CHANGING COUPLING PATTERN OF THE ON–OFF DIRECTION-SELECTIVE GANGLION CELLS IN EARLY POSTNATAL MOUSE RETINA

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Abstract—In the adult rabbit and mouse retina, about 30% of the ON–OFF direction selective ganglion cells (DSGCs) are coupled via gap junctions. In early postnatal rabbit retina, a greater proportion of morphological ON–OFF DSGCs shows coupling with a larger number of nearby somas. It is not clear whether the coupled ON–OFF DSGCs belong to the same subtype, or how coupling patterns change during development. In this study, we showed that in adult mouse retinas, all coupled ON–OFF DSGCs exhibited preferred directions (PDs) to superior, and this pattern emerged at postnatal day 15 (P15). At P13, the ON–OFF DSGCs with PDs to posterior were also coupled. Every ON–OFF DSGC in every subtype injected at P12 exhibited coupling. Therefore, a rapid decoupling process takes place in DSGCs around eye opening. Light deprivation delayed but did not halt the decoupling process. By using a transgenic mouse line in which green fluorescent protein (GFP) is selectively expressed in DSGCs with PDs to posterior and by performing in situ hybridization of cadherin-6, a marker for the coupled DSGCs, we showed that heterologous coupling existed between DSGCs with PDs to anterior and posterior till P12, but this heterologous coupling never spread to DSGCs positive for cadherin-6. It shows coupling with a larger number of nearby somas. It is not clear whether the coupled ON–OFF DSGCs belong to the same subtype, or how coupling patterns change during development. In this study, we showed that in adult mouse retinas, all coupled ON–OFF DSGCs exhibited preferred directions (PDs) to superior, and this pattern emerged at postnatal day 15 (P15). At P13, the ON–OFF DSGCs with PDs to posterior were also coupled. Every ON–OFF DSGC in every subtype injected at P12 exhibited coupling. Therefore, a rapid decoupling process takes place in DSGCs around eye opening. Light deprivation delayed but did not halt the decoupling process. By using a transgenic mouse line in which green fluorescent protein (GFP) is selectively expressed in DSGCs with PDs to posterior and by performing in situ hybridization of cadherin-6, a marker for the coupled DSGCs, we showed that heterologous coupling existed between DSGCs with PDs to anterior and posterior till P12, but this heterologous coupling never spread to DSGCs positive for cadherin-6.

Key words: retinal ganglion cell, mouse, tracer coupling, gap junction, cadherin-6.

INTRODUCTION

Many types of neurons are connected via gap junctions in the mammalian retina. Interesting coupling patterns were first revealed when Neurobiotin was injected into different retinal neurons in the rabbit (Vaney, 1991). About 30% of ON–OFF direction-selective ganglion cells (DSGCs) injected exhibited gap junction coupling to neighboring DSGCs (Vaney, 1994). The gap junctions between DSGCs do not seem to conduct electric current under physiological conditions because the size of the receptive fields closely matched the size of the dendritic fields (Yang and Masland, 1992, 1994). Three lines of evidence suggest that the coupling between DSGCs is homologous. First, the coupled somas formed a regular mosaic and were mostly located outside the dendritic field of the injected cell. Second, in some strongly coupled cases where dendrites of the coupled cells can also be traced, they tiled the retina in a seamless fashion with very little overlap (Vaney, 1994). Third, electrophysiological recording demonstrated that neighboring DSGCs with extensively overlapping dendritic fields exhibited different preferred directions (PDs) (Amthor and Oyster, 1995).

More complex patterns emerged when the coupling was investigated in the early postnatal rabbit retina. Around postnatal day 3 (P3), over 90% of injected DSGCs exhibited coupling, and many surrounding DSGCs were coupled. At P5, the percentage of coupled DSGCs was reduced, and fewer cells were coupled and the coupling pattern appeared more regular. By P10, the number of coupled cells was further reduced to form a pattern similar to that observed in adults (DeBoer and Vaney, 2005). These results suggest that decoupling may reflect the process of DSGC maturation, and perhaps, the formation of functional circuitry.

ON–OFF DSGCs in the mouse retina are very similar to the rabbit counterparts in physiology, pharmacology, dendritic morphology and coupling patterns (Weng et al., 2005). It has been shown that DSGCs form gap junctions with neighboring cells using connexin45 (Schubert et al., 2005b; Volgyi et al., 2009; Pan et al., 2010). However, there is no direct evidence whether the coupled DSGCs exhibit the same preferred directions. If the coupling is homologous, which of the four subtypes (Oyster, 1968; Elstrott et al., 2008) is coupled? Although one coupled rabbit DSGC has been shown to exhibit the preferred direction to superior (Kanjhan and Vaney, 2008), it is not sufficient to answer these questions. Furthermore, in early development, is the coupling heterologous to begin with, or the coupling is always homologous?
We showed here that after P15, all coupled DSGCs in the mouse retina exhibited PDs to superior. At P13, the DSGCs with PDs to posterior also showed tracer coupling. At P12, every DSGC recorded and injected with Neurobiotin showed coupling. Dark-rearing appeared to postpone the decoupling process; nevertheless, complete decoupling was still achieved by P30. We also showed that heterologous coupling can be seen early in development between subtypes exhibiting PDs to anterior and posterior but the heterologous coupling never spread into DSGCs exhibiting PDs to superior or inferior.

**EXPERIMENTAL PROCEDURES**

**Whole-mount retina preparation**

C57BL/6N mice, aged P12, 13, 15 and adulthood (> P30), reared with normal visual experience (12 h light/dark cycle), or completely deprived of light from birth to the day of experiment, were used in this study. A transgenic line in which Enhanced GFP is expressed under the control of Dopamine Receptor D4 promoter (DRD4-EGFP) (Huberman et al., 2009) aged P3, 8 and 12 were also used. The transgenic mice were obtained via in vitro fertilization (sperms from DRD4-EGFP transgenic mice, eggs from FVB/NJ mice) and subsequent crossing into a C57BL/6N mice background. Detailed information about DRD4-EGFP mice and links to endogenous DRD4 gene expression pattern are available at http://www.mmrrc.org/strains/231/0231.html (see Gong et al., 2003). Formal approval to conduct animal experiments described in the article had been obtained from the Institute of Biophysics, Chinese Academy of Sciences. Use and handling of animals were strictly in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23).

Animals were dark adapted for at least 1 h, deeply anaesthetized with an i.p. injection of a mixture of ketamine (50 mg/kg) and xylazine (10 mg/kg), and decapitated. For P3 and P8 mice, diethyl was used as an anesthetic. A scald was made on the temporal side of the cornea via a scorching needle and the eyes immediately enucleated under very dim red light. A cut of the cornea via a scorching needle and the eyes anaesthetized with an i.p. injection of a mixture of ketamine (50 mg/kg) and xylazine (10 mg/kg), and flat-mounted, ganglion cell layer up, on a coverslip coated with 0.1% poly-L-lysine (P6282-5MG, Sigma–Aldrich, St. Louis, MO). Sometimes the retina was attached to a piece of black membrane filter (AABP02500, Millipore, Billerica, MA) with a 2 mm diameter hole to allow adequate infrared illumination and visual stimulation onto the retina during the prolonged electrophysiological recording. The whole-mount retina was then transferred into a recording chamber (0.5 ml in volume) on the fixed stage of an upright microscope (DMLFSA, Leica) equipped with epifluorescence and a ×40 water-immersion objective (Leica HCX APO L 40×/0.80 W UV-I) configured for differential interference contrast (DIC). The preparation was continuously superfused with oxygenated bicarbonate-buffered Ames’ medium at 35 °C.

**Patch clamp recording**

Micropipettes were manufactured from thick-walled borosilicate filament glass tubing (1.5 mm outer diameter, 0.86 mm inner diameter; Sutter Instruments, San Rafael, CA) using a Flaming–Brown P97 puller (Sutter, Inc.). Under infrared illumination and visual control using a cooled CCD camera (CoolSNAP HQ, Photometrics, Atlanta, GA), a pipette was advanced to the retina using an MP 285 micromanipulator (Sutter, Inc.), and the inner limiting membrane was dissected to expose the somas of several retinal ganglion cells (RGCs). RGCs within a radius of about 0.5 mm from the optic disk were targeted (to avoid distortion of PDs of ON–OFF DSGCs caused by flat-mounting of the retina) and their spike activities recorded in the loose-patch mode with a pipette (5 ~ 8 MΩ) filled with Ames’ medium. Using a flashing spot and a moving bar, the ON–OFF DSGCs could be identified, their receptive fields mapped and the preferred-null axes determined. The formulation of intracellular (pipette) solution used was as follows, in mM: 120 potassium gluconate, 5 NaCl, 10 KCl, 1 MgCl₂, 1 EGTA, 10 HEPES, 2 adenosine triphosphate (ATP), and 0.5 guanosine-5’-triphosphate (GTP), adjusted to pH 7.2 with 1 M KOH. For voltage clamp recording, 120 mM cesium methanesulfonate and 10 mM CsCl were used in substitution of potassium gluconate and KCl, and 5 mM lidocaine N-ethyl bromide (QX314-Br, L5783, Sigma–Aldrich) was included; pH was adjusted to 7.2 with 1 M CsOH. To reveal coupling pattern of the recorded ON–OFF DSGCs, 0.4% Neurobiotin (SP-1120, Vector Laboratory, Burlingame, CA) was added in the pipette solution and was infused into the cell for at least 20 min during whole cell recording. Sometimes 0.1% Lucifer Yellow (L-0258, Sigma–Aldrich) was also included in the pipette solution for studying the dendritic morphology during recording. Data acquired from the Axopatch 200B amplifier (Molecular Device, Sunnyvale, CA) were low-pass filtered at 2 kHz, digitized simultaneously with an A/D converter (Digidata 1322A, Molecular Device), and stored on a personal computer. Offline data analysis was done using Clampfit (Molecular Device), Matlab 2006b (The MathWorks, Inc., Natick, MA), and plotted with OriginPro 7.5 (MicroCal Software Inc., Northampton, MA).

**Light stimulation**

Stimuli were generated using a program written in VC ++ and Directx 8 Software Development Kit (SDK), displayed on a monitor (Sony E230) and focused onto the retina through a microscope condenser. Two types of light stimuli were generated: (1) A spot of 25–1000 μm in diameter flashed on for 0.5–5 s, was used to determine the size of the receptive field and response polarity, (2) A rectangle of 100 × 500 μm moving parallel to its long axis in one of 12 directions with 30° intervals at speeds from 300 to 750 μm/s over a 1500 × 1500 μm area was
used to determine the directionality. The elongated bar allowed clear separation of leading edge (ON) and trailing edge (OFF) responses.

Visualization of recorded cells

Retinas were fixed with 4% paraformaldehyde at room temperature for 1 h, washed 3 times in 0.01 M Phosphate Buffered Saline (PBS) (pH 7.4), and incubated in blocking solution (1% bovine serum albumin and 0.1% Triton-X in 0.01 M PBS) at room temperature for an hour. Neurobiotin injected into cells was visualized after an overnight incubation at 4 °C, in streptavidin-fluorescein isothiocyanate (FITC) or streptavidin Texas Red (SA-5001 or SA-5006 from Vector Laboratory), diluted 1:400 in blocking solution.

To enhance fluorescence intensity of GFP-expressing cells in the DRD4-EGFP mouse retinas, the fixed retinas were incubated with rabbit anti-GFP antibody (1:1000 in blocking solution; Ab6455 from Life Technologies, Grand Island, NY) at 4 °C for 72 h, washed 1 h for 3 times at room temperature in 0.01 M PBS, transferred into solution with donkey anti-rabbit FITC antibody (1:400 diluted in blocking solution; 711-095-152 from Jackson ImmunoResearch Laboratories, Tucker, GA) at 4 °C for 1 day, washed 1 h for 3 times in 0.01 M PBS at room temperature, flat-mounted, coverslipped in Vectashield mounting media (H-1000, Vector Laboratory), and sealed with nail polish.

In situ hybridization

Riboprobes against cadherin-6 (Genebank accession number D82029) were synthesized with digoxigenin-labeled uridine triphosphate (UTP) (11277073910, Roche Diagnostics, Rotkreuz, Switzerland) and detected by anti-digoxigenin antibodies conjugated with alkaline phosphatase (1109327491, Roche Diagnostics). Briefly, total mRNA was extracted from P8 DRD4-EGFP mouse retinas, and the primer sequences used to amplify cadherin-6 mRNA fragments were 5'-AAGGAGTGGG ATGTGGAACTC-3' (forward primer) and 5'-ACGGT CTCTGATGATACC-3' (reverse primer). The predicted product length was 506 bp, spanning from exon2 to exon5 of the cadherin-6 gene. The reverse transcription polymerase chain reaction (RT-PCR) product was extracted from the agarose gel and subcloned into the pGEM-T vector (A3600, Promega, Madison, WI). After Apal (R6361, Promega) and SacI (R6061, Promega) restriction endonuclease digestion and gel electrophoresis of the vectors, the antisense and sense probe templates were harvested from the gel, respectively. The antisense and sense probes were then synthesized using Sp6 and T7 transcriptases (10810274001 and 10881767001, Roche Applied Science), respectively, provided with digoxigenin-labeled UTP. The final working concentration of the probes was 1 ng/ml.

Before in situ hybridization, DRD4-EGFP retinas with Neurobiotin-injected ON–OFF DSGCs were incubated in streptavidin Texas Red solution (1:400 in PBS with 1% Tween-20) at 4 °C overnight, counterstained with DAPI (D9542, Sigma–Aldrich) at room temperature for 2 h (1:5000 diluted in 0.01 M PBS, PH7.4 to label the nuclei of retinal cells, which served as landmarks to locate the coupled somas before and after in situ hybridization). In situ hybridization process was based on the protocols from the website of Cepko CL’s lab.

Fig. 1. Tracer coupling is restricted to the ON–OFF DSGCs exhibiting preferred directions to superior in the adult mouse. (A) Distribution of preferred directions (PDs) of ON–OFF DSGCs recorded from adult mouse retinas. Boundary lines (red), separating the recorded PDs into four subgroups (A, anterior; P, posterior; I, inferior; S, superior), were obtained from a cluster analysis in B. All ON–OFF DSGCs with PDs to superior (S) showed tracer coupling. The number of recorded cells in each subgroup was shown. The solid arrows represented DSGCs showing tracer coupling, and the dashed arrows represented DSGCs without tracer coupling. (B) Cluster analysis of PDs in A showed that the distribution of PDs of adult mouse ON–OFF DSGCs clustered into four clusters (marked by red lines). (C) PDs recorded in A showed that the distribution of PDs of adult mouse ON–OFF DSGCs clustered into four clusters (marked by red lines). (D–G) Examples of recorded ON–OFF DSGCs belonging to different subtypes. Only the cells in the superior group showed tracer coupling (arrowheads indicated coupled somas). Scale bar = 25 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
hybridization, retinas were flat-mounted, coverslipped with PBS, and sealed with nail polish. Images were collected using a Nikon Eclipse E800 microscope or Olympus Fluoview FV500/FV1000 confocal microscope. The contrast and brightness of the images were adjusted using Photoshop 8.0 (Adobe).

Data analysis

For the recorded cells, their spiking responses to light stimulus moving in 12 directions at 30° intervals were quantified and polar plots generated. The vector sum of spike responses in 12 directions was calculated and superposed on the polar plots in the corresponding cells in figures. The direction selective index (DSI) was calculated as:

\[
\text{DSI} = \frac{R_p - R_o}{R_p + R_o}
\]

where \(R_p\) is average response (10 trials) in the preferred direction, \(R_o\) is the average response in the opposite direction. Cells with a DSI > 0.3 were considered as direction selective.

Cluster analysis was performed to examine the distribution of PDs of adult mouse ON–OFF DSGCs using Matlab. For arbitrary two recorded cells (e.g., cell 1 and cell 2), their preferred directions were designated as \(X_1\) and \(X_2\), and were converted into a pair of coordinates: \((\cos X_1, \sin X_1)\) and \((\cos X_2, \sin X_2)\). The Euclidean distance between preferred directions of cell 1 and cell 2 can be calculated as:

\[
\sqrt{(\cos X_1 - \cos X_2)^2 + (\sin X_1 - \sin X_2)^2}
\]

Clusters were generated by linking preferred directions/sub-clusters with the shortest pair-wise distance using the average linkage clustering method.

RESULTS

Distribution of the preferred directions and tracer coupling

We performed whole cell recordings on 58 ON–OFF DSGCs from 36 adult mouse retinas, and found the PDs distributed in four cardinal directions (Fig. 1A). This finding is consistent with what was found in rabbits (Oyster, 1968) and in mice (Elstrott et al., 2008). Since in mouse retinas, the distribution of the PDs was not as tight as that in the rabbit retina, we performed a cluster analysis. Results showed that the four subgroups were highly distinguishable from each other (Fig. 1B). When recordings were made from nearby ON–OFF DSGCs, the PDs were almost perpendicular or opposite (Fig. 1C), suggesting the dispersion was mostly caused by pooling data from different retinas. We used the border lines in Fig. 1A to determine the PDs in the following study.

When intracellularly infused Neurobiotin was visualized in adult mouse retinas, 34% of the recorded ON–OFF DSGCs showed tracer coupling. This is very similar to what was found in rabbits (Vaney, 1994). All 20 well labeled cells with the PDs to superior showed tracer coupling (Fig. 1A, D); none of the 38 cells in the other three groups were coupled (Fig. 1A, E, F, G). This finding provided substantial support to the earlier report that a single DSGC recorded in the rabbit retina exhibiting the preferred direction to superior showed...
coupling (Kanjhan and Vaney, 2008). To further verify this pattern, we screened many RGCs on the same retina to find neighboring ON–OFF DSGCs with PDs to superior and infused Neurobiotin into one of them. Superposition of the tracer coupling pattern onto the DIC image confirmed that the neighboring DSGCs with PDs to superior were indeed tracer coupled (Fig. 2 A, B). Dual patch clamp recording showed that current injection into one of the cells induced current responses in the other (Fig. 2 C).

Rapid decoupling around eye opening

When P12 DSGCs were recorded and recovered, every cell in every subtype showed coupling (Fig. 3A1–A5). At P13, all seven DSGCs with PDs to posterior showed tracer coupling (Fig. 3B1, B5), in addition to six cells with PDs to superior (Fig. 3B2, B5). None of the 12 DSGCs in the other two groups showed tracer coupling (Fig. 3B3–B5). Since none of the DSGCs in anterior and inferior groups showed coupling, we paid attention only to the DSGCs in the posterior group. Adult-like coupling pattern emerged at P15: none of the 12 DSGCs with PDs to posterior showed coupling (Fig. 3C1, C2, C4). Therefore, a rapid decoupling process took place around eye opening.

Light regulation of decoupling

The decoupling in early postnatal development appears to be regulated by light input. In the dark-reared P15 mice, many of the injected cells with PDs to anterior (40%), inferior (66%) and posterior (77%) still showed coupling (Fig. 4A1–A4). Nevertheless, the number of coupled somas per injected cell underwent a significant reduction. For the anterior and inferior groups, no cells were coupled in the control P13 retina, where clear coupling could still be seen in dark-reared P13 and dark-reared P15 retina (Fig. 4B). However, a significant reduction in the number of coupled cells was detected in dark-reared P15 retina (Fig. 4B). For the posterior group, significant coupling was observed on normal and dark-reared P13 retinas; although coupling was still clearly present, the number of coupled cells was significant reduced in dark-reared P15 retinas (P = 0.007, K–S test). By P30, a coupling pattern very similar to that of the control mice still emerged in the dark-reared animals (Fig. 4C). Therefore, decoupling
Fig. 4. Dark rearing postpones the decoupling process. (A1–A3) Examples showing the morphology of ON–OFF DSGCs in animals dark-reared to P15. Some cells with PDs to anterior, inferior, and posterior remained coupled. Arrowheads indicated coupled somas. (A4) Distribution of PDs and coupling pattern of ON–OFF DSGCs in animals dark-reared to P15. Since the ON–OFF DSGCs to superior remained coupled throughout development, we did not target them in these experiments. (B) The number of coupled somas per injected cell decreased significantly from P13 to P15 in dark–reared animals (P < 0.05 for the inferior and posterior subtypes). (C) The coupling pattern of ON–OFF DSGCs in mice dark-reared to adulthood (>P30) was very similar to that observed in normal animals. Scale bar = 25 μm.

Fig. 5. Heterologous coupling between DSGCs with PDs to anterior and posterior at P12 retina. (A) An injected GFP-positive cell in a P12 DRD4-GFP transgenic mouse retina. Most coupled cells were GFP positive (arrow heads); there was a GFP-negative cell also coupled to the injected cell (square and inset). (B, C) GFP-negative ON–OFF DSGCs with PDs to superior and inferior never coupled to GFP-positive cells. (D) A DSGC with the PD to anterior showed weak coupling to GFP-positive cells (square and inset). (E) None of the injected DSGCs with PDs to superior and inferior showed coupling to GFP-positive cells, while a majority of DSGCs with PDs to anterior (9 out 11) exhibited weak coupling to GFP-positive cells. (F) Stack histogram showing the numbers of coupled GFP-positive and -negative somas for each subtype of ON–OFF DSGCs. (G) Weak coupling between electrophysiologically identified DSGCs with PDs to anterior (GFP negative) and to posterior (GFP positive). Polar plots and vector sums were shown on recorded cells using the same convention. Scale bar = 25 μm.
process appeared delayed, but not stopped by light deprivation.

Changing coupling pattern throughout development

To address the question whether the coupling was homologous, we took advantage of a transgenic mouse line in which the DSGCs with PDs to posterior express GFP specifically. If coupling is homologous, then somas coupled to a GFP-positive cell should confine to GFP-positive cells. However, in every case \((n = 6)\) when a GFP-positive cell was injected, some GFP-negative somas were labeled (Fig. 5A, F). It is possible that these GFP-negative cells belong to the same subtype, but they express GFP at a level below detection limits, or they are truly heterologically coupled DSGCs, in other words, DSGCs with different PDs. To distinguish these possibilities, we injected physiologically identified DSGCs with PDs to superior, inferior, and anterior in P12 retinas. For 6 injected DSGCs with PDs to superior and 5 to inferior, 87 and 31 coupled somas were detected, and no GFP-positive somas were ever observed (Fig. 5B, C, E, F). For 11 injected DSGCs with PDs to anterior, 63 somas were coupled, among which 13 were GFP positive (Fig. 5D–F). To rule out that these GFP-positive cells are DSGCs with PDs to anterior but somehow mis-express GFP, we performed electrophysiological recording to screen the PDs of GFP-positive cells near the injected DSGCs with PDs to anterior. In all three cases, we confirmed that the GFP-positive cell, coupled to the injected DSGC with PD to anterior, indeed exhibited PD to posterior (Fig. 5G). Therefore, DSGCs with PDs to anterior and posterior are weakly coupled at P12, whereas DSGCs with PDs to superior and inferior never couple heterologously with DSGCs showing PDs to posterior.

We went on to examine whether the coupling pattern remained the same earlier in development. Before P10, the RGCs are not light sensitive; therefore, it is not possible to distinguish GFP-negative DSGCs. Four possible cases exist for a GFP-negative DSGC: (1) superior, (2) inferior, (3) anterior, and (4) posterior but the level of GFP expression is below detection threshold. The fourth case should be very rare compared with other three. If the number of DSGCs in each subpopulation is similar and if the coupling pattern seen at P12 is maintained throughout development, then the probability of GFP-negative cells heterologically coupled to GFP-positive cells should be about 1/3 (only DSGCs with PDs to anterior showed heterologous coupling to GFP-positive DSGCs in P12). We injected 11 GFP-negative DSGCs at P3, and found eight injected cells showed coupling to 88 neighboring cells, but no GFP-positive cells (Fig. 6A1, A3). In the other three cases, among 49 coupled cells, seven cells were GFP positive (Fig. 6A2, A3). So 27.3% (3/11) of...
the injected GFP-negative DSGCs showed heterologously coupling. Similar results were obtained from P8 retinas. Among 15 injected GFP-negative morphological DSGCs, 10 cells were coupled to 138 somas, none of which were GFP positive; the other five cells coupled to 67 somas, out of which, 11 were GFP-positive cells (Fig. 6B1–B3). So 33% (5/15) of the injected GFP-negative DSGCs showed heterologous coupling. The proportion of heterologously coupled DSGCs supported that the pattern of coupling in earlier development is similar to that seen in P12 retinas.

A recent finding reported that cells positive for cadherin-6 (Cdh6) and collagen25α1 (Col25α1) are DSGCs with PDs to superior and inferior (Kay et al., 2011), therefore cadherin-6 and collagen25α1 can be used as markers for DSGCs with PDs to superior and inferior to examine whether heterologous coupling exist between these DSGCs and DSGCs with PDs to posterior. We first confirmed the finding by injecting DSGCs with PDs to superior and inferior in P12 retinas, and performed in situ hybridization on these preparations (Fig. 7). Nineteen out of 24 (77%) somas coupled to a DSGC with the PD to superior (Fig. 7A1–A3) and five out of eight (62.5%) somas coupled to a DSGC with the PD to inferior (Fig. 7B1–B3) were positive for Cdh6.

We went on to test whether heterologous coupling only existed between DSGCs with PDs to anterior and posterior by combining in situ hybridization of Cdh6 with intracellular injection. First, we injected five GFP-positive cells in three P3 retinas and labeled these retinas with Cdh6 probes. We carefully examined the 68 coupled somas (Fig. 8A1–A3, D); none of these somas were positive for Cdh6. This result showed that even at this early postnatal stage, DSGCs with PDs to posterior do not exhibit heterologous coupling with DSGCs showing PDs to superior or inferior. We further analyzed the coupling pattern of GFP-negative morphological DSGCs. In four injected GFP-negative DSGCs where heterologous coupling was detected, none of the 56 coupled somas were Cdh6 positive (Fig. 8B1–B3, D); but in the other six injected GFP-negative DSGCs the coupling did not spread to GFP-positive cells, and the coupled somas were predominantly positive for Cdh6.

Fig. 7. The majority of coupled somas of ON–OFF DSGCs with PDs to superior/inferior are positive for Cdh6. (A1, B1). Dendritic morphology of the physiologically identified ON–OFF DSGCs with PDs to superior (A1) and inferior (B1), respectively. Coupled somas were indicated with arrowheads. (A2, B2) Cdh6 mRNA signal in A1 and B1, with red dots indicating soma positions. Coupled somas positive or negative for Cdh6 were indicated with black or red numbers respectively. The red stars indicated the injected cells. (A3, B3) High-magnification images showing Cdh6-positive (black) and -negative (red) somas in A2 and B2. Scale bar = 50 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
Fig. 8. *In situ* hybridization of cadherin6 supports that posterior and anterior subtype ON–OFF DSGCs are not coupled to superior or inferior subtype ON–OFF DSGCs at P3. (A1) An injected GFP-positive cell, showing coupling to a number of GFP-positive (yellow arrow heads and yellow numbers) and GFP-negative cells (red arrow heads and red numbers). (A2) Cdh6 *in situ* hybridization of the same preparation shown in A1. (A3) None of coupled cells were Cdh6-positive. P (black rectangle) is a non-coupled Cdh6-positive soma on the same retina. (B1) An injected GFP-negative cell showing weak coupling to GFP-positive cells. (B2) Cdh6 *in situ* hybridization of the same preparation shown in B1. (B3) None of the coupled cells were positive for Cdh6. This cell is presumably a DSGC with the PD to anterior. P (black rectangle) is a non-coupled Cdh6-positive soma on the same retina. (C1) An injected GFP-negative cell without coupling to GFP-positive cells. (C2) Cdh6 *in situ* hybridization of the same preparation shown in C1. (C3) Cdh6 mRNA signals were detected in a large proportion of coupled somas (black numbers). This cell is presumably a DSGC with the PD to superior or inferior. The red star in A2, B2 and C2 designate the injected cell. Scale bar = 25 μm. (D) A stack histogram showing the number of coupled Cdh6-positive and -negative somas for all injected ON–OFF DSGCs at P3. Representative cases were shown in A, B, and C. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
one subtype of ON DSGCs also coding the upward motion in the rabbit retina extensively studied ON–OFF DSGCs, a subtype of the represented repeatedly in the retina. In addition to the subtype with PDs to superior and the other to inferior, the coupling never showed heterologous coupling with DSGCs to anterior before but never with those to superior or inferior, indicates that connexins from different subfamilies might be used to form gap junctions between DSGCs with horizontal and vertical PDs. We do not know whether heterologous coupling existed between DSGCs with PDs to superior and inferior; more genetic markers for specific populations are required for answering this question (e.g., Trenholm et al., 2013).

The functional significance of coupling and the rapid decoupling around eye opening is not clear. Perhaps, gap junctions help dendrites of the same subtype of DSGCs to cofasciculate more tightly to facilitate the formation of appropriate synaptic connections between DSGCs and starburst amacrine cells. When the circuitry is established, the expression of connexins is no longer necessary. The timing of decoupling is consistent with this speculation, with the earliest DS responses detected at P11 (Chen et al., 2009). However, dendritic morphology and stratification of bistratified cells in Cx45 knockout animals appeared quite normal (Schubert et al., 2005b); whether there is any functional change awaits further investigation.

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REFERENCES


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