FLRT3 Marks Direction-Selective Retinal Ganglion Cells That Project to the Medial Terminal Nucleus

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The mammalian retina extracts a multitude of diverse features from the visual scene such as color, contrast, and direction of motion. These features are transmitted separately to the brain by more than 40 different retinal ganglion cell (RGC) subtypes. However, so far only a few genetic markers exist to fully characterize the different RGC subtypes. Here, we present a novel genetic Flrt3-CreERT2 knock-in mouse that labels a small subpopulation of RGCs. Using single-cell injection of fluorescent dyes in Flrt3 positive RGCs, we distinguished four morphological RGC subtypes. Anterograde tracings using a fluorescent Cre-dependent Adeno-associated virus (AAV) revealed that a subgroup of Flrt3 positive RGCs specifically project to the medial terminal nucleus (MTN), which is part of the accessory optic system (AOS) and is essential in driving reflex eye movements for retinal image stabilization. Functional characterization using ex vivo patch-clamp recordings showed that the MTN-projecting Flrt3 RGCs preferentially respond to downward motion in an ON-fashion. These neurons distribute in a regular pattern and most of them are bistratified at the level of the ON and OFF bands of cholinergic starburst amacrine cells where they express the known ON-OFF direction-selective RGC marker CART. Together, our results indicate that MTN-projecting Flrt3 RGCs represent a new functionally homogeneous AOS projecting direction-selective RGC subpopulation.

Keywords: FLRT3, RGC, MTN, ON direction selective, downward movement

INTRODUCTION

Retinal ganglion cells (RGCs) transmit visual information captured by the eyes to different regions in the brain. Distinct RGC subtypes respond with different activity patterns to the same set of visual stimuli (Baden et al., 2016). The feature selectivity of each RGC subtype is established by the various patterns of connections with different types of amacrine and bipolar cells in the inner plexiform layer (IPL). One such feature is the selective activity in response to motion stimuli into a certain...
direction (Vaney et al., 2012). The detection of motion direction in the visual system is essential to drive the optokinetic reflex to stabilize the retinal image in response to the slow head or eye movements (Yoshida et al., 2001; Yonehara et al., 2016). In the brain, these stabilization signals are processed by different nuclei of the accessory optic system (AOS): the medial and lateral terminal nuclei (MTN and LTN), and the optic tract and the dorsal terminal nucleus (NOT/DTN) (Yonehara et al., 2009). It has been shown that three types of ON and one type of ON-OFF direction-selective RGC that project to the AOS are tuned to either upward (ON), downward (ON), or forward (ON, OFF) direction (Dhande et al., 2013). To fully understand how each channel contributes to the smooth adaptive eye movements of the optokinetic reflex, it is essential to gain specific genetic access to each of the four input channels. Generally, all four types of AOS projecting RGCs are genetically accessible using the Hoxd10 mouse line (Dhande et al., 2013). So far only the SPIG1 and the Pcdh9-Cre line were shown to each specifically access to each of the remaining two AOS projecting RGCs.

Previous work from our lab using the fibronectin leucine rich-repeat transmembrane (Flrt) protein, showed that these cell adhesion molecules lead to cortex folding and altered distribution of pyramidal neurons during cortical development (Seiradake et al., 2014) in the mouse (del Toro et al., 2017). We observed that Flrt3 is expressed in early postnatal stages (Seiradake et al., 2014), and in the adult retina (Visser et al., 2015), and thought it could thus be interesting to evaluate it as a genetic marker. Here, using a novel, inducible CreERT2 knock-in line inserted into the Flrt3 locus on chromosome 2 and a combination of morphological, molecular, and electrophysiological characterizations, we identified four morphologically distinct RGC types. Anterograde tracings revealed that one Flrt3-positive RGC subtype specifically projects to the medial terminal nucleus (MTN) of the AOS. Functional characterizations of the MTN-projecting Flrt3-subtype revealed that they are ON-RGCs and respond preferentially to downward moving stimuli. Interestingly, most MTN-projecting Flrt3-RGCs are bistratified in the ON and OFF-ChAT band, which has not been described before. Thus, our results indicate the presence of an additional fifth AOS projecting direction-selective RGC subtype.

**MATERIALS AND METHODS**

**Mouse Lines**

The following mouse lines used in this work have been previously described and were back-crossed to C57BL/6J mice: FLRT1 LacZ (EUCOMM), Flrt2 LacZ (EUCOMM), Flrt3 LacZ (Egea et al., 2008), Aig^{Bl−}tdTomato [B6.Cg-Gt(Rosa)26Sortm9(CAG-tdTomato)Hze/+] (Madisen et al., 2010).

Briefly, the Flrt3 Cre/ERT2 knock-in line used in this study was generated by homologous recombination in embryonic stem (ES) cells using a replacement-type targeting vector. The targeting construct for Flrt3 was generated by inserting a sequence containing part of the 5′UTR of the exon III, the CreERT2 sequence, an SV40 polyadenylation signal and a loxP-flanked neo cassette into a vector containing 3.8 kb and 6 kb homology arms surrounding exon III of the Flrt3 gene and the thymidine kinase (TK) cassette. The sequence flanking the insertion site in the 5′UTR is 5′ TAACAGAAGCTACTGCTATAAT 3′. The sequence flanking the insertion site in the 3′UTR is 5′ TGAGGAAGCATGTACTGTCACATT 3′.

Embryonic stem cell cultures, electroporation, and selection were carried out according to standard protocols. Screening and homologous recombination on both arms of the constructs was assessed by Southern blot in the targeted ES cells. Germline transmission was achieved with at least two independent ES cell clones. Mice were maintained in a C57BL/6J background. They will be deposited in the Mutant Mouse Resource and Research Centers (MMRRC) for distribution. Animals were kept and used according to the regulations of the Regierung von Oberbayern.

The FLRT3-CreERT2 line was crossed to the R26-tdTomato (Ai9) line [B6.Cg-Gt(Rosa)26Sortm9(CAG-tdTomato)Hze/+] (Madisen et al., 2010).

**4-Hydroxytamoxifen Administration**

4-hydroxytamoxifen (Sigma, ref: H-7904) solution was prepared according to the manufacturer’s protocol (Chevalier et al., 2014). To induce Cre expression in the Flrt3-CreERT2 line, adult mice (>P60) of both sexes were injected intraperitoneally with 60 µg/g 4-Hydroxytamoxifen.

**Retina Histology**

For retina whole-mount preparation, eyes were first enucleated and a small hole was made in the dorsal side to maintain correct orientation after dissection. Next, the cornea and lens were removed by cutting along the ora serrata and the retina cleaned of any residues from the vitreous. Finally, the retina was detached from the eyeball and transferred onto a cell culture insert (Millipore, Cat. no. PIMCOSG50). To allow a flat mount of the retina, 4 incisions were made. The deepest incision marked the dorsal side. Retinas were fixed on the membrane for at least 30 min in 4% paraformaldehyde (PFA).

**Brain Histology**

For analysis of retinofugal projections, the animals were deeply anesthetized with an overdose of Ketamine (medistar)/Xylazine (Bernburg) (1.6%/0.08%). Mice were transcardially perfused for 10 min at a speed of 1 ml/min with ice-cold PBS followed by 10 min of ice-cold 4% PFA (10% stock solution, Electron Microscopy Sciences, 15712) in PBS. Brains were post-fixed in 4% PFA for 24–48 h and subsequently stored in PBS (0.02% Sodium azide). Brains were cut at 100 µm slices using a vibratome (Leica). Retinas were dissected after transcardial perfusion.

**Immunohistochemistry**

For immunostainings, retinas were fixed in 4% PFA and transferred to a permeabilizing and blocking solution containing
and fixed for 30 min in an X-Gal fixative solution (0.2% X-Gal Staining (in 0.3% Triton X-100, 3% donkey serum in PBS) was added azide in PBS). After 3 washes in PBS, the secondary antibody was incubated for 1–3 days with the primary antibodies (in 0.3% Triton X-100, 2% bovine serum albumin, 0.02% sodium azide in PBS). After 3 washes in PBS, the secondary antibody (in 0.3% Triton X-100, 3% donkey serum in PBS) was added for at least 1 day.

**X-Gal Staining**

For X-Gal staining, the retina was dissected from the eye and fixed for 30 min in an X-Gal fixative solution (0.2% Glutaraldehyde, 1% PFA in PBS, 5 mM EGTA, and 2 mM MgCl2 and 0.02% NP40). Brains were fixed for 1–3 h. Retinas and brain regions were stained for beta-galactosidase activity by incubating them for 5–10 h in a 1 mg/ml X-gal solution containing 5 mM Fe(CN)6− and 5 mM K3Fe(CN)6. Retinas were postfixed for 10 min in 4% PFA.

**Single Retinal Ganglion Cell Injections**

For single-cell reconstructions, retinas of FLRT3-CreERT2 × R26-tdTomato (Ai9) mice were dissected from the eye and mildly fixed in 4% PFA for 15 min. FLRT3+ neurons were identified by Tomato expression and injected with 4% Lucifer yellow dissolved in ACSF using a sharp electrode. Sharp electrodes were pulled from borosilicate glass capillaries to a final resistance of 200 Ω (Science Products GP150F-8P) and mounted on a motorized patch-clamp manipulator. The dye was expelled using a positive current of 1–2 nA at 100 ms pulses for about 2 min (90% duty cycle).

**Intravitreal Virus Injections**

Mice were anesthetized intraperitoneally with a mixture of fentanyl [0.05 mg/kg, SIGMA-ALDRICH Chemie GmbH (800021)], midazolam [5 mg/kg, SIGMA-ALDRICH Chemie GmbH (800021)], and medetomidine [5 mg/kg, TCI Deutschland GmbH (800142)]. On the day of surgery, and on two subsequent days, carprofen [5 mg/kg, Zoetis (Rimadyl)] was also administered as an additional analgesic. Surgical instruments were heat-sterilized and washed with ethanol. The 7000 series Neuros Hamilton syringe with a 32G blunt needle was rinsed several times with distilled H2O, then ethanol, and again with distilled H2O. The animal was then fixated in a stereotaxic setup (Kopf instruments) and the eye that was protruding the eye from the eye socket and punctured at the ora serrata using a 30-gauge needle. Next, 1 µl of the adenovirus vector (rAAV2/CAG-GFP) was injected using a Hamilton syringe controlled by a micromanipulator (M3301R, WPI) and inserted into this opening at an oblique angle to avoid damaging the lens. After injection, the syringe was left in place for 4 min to allow the virus to disperse. After removing the Hamilton syringe, the eye was covered with eye cream (Isopto-Max, Novartis) and the procedure was repeated with the other eye. After 3 weeks, 2 µl of Cholera Toxin Subunit B conjugated with Alexa Fluor 647 (CTB647, C34778, Invitrogen, United States) (1%) was injected using a similar procedure. For histological analysis the animals were sacrificed as described previously in “brain histology” and perfused using 4% PFA 24 h later. The brain and retina were postfixed in 4% PFA for 24 and 1 h, respectively.

**Stereotoxic Surgeries**

Mice were anesthetized using isoflurane (Cp-pharma, 1.5–2%) and placed in a stereotoxic setup (Kopf Instruments). Body temperature was maintained using a heating pad at 37°C, and an analgesic was injected subcutaneously (5 mg/kg, Rimadyl, Zoetis). Viruses (pAAV-FLEX-tdTomato (Addgene), rAAV5-hsyn-EYFP (UNC GTC vector core, United States), or CTB (cholera toxin subunit B, Invitrogen) were injected into the MTN (AP −2.65, ML ± 0.95, DV −5) using glass pipettes (#708707, BLAUBRAND intraMARK).

**Electrophysiology**

Mice were dark-adapted overnight and sacrificed by cervical dislocation. The retinas were dissected immediately after and transferred to oxygenated (95% O2 and 5% CO2) Ames solution at room temperature (~22°C). The retina was placed onto an Anodic filter membrane (Whatman, WHA68096022) with the ganglion cell layer facing upward and transferred into the recording chamber. The ventral part of the retina was marked for orientation. Flrt3-tomato neurons were visualized under brief fluorescent illumination using a microscope equipped with IR–DIC optics (Olympus BX51). All electrophysiological recordings were performed with constant superfusion of carbogenated Ames solution at 30–32°C. Whole-cell-current-clamp or cell-attached recordings were performed with a MultiClamp 700B amplifier and a Digidata 1550 digitizer (Molecular Devices). For current-clamp recordings, a patch pipette with a resistance of 4–6 MΩ was filled with an intracellular recording solution (130 mM potassium gluconate, 10 mM KCl, 2 mM MgCl2, 10 mM HEPES, 2 mM Na-ATP, 0.2 mM Na2GTP, 0.2% neurobiotin, pH 7.35, and 290 mMso). For cell-attached recordings, the patch pipette was filled with Ames solution.

Blue light stimuli were presented using a DLP projector (DLP LightCrafter 4500) through a 4x objective. Direction selectivity was analyzed by presenting a moving grid (bar width = 285 µm, speed = 570 µm/s, time = 2 s) in eight different directions. Only a few neurons were unresponsive to visual stimuli and thus discarded. Responses to stationary centered flashing dots of increasing size ([50–1,200 µm], time = 2 s) were recorded.
For firing adaptation calculation, we used the formula
\[ 1 - \left( \frac{T_{\text{last}}}{T_{\text{initial}}} \right) \], where \( T_{\text{last}} \) is the time interval between the last two action potentials and \( T_{\text{initial}} \) is the time interval between the first two action potentials. Consequently, a value of 0 represents no change in spike frequency, while higher values represent a decay in the spiking rate of a neuron.

**RESULTS**

**Flrt3 Is Expressed in a Subpopulation of Retinal Ganglion Cells**

In previous work, we observed Flrt3 expression in the early postnatal retina (Seiradake et al., 2014). Additionally, all three members of the Flrt family of cell-adhesion molecules (Flr1, 2, and 3) were found to be expressed in the adult retina (Visser et al., 2015). Using our Flrt1\(^{lacZ}\), Flrt2\(^{lacZ}\), and Flrt3\(^{lacZ}\) reporter mouse lines, we confirmed that all three Flrts are expressed in a subpopulation of neurons in the ganglion cell layer (GCL) (Supplementary Figures 1A–F and data not shown). Since Flrt3 showed a sparser expression pattern in the GCL than Flrt1 and 2, we decided to focus on characterizing the Flrt3 population (Supplementary Figures 1A,B). To gain genetic access to the Flrt3-RGCs, we generated a tamoxifen-inducible Flrt3-CreERT2 knock-in line (Figure 1A). By crossing the Flrt3-CreERT2 line to a tdTomato reporter line, we confirmed that Flrt3-Cre expression matched Flrt3\(^{lacZ}\) expression in the brain (Supplementary Figures 2A–C). Immunostainings using the pan RGC marker RBPMS [RNA-binding protein (Rodriguez et al., 2014)] showed that 22.7% of all Flrt3+ neurons in the GCL were RGCs, while 77.3% were amacrine cells (Figures 1B,C). Conversely, Flrt3-RGCs represented a sparse population of about 4.6% of all RGCs. Retinal sections (vibratome cut sections orthogonal to the retinal cell layers) of the Flrt3-CreERT2-Tomato line showed that Flrt3 was not only expressed in the GCL but also in the inner nuclear layer (INL) (Figure 1D and Supplementary Figure 1F). Flrt3+ amacrine cells, RGCs, and possibly also bipolar cells of the INL and GCL contribute to the stratification pattern observed within the IPL. The peak stratification is located between the two ON and OFF ChAT bands (Voigt, 1986) (Figure 1D). Moreover, the Flrt3-CreERT2 line also labels a population of horizontal cells in the OPL (data not shown).

A relatively homogeneous distribution and small fraction of Flrt3-RGCs suggested that only a limited number of functionally specific RGC types were labeled (Figures 1E,F). To examine if Flrt3-RGCs represented an anatomically homogeneous subpopulation with a regular spacing, we analyzed their spatial arrangement based on density recovery profiles (DRP) (Supplementary Figure 2D) (Rodieck, 1991). We found that Flrt3-RGCs were distributed across the whole retina, with a peak density in the ventral regions (Figure 1E). However, DRP analysis showed random distribution in both Flrt3-RGCs (Figure 1F) and Flrt3-amacrine cells (Supplementary Figure 2E). In summary, these results suggest that Flrt3 labels more than one functional RGC and amacrine subpopulation.

**Morphological Characterization Indicates at Least Four Flrt3-RGC Subpopulations**

The depth of dendritic stratification in the IPL is a good feature to classify functional RGC subpopulations (Seung and Sümbül, 2014). Thus, we used the Flrt3-CreERT2-Tomato line to label the morphology of Flrt3\(^+\) RGCs and amacrine cells by...
We found that Flrt3-RGCs consist of 4 subtypes: 53% of all Flrt3-RGCs were bistratified in the ON and OFF layers S2 and S4, 37% stratified in the ON layers (2 subtypes) S4 or S5, and a small fraction (~10%) stratified only in the OFF layer S1 (Figure 2C).
FIGURE 2 | Dye-filled RGCs revealing their morphology and stratification. (A) Injection of a fluorescent dye [Alexa 488 or Lucifer yellow (LY)] in Flrt3-RGCs with a borosilicate glass pipette using a patch-clamp setup and filling the whole neuron to assess the morphology of the cells. (B) Dendritic stratification pattern of Flrt3-RGCs in the IPL for ON, ON-OFF, and OFF cells. Stratification depth between the inner limiting borders of the GCL (=0%) and INL (=100%) defined by DAPI staining. \( N = 5 \) retinas from 4 mice. (C) Scheme summarizing the stratification pattern and their percentages. (D) Representative morphologies of Flrt3 RGCs stratifying in different IPL layers that were filled with Lucifer yellow and reconstructed using the Fiji Simple Neurite Tracer plugin. (E) Different morphologies of Flrt3 amacrine cells of the INL. Scale bars = 50 \( \mu m \).

To further characterize the Flrt3-RGC subpopulations, we performed immunostainings against known RGC markers. The strongest co-labeling of Flrt3+ RGCs (RBPMS+) was found with Calbindin (28 ± 2.7%), followed by Parvalbumin (19 ± 3.6%), and then the ON-OFF direction selective ganglion cell marker CART (22 ± 4.8) (Supplementary Figures 3A–F, mean ± SEM). Overall, none of the markers labeled all Flrt3+ RGCs, suggesting that Flrt3 labels a new combination of RGC subtypes. Importantly, it has been shown in mice that parvalbumin and calbindin label at least eight and ten (PV1–PV8), respectively, morphologically different RGC types differing in their stratification pattern and dendritic field size (Kim and Jeon, 2006; Gu et al., 2016). CART positive RGCs are known to label the four ON-OFF direction selective ganglion cells (oO-DSCG) that can be distinguished using combinatorial staining of Cadherin 6, MMP17, and Collagen 25a1 (Kay et al., 2011).

Electrophysiological Characterization Revealed Three Major Response Patterns

To physiologically characterize Flrt3-RGCs, we performed patch clamp recordings from Flrt3-Tom mice, showing different movie patterns (e.g., moving bars, stripes, or dots) directly onto the retina to monitor light-evoked firing activities (Figure 3A). In an initial evaluation, we analyzed the firing properties of Flrt3-RGCs by injecting current pulses with different intensities in whole-cell current-clamp mode. We recorded 21 Flrt3-negative and 16
FIGURE 3 | Flr3-Tom-RGCs are mainly ON neurons. (A) The left panel shows a schematic of the experiment in which an RGC is patch-clamped while different movie patterns are shown with a projector. The right panel shows representative whole-cell current-clamp recordings displaying firing after a step of current injection in Flr3\textsuperscript{+} and Flr3\textsuperscript{−} RGCs. (B) Plot showing the firing rate (Hz) after increasing current steps. (C) Firing adaptation from the recordings in (A). A value for firing adaptation closer to 1 represents cells with a higher adapting firing, while a value closer to 0 is more regular (see section “Materials and Methods”). 

N = 16–21 cells

(Continued)
Flrt3-positive RGCs from random retina regions. Interestingly, Flrt3-RGCs showed a higher firing rate than those of Flrt3-negative RGCs (Figures 3A,B). Moreover, when comparing the temporal features of firing patterns, Flrt3-RGCs showed non-adaptive and regular firing patterns (Flrt3-negative 0.46 ± 0.08, Flrt3-Tom 0.18 ± 0.11, t-test \(^* p < 0.05\) (Figure 3C). The membrane potential of Flrt3 and Flrt3-negative RGCs did not show any significant differences (Flrt3-negative −53.21 ± 0.63, Flrt3-Tom −53.69 ± 0.74) (Figure 3D).

To evaluate the visual responses of Flrt3-RGCs, we performed cell-attached recordings to analyze the light-evoked firing activity in response to a set of different movie patterns: static flashing spot with different sizes (50–1,200 µm), and moving bars (Figures 3E,F). The responses to static flash stimulus revealed that Flrt3-RGCs included ON (50%), OFF (25%), and ON-OFF (25%) cells. A subset of ON cells showed direction selectivity to upward movements (2 out of 10 cells) (Figure 3E). Within our sample size no OFF and ON-OFF cells showed any direction selectivity (Figure 3E).

**Flrt3-RGCs Project to Nuclei in the Accessory Optic System**

Retinal ganglion cells project to one of more than 50 different target regions in the mouse brain (Martersteck et al., 2017), and the projection patterns vary depending on RGC subtypes (Lawrence and Studholme, 2014). To analyze the projection pattern of Flrt3-RGCs, we performed intravitreal virus injections using a Cre-dependent GFP expressing AAV to specifically label the projection targets of Flrt3 RGCs, and compared it to the overall projection pattern of all RGCs using CTB647 (Figures 4A–C). The expression pattern showed non-specific innervation of the superior colliculus and LGN (Figures 4D,E). No Flrt3-RGCs axons were found in the intergeniculate leaflet (IGL) or the suprachiasmatic nucleus (SCN) (Figures 4E,F). Interestingly, Flrt3 RGCs projected to the medial terminal nucleus (MTN) and nucleus of the optic tract (NOT) (Figures 4G,H) which are part of the AOS and are known to be essential in driving the optokinetic reflex.

**Medial Terminal Nucleus Projecting FLRT3 Retinal Ganglion Cells Are ON-DSGC**

Based on the finding that Flrt3-RGCs project to the MTN of the AOS, we hypothesized that Flrt3-RGCs contain a subpopulation of direction selective ganglion cells (DSGC) encoding one of the four cardinal axes. To specifically label only Flrt3-RGCs projecting to the MTN, we injected a retrograde Cre-dependent AAV-Tomato into the MTN of Flrt3-CreERT2 mice (Figure 5A). After 3 weeks of viral expression, we were able to identify MTN-projecting Flrt3-RGCs in the retina (Figure 5B). Density recovery profile analyzes revealed a regular distribution of MTN projecting Flrt3-RGCs, indicating a homogenous subpopulation (Figure 5C). Indeed, the regularity index (RI) (see section “Materials and Methods”) and mean nearest neighbor distance (NND) showed higher values for MTN-projecting Flrt3-RGCs (RI = 2.08 ± 0.07; NND = 111.4 ± 5.29) than for total Flrt3-RGCs (RI = 1.7 ± 0.17; NND = 54.04 ± 6.44) and Flrt3-amacrine cells (RI = 1.92 ± 0.04; NND = 31.57 ± 2.22) (Figures 5D,E), suggesting that MTN projecting Flrt3-RGCs consist of a single RGC subtype.

The majority of MTN-projecting Flrt3-RGCs bistratified in the S4 ON and S2 OFF layer (∼74%) of the inner plexiform layer (IPL) and overlapped with the two outer bands of Calbindin (Figure 5F). The remaining MTN-projecting Flrt3-RGCs mono-stratified in the S4 layer and overlapped with the inner most band of Calbindin (Figure 5I). Immunostainings showed that most MTN-projecting Flrt3-RGCs are positive for the ON-OFF DSGC marker CART (89 ± 11%, n = 69 cells) and Calbindin (97 ± 2%, n = 98 cells) (Figures 5G,H), which have been shown to label at least 10 morphologically distinguishable RGC types (Gu et al., 2016). Interestingly, among Calbindin-containing RGCs, Gu et al. (2016) reported that calretinin-containing RGCs include only 1 type (“CB3” type) that is bistratified in the S2/S4 layers of the IPL, similar to what we show with our MTN-projecting Flrt3-RGCs (Gu et al., 2016). Approximately 56 ± 5% (n = 49 cells) co-labeled for the calcium-binding protein parvalbumin (Figures 5G,H), which has been shown to label 8 morphologically distinct RGC types (PV1-8), and only the PV3 subtype shows the same bistratification pattern as most of the MTN-projecting Flrt3-RGCs (Yi et al., 2012). These data indicate that the known CB3 and PV3 RGCs are possible candidates for the cell types of MTN-projecting Flrt3-RGCs.

Although the majority of MTN-projecting Flrt3-RGCs had bistratified dendrites in the ON and OFF layers, the light-evoked firing activity monitored by cell-attached recordings revealed that MTN-projecting Flrt3-RGCs are physiologically ON cells, responding to luminance increments of static flashes (50–1,200 µm) (Figure 6A). Interestingly, almost all MTN-projecting Flrt3-RGCs (14 out of 15) were direction selective cells, and preferentially responded to an upward-moving grating (Figures 6B,C,E), corresponding to ventrally moving objects in the living animal (Figure 6D). Together, these results indicate that MTN-projecting Flrt3 RGCs represent a homogeneous AOS projecting direction-selective RGC subpopulation.
Flrt3 ON DSGCs

**FIGURE 4** | Flrt3-RGCs projections in the mouse brain. (A) Scheme showing intravitreal injection of CTB647 and the cre-dependent AAV2-flex-GFP virus in Flrt3-CreERT2 mice. Representative images from one animal. (B,C) Representative whole mount retinas showing CTB647 labeled RGCs (magenta) and GFP expressing RGCs (green). (B) Shows an overlay of CTB647 and GFP. The right panel shows a zoom from each of (B',C'), respectively. (D–H) Specific projections of Flrt3 RGCs (green) and all RGC (magenta) axons in the (D) superior colliculus (SC), (E) SCN and optic tract (ot), (F) dLG, IGL, and vLG, (G) MTN, (H) NDT and OPT.
FIGURE 5 | MTN-projecting Flrt3-RGCs are a homogeneous downward ON-DSGC subpopulation. (A) Stereotaxic injection of AAV2retro-cre-Tomato in the MTN of Flrt3-CreERT2 mice to identify Flrt3-RGCs projecting to the MTN. The image on the right shows a representative MTN-injection site after 3 weeks of viral expression. N = 4 mice. (B) Whole mount retina showing the distribution of MTN-projecting Flrt3-RGCs. (C) Normalized density profile of MTN-projecting Flrt3-RGCs. N = 50 ROIs from 11 retinas and 8 animals. (D) Regularity index plot for Flrt3-RGC, Flrt3-MTN-projecting-RGC and Flrt3-amacrine cells. N = 4–11 ROI from different retinas of 3–4 mice (as shown in (B). One-way ANOVA Tukey’s Multiple Comparison Test, *P < 0.05. (E) Average nearest neighbor distance (µm), calculated from same ROIs as in (D). One-way ANOVA Tukey’s Multiple Comparison Test, **P < 0.001. (F) Orthogonal section through the IPL to visualize the stratification pattern of
FIGURE 5 | MTN-projecting Flrt3-RGCs. The right panel shows the average normalized stratification pattern of Calbindin (orange), mono- (green, $n = 16$ cells from 3 animals, 26%) and bistriated MTN-projecting Flrt3-RGCs (magenta, $n = 46$ cells from 3 animals, 74%). (G) Immunostaining for CART, Calbindin and Parvalbumin in a retinal sections of MTN injected mice from (A). Tomato signal (magenta) shows MTN-projecting Flrt3-RGCs. (H) Fraction (%) of MTN-projecting Flrt3-RGCs positive for CART, Calbindin and Parvalbumin. $N = 60–66$ ROIs for each marker from 3 retinas. (I) Normalized stratification patterns of mono- (green) and bistriated (red) MTN-Flrt3-RGCs in the inner plexiform layer, from (F). $N = 46$ cells from 3 animals.

FIGURE 6 | MTN-projecting Flrt3-RGCs are a homogeneous downward ON-DSGC subpopulation. (A) Heatmap plot showing the frequency of firing in cell-attached MTN-projecting Flrt3-RGCs while showing a flashing dot with different diameters (50–1,200 $\mu$m). (B) Top: Scheme representing the recordings of MTN-projecting Flrt3-RGCs firing rate while displaying a moving grid in eight different directions. The frequency plot shows the average frequency of firing. Bottom: The heatmap plot shows the individual frequencies for each neuron patched for each moving grid displayed. $N = 15$ RGCs from 4 animals. (C) Polar plot showing the direction selectivity of the RGCs recorded from the retina’s perspective. $N = 15$ RGCs from 4 animals. (D) Cartoon showing the mouse’s perspective of the moving object. (E) Direction selectivity plots of fifteen different MTN-Flrt3-RGCs, $N = 15$ cells from 4 animals.

DISCUSSION

In this work, we generated a new Flrt3-CreERT2 knock-in mouse line to genetically target and characterize Flrt3 positive RGCs in the mouse retina. We found that Flrt3-RGCs represent a small subpopulation of $\sim 4.6\%$ of all RGCs. Based on their stratification patterns, Flrt3-RGCs can be subdivided into four morphologically and three functionally distinct subtypes. Electrophysiological recordings showed that Flrt3-RGCs have more non-adaptive and higher firing rates than Flrt3-negative RGCs. Fifty percent of Flrt3-RGCs are ON-responding cells when they are stimulated with a static flashing light. Retinofugal projection and electrophysiological analyses revealed that the functional ON subtype of the Flrt3 population projects to the MTN. Histologically, MTN-projecting Flrt3-RGCs bistriatify in the IPL and express the ooDSGC marker CART ($\sim 89\%$) and Calbindin ($\sim 97\%$). Further analysis showed that MTN-projecting Flrt3-RGCs are direction selective cells, preferring downward direction.

Prior research on MTN-projecting direction-selective RGCs (Dhande et al., 2013) revealed that the Hoxd10-EGFP mouse line includes three ON subtypes, each of which encodes upward, downward or forward motion, and one ON-OFF type encode forward movements. So far, the Fstl4$^{TM1Mno}$ (here referred
to as SPIG1) mouse line is the only mouse model that specifically labels the upward direction-selective ON-mono-stratifying MTN-RGCs. For the remaining 3 subtypes, no genetic marker exists (Yonehara et al., 2009; Dhande et al., 2013). In contrast to our MTN-projecting Fltr3-RGCs, downward selective cells in Hoxd10 mice show an ON mono-stratifying pattern in the IPL. Interestingly, Dhande et al. described a small (11%) population of RGCs in the Hoxd10 line that co-localized with the ON-OFF DSGC marker CART. However, the cell type was not further characterized. Another genetic line, Pcdh9-Cre, also labels MTN projecting ON RGCs. Like the MTN-projecting Fltr3+ cells, the Pcdh9-Cre line labels 2 subtypes that either stratify mostly in the ON S4 layer, or show weaker stratifications in the S2 OFF layer. Both subtypes, however, respond to upward moving stimuli. In contrast to the MTN projecting Fltr3+ RGCs, the Pcdh9 ON-DS cells show a transient OFF response to the trailing edge of the moving stimulus (Lilley et al., 2019). Overall, this data suggests that Fltr3-RGCs could represent the CART positive RGCs of the Hoxd10 mouse line and thus describe a new and as yet uncharacterized MTN-projecting RGC population.

The co-stratification of MTN projecting Fltr3-RGCs within the same layer of the ON and OFF starburst amacrine (SAC) dendrites suggests that their direction selectivity toward downward moving objects is mediated by modulating GABAergic and cholinergic input from SACs (Mauss et al., 2017). However, we found a discrepancy between the stratification pattern and physiological response properties. Although MTN-Fltr3-RGCs showed a bi-stratifying ON-OFF morphology within the IPL, they lacked any functional OFF response. The small OFF responses seen despite the ON-OFF dendritic bistratification could be caused by the offset of OFF excitatory inputs by inhibitory inputs or presynaptic inhibition of OFF bipolar cells (Jacoby, 2015; Nath and Schwartz, 2016). Indeed, the same discrepancy between dendritic bi-stratification and ON dominant responses has been revealed in orientation selective RGCs (Nath and Schwartz, 2016), suppressed-by-contrast RGCs (Jacoby, 2015) and MTN/SC projecting ON DSGCs (Gauvain and Murphy, 2015), in which OFF dendrites did not receive any excitatory inputs. The absent OFF response could also be explained by the shorter dendritic ramification in the OFF layer in comparison with the longer projection into the ON layer (Figure 5F; Nath and Schwartz, 2016).

CONCLUSION

Based on the light responses of RGCs and basic anatomical criteria, we found a new genetic marker to identify a novel FLRT3+ cell-type in the mouse retina, corresponding to an ON downward-selective MTN-projecting RGC. Like the Hoxd10 mouse, which identified an additional ON-OFF RGC that projects to the NOT of the AOS, our findings add a homogeneously distributed ON RGC-type that projects to the MTN of the AOS. Interestingly, this MTN-projecting FLRT3+ cell-type is positive for CART, a marker for ON-OFF RGCs. Moreover, this RGC-type bistratified in the ON and OFF layer, but the electrophysiological recordings showed that they fire only when the light is ON, probably because the stratification pattern is larger in the ON layer compared to the OFF layer of the IPL.

In conclusion, the current work shows that almost all MTN-projecting Fltr3 cells are: (1) bistratified, (2) ON-dominant response, and (3) direction selective RGCs. Finally, using intersectional genetics, our Fltr3 line in combination with the Parvalbumin, Calbindin or CART transgenic mouse lines could provide functional access to these RGCs, which would enable us to study the function of the downward selective Fltr3-RGCs in vivo.

AUTHOR’S NOTE

The retinal ganglion cells (RGCs) in the retina are the only cells that transmit visual information into the brain. Until now, more than 40 different RGC type has been identified which respond in a very specific way to the same visual stimuli. However, only a small fraction of these can be studied using specific genetic markers. We found that Fltr3 labels a new RGC subpopulation that projects to the medial terminal nucleus (MTN) which is essential to drive reflexive eye movements for retinal image stabilization. Fltr3 labels MTN projecting RGCs that preferentially respond to downward moving objects by increasing their firing rate (ON-response). Thus, Fltr3 provides the first genetic tool for studying downward motion selective RGCs projecting to MTN.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The animal study was reviewed and approved by the Regierung von Oberbayern under the license 55.2-2532.Vet_02-20-10.

AUTHOR CONTRIBUTIONS

RK, DT, and TR initiated and conceived the project. CP performed electrophysiological recordings and intracranial injections. AM helped with electrophysiological recordings and intracranial injections. PM and LG performed ES cell gene targeting. SI wrote the python analysis script to straighten the curved retina sections for stratification analysis. TR and CP wrote the manuscript with support from all other authors. LG checked the English grammar of the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnmol.2021.790466/full#supplementary-material

Supplementary Figure 1 | Flrt2 and Flrt3-lacZ lines express Flrt3 in a subpopulation of ganglion cells in the retina. (A) Representative retina from Flrt3-LacZ animals. The immunostaining in the right panel shows Flrt3 cells (light blue, Xgal) and RGCs (green, RBPMS). White astersisks indicate Flrt3+ RGCs. Scale bar = 25 μm. (B) Top: percentage of Flrt3+ RGCs and amacrine cells in the retina. (C–D) Same figures as (A,B), but with Flrt2-LacZ mice. White astersisks indicate Flrt2+ RGCs. (E,F) Retina sections from Flrt2-LacZ and Flrt3-LacZ mice, respectively, with Xgal staining at different developmental time points.

Supplementary Figure 2 | Flrt3-CreERT2 expression matches Flrt3-lacZ expression in the brain. (A) Coronal brain slices of Flrt3-CreERT2; Tom2 mice, with anterior from posterior to anterior. (B) Zoomed in view of different regions from (A) (dashed boxes). (C) Comparison between Flrt3-Tom and Flrt3-LacZ mice. (D) Algorithm showing how the DRP values were calculated. Rectangular regions of the retina were selected to create an autocorrelation. Annuali around the center of the autocorrelation were drawn to calculate the binned density from the center to the periphery and represented as the density recovery profile. (E) Random distribution of Flrt3-amacrine cells. Left panel: Flrt3-amacrine cell distribution in a ROI. Each dot represents a Flrt3+ amacrine cell. Right panel: Density recovery profile of Flrt3+ amacrine cells showing a random distribution. Graph shows mean value ± SEM. (F) Same Supplementary Figure 3 Immunostainings showing some overlap with other known RGC markers. (A–F) Staining for various known markers: Safb1, SM32, Calbindin, Brm3c, Parvalbumin, and CART (blue) in Flrt3-RGCs (Tom, magenta) and RBPMS (green). The blue arrowhead shows an example of RGC that co-localizes with Flrt3 and the different markers. (G) Quantification of the percentage of Flrt3-RGCs expressing the markers shown in (A–F). Mean value ± SEM.

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