RESEARCH ARTICLE

Functional, molecular and morphological heterogeneity of superficial interneurons in the larval zebrafish tectum

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Abstract
The superficial interneurons, SINs, of the zebrafish tectum, have been implicated in a range of visual functions, including size discrimination, directional selectivity, and looming-evoked escape. This raises the question if SIN subpopulations, despite their morphological similarities and shared anatomical position in the retinotectal processing stream, carry out diverse, task-specific functions in visual processing, or if they have simple tuning properties in common. Here we have further characterized the SINs through functional imaging, electrophysiological recordings, and neurotransmitter typing in two transgenic lines, the widely used Gal4s1156t and the recently reported LCRRH2-RH2-2:GFP. We found that about a third of the SINs strongly responded to changes in whole-field light levels, with a strong preference for OFF over ON stimuli. Interestingly, individual SINs were selectively tuned to a diverse range of narrow luminance decrements. Overall responses to whole-field luminance steps did not vary with the position of the SIN cell body along the depth of the tectal neuropil or with the orientation of its neurites. We ruled out the possibility that intrinsic photosensitivity of Gal4s1156t+ SINs contribute to the measured visual responses. We found that, while most SINs express GABAergic markers, a substantial minority express an excitatory neuronal marker, the vesicular glutamate transporter, expanding the possible roles of SIN function in the tectal circuitry. In conclusion, SINs represent a molecularly, morphologically, and functionally heterogeneous class of interneurons, with subpopulations that detect a range of specific visual features, to which we have now added narrow luminance decrements.

KEYWORDS
cell type, interneurons, luminance detection, optic tectum, zebrafish

1 | INTRODUCTION

Ensuring behavioral reliability across variable environmental conditions requires our brains to extract relevant features from the visual landscape, a process which is refined over multiple levels of visual processing. Due to their robust visually mediated behavioral repertoire and their amenability to genetic and imaging tools, the larval zebrafish is a highly tractable model for interrogating the neural circuits underlying visual behavior (Baier & Scott, 2009; Nikolaou & Meyer, 2012; Nikolaou et al., 2012; Orger et al., 2008; Walker et al., 2013; Thiele et al., 2014; Bianco & Engert, 2015; Portugues

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et al., 2014; Portugues & Engert, 2009; Kubo et al., 2014; Mearns et al., 2020).

In the zebrafish brain, the optic tectum is the main center for visual processing, and significant strides in mapping the circuitry of the tectum have been achieved with the identification of tectal cell types (DeMarco et al., 2020; Förster et al., 2020; Förster et al., 2018; Gabriel et al., 2012; Helmbruch et al., 2018; Kramer et al., 2019; Robles et al., 2011; Scott & Baier, 2009) and with the publication of a comprehensive single-cell atlas of the larval zebrafish brain (Kunst et al., 2019). Retinal ganglion cells (RGCs) send axonal projections into the tectum, where they each arborize in one of nine sublayers, the stratum opticum (SO), six laminae of the stratum fibrosum et griseum superficiale (SFGS1 through 6), the stratum griseum centrale (SGC), and the boundary region between stratum album centrale and the stratum periventriculare (SAC/SPV) (Robles et al., 2013, 2014; Xiao et al., 2011). In the tectum, the majority of cell bodies reside in the periventricular layer (SPV), a dense collection of periventricular projection neurons and interneurons (PVNs) below the neuropil (Nevin et al., 2010).

A third, much less abundant class of tectal neurons, named the superficial interneurons (SINs), is unique in that their cell bodies lie in the most superficial layers of the tectal neuropil, SO and SFGS1 (Del Bene et al., 2010). SINs have generally been considered to be GABAergic. Due to their spatial segregation, these cells offer a unique foothold for dissecting visual processing in the optic tectum. The sparseness of cell bodies in the neuropil has allowed for targeted electrophysiology, functional imaging, and ablation.

In the last decade, SINs have been implicated in several tectum-dependent behaviors, including prey capture and looming-mediated escape (Abbas et al., 2017; Barker & Baier, 2015; Del Bene et al., 2010; Dunn et al., 2016; Hunter et al., 2013; Preuss et al., 2014; Yin et al., 2019). This broad involvement suggests that SINs either occupy a global, “general-purpose” inhibitory role in visual processing, or that functional subsets with distinct feature selectivities and specific neural connectivities exist. In support of the latter scenario, subpopulations of SINs differentially tuned to size, direction, orientation, and looming speeds have been observed (Abbas et al., 2017; Dunn et al., 2016; Förster et al., 2020; Hunter et al., 2013; Preuss et al., 2014; Yin et al., 2019). Here we contribute to this growing literature by using functional imaging and electrophysiology to investigate the tuning of SINs to whole-field luminance changes. We find that subsets of SINs encode narrow luminance steps, mostly in the OFF direction. We also characterize a new transgenic line, Tg(lcrR2:R2-H2-2:GFPpt115-c) (called lcrR2-H2:R2:GFP here; Fang et al., 2013) with a broader SIN labeling than the commonly used Gal4s1156t line, and investigate overlap with other molecular markers. Unexpectedly, we discovered that a substantial portion of SINs are glutamatergic. The rich diversity in feature selectivity, morphology, marker expression, and transmitter use argues in favor of the existence of multiple SIN types, each serving in parallel in feature-selective microcircuits, similar to the amacrine cells of the retina.

2 | METHODS

2.1 | Zebrafish care and maintenance

Zebrafish (Danio rerio) were maintained at 28°C on a 14 h light/10 h dark cycle following standard procedures. All animal procedures were performed in accordance with regulations set forth by the Max Planck Society and the Regierung von Oberbayern. The following transgenic lines were used and maintained in a TLN background (Tüpel long-fin wild-type strain carrying mutations in mitfa (nacre, N); EtGal44VP16, s1156t, Tg(UAS:Kaede)15pppp, Tg(UAS:RFP, cry:EGFP), Tg(UAS:GCoMP6s), mnpn101, Tg Isl2b:Gal44VP16:265, Tg atoh7:Gal44VP16, TgUas:EGFP, and Tg(UAS: mCherry)s1984t. The Tg(lcrR2:R2-H2:2:GFPpt115-c) line was obtained from X. Wei (Fang et al., 2013). Tg(vglut2a:loxP-DsRed-loxP-GFP) and Tg(gad1b:loxP-DsRed-loxP-GFP) lines were acquired from S. Higashijima (Satou et al., 2012). The reelin:Gal4, UAS:RFP line was acquired from F. Del Bene (Auer et al., 2014). All fish used in experiments were 6–10 days post fertilization (dpf).

2.2 | Enucleation experiments

Bilateral eye removal was performed 1–2 days prior to imaging experiments. Larvae were anesthetized with 0.02% tricaine and embedded in 2% low melting point agarose (In VitroGen, Carlsbad, CA, USA). Eyes were removed surgically with a sterile 25G needle and #55 forceps (Fine Science Tools). Following the enucleation procedure larvae were removed from agarose and allowed to recover overnight. The overall health of the larvae was assessed before proceeding to imaging experiments.

2.3 | Immunostaining

Larvae (8 dpf, Gal4s1156t × UAS:EGFP) were fixed in 4% PFA in PBS overnight at 4°C followed by a 12 h incubation in 30% sucrose in PBS. Larvae were embedded in O.C.T. (Tissue-Tek, Torrance CA, USA) and sectioned into 12 μm sections. Sections were allowed to dry overnight and subjected to the following immunostaining protocol: 2 min rehydration in PBS, 30 min incubation in 0.1% sodium citrate buffer, a brief wash in PBS (~2 min), 5 min RT incubation in 1% SDS, 15 min washes in PBS, a 1 h RT incubation in blocking solution (3% Donkey Serum (Sigma-Aldrich, St. Louis, MO, USA) and 0.3% Triton X-100 in PBS) and an overnight primary incubation (Mouse anti-Reelin [1:500 dilution, Calbiochem #553730]) and chicken anti-GFP (1:1000 dilution, Invitrogen A10263) in blocking solution at 4°C. The following day, sections were washed three times with PBS (~15 min per wash) and incubated in secondary antibody (1:200, Invitrogen), goat anti-mouse IgG (H+L), Alexa Fluor 546 (A-11030), and goat anti-chicken IgY (H+L), Alexa Fluor 488 (A-11039) diluted in blocking solution.

Sections were washed three times with PBS (~15 min per wash) and mounted in Fluoromount (Sigma-Aldrich) prior to imaging.
2.4 | Statistics

All statistical tests were performed using GraphPad Prism 6.0. When appropriate, unpaired t-tests or one-way and two-way ANOVA tests with Tukey’s correction for multiple comparisons were performed. In all figures * denotes p < 0.05, ** denotes p < 0.005, and *** denotes p < 0.0005, n.s. denotes “not statistically significant.” All error bars are standard error of the mean (SEM).

2.5 | Confocal imaging

Confocal images were acquired with Zeiss LSM700 or LSM780 microscopes with associated ZEN software. A minimum of four larvae were imaged for each condition.

2.6 | Colocalization analysis

Colocalization analysis was performed using the Coloc2 custom plug-in for ImageJ (Schneider et al., 2012). In brief, corresponding z stacks for green and red fluorescence were split. ROIs for each GFP+ cell (SINs) were defined and colocalization analysis runs for both channels within the ROI in the sub-stack where the GFP+ cell body was present. A Pearson’s r value coefficient for colocalization of pixel intensity within each ROI was generated. Pearson coefficients ≥0.2 were counted as colocalized in crosses of transgenic lines, and ≥0.5 for immunostainings.

2.7 | Calcium imaging and analysis

Calcium imaging was performed using either a custom-built moveable objective 2-photon microscope (MOM, Sutter Instruments, Novato, CA, USA) with ScanImage software for image acquisition (Pologruto et al., 2003) or a customized commercial 2P microscope (Femtonics 3DRC, Femtonics, Hungary; Dal Maschio et al., 2017) with associated software for image acquisition. Both microscopes used 20x objectives (Olympus, NA 1.0) and 920 nm excitation for GCaMP6s. Prior to imaging, larvae were embedded in 2.5% low melting point agarose (Invitrogen). All larvae were raised on a normal light/dark cycle and kept in a dark room prior to and during imaging (~0.2 lux). Imaging

**FIGURE 1** Many Gal4s1156t+ SINs are strongly tuned to changes in whole-field luminance. (a) Example SIN from a 7dpf Gal4s1156t × UAS:GCaMP6s larva shows consistent responses (shown as ΔF/F) to whole-field changes in luminance. (b) Responses to whole-field luminance are highly consistent across larvae. Responses from five SINs from four Gal4s1156t × UAS:GCaMP6s larvae. (c) Gal4s1156t+ SINs are more responsive to darkening (decreasing luminance) whole-field flashes than brightening (increasing luminance) whole-field flashes (unpaired t-test, p < .0001). (d, e) Electrophysiological recordings show increased excitatory synaptic input in response to transitions between light ON and light OFF conditions. (d) Four example traces from neurons targeted in Gal4s1156t × UAS:Kaede. EPSCs occur with greater frequency during low luminance (light OFF) conditions. (e) Total excitatory postsynaptic current is plotted for OFF and ON light conditions. For (c), n = 47 cells from 17 larvae. *** denotes p < .0005; Scale bar in (d), 20 pA, 200 ms. Error bars are SEM. Visual stimulus is shown above the corresponding example traces in (a), (b), and (d).
acquisition was performed between 2.94 and 3.37 Hz (frames per second, 256 × 256 pixel dimensions). For analysis of whole-field flash and looming stimuli calcium imaging data, raw fluorescence traces were analyzed using the Time Series Analyzer V3.0 plug-in for Fiji (Pologruto et al., 2003) as previously described (Barker & Baier, 2015). In brief, ROIs were manually defined for all cell bodies and ΔF/F values calculated as follows: \( \Delta F/F = (F_t - F_0)/F_0 \), where \( F_t \) is the fluorescence at time \( t \) and \( F_0 \), the baseline fluorescence, calculated as the mean raw \( F \) value across all frames in the 40 frames (∼13 s) preceding stimulus onset and after background subtraction of stimulus presentation artifacts. SINs were considered unresponsive and excluded from further analysis if they did not show responses across two trials, did not have a minimum \( \Delta F/F \) response of 0.8 for all trials, or if the baseline \( F_0 \) (40 frames) before the onset of the visual stimuli had a \( \Delta F/F \) value greater than 0.2. Responses to stimuli were taken as an average of the four frames immediately following stimulus offset. Responses were averaged across trials for each fish and then across individual fish. Fifty-seven cells (47 SINs) in 17 Gal4s1156t × UAS:GCaMP6s larvae were analyzed in Figures 1 and 2. Six of these cells were also tested with looming stimuli.

**FIGURE 2**  
Gal4s1156t+ SIN responses to whole-field luminance changes are consistent across tectal neuropil layers. (a) Imaging plane in a 7 dpf Gal4s1156t × UAS:GCaMP6s larva. (b) Example of a superficially positioned SIN (layer 1, magenta and indicated by magenta circle in (a), demonstrating a characteristic response to all luminance transitions. (c) Example trace of a SIN with its cell body positioned in an intermediate layer (layer 2, blue and indicated by blue circle in (a). (d) Example trace from a SIN with its cell body positioned in the deepest layer (layer 3, green and indicated by green circle in (a). (e) Gal4s1156t+ PVNs display more narrowly tuned response profiles to whole-field luminance transitions. An example PVN (layer 4, orange and indicated by orange circle in (a)) trace is shown here. (f) Nearly all Gal4s1156t+ SIINs (same cells as shown in Figure 1 plus 10 PVNs, \( n = 57 \) cells in 17 larvae) show greater responses to OFF than to ON, across all neuropil layers. (g) Gal4s1156t+ SIINs either have upward- or downward-oriented neurites. (h) OFF or ON responses are not significantly different between Gal4s1156t+ SIINs with upward- or downward-oriented neurites (unpaired t-tests, \( p = .13 \) for OFF, \( p = 0.22 \) for ON). Scale bar in (a) = 20 \( \mu \)m, (g) = 10 \( \mu \)m. A, anterior; L, lateral; M, medial; P, posterior. Error bars are SEM.
2.8 Visual stimulus presentation

Visual stimuli were presented using an OLED screen (eMagin) with three magenta filters (Figures 1, 2, 5) or a portable LED projector using only the red channel (LG, Model No. PA72G, Seoul, South Korea), Figures 3 and 4). Looming stimuli were presented using an expanding disk of constant radial velocity (expanding at \( 20 \text{ ms}^{-1} \)). Stimuli for full-screen flash and looming experiments were generated with a custom programmed graphical interface for Vision Egg (Straw, 2008). The bright loom stimulus consisted of a white expanding disc on a gray background, and the dark loom stimulus consisted of a black expanding disc on a gray background. Looming stimuli and whole-field flash stimuli were presented binocularly. Whole-field flash stimuli were presented for 0.5 or 1.0 s and maintained for 4 or 10 s before the next luminance change (a whole-field flash) was initiated.
2.9 | Luminance step stimuli

Luminance steps were generated using custom scripts for PsychoPy (Peirce, 2008). Lux values for luminance steps were: 2.7, 10.9, 29.8, 67.2, and 92.8 lux. Each luminance step was displayed for 10 s. For analysis a custom written python script was used to select single cell ROIs, their ΔF/F values calculated and baseline changes in the traces normalized using asymmetric least square smoothing (ALS, Eilers & Boelens, 2005). For each luminance step the baseline was calculated as a mean of 2–7 frames before stimulus onset and the response as the maximum value in the first 5 frames after the luminance change (baseline subtracted). The stimulus was presented twice, and an average value from the two repetitions was calculated.

2.10 | Receptive field mapping

Receptive fields (RFs) were mapped using a checkerboard stimulus with 4 × 3 square grids of ~20° or 10 × 6 square grids of ~9° of the larva’s visual field (total area 78° × 60°) generated using custom scripts in PsychoPy (Peirce, 2008). Analysis was performed using a custom written python script, where single cell ROIs were selected and ΔF/F values with corrected baseline calculated. The RF was calculated using the maximum response during the presentation of each grid square and averaged across two trials. In order to calculate the size of the RF, the calculated array was interpolated by a factor of 100 for a smoothed fit, the array was thresholded to the half-maximum, and the contour diameter calculated. Ten SINs were exclusively tested with RF mapping, 6 cells with both RF mapping and luminance stimuli, and 8 cells with luminance stimuli only.

2.11 | Electrophysiology

Whole-cell patch clamp recordings were performed on 6–10 dpf Gal4s1156t × UAS:Kaede larvae. Larvae were anesthetized on ice and embedded in 2% low melting point agarose (Invitrogen) in external solution. To allow for electrode access to the tectum, a small incision was made with a sharp electrode and used to remove the skin above both tecta immediately prior to recordings. SINs were visualized using the GFP filter on the Zeiss AxioScope2. Recording solutions were as follows: external solution (in mM): 115 NaCl, 2 KCl, 10 HEPES, 2 CaCl2, 10 glucose, 1.5 MgCl2, pH 7.4; internal solution (in mM): 110 potassium gluconate (C6H11KO7), 10 KCl, 5 NaCl, 1.5 MgCl2, 20 HEPES, 0.5 EGTA.
pH 7.3. Patch pipettes were pulled from borosilicate glass (OD: 1.5 mm, I.D. 0.86 mm; Sutter Instruments BF 150-86-10). The resistance of patch pipettes ranged from 8 to 10 M. Data were low-pass filtered (2 kHz, Axopatch 200B, Axon Instruments), digitized (10 kHz, Digidata 1440A, Axon Instruments), and analyzed using pClamp software (Molecular Devices). Visual stimuli during recordings were presented using a miniature OLED screen (eMagin).

3  | RESULTS

3.1  | Gal4s1156t+ SINs are tuned to changes in whole-field luminance

The SINs were first described in the Gal4s1156t enhancer trap line (Del Bene et al., 2010; Scott et al., 2007; Scott & Baier, 2009). To further investigate the functional properties of these SINs, we measured changes in calcium as a proxy for neural activity by crossing the Gal4s1156t driver line to a UAS:GCaMP6s sensor line. The original characterization of the Gal4s1156t line employed early generations of genetically encoded calcium indicators (GECIs), GCaMP1.6 and GCaMP3, and subsequent improvements in the detection capabilities of the GECIs (Chen et al., 2013) made it of potential interest to reinvestigate the whole-field luminance tuning in the Gal4s1156t + SINs. Consistent with previous reports (Del Bene et al., 2010; Yin et al., 2019), we identified strong responses to whole-field luminance changes in SINs in the Gal4s1156t line (Figure 1). We then expanded this stimulus set, presenting flashes with a range of six moderate-to-strong whole-field luminance steps, and varied the duration of stimulus transitions. To exclude the confounding influence of retinal light adaptation on response properties, we kept the overall light levels constant (ca. 0.2 lux) just prior to and during imaging sessions. At this larval age, zebrafish rely exclusively on cone photoreceptors for vision—rods become functional only several days later (Branchek, 1984; Branchek & Bremiller, 1984). We performed imaging experiments at the same time every day, in the early afternoon of the larva’s subjective day, to account for the well-documented circadian oscillations in light sensitivity (Moore & Whitmore, 2014).

We found that any whole-field luminance step tested was sufficient to drive responses in ~30% of Gal4s1156t+ SINs (Figure 1a,b), and this response profile was highly stereotyped across larvae (Figure 1, n = 47 SINs out of 146 labeled cells in 17 larvae). We tested several flash durations (0.5, 1, 4, and 10 s), as shown in Figure 1a,b, and found that cells that showed luminance responses, responded consistently to all luminance transitions. However, we observed differences in the amplitude of responses when the stimulus was darkening (decreasing overall luminance) or brightening (increasing overall luminance). Significantly greater responses were observed to overall luminance decrements (Figure 1c, unpaired t-test, p < .0001), suggesting that the majority of luminance responsive Gal4s1156t+ SINs are OFF responsive cells.

3.2  | Gal4s1156t+ SINs receive increased excitatory synaptic input in response to decreases in whole-field luminance

To corroborate the calcium imaging data with electrophysiology, we carried out voltage clamp recordings from SINs, targeting fluorecently labeled SINs in the Gal4s1156t × UAS:Kaede line. Across seven cells in seven individual larvae, we observed repeated and sustained membrane depolarizations following an offset of the light stimulation. The total current from these excitatory postsynaptic currents was greater for whole-field decreases in luminance (OFF flashes) than for whole-field luminance increases (ON flashes), except for one cell where the ON and OFF currents were approximately equal (Figure 1d,e). This result is consistent with the increased calcium responses observed during stimulus darkening in our GCaMP6 imaging experiments.

3.3  | Responses to whole-field luminance steps do not vary with cell body position or with orientation of dendrites

In the Gal4s1156t line, cell bodies are labeled in all layers of the neuropil (Figure 2a) as well as in a subset of neurons within the cell-body layer of the tectum, the SPV. Thus we were able to investigate to what extent, if any, response profiles of cells tuned to whole-field luminance transitions varied across the layers of the neuropil. For this analysis, we assigned each of our luminance-responsive 47 SINs (from Figure 1) and 10 Gal4s1156t+ PVNs, as a control, to one of four tectal subdivisions, roughly corresponding to SO and SFGS1 and 2 (layer 1), SFGS3–6 (layer 2), SGC and SAC (layer 3), and SPV (layer 4). We assigned 25 SINs to layer 1, 15 SINs to layer 2, and 7 SINs to layer 3. The 10 PVNs were all located in layer 4. We observed consistent responses in all three subdivisions of the neuropil (Figure 2a–e). Regardless of layer, most SINs showed greater calcium responses to overall decreases in the whole-field luminance (Figure 2f). The PVNs, on the other hand, showed responses that were more narrowly tuned to specific portions of the visual stimulus (Figure 2e,f).

In some cases, we observed Gal4s1156t+ SINs with neurites extending upward, that is, toward the superficial surface of the tectum (Figure 2g). We found no significant differences in the whole-field flash responses between Gal4s1156t+ SINs, regardless of an upward or downward orientation of their neurites (Figure 2h, unpaired t-tests, p = .13 for OFF, p = .22 for ON, n = 12 upward-pointing SINs, n = 29 downward-pointing SINs, from 17 fish).

3.4  | Gal4s1156t+ SIN subpopulations respond selectively to discrete OFF transitions, but not to looming

We next asked how sensitively tuned the Gal4s1156t+ SINs were to discrete luminance steps. The full set of luminance values
extended from 2.7 to 92.8 lux (measured at the surface of the screen). Interestingly, we observed Gal4s1156t+ SINs with selective responses to small, intermediate, and large luminance steps (Figure 3a–c).

We next tested whether Gal4s1156t+ SINs were tuned to a looming stimulus, which also produces an overall luminance change, but in a defined spatiotemporal pattern. We presented both white, expanding discs on a gray background (ON looming) and black, expanding discs on a gray background (OFF looming; Figure 3d,e). Expansion velocity was matched to that previously reported to elicit maximal escape probability (constant radial expansion of 20°/s) (Temizer et al., 2015). We found no differences in responses to bright or dark looming stimuli when compared to full-field flashes (Figure 3d–f, n = 11 cells from five larvae, one-way ANOVA, Tukey’s correction for multiple comparisons, p = .787). This suggests that Gal4s1156t+ SINs are generally tuned to luminance steps without regard for their spatiotemporal characteristics and that the Gal4s1156t+ SINs may not overlap with SIN populations previously implicated in looming computations (Dunn et al., 2016).

### 3.5 | SIN RFs sample over a large visual area

In order to assess the spatial area over which SINs are responsive, we mapped their RFs using a classic mapping strategy (e.g., Smear et al., 2007). We again used double-transgenic fish carrying Gal4s1156t and UAS:GCaMP6s. A coarse grid, composed of an array of 12 or 60 squares, each filling about 20° or 9° of the larva’s visual field was presented to one eye of an embedded fish larva positioned on the stage of a two-photon microscope (Figure 4). We found that all SINs had large RFs (23° or greater, Figure 4h). Two SINs tested with a 60-square grid (with each square covering 9° of the larva’s visual field) showed RFs similar in size and position to those mapped more coarsely (Figure 4a,b). Ten of 16 SINs mapped had contiguous RFs in the range of 40–55° (Figure 4c–f). Five SINs displayed irregular RFs, with foci of peak activity located in spatially incoherent pixels of the grid (Figure 4g). The irregularity could be due to the noise inherent to the small number of stimulus repetitions or could be caused by a fragmented RF. Together these studies confirm previous work that individual SINs sample over a large portion of the visual field, although their preferred stimulus may be

**FIGURE 5** Gal4s1156t+ SIN whole-field luminance responses require the retina. (a–c) 7 dpf Gal4s1156t × UAS:GCaMP6s larva with bilateral removal of retinal input. Enucleation sites and optic tecta are denoted in the brightfield image (a). Following enucleation, GCaMP6s+ SIN cell bodies are still clearly visible (b, c). (d) Example SIN from the larva shown in (a–c). Responses to luminance transitions are not consistent across trials and are not closely synced to the visual stimuli (shown above trace). Scale bar (a–c) = 100 μm.
substantially smaller than their RF (Preuss et al., 2014; Yin et al., 2019).

3.6 | Gal4s1156t+ SIN responses to whole-field luminance changes require the retina

One possibility that had not been tested previously is that some SINs may possess endogenous light detection capacity. Expression of opsin has been reported in the brains of many teleost species (Cavallari et al., 2011; Fischer et al., 2013; Whitmore et al., 2000). We directly tested this possibility by surgical removal of both eyes. In the absence of retinal input, Gal4s1156t+ SINs did not show robust endogenous responses to luminance changes (Figure 5). In only 3 of 12 SINs from enucleated larvae (n = 3 larvae) were any calcium responses observed (Figure 5). These signals likely originated from spontaneous discharges, as they were not consistent across trials and were not synchronized to the visual stimulus. Responses to flashes in control larvae, with eyes present, often occurred within ~300 ms (1–2 frames of imaging acquisition) of stimulus offset, whereas, in enucleated larvae, responses...

FIGURE 6 | SINs are strongly and stably labeled by the LCRB2-RH2-2:GFP transgene. (a) Top view of a 7dpf isl2b:Gal4 × UAS:mCherry × LCRB2-RH2-2:GFP larva. GFP expression is observed in putative SINs as well as in the pineal gland and a subset of photoreceptor cones. (b) Rotated confocal volume of the same larva shown in (a). Note LCRB2-RH2-2:GFP+ SIN cell bodies (green) line are anatomically restricted to SO (RGC axons labeled by mCherry, magenta). (c, d) Fluorescence intensity profile measurements along the yellow lines shown in (c). mCherry signal peaks correspond to SFGS, SGC, and SAC/SPV. (e) GFP expression, analyzed across multiple larvae crossed to either isl2b:Gal4 or atoh7:Gal4 × UAS-mCherry, shows that LCRB2-RH2-2:GFP+ SINs are restricted to SO (n = 20 larvae for isl2b:Gal4, 18 larvae for atoh7:Gal4, 7 dpf). (f) Label of LCRB2-RH2-2:GFP+ SINs is stable over time. Number of GFP+ cells shown in 5 larvae over for each developmental age, respectively. (g–j) A single LCRB2-RH2-2:GFP larva at 5 dpf (g), at 6 dpf (h), at 7 dpf (i), at 8 dpf (j). Scale bars in (a, j) = 50 μm. A, anterior; L, lateral; M, medial; P, posterior. Error bars are SEM.
occurred, if at all, >3 s (>10 frames of imaging acquisition) after the stimulus presentation (Figure 5d). Together, these results suggest that Gal4s1156t SINs are not intrinsically photosensitive.

3.7 The LCRRH2-RH2-2:GFP transgene labels more SINs than Gal4s1156t

The Gal4s1156t line has two significant limitations: it does not label all SINs, and its labeling is not restricted exclusively to the SINs; sparse labeling throughout the brain is also observed in non-SIN cells. Thus, we sought to identify new lines with a broader label of the SIN population and less exogenous expression in non-SIN populations. To this end, we examined LCRRH2-RH2-2:GFP, in which a regulatory element of the zebrafish RH2-2 green opsin drives GFP expression (Fang et al., 2013). This opsin promoter line had been previously reported to label only a subset of tectal neurons, along with restricted expression in green cone photoreceptors and the pineal gland (Fang et al., 2013). Intriguingly, the labeled tectal neurons were similar in morphology and position to the SINs (Figure 6a,b) with a more ubiquitous expression pattern than that observed in the Gal4s1156t line.

As an initial anatomical characterization, we crossed LCRRH2-RH2-2:GFP transgenic fish to fish carrying both isl2b:Gal4 and UAS:mCherry. This allowed us to label RGC axons and the retinorecipient laminae in the tectum. We confirmed that the majority of GFP+ cells in the tectum of LCRRH2-RH2-2:GFP larvae are positioned in the SO and the upper layers of the SFGS (n = 20 fish; Figure 6c–e). This result was confirmed through additional anatomical analysis with a second pan-RGC transgenic line, atoh7:gal4 × UAS:mCherry (Figure 6e). Based on overall morphology and anatomical position, we could confidently identify the LCRRH2-RH2-2:GFP+ cells in the tectum as SINs. Strikingly, these SINs are the only neurons labeled in the brain outside the retina.

GFP expression in the SINs was stable within a single larva over development and consistent among different larvae (Figure 6f–j). Further developmental characterization of the LCRRH2-RH2-2:GFP line demonstrated an increase in the total number of GFP+ SINs from 4 to 15 dpf, likely concomitant with increasing brain size during this developmental window. Taken together, these results position LCRRH2-RH2-2:GFP as a valuable new marker for reliably and specifically targeting SIN populations.

**FIGURE 7** SINs are molecularly heterogeneous. (a–d) Representative examples of LCRRH2-RH2-2:GFP+ SINs, colabeled with previously identified SIN markers. (a) Crossing LCRRH2-RH2-2:GFP (green) to vglut2a:loxP-DsRed-loxP-GFP (magenta) shows a small number of SINs express Vglut2a. Some, but not all, vglut2a+ SINs colocalize with GFP. (b) Crossing LCRRH2-RH2-2:GFP (green) to gad1b:loxP-DsRed-loxP-GFP (magenta) shows that some, but not all, gad1b+ SINs colocalize with GFP. (c) Crossing LCRRH2-RH2-2:GFP (green) to reelin:Gal4 × UAS:RFP (magenta) shows that most GFP+ cells are reelin+. (d) Crossing LCRRH2-RH2-2:GFP to Gal4s1156t × UAS:RFP shows that some, but not all, GFP+ SINs are Gal4s1156t+. (e) Percentage of colocalized LCRRH2-RH2-2:GFP+ SINs with each of the marker lines tested in (a–d) (n = 5 larvae, each condition). (f1–f3) Some, but not all, Gal4s1156t+ SINs colocalize with vglut2a:loxP-DsRed-loxP-GFP (magenta). Individual channels are shown for vglut2a (f1) and Gal4s1156t (f2) signal in addition to the merged image (f3). White arrows denote three examples of colocalization. (g) Some, but not all, Gal4s1156t+ SINs are reelin+. Representative immunostaining of a 8 dpf Gal4s1156t × UAS:EGFP larva stained with anti-GFP (green) and anti-Reelin (magenta). (h) Percentage of colocalized GFP+ SINs in Gal4s1156t with each reelin or vglut2a (reelin, n = 39 SINs in 4 larvae; vglut2a n = 79 SINs in 8 larvae). Scale bar (a–d) = 20 μm, (f, g) = 10 μm. A, anterior; L, lateral; M, medial; P, posterior. White hatched line indicates the neuropil and SPV boundary. Error bars, SEM.
3.8 | SIN population is composed of GABAergic and glutamatergic cells

To further investigate the molecular and neurotransmitter profiles of the LCR^{Rl2}.RH2-2:GFP+ SINs, we crossed it into a genetic background in which glutamatergic, GABAergic or Reelin-positive neurons are labeled (vglut2a:loxP-DsRed-loxP-GFP, gad1b:loxP-DsRed-loxP-GFP, and reelin:Gal4). Analyzing co-expression patterns, we observed that 16% (±3% SEM) of these SINs express Vglut2a (Figure 7a,e), 26% (±5% SEM) Gad1b (Figure 7b,e), and 73% (±3% SEM) Reelin (Figure 7c,e). In addition, 26% (±3% SEM) of the LCR^{Rl2}.RH2-2:GFP+ SINs were also labeled in Gal4s1156t×UAS:RFP fish (Figure 7d,e).

We next examined to what extent Gal4s1156t+ SINs express the vglut2a:loxP-DsRed-loxP-GFP transgene. Again, we found that ~30% of Gal4s1156t+ SINs expressed the DsRed reporter, indicative of glutamatergic identity (Figure 7f,h; n = 23 cells of 79 SINs, 8 larvae). Reelin signal was found in 38% of the Gal4s1156t+ SINs examined (SEM ± 14% in 39 cells from 4 larvae). Conversely, many Reelin-positive SINs were Gal4s1156t-negative (Figure 7g,h). Taken together, these findings show that SINs are highly diverse in their patterns of marker expression and transmitter choice.

4 | DISCUSSION

The SINs were first described in larval zebrafish by Del Bene et al. (2010), who reported that most of them were tuned to detect large (>20°) or whole-field visual stimuli, as opposed to PVNs or RGCs, which responded as a population to stimuli of all sizes. These authors also noted that SINs expressed GABA and gad2b and concluded that they were inhibitory. Ablation studies suggested that inhibition from the SINs contributed to small-size selectivity of PVNs and thus helped tune responses to small prey items by means of a subtractive filter (Del Bene et al., 2010). In the past decade, a substantial body of work has been published, making the SINs one of the best-characterized cell classes in the zebrafish brain. Here we confirm and extend the emerging view that SINs are functionally, morphologically, and molecularly more diverse than initially considered.

4.2 | Morphological heterogeneity of SINs

SINs are often defined by the position of their cell body on the surface of the tectum. Their dendrites have been reported to extend into the SO or the superficial SFGS, where they arborize in a single sublayer and are assumed to receive input from RGC axons terminating in this layer (Del Bene et al., 2010; Preuss et al., 2014). Preuss et al. (2014) identified subtypes of SINs with distinct size tuning based on their neurite stratification pattern: superficially stratified SINs preferentially responded to small moving objects, and the more deeply stratified SINs preferentially responded to large moving objects. Here we describe two separate classes of SINs from the Gal4s1156t+ population, one with a downward and another with an upward-pointing orientation of their dendritic arbor. The latter SIN population resides in SFGS, SGC, or SAC, arborizing in specific SFGS sublayers and does not appear to overlap with any of the previously described SIN populations.

Other authors have also expanded the original SIN definition. Avitan et al. (2017) distinguished between superficial and deep SINs, showing that the superficial population (SINs sensu stricto) showed a lower frequency of spontaneous discharges during development than the deep population (Avitan et al., 2017). Förster et al. (2020) described seven morphotypes of neurons with cell bodies in the deeper layers of the neuropil, often with bistratified or tristratified arbors. These so-called NINs, which correspond roughly to SINs with upward-oriented dendrites described here, showed graded differences in their population tuning compared to the SINs sensu stricto, although both cell classes were preferentially OFF-selective. In the present study, responses to whole-field luminance flashes did not vary measurably across neuropil layers, although the sample size may have been too small to detect a systematic difference. Interestingly, the new LCR^{Rl2}.RH2-2:GFP line labels exclusively SINs sensu stricto and is excluded. Taken together, the available evidence points to at least ten morphological classes of SINs/NINs in the larval zebrafish tectum, three of which are classical SINs (Förster et al., 2020).
| Publication          | Transgenic line | Recording technique         | Stimuli                                      | Responses                                      | Fraction of SINs | Number of SINs |
|---------------------|-----------------|-----------------------------|----------------------------------------------|------------------------------------------------|------------------|----------------|
| Del Bene et al. (2010) | Gal4s1156t      | GCaMP1.6 and GCaMP3 imaging | White bar of varying width, moving forward or backward, ON and OFF flash | Preference for wide over narrow bars          | ND               | 4              |
| Preuss et al. (2014)  | Oh:GCaMP6s      | Electrophysiology plus GCaMP6s imaging | White rectangle of varying size, moving forward or backward | Small-size (4–16") | 0.19             | 133            |
| Yin et al. (2019)    | Gal4s1156t      | Electrophysiology plus GCaMP-HS imaging | Dot of varying size, moving or static       | Preference for large over small dots; unresponsive to static dot | ND               | 338            |
| Hunter et al. (2013) | —               | Oregon Green 488 BAPTA imaging | Black bar (10"), moving in 12 directions     | Non-DS response                                | 0.77             | 180            |
| Abbas et al. (2017)  | Gal4s1156t      | GCaMP5G imaging             | Grating (10"), moving in 12 directions       | Non-DS response                                | 0.93             | 190            |
| Yin et al. (2019)    | Gal4s1156t      | GCaMP-HS imaging            | Black bar (10"), moving in 8 directions      | Non-DS response                                | 0.64             | 304            |
| Dunn et al. (2016)   | elavl3:H2B-GCaMP6s | GCaMP6s imaging         | Black looming disk, expanding at 3 speeds    | Cluster #1: sustained                          | 0.30             | 541            |
| Yin et al. (2019)    | Gal4s1156t      | Electrophysiology          | Dimming flash                                | Transient ON/OFF                               | 0.84             | 92             |
| Barker et al. (this study) | Gal4s1156t      | GCaMP6s imaging            | ON, OFF whole-field steps; dark or bright looming disk (20"/s) | OFF preference | 0.29             | 146            |
| Publication            | Transgenic line | Recording technique   | Stimuli | Responses                  | Fraction of SINs | Number of SINs |
|-----------------------|-----------------|-----------------------|---------|----------------------------|------------------|----------------|
| Barker et al. (this study) | Gal4s1156t      | Electrophysiology     | ON and OFF flash | OFF preference | 0.86            | 7              |
|                       |                 |                       |         | Equal response to ON and OFF | 0.14            |                |
| **Combined responses** |                 |                       |         |                            |                  |                |
| Förster et al. (2020) | elavl3:H2B-GCaMP6s | GCaMP6s imaging of SINs | Dark ramp | 5° dot forward/backward | 0.05            | 63             |
|                       |                 |                       | Bright ramp | 5° dot backward | 0               |                |
|                       |                 |                       | Dark flash | 5° dot forward | 0.02            |                |
|                       |                 |                       | Bright flash | 5°/30° dot forward/backward | 0.05            |                |
|                       |                 |                       | 5° dot forward | 30° dot backward | 0.03            |                |
|                       |                 |                       | 5° dot backward | 5°/30° dot backward | 0.03            |                |
|                       |                 |                       | 30° dot forward | Looming slow | 0.1             |                |
|                       |                 |                       | 30° dot backward | 30° dot forward | 0               |                |
|                       |                 |                       | 5° grating forward | 30° dot fwd/bwd + looming slow | 0.43            |                |
|                       |                 |                       | 5° grating backward | Dark flash | 0.08            |                |
|                       |                 |                       | Looming fast | Dark ramp | 0.08            |                |
|                       |                 |                       | Looming slow | Looming fast/slow + dark ramp/ flash | 0.11            |                |
|                       |                 |                       | Bright ramp | 0.03            |                |
|                       |                 |                       | Bright flash | 0               |                |
| Förster et al. (2020) | elavl3:H2B-GCaMP6s | GCaMP6s imaging of NINs | Dark ramp | 5° dot forward/backward | 0.06            | 87             |
|                       |                 |                       | Bright ramp | 5° dot backward | 0.01            |                |
|                       |                 |                       | Dark flash | 5° dot forward | 0.03            |                |
|                       |                 |                       | Bright flash | 5°/30° dot forward/backward | 0.06            |                |
|                       |                 |                       | 5° dot forward | 30° dot backward | 0.03            |                |
|                       |                 |                       | 5° dot backward | 5°/30° dot backward | 0               |                |
|                       |                 |                       | 30° dot forward | Looming slow | 0.03            |                |
|                       |                 |                       | 30° dot backward | 30° dot forward | 0.01            |                |
|                       |                 |                       | 5° grating forward | 30° dot fwd/bwd + looming slow | 0.17            |                |
|                       |                 |                       | 5° grating backward | Dark flash | 0.05            |                |
|                       |                 |                       | Looming fast | Dark ramp | 0.08            |                |
|                       |                 |                       | Looming slow | Looming fast/slow + dark ramp/ flash | 0.33            |                |
|                       |                 |                       | Bright ramp | 0.07            |                |
|                       |                 |                       | Bright flash | 0.06            |                |
| **Spontaneous activity** |                 |                       |         |                            |                  |                |
| Avitan et al. (2017)  | elavl3:H2B-GCaMP6s | GCaMP6s imaging       | Spontaneous activity | Low frequency (superficial) | ND              | ND             |
|                       |                 |                       |         | High frequency (deep) = NINs | ND              |                |
4.3 Marker and transmitter heterogeneity of SINs

In retina and visual cortex, functional diversity is mirrored by an abundance of genetically defined cell types. The commonly used Gal4s1156t enhancer-trap line labels only a subpopulation of the SINs (approx. 10%; Abbas et al., 2017). It is unclear if this pattern is entirely due to variegation (stochastic gene expression, a commonly observed phenomenon with randomly integrated transgenes; Goll et al., 2009) or if different SIN cell types exist, only some of which stochastically express Gal4s1156t. Previous work has discovered additional SIN markers. Here we confirm that many SINs express the GABA-synthesizing enzyme Gad1b (Del Bene et al., 2010), but report for the first time the presence of a substantial minority of Vglut2a+ SINs, an indicator of glutamatergic identity. Glasauer et al. (2016) identified a SIN population expressing an mGluR6b:EGFP transgene. The metabolotropic glutamate receptor mGluR6b inverts the sign of excitatory transmission; mGluR6b+ SINs are thus expected to be inhibited by RGC input (Glasauer et al., 2016). These findings suggest a diversity of signaling mechanisms: SINs may either inhibit or excite their postsynaptic cells, or be inhibited or excited by presynaptic cells.

Del Bene et al. (2010) already showed that a large proportion of SINs express reelin, a finding corroborated by labeling in the reelin: Ga4 transgenic line investigated here. Preuss et al. (2014) reported that their Oh:Ga4 enhancer-trap line labeled two populations of differentially tuned SINs with divergent stratification patterns. In our quest for more comprehensive markers, we found that a LCRRH2-RH2-2:GFP transgene (Fang et al., 2013) labels substantially more, but still not all, SINs. Significantly, this line shows robust SIN labeling which is highly stable within a single larva over the first two weeks of development and across larvae, making it particularly attractive for targeting in future electrophysiological studies. The lack of complete overlap between molecularly defined populations of SINs may be due in part to variegation within the Gal4 lines (i.e., reelin:Ga4 and Gal4s1156t) or the incomplete labeling of glutamatergic and GABAergic populations within the vglut2a:loxP-DsRed-loxP-GFP and gad1b:loxP-DsRed-loxP-GFP lines. However, stochastic labeling cannot fully account for the molecular diversity of SINs observed. We found that none of the markers labels all SINs, and none of the markers appears to completely overlap with any of the other markers, suggesting that SINs are molecularly more heterogeneous than previously anticipated.

4.4 Retinal origin of visual responses

Extraretinal opsins are found in the zebrafish brain (Fischer et al., 2013), and deep brain photoreceptors have been shown to influence light-mediated behavior (Fernandes et al., 2012; Fischer et al., 2013; Fontinha et al., 2020). Moreover, the specific SIN marker LCRRH2-RH2-2:GFP was generated by linking GFP to the green cone opsin enhancer. We tested the possibility of intrinsic light responses by enucleation. In the absence of retinal input, Gal4s1156t+ SINs did not respond to whole-field ON or OFF flashes, suggesting they are not intrinsically photosensitive, at least under the experimental conditions employed here.

This result raises the question of how tuning to luminance changes emerges in the SIN population. We propose that a direct retinon synaptic input from OFF-selective RGCs is likely, although this remains hypothetical. Electrophysiological recordings can give us a clue—we see delays between visual stimulus presentation and onset of activity on the order of 100 ms. In other species, monosynaptic delays in the millisecond range have been reported between retina and tectum, and between retina and thalamus (Matsumoto & Bando, 1980; Usrey et al., 1998). However, delays between visual stimulus onset and OFF RGC spiking can be 100 ms in mouse retina (Gollisch & Meister, 2008), similar to the response latencies observed in SINs. One possibility for testing a direct RGC-SIN synapse would require electrical stimulation of RGC axons combined with electrophysiological recordings of SINs, but would likely prove difficult due to the small size of the optic tract and the short distance to the tectum in the zebrafish larva. Better still, electron microscopy reconstruction of the larval zebrafish, which has been performed for other circuits (Helmstaedter et al., 2013; Wanner et al., 2016), should provide a definitive answer regarding the presynaptic connectivity of the SINs.

4.5 Are SINs more like the horizontal or the amacrine cells of the retina?

Based on their positions close to the retinal input layers and their monostratified morphologies, SINs have been likened to tectal horizontal cells in the older literature (Langer & Lund, 1974; Luksch & Golz, 2003; Meek & Schellart, 1978). Retinal horizontal cells allow for adaptation to overall luminance levels by acting directly on photoreceptors via feedback inhibition and on bipolar cells via feedforward inhibition (Perlmutter et al., 1995). In the mouse retina, targeted ablation of horizontal cells results in reductions of contrast sensitivity across all spatial frequencies tested in an optomotor assay and deficits in overall visual acuity (Sonntag et al., 2012). Similar gain control functions may be important for all levels of visual processing, and the SINs may play such a general-purpose role in the tectum, for instance, allowing it to operate under different luminance conditions.

Judging by the accumulated evidence over the past decade, however, we favor a more nuanced view of SIN function. Their tremendous molecular, functional, transmitter, and morphological diversity suggests that they act as highly specialized filters for the visual feature channels that reach the nine neuropil layers of the tectum (for a review of the functional anatomy, see Baier, 2013). A comparison to cortex or retina may be instructive, where diverse interneuron populations have been shown to operate in specialized microcircuits (Hangya et al., 2014; Jadzinsky & Baccus, 2013; Masland, 2012). In this scenario, different SIN types may be dedicated to the parallel processing of distinct visual features, more similar to the amacrine cells than the horizontal cells of the retina.
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CONFLICT OF INTEREST

The authors declare no competing financial interests.

AUTHOR CONTRIBUTIONS

AJB and HB designed the research; AJB, TOH, and AG performed experiments and analyzed data; AJB and HB wrote the article with input from the other authors.

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DATA AVAILABILITY STATEMENT

All raw data included in this manuscript will be made available by the authors upon request.

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