphologies were similar in the two mouse lines. At any rate, developmental alteration could not have caused the reduction in M2-amacrine and M4-amacrine coupling because all ipRGC types receive rod/cone input, and so it is inconceivable that disrupting rod-cone interaction would dramatically affect M2 and M4 but have no impact on M3.

Since Cx45 has been detected in certain bistratified ganglion cells and Cx30.2 in some melanopsin-immunopositive cells (presumably M1, M2, and/or M3), one or both of these connexins could mediate amacrine-cell coupling with M3 ipRGCs, which are bistratified. By contrast, eliminating Cx36 in M2 and M4 dramatically reduced their coupling with amacrine cells. Three prior studies on the coupling of ON α-like cells in Cx36-deficient mice produced conflicting results: whereas Schubert et al. and Roy et al. saw an abolition of coupling, Pan et al. saw normal coupling in their “G” ganglion cells, which correspond to the ON α-like “RG1”, “cluster 11”, and “M10” types. Reinforcing the latter finding, Müller et al. showed that RG1-amacrine coupling was unaffected in Cx36-deficient mice but abolished in Cx30.2-deficient mice. However, a more recent study proposed that RG1 corresponds to M2 ipRGCs rather than ON α and that M2-amacrine coupling uses Cx30.2 exclusively, which would contradict our result, although in our opinion, RG1-amacrine somas are too large for them to be M2 ipRGCs. One potential explanation for these divergent results is that the somewhat similar morphologies of several ganglion cell types could cause misclassification, whereas our Opn4Cre+/− lines should have helped mitigate this problem by ensuring all injected cells were ipRGCs. Nonetheless, we detected residual tracer coupling in Cx36-knockout M2 and M4 cells, so these ipRGC types’ utilization of Cx30.2 remains possible.

It is unknown whether ipRGCs transmit photoresponses to all or only some coupled cells, although ipRGCs generally have lower membrane resistances than amacrine cells, and when cells with different membrane resistances couple electrically, this mismatch favors transmission from the lower-resistance to the higher-resistance partners. Since all ipRGCs generate sustained, excitatory light responses and gap junction transmission is typically sign preserving, one potential explanation for these divergent results is that the somewhat similar morphologies of several ganglion cell types could cause misclassification, whereas our Opn4Cre+/− lines should have helped mitigate this problem by ensuring all injected cells were ipRGCs. Nonetheless, we detected residual tracer coupling in Cx36-knockout M2 and M4 cells, so these ipRGC types’ utilization of Cx30.2 remains possible.

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should cause them to secrete GABA\textsuperscript{11} and neuromodulators, and the latter may broadly influence retinal function in a paracrine manner.\textsuperscript{7} Numerous modulatory effects have been documented for nitric oxide, NPY, and serotonin,\textsuperscript{70–72} and ipRGC-to-amacrine signaling could induce any of them.

**Acknowledgments**

The authors thank Sara Aton, Richard Hume, and Shawn Xu for comments.

Supported by NIH grants EY023660 and EY007003, a Research to Prevent Blindness Special Scholar Award, and a Brain Research Foundation Fay/Frank Seed Grant.

Disclosure: K.R. Harrison, None; A.P. Chervenak, None; S.M. Resnick, None; A.N. Reifler, None; K.Y. Wong, None

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