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Zebrafish *Tg(7.2mab21l2:EGFP)ucd2* Transgenics Reveal a Unique Population of Retinal Amacrine Cells

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Abstract

Purpose—Amacrine cells constitute a diverse, yet poorly characterized, cell population in the inner retina. Here, the authors sought to characterize the morphology, molecular physiology, and electrophysiology of a subpopulation of EGFP-expressing retinal amacrine cells identified in a novel zebrafish transgenic line.

Methods—After 7.2 kb of the zebrafish *mab21l2* promoter was cloned upstream of EGFP, it was used to create the *Tg(7.2mab21l2:EGFP)ucd2* transgenic line. Transgenic EGFP expression was analyzed by fluorescence microscopy in whole mount embryos, followed by detailed analysis of EGFP-expressing amacrine cells using fluorescence microscopy, immunohistochemistry, and electrophysiology.

Results—A 7.2-kb fragment of the *mab21l2* promoter region is sufficient to drive transgene expression in the developing lens and tectum. Intriguingly, EGFP was also observed in differentiated amacrine cells. EGFP-labeled amacrine cells in *Tg(7.2mab21l2:EGFP)ucd2* constitute a novel GABA- and glycine-negative amacrine subpopulation. Morphologically, EGFP-expressing cells stratify in sublamina 1 to 2 (type 1 OFF) or sublamina 3 to 4 (type 1 ON) or branch diffusely (type 2). Electrophysiologically, these cells segregate into amacrine cells with somas in the vitreal part of the INL and linear responses to current injection or, alternatively, amacrine cells with somas proximal to the IPL and active oscillatory voltage signals.

Conclusions—The novel transgenic line *Tg(7.2mab21l2:EGFP)ucd2* uncovers a unique subpopulation of retinal amacrine cells.

Amacrine cells constitute approximately 40% of all neurons in the inner nuclear layer (INL).¹ They are retinal interneurons, predominantly located in the INL, that synapse in the adjacent inner plexiform layer (IPL). In birds and mammals, displaced amacrine cells are also found in the ganglion cell layer (GCL). In general, amacrine cells function in retinal circuitry, mediating horizontal processing of neuronal signals delivered from bipolar to ganglion cells.² However, retinal amacrine cells can be divided into many subtypes, and the precise function of only a few is understood.² For example, rodent starburst amacrine cells

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are responsible for directional selectivity of ganglion cells and the optokinetic response, whereas AII amacrine cells transmit rod signals to cone bipolar cells.³⁻⁵

The amacrine subtypes are classified based on morphology, physiology, and molecular markers.^{6,7} Approximately 24, 28, and 28 morphologic subtypes are distinguished in human, rabbit, and zebrafish retinas, respectively⁸⁻¹¹ (Connaughton VP, et al. *IOVS* 2007;48:ARVO E-Abstract 5945). Morphologic distinctions are based on soma size, number of dendritic stratifications (mono-, bi-, or tri-stratified or diffuse), the size of dendritic fields (narrow-, medium-, and wide-field), and the location of dendritic stratifications within the IPL (sublamina s1-5).^{8-10,12} This diversity may restrict visual signaling by limiting the synaptic interactions of specific subtypes or may reflect the dedication of subtypes to specific tasks.^{2,10} Physiologically, amacrine cells segregate into two groups based on response to synaptic input: cells that spike and cells that show only graded changes in potential.¹³ Based on dendritic tree stratifications, amacrine cells can also be classified as OFF and ON subtypes. These stratify within sublamina s1-2 or s3-5 of the IPL, respectively. However, many narrow- and medium-field amacrine cells synapse in both layers.^{9,10} Finally, amacrine cells can be subdivided based on the expression of molecular markers. Known neurotransmitters include γ -aminobutyric acid (GABA), glycine, acetylcholine, serotonin, and dopamine.^{14,15} GABA and glycine are the main inhibitory neurotransmitters in amacrine cells. For example, in zebrafish, the relative proportion of GABAergic to glycinergic amacrine cells is 64:35, with a small percentage negative for both.¹⁴ Other molecular markers include the calcium-binding proteins parvalbumin and calretinin, glutamate vesicular transporter 3, and aquaporin 1 and 9.¹⁴⁻¹⁷

Vertebrate *mab21-like* genes (*mab21l1* and *mab21l2*) are expressed in the eye primordia, midbrain, branchial arches, neural tube, and limb buds during development.¹⁸⁻²² The function of vertebrate *Mab21-like* genes is largely unknown, but loss-of-function studies in mice and zebrafish suggest a role in cell proliferation.^{18,22,23} Both murine *Mab21l1* and *Mab21l2* have essential, but distinct, developmental roles—particularly in eye development. *Mab21l2* mouse knockouts are embryonic lethal and have severely underdeveloped retinas.¹⁸ In contrast, *Mab21l1* mouse knockouts survive to adulthood but fail to develop lenses.²² Here we isolated a 7.2-kb fragment of the zebrafish *mab21l2* promoter region and used it to generate a novel transgenic zebrafish line expressing EGFP. *Tg(7.2mab21l2:EGFP)ucd2* zebrafish discriminate a unique GABA-/glycine-negative subpopulation of EGFP-positive amacrine cells with distinct morphology and electrophysiology. This was unexpected because *mab21l2* is not known to be expressed in retinal amacrine cells, and 99% of all amacrine cells express either GABA or glycine.¹⁴

Methods

Creation of the *mab21l2* Promoter/EGFP Construct

A 7.2-kb fragment of the zebrafish *mab21l2* promoter region was PCR amplified from BAC clone zk257N17 (Imagenes, Nottingham, UK) using a proofreading polymerase (forward primer, mc065 5'-ACG TCG ACA TTC AAC TCA CTG TAT GCC-3'; reverse primer, mc032 5'-TGG GCA CAG ATC CGG ACT GTA GAC-3'). PCR products were digested with *SalI* and *BamHI* and were subcloned into the *pT2KXIGΔ* Tol2 transposon vector digested with *XhoI* and *BamHI* generating the *Tg(7.2mab21l2:EGFP)* transgene.^{24,25} The integrity of the PCR insert in the recombinant vector was confirmed by DNA sequencing.

Generation and Analysis of *Tg(7.2mab21l2:EGFP)ucd2*

Tg(7.2mab21l2:EGFP) was injected into zebrafish embryos at the single-cell stage with 2.5 ng/ μ L Tol2 transposase mRNA added to the injection mix. The transgenic line was created

by injecting the *Tg(7.2mab21l2:EGFP)* transgene and using the Tol2 transposon system. Transposase mRNA was synthesized using a kit (mMESSAGE mMACHINE; Amersham Biosciences, Piscataway, NJ) according to the manufacturers' instructions using *NotI*-digested *pCS-TP* vector as template. The final concentration of synthesized RNA was adjusted to 100 ng/ μ L. After each injection day, approximately five embryos were removed to confirm excision of the *mab21l2* promoter fragment by the transposase.²⁶ Sixty to 100 offspring from nine putative founders were screened under a fluorescence microscope at approximately 24 hpf, and a carrier was identified that transmits the transgene to approximately one-third of its offspring. The *EGFP* expression pattern in the confirmed transgenic line, *Tg(7.2mab21l2:EGFP)ucd2*, was analyzed from larval stages to adulthood. All analyses were performed using a stereomicroscope (Lumar V12; Zeiss, Thornwood, NY) or a confocal microscope (LSM S10; Zeiss).

Immunohistochemistry

Adult eyes from *Tg(7.2mab21l2:EGFP)ucd2* were fixed for 30 minutes in 4% paraformaldehyde (PFA) at room temperature. The front of the eye was removed and the eyes were left in 4% PFA at 4°C overnight before infiltration with 20% sucrose and OCT embedding medium. Embryos at 5 days postfertilization (dpf) were fixed in 4% PFA at 4°C overnight before OCT embedding. Blocks were sectioned at 12 μ m, and sections allowed to air dry for 30 minutes. Sections were rehydrated in PBS for 15 minutes before incubation in blocking buffer (5% goat serum, 0.5% Tween-20, 0.5% Triton X-100 in PBS) for all antibodies except anti-choline acetyltransferase (5% calf serum, 0.5% Tween-20, 0.5% Triton X-100 in PBS). Before blocking solution was added, sections to be stained with either choline acetyltransferase or serotonin antibodies were boiled in 10 mM sodium citrate (pH 6) for 10 minutes for antigen retrieval.²⁷ Sections were incubated in primary antibodies in blocking buffer overnight at 4°C. The following antibodies and dilutions were used: anti-5E11 (1:50; a kind gift from James Fadool, Florida State University), anti-GAD65/67 (1:800; Abcam, Cambridge, MA), anti-calretinin (1:5000; a kind gift from Marius Ader University of Technology [TU], Dresden, Germany), anti-parvalbumin (1:500; Chemicon, Temecula, CA), anti-tyrosine hydroxylase (1:100; Chemicon), anti-choline acetyltransferase (1:30; Chemicon), and antiseroitonin (1:250; Chemicon). Sections were incubated in secondary antibodies for 1 to 2 hours at room temperature. Secondary antibodies used were Cy3-labeled anti-mouse, anti-rabbit, anti-goat, or anti-rat (1:200; Jackson Immuno Research, West Grove, PA). Sections were analyzed with a confocal microscope (LSM S10; Zeiss).

Cell Morphology Analyses

Adult whole mount retinas were dissected and fixed in 4% PFA at 4°C overnight before being placed on depression slides. Adult *Tg(7.2mab21l2:EGFP)ucd2* retinal sections were prepared as for the immunohistochemistry analysis. Mosaic larvae were generated by injecting the *Tg(7.2mab21l2:EGFP)* construct as described, but without transposase mRNA. Larvae 5 dpf were processed as described for immunohistochemistry and sectioned at 40 μ m. All samples were analyzed with a confocal microscope (LSM 510; Zeiss). For IMARIS analysis, confocal z-stacks were imported into IMARIS 7.2, and 3D reconstructions of cell dendrites were performed using the filament tracing module.

Single-Cell Electrophysiology and Imaging

Fish were dark adapted for at least 20 minutes, anesthetized in a 0.02% tricaine solution until gill movements stopped, and then decapitated. Retinas were removed from the eyes. For whole-cell voltage-gated recordings, retinas were treated with hyaluronidase in Ringers for 20 minutes before being placed vitreal-side down on a filter paper. For whole-cell current clamp recordings, retinas were placed directly vitreal-side down on filter paper.

Filter with retina was mounted in the recording chamber between two petroleum jelly (Vaseline; Unilever, London, UK) strips and submerged in the standard extracellular (Ringers) solution. The filter with retina was then cut into 100- μ m sections. Each section was rotated 90° and viewed in cross-section.²⁸

Retinal slices were perfused with extracellular Ringers supplemented with 2.5 CaCl₂ at 0.5 mL/min and visualized under oblique illumination through a 60× objective on an upright microscope (Ex51WI; Olympus, Melville, NY) for current clamp recordings or a 40× water immersion lens and microscope (BX50WI; Olympus) for voltage clamp recordings. Whole cell current clamp recordings were obtained from EGFP-positive somata using 8 to 12 M Ω patch electrodes pulled from borosilicate glass (GC120F-10; Harvard Apparatus, Kent, UK). The intracellular solution contained 104 mM K-gluconate, 8 mM KCl, 2 mM MgCl₂, 4 mM HEPES, 0.5 mM EGTA, 2 mM MgATP, 1 mM NaGTP, 1 mM NacGMP (pH 7.4, 250 Osm), and 50 μ M Alexa 594 (Molecular Probes, Invitrogen Ltd., Paisley, UK) to reveal the morphology of recorded cells. Series resistance ranged from 8 to 15 M Ω , whereas input resistance was >1 G Ω at -70 mV. Electrophysiological recordings were corrected for junction potentials, calculated to be -11.95 mV. Signals were recorded using an amplifier (Axopatch 200B; Molecular Devices, Sunnyvale, CA) with an ITC-16 interface (HEKA, Lambrecht, Germany) and digitized at 20 kHz in Pulse Control (developed by Richard Brockman) running under graphing and data analysis software (Igor-Pro 6.1; Wavemetrics, Lake Oswego, OR). A water-cooled EM-CCD camera (ImageM; Hamamatsu, Iwata City, Japan) was used to record fluorescence. A 1-A 470 nm light-emitting diode (LED) and a 300-mA 590 nm LED (K2; Luxeon, San Jose, CA) served as light sources for GFP and Alexa 594, respectively.

Whole-cell voltage-gated currents were recorded in response to voltage steps from a holding potential of -60 mV (-80 to +60 mV, 10-mV increments). Recordings were made using a standard external Ringers solution, and a K-based pipette solution (12 mM KCl, 104 mM K-gluconate, 1 mM EGTA, 4 mM HEPES, and 100 μ M CaCl₂, to pH 7.4–7.5 using KOH). Alexa Red was present in the patch pipette to label recorded neurons. Labeled cells were photographed using a camera (Oly-150; Olympus) and video frame-grabber software (Flash-Bus; Integral Technologies, Indianapolis, IN). Patch electrodes were made of thin-walled borosilicate glass pulled to 1- μ m tip diameter. Recordings were made using a patch clamp amplifier (AxioPatch 1D; Molecular Devices) and software (pCLAMP, version 9.0; Molecular Devices) software. Traces were analyzed (pCLAMP, version 9.0; Molecular Devices); graphs were made with statistical software (SigmaPlot, version 6; Sigma, St. Louis, MO).

Animal Use

All procedures involving animals were carried out in accordance with the ARVO Statement for Use of Animals in Ophthalmic and Vision Research and were approved by the University College Dublin (Dublin, Ireland) Animal Research Ethics Committee, the American University (Washington, DC) Animal Care and Use Committee, and the MRC Laboratory of Molecular Biology (Cambridge, UK) Ethical Review Committee.

Results

***Tg(7.2mab21l2:EGFP)ucd2* Drives EGFP Expression in *mab21l2* Expression Domains**

An approximately 7.2-kb fragment of the zebrafish *mab21l2* promoter region was cloned upstream of *EGFP*. A stable transgenic line, *Tg(7.2mab21l2:EGFP)ucd2*, was generated using this construct to characterize the expression patterns directed by the promoter fragment (Fig. 1). During development, *Tg(7.2mab21l2:EGFP)ucd2* larvae exhibited

transgene expression in known *mab2l12* expression domains, including the midbrain/tectum starting at approximately 13 somites (~14 hpf), the neural tube starting at approximately 24 hpf, and branchial arches starting at approximately 48 hpf (Figs. 1A, 1C, 1D, 1G, 1I, 1J, 1L). Unexpectedly, *Tg(7.2mab2l12:EGFP)ucd2* larvae do not direct EGFP expression in the primitive retina at approximately 14 hpf, a stage at which endogenous *mab2l12* is strongly expressed in this tissue (Figs. 1A–C).²¹ Other unanticipated results were that *Tg(7.2mab2l12:EGFP)* directs expression in the developing lens and olfactory bulbs starting at approximately 24 hpf (Figs. 1E, 1F) and, of most relevance here, directs robust reporter expression in a subset of inner retinal neurons at 3 to 5 dpf and in adult retinas (Figs. 1H, 1K, 2, 3). These are not known expression domains of *mab2l12* and likely reflect the activity of the promoter fragment in isolation.

***Tg(7.2mab2l12:EGFP)*-Labeled Amacrine Cells Colocalize with Calretinin and Parvalbumin**

Confocal microscopy of retinal sections from 5 dpf *Tg(7.2mab2l12:EGFP)ucd2* zebrafish demonstrated that the EGFP-positive cells in the INL had dendrites primarily localizing to two bands within the IPL (Fig. 1K), suggesting they were amacrine cells; this was subsequently confirmed (Figs. 2–4). To characterize these cells, *Tg(7.2mab2l12:EGFP)ucd2* retinal sections were labeled with antibodies that distinguish amacrine subtypes. EGFP-positive soma do not colocalize with GABAergic (anti-GAD65/67)–, glycinergic (anti-Gly)–, dopaminergic (anti-TH)–, serotonergic (anti-5HT)–, or cholinergic (anti-ChAT)–positive amacrine cell soma or with anti-5E11, an unknown epitope expressed by most amacrine cells (Figs. 2C–H; Table 1). However, the calcium transporters calretinin and parvalbumin do colocalize with approximately 76% and approximately 70% of EGFP-positive soma, respectively (Figs. 2A, 2B; Table 1). Less than 10% of the calretinin or parvalbumin positive cells coexpressed EGFP.

EGFP-Expressing Amacrine Cells Can Be Subclassified Based on Morphology and Electrophysiology

To further characterize the EGFP-positive amacrine cells, morphologic analyses were performed by confocal microscopy and 3D dendrite tracing. Flat mount images of adult *Tg(7.2mab2l12:EGFP)ucd2* retinas indicated that the EGFP-positive cells were evenly distributed across the retina and accounted for <5% of INL cells (Fig. 3A). In the retinal flat mounts, 3D reconstruction of dendritic morphology showed that the EGFP-positive amacrine cells contained processes that collectively spanned the length and width of the area of retina examined (Fig. 3B). Confocal images of retinal sections from *Tg(7.2mab2l12:EGFP)ucd2* adults identified three characteristic morphologies of the EGFP-expressing amacrine cells. Their dendrites projected only from the soma in the INL toward the synaptic layers in the IPL, typical of amacrine cells. Two morphologic subtypes were distinguished based on location of the EGFP-labeled cell soma (Figs. 3C, 4K). Type 1 cells had somas in the middle of the INL, and type 2 cells had somas in proximity to the IPL (Figs. 3C, 4K). Type 1 amacrine cells could be further subtyped into type 1 OFF cells, which primarily stratified in sublamina 1 to 2, and type 1 ON cells, which primarily stratified in sublamina 3 to 4 (Fig. 3C). Type 2 cells are diffuse amacrine cells with dendrites throughout the IPL (Fig. 3C). The somas of EGFP-labeled amacrine cells have an average soma length of ~6.4 μm (6.0 – 6.7 μm) and an average soma width of approximately 5.0 μm (4.6–5.4 μm). Compared with the stratification pattern for ChAT, the main EGFP-positive stratifications are located at the same level as the upper ChAT-positive band and just above the lower second band (Figs. 4A–C). It is likely that type 1 ON cells corresponded to cells coexpressing EGFP and calretinin, with somas in the mid-INL and monostratified in sublamina 3 to 4 (Fig. 4K).

To analyze the morphology of individual EGFP-expressing amacrine cells, embryos were injected with the *Tg(7.2mab21l2:EGFP)* transgene. At 5 dpf, retinal sections from the corresponding mosaics were analyzed by confocal microscopy and 3D dendrite tracing (Figs. 4D–J). Although soma location could not be used to predict cell morphology, stratification patterns corresponding to the adult type 1 OFF (Figs. 4D, 4E, 4G, 4J, Supplementary Movie S1, <http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-5376/-/DCSupplemental>), type 1 ON (Figs. 4D, 4E, 4H, 4J, Supplementary Movie S2, <http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-5376/-/DCSupplemental>), and type 2 (Figs. 4D, 4F, 4I, 4J, Supplementary Movie S3, <http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-5376/-/DCSupplemental>) EGFP-positive amacrine cells were observed in the mosaic larval retinas. These analyses confirmed that this labeled subset of amacrine cells have either medium-field stratifications (type 1) or narrow-field stratifications (type 2; Figs. 4G, 4H, Supplementary Movies S1–S3, <http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-5376/-/DCSupplemental>).¹¹ An intriguing morphology is revealed for one type 1 ON cell that largely stratifies in the INL but then takes an acute turn through a gap in the ganglion cell layer that opens onto the lens (Fig. 4E).

Electrophysiological recordings of EGFP-positive amacrine cells in adult *Tg(7.2mab21l2:EGFP)ucd2* zebrafish indicate distinct physiological properties of type 1 and type 2 cells (Figs. 5A, 5B). Type 1 cells have a resting membrane potential of approximately -40 mV and respond linearly to depolarizing current injection in current clamp mode (data not shown). Voltage clamp recordings from five type 1 cells identify two depolarization-elicited outward currents: slowly activating, sustained currents (I_K) and rapidly activating, transient currents (I_A) (Fig. 5A). In contrast, current clamp recordings of seven type 2 cells reveal active voltage signals. These cells have a resting membrane potential of approximately -70 mV and exhibit fast (100–150 Hz), large (> 30 mV), and regular membrane potential oscillations on depolarizing current injection (Fig. 5B). Overall, the morphologic and physiological analyses of the EGFP-positive amacrine cells indicate that they are composed of three unique amacrine subtypes.

Discussion

Our results identify a *mab21l2* promoter fragment that distinguishes a unique subpopulation of retinal amacrine cells. Amacrine cells are a diverse and poorly understood cell population in the retina. Most zebrafish amacrine cells express GABA or glycine neurotransmitters, though dopaminergic interplexiform cells are a known exception.¹⁴ Interestingly, the EGFP-positive amacrine cells in *Tg(7.2mab21l2:EGFP)ucd2* do not coexpress GABA, glycine, or dopamine, indicating they are a unique subtype. GABA- and glycine-negative amacrine cells are present in other vertebrate retinas, but their function is unclear and their characterization limited.^{29,30} Here, we characterize the molecular biology, morphology, and electrophysiology of the GABA- and glycine-negative amacrine subpopulation labeled in *Tg(7.2mab21l2:EGFP)ucd2*.

Zebrafish amacrine cells have been classified based on molecular markers, morphology, and electrophysiology. However, in general, these studies are performed in isolation, and it is difficult to correlate morphologic and molecular subclasses of amacrine cells with physiological properties. Molecularly, the *Tg(7.2mab21l2:EGFP)*-labeled amacrine cells described here co-label with calretinin and parvalbumin but not GABA or glycine. Morphologically, these EGFP-expressing amacrine cells can be divided into medium-field, monostratified type 1 cells and narrow-field, diffuse type 2 cells. Type 1 amacrine cells can be further divided into subtypes that predominantly stratify in either sublamina 1 to 2 (type 1 OFF) or in sublamina 3 to 4 (type 1 ON). Type 1 ON amacrine cells have a morphology similar to that of the previously described A_{on-s4} zebrafish amacrine cell.¹² This cell is monostratified in

sublamina 3 to 4, though the soma appears to be located closer to the IPL.¹² Type 1 ON cells also have a morphology similar to that of the previously described pyriform, calretinin-positive amacrine cells that monostratify in sublamina 3 to 4 and whose somas are in the vitreal aspect of the zebrafish INL.¹⁵ Type 2 cells potentially correspond to the previously described diffuse A_{diffuse}-1 to 2 cells.¹² As with type 2 cells, their somas are located proximal to the IPL. A recent morphologic study of zebrafish amacrine cells revealed 28 inhibitory cell types.¹¹ Several of these have somas either in the middle INL or proximal to the IPL and dendrites branching in soma 1 to 2 or 3 to 4. Further characterization will be required to reveal which, if any, of these correspond to the EGFP-expressing cells described here.¹¹ Similarly, parvalbumin-expressing amacrine cells have been described in the zebrafish retina and appear to stratify mainly in either sublamina 3 or 4 to 5 of the IPL.³¹ Although approximately 70% of the EGFP-positive amacrine cells in *Tg(7.2mab2112:EGFP)ucd2* zebrafish coexpress parvalbumin, we find no evidence that the parvalbumin-positive, EGFP-positive cells correspond to any of these described populations. In summary, we define molecular markers and cell morphologies associated with the poorly characterized GABA- and glycine-negative subpopulation of amacrine cells.

Electrophysiologically, type 1 cells with somas in the INL respond linearly to current injection, whereas type 2 cells with somas in proximal INL generate active voltage oscillations. I_K is a delayed rectifying potassium current found in type 1 amacrine cells. It is a slowly activating current that is sustained. The transient I_A current found in type 1 cells is a rapidly activating and inactivating outward potassium current. Both are elicited in response to membrane depolarization. Both current types have been identified in zebrafish bipolar cells.³² There are no previous reports of zebrafish amacrine cell recordings matching those of type 1 or 2 cells. However, out-ward K^+ currents have been identified in mouse, rat, and salamander amacrine cells.³³⁻³⁶ In particular, mouse cholinergic amacrine cells express either I_K or I_A currents,³³ similar to our findings here. Voltage oscillations similar to those exhibited by type 2 cells have been recorded in wide-field amacrine cells in the bass retina.^{37,38} In conclusion, we demonstrate that the two morphologic subtypes of GABA- and glycine-negative amacrine cells, labeled in *Tg(7.2mab2112:EGFP)ucd2* zebrafish, have distinct electrophysiological properties that correlate with previous recordings of amacrine cell subtypes.

Several transgenic zebrafish lines with reporter gene expression in amacrine cells have been created. The *ptf1a::GFP* transgenic line, in which GFP replaces pancreas transcription factor 1a (*ptf1a*) in a genomic clone, expresses GFP in most amacrine cells.³⁹ *Tg(eno2:GFP)*, under control of an approximately 12-kb promoter fragment of the enolase-2 gene, expresses GFP in many central nervous system cells, including retinal ganglion cells, amacrine cells, and rod photoreceptors.⁴⁰ Some of the GFP-expressing amacrine cells coexpress tyrosine hydroxylase. *Tg(-12th:MmGFP)*, under control of an approximately 12-kb promoter fragment of the tyrosine hydroxylase, expresses GFP in two cell subpopulations in the INL, of which approximately 30% are dopaminergic neurons.⁴¹ *Pax6-DF4:mGFP* transgenic zebrafish express membrane-targeted GFP in subpopulations of amacrine cells whose dendrites localize to sublamina 2 and 4 of the IPL and that coexpress marker 5E11.^{39,42} However, none of these lines demarcate the same subset of GABA-, glycine-, dopamine-, and 5E11-negative amacrine cells uncovered in the *Tg(7.2mab2112:EGFP)ucd2* transgenic line. Thus, the *Tg(7.2mab2112:EGFP)ucd2* line is a novel tool enabling the characterization of a unique population of amacrine cells. It will be interesting to characterize the molecular genetics of the EGFP-expressing amacrine cells in the *Tg(7.2mab2112:EGFP)ucd2* line. One approach enabled by our transgenic line is to characterize their transcriptome and compare it to the molecular signatures of individual amacrine cells recently analyzed in mice by single-cell microarrays.²⁹ Preliminary analyses indicate that EGFP-expressing amacrine cells can be purified by fluorescence-activated cell sorting from dissociated

Tg(7.2mab2112:EGFP)ucd2 retinas and that they represent approximately 0.6% of sorted retinal cells (Vendrell V, et al., unpublished data, 2009).

Even though the isolated promoter fragment drives expression in adult amacrine cells, these cells do not appear to express *mab2112*.⁴³ The integration site of the transgene is unlikely to be a determinant of the transcriptional activity of the 7.2 *mab2112* promoter fragment in amacrine cells because mosaic fish transiently injected with the *Tg(7.2mab2112:EGFP)* transgene also exhibit expression in these cells. We therefore hypothesize that the isolated fragment contains regulatory elements that drive expression in amacrine cells, but additional gene regulatory mechanisms prevent endogenous *mab2112* expression in this area. These could be *cis*-silencers located further upstream or downstream of the *mab2112* coding exon.

In summary, this study describes a new zebrafish transgenic line, *Tg(7.2mab2112:EGFP)ucd2*, within which the morphology, molecular biology, and electrophysiology of a novel subpopulation of GABA- and glycine-negative amacrine cells was characterized.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

EGFP expression in the stable transgenic line *Tg(7.2mab2l2:EGFP)ucd2*. Epifluorescent images of whole mount transgenic larvae (A–L). At approximately 13 somites, *EGFP* expression is observed prominently in the optic tectum and weakly in the lens and is missing in the retinal primordia (A–C). At approximately 28 hpf, *EGFP* expression remains strong in the tectum (D, *inset*), has increased in the central lens (E), and is initiated in olfactory bulbs (F). At approximately 3 to 5 dpf, strong *EGFP* expression is observed in the branchial arches, pectoral fins, eye, and optic tectum (G–L). An optical section through the eye at approximately 5 dpf identifies *EGFP*-expressing cells in the INL, subsequently identified as amacrine cells (K). NT, neural tube; AC, amacrine cell; BA, branchial arch; OB, olfactory

bulb. *Yellow arrowhead*: tectum. (**A**, **C**, *inset* in **D**, **I**, **K**, **L**) Dorsal views. (**F**) Front view. (**B**, **D**, **E**, **G**, **H**, **J**) Lateral views.

Figure 2.

Tg(7.2mab2l12:EGFP)-labeled amacrine cells constitute a unique subpopulation. Confocal images of transgenic adult (A–D) and 5 dpf larvae (E–H) retinal sections. Most *EGFP*-expressing cells co-label with anti-parvalbumin (A) and anti-calretinin (B). The *Tg(7.2mab2l12:EGFP)*-labeled amacrine cells do not colocalize with dopaminergic (anti-TH) (C), glycinergic (anti-Gly) (D), serotonergic (anti-5HT) (E), cholinergic (anti-ChAt) (F), GABAergic (anti-GAD65/67) (G), or 5E11-positive amacrine cells (anti-5E11) (H). Arrowheads: colocalization. Scale bar, 50 μm. (D) Lower magnification than for other adult retinas.

Figure 3.

Morphologic characterization of the *EGFP*-positive amacrine cells. **(A)** Confocal image of a flatmounted retina from an adult *Tg(7.2mab2l12:EGFP)ucd2* shows a random distribution of *EGFP*-labeled amacrine cell across the retina. **(B)** 3D reconstruction of a portion of the flat mounted retina showing that the *EGFP*-labeled amacrine cells send processes throughout the retina. **(C)** Confocal Z-stack of a section through the retina of a *Tg(7.2mab2l12:EGFP)ucd2* adult. Type 1 *EGFP*-labeled amacrine cells have cell somas more distal from the IPL than type 2 amacrine cells. Computerized tracing of the dendritic trees identified *EGFP* cells stratifying in sublamina 1 to 2 (type 1 OFF) or sublamina 3 to 4 (type 1 ON) of the IPL and cells with a diffuse branching pattern (type 2). Scale bars: 50 μm (A); 20 μm (C).

Figure 4.

Tg(7.2mab2ll2:EGFP)-labeled amacrine cells segregate into monostratified or diffuse subtypes. (A–C) Comparison of the stratification patterns of EGFP-labeled amacrine cells (green) and ChAT-positive amacrine cells (red) in the IPL of 5 dpf *Tg(7.2mab2ll2:EGFP)ucd2* embryos. EGFP-expressing cells stratify on the first and just above the second ChAT bands. (D–I) Cellular morphology of individual EGFP-labeled amacrine cells in mosaic embryos injected with *Tg(7.2mab2ll2:EGFP)*. (D) Confocal z-stack of a retinal section from a 5 dpf embryo injected with *Tg(7.2mab2ll2:EGFP)* showing several EGFP-positive amacrine cells. (E', F') show the location from which images are

shown in (E) and (F). (E, F) Single-plane confocal images showing the cellular morphologies of a type 1 OFF cell monostratified in sublamina 1–2, a type 1 ON cell monostratified in sublamina 3 to 4 (the cell soma is not in this plane), and a diffuse type 2 cell. (G–I), Confocal z-stacks showing representative morphologies of type 1 OFF, type 1 ON, and type 2 amacrine cells in mosaic 5 dpf larvae injected with *Tg(7.2mab2ll2:EGFP)*. (J) Schematic representation of the morphology of EGFP-labeled amacrine cells deduced from confocal microscopy and IMARIS reconstruction. Type 1 OFF cells branch in sublamina 1 to 2. Type 1 ON cells branch in sublamina 3 to 4. Type 2 cells are diffuse with dendrites throughout the IPL. (K) An adult transgenic retina labeled for calretinin (*red*) and DAPI showing a type 1 OFF and a type 2 cell. The double-labeled cell has a single dendrite that branches in sublamina 3 to 4 of the IPL. *White arrows*: EGFP/ChAT-labeled dendrites. IPL, inner plexiform layer; INL, inner nuclear layer. Scale bars, 20 μm (E–I).

Figure 5.

Electrophysiologically, *EGFP*-positive amacrine cells belong to two subpopulations. (A) Representative I_K current trace for type 1 cells ($n = 5$ cells), voltage-gated currents were recorded in response to voltage steps from a holding potential of -60 mV (-80 to $+60$ mV; 10 -mV increments). (B) Representative current clamp recording of type 2 cell ($n = 7$ cells). A ramp depolarization (0 - 5 pA over 2 seconds) was applied from a zero holding current. A membrane potential oscillation of approximately 25 -mV amplitude was elicited at approximately 45 mV (*inset*).

Table 1Colocalization of *Tg(mab2112:EGFP)* Amacrine Cells with Known Amacrine Cell Markers

Antibody	No. EGFP-Positive Cells	Double-Labeled Cells (% of EGFP)
Anti-parvalbumin (adult)	10	7 (70)
Anti-calretinin (adult)	25	19 (76)
Anti-TH (adult)	18	0 (0)
Anti-gly (adult)	27	0 (0)
Anti-5E11 (adult)	17	0 (0)
Anti-5HT (5 dpf)	42	0 (0)
Anti-chat (5 dpf)	27	0 (0)
Anti-GAD65/67 (5 dpf)	42	0 (0)
Anti-5E11 (5 dpf)	37	0 (0)