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CELL TYPE-SPECIFIC BIPOLAR CELL INPUT TO GANGLION CELLS IN THE MOUSE RETINA

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Abstract—Many distinct ganglion cell types, which are the output elements of the retina, were found to encode for specific features of a visual scene such as contrast, color information or movement. The detailed composition of retinal circuits leading to this tuning of retinal ganglion cells, however, is apart from some prominent examples, largely unknown. Here we aimed to investigate if ganglion cell types in the mouse retina receive selective input from specific bipolar cell types or if they sample their synaptic input non-selectively from all bipolar cell types stratifying within their dendritic tree. To address this question we took an anatomical approach and immunolabeled retinae of two transgenic mouse lines (GFP-O and JAM-B) with markers for ribbon synapses and type 2 bipolar cells. We morphologically identified all green fluorescent protein (GFP)-expressing ganglion cell types, which co-stratified with type 2 bipolar cells and assessed the total number of bipolar input synapses and the proportion of synapses deriving from type 2 bipolar cells. Only JAM-B ganglion cells received synaptic input preferentially from bipolar cell types other than type 2 bipolar cells whereas the other analyzed ganglion cell types sampled their bipolar input most likely from all bipolar cell terminals within their dendritic arbor.

Key words: bipolar cell, ganglion cell, connectivity, selective input, mouse retina.

INTRODUCTION

The retina filters and processes light signals deriving from external visual scenes by extracting several features of these scenes before the information is sent to higher visual brain areas by the retinal output elements, the retinal ganglion cells (RGCs).

Accordingly, distinct RGC types have been found to be tuned to signal specific aspects of a visual scene, such as contours, color, contrast, or motion direction (for review see: Wässle, 2004; Berson, 2008; Sanes and Masland, 2015). Many of these functionally identified RGC types can directly be matched to morphologically described RGC types for example ON/OFF direction-selective ganglion cells (ON/OFF DSGCs) or sustained and transient responding alpha-like RGCs in the mouse (Weng et al., 2005; van Wyk et al., 2009), the "local edge detector" in rabbit (Amthor et al., 1989) or small-bistratified RGCs in primate retina, which mediate blue/yellow opponent signals (Dacey and Lee, 1994).

Despite the knowledge of the correlation between an RGC's morphology and function (Famiglietti and Kolb, 1976; Berson, 2008) only little is known about the retinal circuits that shape the RGC's responses and lead to the tuning of the RGC types in mice.

Traditionally, the mammalian inner plexiform layer (IPL) is subdivided into five strata (S1–S5) of equal thickness (Cajal, 1893). OFF bipolar cells (BCs) depolarize at light-offset and stratify in the outer (OFF) layers S1 and S2, whereas ON BCs depolarize at light-onset and stratify in the inner (ON) layers S3–S5 (Hartveit, 1997; McGillem and Dacheux, 2001). Five types of OFF BCs (type 1, 2, 3a, 3b and 4) have been morphologically and physiologically described in the mouse retina (Ghosh et al., 2004; Mataruga et al., 2007; Haverkamp et al., 2008; Wässle et al., 2009; Breuninger et al., 2011; Baden et al., 2013; Puller et al., 2013). Type 3a and 3b stratify in S2, type 4 terminals branch more broadly in S1 and S2, whereas type 1 and 2 are restricted to S1. Based on the number and types of cones contacted, the dendritic glutamate receptor types expressed, and distinct modulations through inhibitory interneurons, the different BC types convey signals from different aspects of a visual scene to RGCs (for review see Euler et al., 2014).

Here we were interested, if certain RGC types are specifically connected with certain BC types, or if they sample their excitatory input from all BC types stratifying in the area of their dendritic arbor. We know from primate retina that midget RGCs receive most of their BC input from midget BCs (Kolb and Dekorver, 1991; Jusuf et al., 2006) and small bistratified RGCs receive dominant ON BC input from blue cone BCs (Calkins et al., 1998). In cat retina, however, small beta RGCs – probably homologous to the primate midget RGCs – receive their driving input less selectively from two types of cone BCs (McGuire et al., 1986). These studies

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Abbreviations: $A_{OFF\text{-}S}$, alpha-like OFF-sustained; $A_{ON\text{-}S}$, alpha-like ON-sustained; BC, bipolar cell; CtBP2, C-terminal binding protein 2; DSGC, direction-selective ganglion cell; GFP, green fluorescent protein; IPL, inner plexiform layer; PB, phosphate buffer; RGC, retinal ganglion cell; S, stratum; SD, standard deviation; SEM, standard error of the mean; Syt2, synaptotagmin-2; YFP, yellow fluorescent protein.

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indicate interesting species related differences in the circuitry of these cells. Hence, we were interested to elucidate the circuitry of RGCs in a model system of growing importance, the mouse retina. We used *thy1*-GFP-O and JAM-B mice (Feng et al., 2000; Kim et al., 2008), in which certain RGC types are fluorescently labeled, and co-immunolabeled the retinae with a synaptic ribbon marker (Ribeye/CtBP2, tom Dieck et al., 2005) and a marker for type 2 BCs (synaptotagmin-2 (Syt2), Wässle et al., 2009). To find out, if the input onto certain RGC types is dominated by a certain BC type, as described for the alpha-like ON-sustained (A_{ON-S}) RGCs, which receive input dominantly from type 6 BCs (Schwartz et al., 2012), we quantified all ribbon synapses deriving from Syt2-labeled BCs. Since the expression of Syt2 is mainly confined to type 2 BCs with axon terminals stratifying in S1 of the IPL, we focused our analysis on RGC types stratifying within S1.

Although many studies addressed the morphological classification of RGC types in the mouse retina (Sun et al., 2002; Badea and Nathans, 2004; Kong et al., 2005; Coombs et al., 2006; Völgyi et al., 2009; Hong et al., 2011; Farrow et al., 2013; Sümbül et al., 2014) the RGC classification within *thy1*-GFP-O mice was challenging. It had been largely accepted that, unlike in rabbit, cat and primates, the density and the dendritic arbor size of RGCs in the mouse retina change only little with eccentricity; and many classification schemes are based on this assumption. However, there is recent evidence that at least alpha-like ON-sustained (A_{ON-S}) and OFF-sustained (A_{OFF-S}) RGCs undergo drastic changes in their dendritic field size depending on their location within the retina (Bleckert et al., 2014).

Therefore we classified the monostratified RGCs in S1 not on the size of the dendritic arbor, but on the density of their dendritic tree. This resulted in two distinct groups of RGC types, one group of densely branched and mostly small cells resembling the B-type cells, and another group of usually larger and sparsely branched C-type cells (according to the classification of Sun et al., 2002). We assume that the larger and sparser C-type cells correspond to the A_{OFF-S} cells in Bleckert et al. (2014). Additionally to monostratified cells we also analyzed the BC input onto the OFF plexus of bistratified RGC types which likely correspond to ON/OFF DSGCs.

EXPERIMENTAL PROCEDURES

Animals and tissue preparation

The retinal tissue used in this study was obtained from two transgenic mouse lines, the *thy1*-GFP-O (Feng et al., 2000) and the JAM-B line (Kim et al., 2008) which express green or yellow fluorescent proteins (GFP or YFP) in certain retinal cell types. Altogether, 23 GFP-O and 5 JAM-B retinae were analyzed for the respective cells.

Animals were deeply anesthetized with isoflurane and decapitated before the eyes were removed. All procedures were approved by the local animal care committee and were in accordance with the law for animal experiments issued by the German government (Tierschutzgesetz). After dissection of the eyes, the posterior eyecup was immediately immersion fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4) for 15–30 min at room temperature. After fixation the retina was cryoprotected in a sucrose gradient (10%, 20% for 1 h and 30% sucrose in PB overnight) and stored at −20 °C. The retinae of JAM-B mice were kindly provided by In-Jung Kim (Joshua Sanes' lab, Harvard University, Cambridge, MA). JAM-B-CreER (Kim et al., 2008) mice were crossed with Thy1-lox-stop-lox-YFP (Buffelli et al., 2003) mice. CreER-mediated excision of the stop signal results in YFP expression in cells expressing the *JAM-B* transgene. A few hundred RGCs per retina were marked after tamoxifen injection (100 μg, Sigma) at postnatal day 0 (P0)-P1.

Antibodies

A monoclonal antibody raised in mouse against synaptotagmin-2 (Syt2 or ZNP-1; 1:1000; Zebrafish International Resource Center (ZIRC), Eugene, OR) was used to label type 2 BCs in the mouse retina. The Syt2 antibody recognizes a single band of 60-kDa protein on Western blots of zebrafish embryos and mouse cerebellum, but not of mouse liver (Fox and Sanes, 2007). In mouse retina, the antibody labels type 2 BCs strongly and type 6 BCs weakly (Wässle et al., 2009).

The rabbit polyclonal antibody against the C-terminal binding protein 2 (CtBP2; 1:2000–1:5000; Synaptic Systems, Göttingen, Germany, 193–003), a RIBEYE homolog, recognizes synaptic ribbons in mammalian retinae (Schmitz et al., 2000; tom Dieck et al., 2005). The antiserum recognizes a 110–120 kDa protein band corresponding to the size of the RIBEYE protein and a 50 kDa protein band corresponding to the transcription repressor CtBP2 (tom Dieck et al., 2005).

The endogenous GFP signal in the transgenic mouse retinae was enhanced using goat anti-GFP (1:1000; Rockland, Gilbertsville, PA, 600-101-215) or chicken anti-GFP antibodies (1:1000; Merck Millipore, Darmstadt, Germany, AB16901). The layering within the inner plexiform layer was revealed by using rabbit (1:2000; Swant, Bellinzona, Switzerland, 7699/3H) or mouse anti-calretinin (1:2000; Merck Millipore, Darmstadt, Germany, MAB1568). Alpha-like RGCs were identified with a polyclonal goat antibody against mouse osteopontin (OPN, 1:1000; R&D Systems, Wiesbaden, Germany, AF808).

Immunohistochemistry and light microscopy

Immunohistochemical labeling was performed using the indirect fluorescence method: Antibodies were diluted in a solution containing 3% normal donkey serum, 1% bovine serum albumin and 1% Triton-X 100 in PB. After several washing steps in PB, retinal whole mounts were incubated in a mixture of primary antibodies for three days at room temperature. Afterward the retinae were incubated overnight in a mixture of secondary antibodies conjugated either to Alexa488 (Molecular Probes, Eugene, OR), Cy3 or Cy5 (Dianova, Hamburg, Germany). After further washing steps the whole