Development and diversification of retinal amacrine interneurons at single cell resolution

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The vertebrate retina uses diverse neuronal cell types arrayed into complex neural circuits to extract, process, and relay information from the visual scene to the higher order processing centers of the brain. Amacrine cells, a class of interneurons, are thought to mediate much of the processing of the visual signal that occurs within the retina. Although amacrine cells display extensive morphological diversity, the molecular nature of this diversity is largely unknown. Furthermore, it is not known how this diversity arises during development. Here, we have combined in vivo genetic labeling, single cell genome-wide expression profiling, and classical birthdating to (*i*) identify specific molecular types of amacrine cells, (*ii*) demonstrate the molecular diversity of the amacrine cell class, and (*iii*) show that amacrine cell diversity arises at least in part through temporal patterning.

amacrine cell | neuronal classification | retinal development | single cell profiling | molecular taxonomy

The vertebrate retina is an excellent system for studying neuronal diversity and how this diversity arises during development. The retina contains 5 major neuronal cell classes and 1 glial cell type. These cell classes may, additionally, contain diverse cell types (1). To investigate how cell diversity arises during development, we focused on amacrine interneurons, the most diverse class of cells in the mammalian retina. Over 30 morphological types of amacrine cells have been characterized to date, each of which is believed to be functionally distinct (2). Amacrine cell types also can be characterized based on their expression of a limited set of molecular markers; however, there are far fewer molecular markers than known morphological types. This paucity of markers is a serious impediment to characterization of this important cell class (3, 4).

To characterize the diversity of amacrine cells and how it arises during development, we first used genetic reporters to label individual amacrine cells within the developing retina and observed a range of distinct morphologies. We then used microarray-based expression profiling of single cells to analyze the distinct transcriptional programs of amacrine cells during development. Single cell profiling allowed us to investigate the extent of molecular diversity within the amacrine cell class and led to the identification of previously unknown molecular markers for known and previously uncharacterized amacrine cells, as well as a classification of single amacrine cells according to transcriptional identity. We also unexpectedly observed that GABAergic amacrine cells emerge before glycinergic amacrine cells in development. We pursued this observation by birthdating GABAergic and glycinergic amacrine cells and found that the 2 groups are born during distinct, but overlapping windows of development. Taken together, this study demonstrates an extensive molecular diversity for the amacrine cell class and shows that this diversity arises at least in part through temporal patterning.

Results

Distinct Morphological Types of Amacrine Cell Are Labeled by Electroporation. To visualize single amacrine cells during development, we introduced genetic reporters into the developing retina by in vivo or ex vivo electroporation (5). We made use of 2 genetic reporters, pNdrg4::GFP (Fig. 1) and pSynapsin::GFP (6, 7), and confirmed that these plasmids specifically label a range of amacrine cell morphologies when electroporated into the retina at birth (p0). Observed cell morphologies included diffusely (Fig. 1*A*) or narrowly stratifying (Fig. 1 *B* and C) amacrine cells with narrow dendritic arbors. Other cells had broader dendritic arbors (Fig. 1*D*). The morphologically identifiable AII amacrine cell was frequently labeled (Fig. 1*A*). Labeled amacrine cells that ramify to distinct sublaminae of the inner plexiform layer (IPL) will contact distinct synaptic partners. For example, an AII amacrine cell (green cell, Fig. 1*E Right*) can synapse with a rod bipolar cell (gray cell with process in the IPL) because their termini occupy the same deep sublamina of the IPL. However, an amacrine cell whose processes are restricted to more apical sublaminae (green cell, Fig. 1*E Left*) cannot. We observed that electroporation of these genetic reporters was sufficient to label diverse amacrine cell types and would allow us to isolate diverse amacrine cells during development.

Isolation and Profiling of Single Amacrine Cells. Single cells, either genetically labeled or randomly chosen from a pool of dissociated cells, were isolated from mouse retinae at 6 developmental ages [supporting information (SI) Table S1] (8). Although these cells had not yet developed their specific morphologies, these time points were chosen to identify transcriptional programs active in amacrine cells at different stages of neuronal differentiation (9). In all, 32 single amacrine cells were isolated and profiled on Affymetrix microarrays. In these profiles the sum total of present probeset calls was 33,057 corresponding to at least 16,435 unique genes. The average number of present probeset calls per cell was 8,335 (SD 2106) representing at least 5,491 (SD 1244) genes.

Identification of Amacrine Cell Class Specific Gene Expression. Profiled cells were confirmed to be amacrine cells by (*i*) expression of genes known to be expressed in every amacrine cell, (*ii*) expression of genes known to be expressed in subsets of amacrine cells, and (*iii*) lack of genes previously determined to be expressed in other retinal cell classes and not in amacrine cells. To assess the robustness of this strategy, we included control cells from other retinal cell classes. *Pax6* is thought to be expressed in all amacrine cells and was present in all 32 single amacrine cells

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The authors declare no conflict of interest.

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Data deposition: The data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, www.ncbi.nlm.nih.gov/geo (accession nos. GSE12601 and GSE9812).

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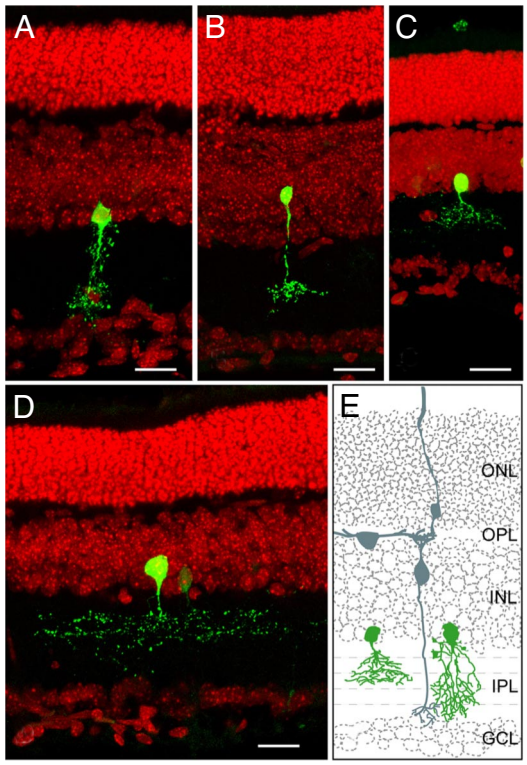

Fig. 1. In vivo electroporation reveals distinct morphologies of amacrine cells. Electroporation of pNdrg4::GFP at p0 labeled diffusely (*A*) or narrowly (*B* and *C*) stratifying narrow-field cells and narrowly stratifying medium-field amacrine cells (*D*) with distinct levels of stratification in the IPL. (*A*) pNdrg4::GFP labels AII amacrine cells which were identifiable according to their distinct morphology. (*E*) A representative schematic of morphologically distinct, genetically labeled amacrine cells (green; *Left*, narrow diffuse cell, and *Right*, AII cell). These cells are predicted to have distinct synaptic partners (gray; *Top*, rod photoreceptor; *Middle*, horizontal cell; *Bottom*, rod bipolar cell) as a consequence of their stratification in particular lamina of IPL. Red, DAPI; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. (Scale bars, 50 μm.)

(Fig. 2*A*). As previously reported, *Pax6* was also expressed in retinal progenitor, Müller glial, and ganglion cells (10). We therefore assessed these 32 cells for expression of other markers expressed within the amacrine cell class. These markers included *Tcfap2*b, *Gad1*, and *GlyT1* (3, 8). All but 6 of the 32 profiled cells expressed *Tcfap2b*, but none of the control cells did. Thirty of the amacrine cells expressed *Gad1* or *GlyT1*, including 5 of the 6 cells that did not express *Tcfap2b* (see below).

Because there are no known perfectly exclusive markers of all amacrine cells, the absence of other cell type-specific genes was useful to establish that a profiled cell belonged to the amacrine cell class. Markers of rod (*Rho* and *Gcap2*), bipolar (*Cabp5* and *Og9x*), Müller glial (*ApoE* and *Aqp4*), ganglion (*Nefl* and *Pou4f2*), and progenitor (*Sfrp2* and *Fgf15*) cells were analyzed for expression in the single amacrine cells and, as a positive control, in previously profiled single cells representing each of these classes (Fig. 2*A*) (8, 10–12). Markers of horizontal (*Lim1*) and cone (*Opmw*) cells were also analyzed, although these cell types were not available for comparison. In the majority of the amacrine cells there was no expression of these markers. In rare cases, where one of these genes was found to be expressed at low levels, no other markers of that same cell class were observed. Notably, 2 amacrine cells expressed *Fgf15*, a marker of retinal progenitor cells. Kurose et al. have reported expression of *Fgf15* in a subset of amacrine cells, suggesting that this expression represents normal amacrine cell type-specific expression (26).

To identify previously unknown markers that were specific to the amacrine cell class, we screened the expression profiles of the 32 amacrine cells against the profiles of 111 single, nonamacrine retinal cells profiled in our other studies (8, 10, 11). We identified 491 probesets corresponding to 467 unique genes that were expressed in at least 1 amacrine cell, but in none of the other retinal cell classes (Fig. 2*B* and Table S2). To confirm the cell type specific expression of these genes, we performed RNA in situ hybridization using EST templates available from the Brain Molecular Anatomy Project, as well as selected others, at developmental and mature time points (Fig. S1 and Table S3). Expression of these candidate genes, when detectable, was found to be distributed in patterns consistent with the amacrine cell class or subsets of amacrine cells. These patterns included expression in the developing inner neuroblastic region of the retina at p0, as well as more restricted expression in the vitreal inner nuclear layer (INL) and the ganglion cell layer (GCL) at later time points, p4, p8, and after p35. Although gene expression in the GCL may reflect expression in ganglion cells, as well as in displaced amacrine cells, we did not observe expression of any of these selected genes in 20 single profiled ganglion cells. The single cell profiles suggest that expression of these genes in the GCL is most likely restricted to displaced amacrine cells.

Intriguingly, many genes that were found to be specific to the amacrine cell class, including *Zfp697* and *Scrt1*, belong to the family of zinc finger protein genes (Fig. S1 and Table S2). *Caln1*, a member of the calcium binding protein family, was also identified as one putative amacrine cell marker. Other members of this family (i.e., calretinin, calbindin, and parvalbumin) were already known to label distinct types or sets of amacrine cells (3). *Caln1* and other genes that were identified in this analysis may represent markers of small subsets or possibly even specific types of amacrine cell. The hallmark of a specific amacrine cell type is the formation of a mosaic, in which the cell bodies of a given neuronal type maintain a minimal distance from one another and are evenly spaced across the horizontal plane of the retina (13). We have identified several markers that appear to label a subset of amacrine cells and are candidate markers for specific amacrine cell mosaics (Fig. 3 and Fig. S1).

Identification of Molecular Diversity Within the Amacrine Cell Class. To determine molecular diversity within the amacrine cell class, we investigated the transcriptional profiles of the 32 single profiled amacrine cells for the expression of molecular markers that would distinguish among different types of amacrine cells. The amacrine cell class can be divided into specific physiological groups or types based on expression of specific molecular markers. *Gad1* and *GlyT1* expression subdivides the amacrine class into GABAergic and glycinergic groups, respectively. Sixteen of the thirty-two profiled amacrine cells expressed *Gad1*, a biosynthetic enzyme for GABA, and fourteen cells expressed *GlyT1*, a transporter necessary for glycine accumulation (Fig. 4 *A* and *B*).

Although *Gad1* or *GlyT1* expression reflects the principle neurotransmitter of an amacrine cell, GABAergic and glycinergic amacrine cells are still broad designations and encompass many distinct cell types. Amacrine cells can be further distinguished by markers of specific types. For example, among the 32 profiled amacrine cells, expression of the vesicular acetylcholine transporter (*VAChT*) and the neuropeptide tachykinin (*Tac1*) distinguished 3 cholinergic and 1 tachykinin amacrine cell (Fig. 4 *A* and *B*). Expression of disabled 1 (*Dab1*) distinguishes the AII physiological type of glycinergic amacrine cell from other neurons in the mature retina. However, *Dab1* is also expressed in other cells of the developing retina. We therefore used the coexpression of *Dab1* and *Cx36*/*Gja9*, a gene known to be expressed in mature AII amacrine cells, to identify 4 AII amacrine cells (Fig. 4 *A* and *B*). The absence of molecular

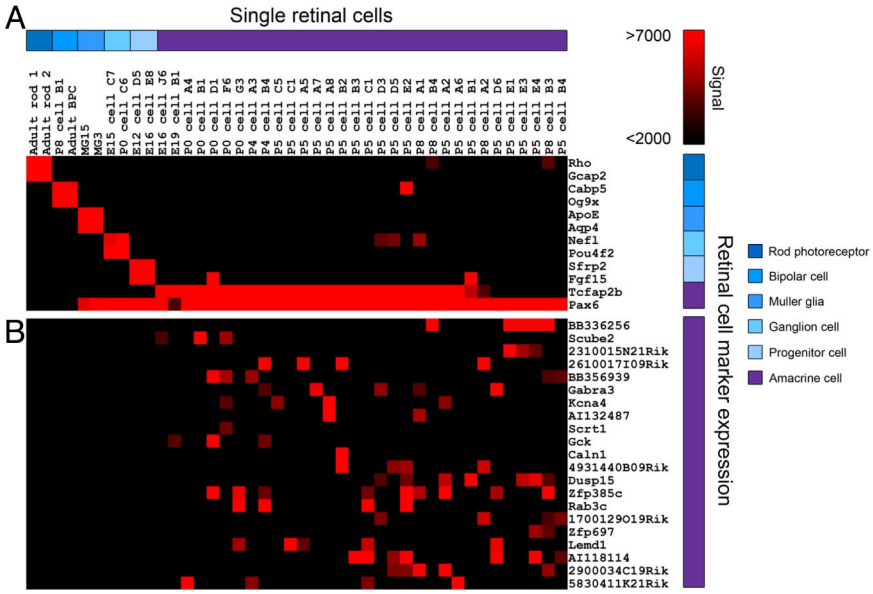

Fig. 2. Identification of individually profiled single amacrine cells and previously unknown amacrine class markers. (*A*) Thirty-two single cells were profiled and classified as amacrine cells according to their expression of retinal cell class markers. A heatmap was generated to display the expression of designated genes in amacrine (purple) or control cells (blue). (*B*) The transcriptional profiles of the 32 amacrine cells were screened against those of 111 other single retinal cells of different types to identify previously unknown molecular markers of the amacrine cell class.

markers for other known amacrine cell types prevented us from further characterizing the remaining 24 amacrine cells. As a consequence, we sought to characterize these cells not by single molecular markers, but through an unbiased approach according to their gene expression profiles.

Classification of Known and Novel Amacrine Cells. To classify the single profiled amacrine cells, we used an unsupervised clustering strategy. We converted expression values for each gene into log space ($\log_2$) and calculated similarity between pairs of cells by Pearson's correlation. Ward's clustering method was then applied to establish an agglomerative dendrogram of the single profiled cells (Fig. 4*C*) (14). This clustering strategy proved robust as the cell clusters remained remarkably similar over the tested range of increasing expression thresholds, from ~15,000 to ~1,300 probesets. For our analysis, we used an expression cutoff of 10.97 and a variance cutoff of 10.97. These thresholds correspond to probesets expressed above a relative signal (RS) of 2000 in at least one cell, and whose variance was greater than 2000 across all of the cells. After thresholding, 4,555 probesets were left for comparison.

To evaluate the accuracy of this clustering approach, we compared the unsupervised dendrogram (Fig. 4*C*) with the molecular taxonomy based on known molecular markers of amacrine cell types (Fig. 4*A*). Among the 32 single profiled amacrine cells, 6 distinct groups were definable according to known molecular markers, including cholinergic, tachykinin, and AII types of amacrine cells. The unsupervised classification of profiled cells captured the dichotomy between GABAergic and glycinergic amacrine cells, with only 3 glycinergic "outliers" in the GABAergic cluster, and 0 GABAergic cells within the glycinergic cluster. Interestingly, one of the glycinergic outliers that clustered with the GABAergic cells had low levels of *Gad1* expression. The unsupervised classification also correctly clustered the 3 *VAChT*+, cholinergic amacrine cells into an exclusive branch of the GABAergic cell group and 3 of the 4 cells identified by conventional markers as AII amacrine cells into an exclusive branch of the glycinergic cells (Fig. 4*C*).

An advantage of this unsupervised clustering technique was that it yielded information about the relatedness of the cells for which there are no known molecular markers. The measure of this relatedness can be described quantitatively by the length of the branches to the nearest shared node between 2 or more cells in a dendrogram (Fig. 4*C*). A possible threshold for determining whether a group of uncharacterized cells within a cluster represents a novel amacrine cell type could be to compare the branch lengths connecting uncharacterized cells with branch lengths between cells that are known to comprise a specific functional type. For example, a horizontal "cut" across the dendrogram at the height of the node that includes all cholin-

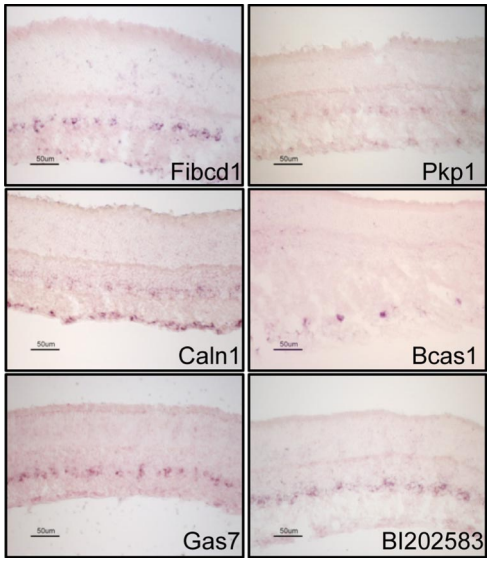

Fig. 3. Previously unknown molecular markers of amacrine cell subsets. In situ hybridization in retinal cross-section was performed by of genes determined to be expressed in small sets of profiled amacrine cells. *Gas7* and BI202583 are from p8 retinae, others >p35. ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer. (Scale bars, 50 μm.)

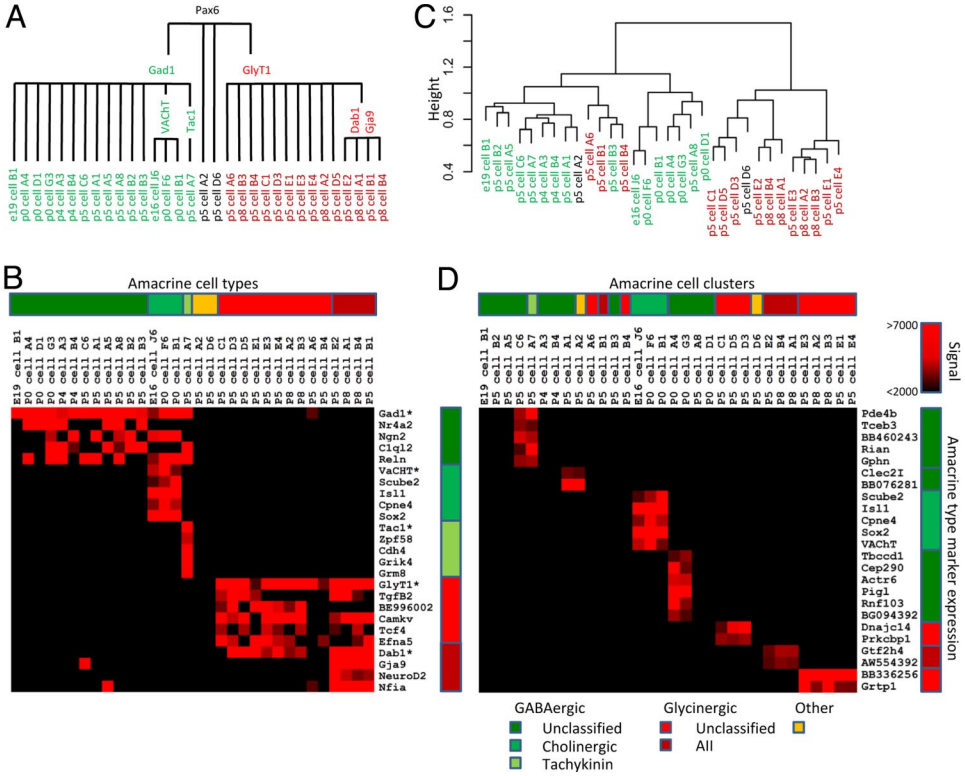

Fig. 4. Molecular taxonomy of single amacrine interneurons. Amacrine cells were classified according to known molecular markers of amacrine cell type (*A*) or according to transcriptional profiles using Pearson's correlation and Ward's clustering method (*C*). These classifications were then used to identify genes specific to molecularly defined amacrine cells, including GABAergic, cholinergic, tachykinin, glycinergic, or AII amacrine cells, as indicated by the color coding (*B*) or unsupervised clusters (*D*). *, established amacrine group or type markers.

ergic amacrine cells grouped the 32 amacrine cells into 7 discrete clusters. These 7 clusters included 2 clusters of known types of amacrine cells, the 3 cholinergic amacrine cells and 3 AII amacrine cells (Fig. 4*C*). Additionally, 5 other clusters of uncharacterized cells were equally or more related to one another than were cells of the cholinergic amacrine type. To test whether these clusters truly represented a single amacrine cell type, we next searched for genes whose expression could serve as molecular markers for specific amacrine cell types.

Identification of Amacrine Type Specific Gene Expression. To identify gene expression that was specific to distinct amacrine cell types, we took several approaches. First, we analyzed the profiles of all single cells that expressed a known amacrine cell type marker and identified genes that were coexpressed in at least 50% of those cells. We then filtered out genes that were expressed in any amacrine cell that did not express the marker of that group. This filtering allowed us to identify transcripts that were restricted within the amacrine class to a particular group of cells (i.e., GABAergic or glycinergic) or to specific amacrine cell types (i.e., cholinergic or AII) (Fig. 4*B*).

We also sought to identify new molecular markers for the types of amacrine cells predicted by unsupervised clustering. We analyzed the transcriptional profiles of all cells within a cluster and identified genes expressed in all members of that cluster. We then screened out all genes expressed in any other amacrine cell that did not fall within this cluster. One measure of the accuracy of this approach was that this unsupervised analysis reproduced the transcriptional signature of the cholinergic amacrine cells. Additionally, this analysis gave previously unknown transcriptional signatures of previously uncharacterized amacrine cells (Fig. 4*D*).

Expression of Genes Relevant to Physiology or Development. We examined the transcriptional profiles of different amacrine cell types for expression of physiologically important genes, including neurotransmitter receptor subunits, neurotransmitter biosynthetic enzymes, neuropeptides, and ion channels. In some cases, we were able to approximate some of the physiological properties of a given cell. In the case of the well-characterized cholinergic amacrine cells, it is known that nicotinic acetylcholine receptors (nAChRs) and GABA receptors on cholinergic amacrine cells are necessary for the propagation of spontaneous retinal waves (15). This amacrine to amacrine cholinergic neurotransmission is transient, however, and the cholinergic amacrine cells undergo a developmental switch where they no longer provide excitatory input onto one another and instead switch to provide laterally inhibitory input. This switch is mediated by the down-regulation of nAChRs in cholinergic amacrines, so that they are no longer sensitive to cholinergic input, and the up-regulation of the potassium/chloride cotransporter, *Slc12a5*, which changes the effect of GABA input from depolarizing to hyperpolarizing (15). By determining whether nAChR subunits or *Slc12a5* were expressed in the cholinergic cell profiles, it was possible to assess the developmental stage and the physiological properties of these neurons. In fact, 2 cholinergic amacrine cells proved to express nAChR subunits, and although other amacrine cells expressed *Slc12a5*, none of the 3 cholinergic amacrine cells had yet begun to express this gene (Figs. S2 and S3). The profiles of these neurons may therefore help to identify the transcriptional programs in early cholinergic amacrine cells that are necessary for the propagation of spontaneous retinal waves.

Another class of functionally relevant genes includes genes that are important for the development of distinct amacrine cell types (i.e., transcription factors, cell adhesion molecules, and

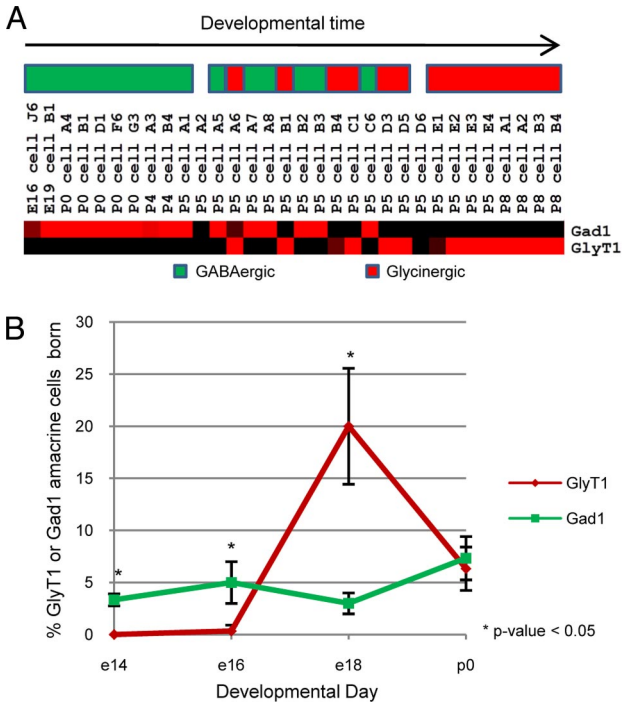

Fig. 5. GABAergic amacrine cells are born and differentiate earlier than glycinergic amacrine cells. (*A*) Among profiled cells, GABAergic cells were present by e16, whereas glycinergic amacrine cells were not detectable until p5, as shown by a heatmap representing RS levels for *Gad1* and *GlyT1*. (*B*) GABAergic amacrine cells were birthdated between e14 and p0, whereas glycinergic amacrine cells were not born until after e16. *, Student's *t* test, two-tailed, homoscedastic.

guidance factors). We identified expression of many of these functionally important transcripts in all of the single profiled amacrine cells (Fig. 4 and Fig. S4). Two transcription factors, Isl1 and Sox2, that have been implicated in development of cholinergic amacrine cells (16, 17) were expressed in all of the profiled cholinergic amacrine cells (Fig. 4). In terms of other amacrine cell types, however, very little else is known about genes that are functionally important for their development. For example, no transcription factors have been implicated, to date, in specification of the AII cell, the most common type of amacrine cell in the mammalian retina. Intriguingly, we found the transcription factor NeuroD2 to be expressed exclusively in 4 of the 4 profiled AII amacrine cells (Fig. 4*B*).

Temporal Patterning of Amacrine Diversity. Through single cell profiling, we observed that among the cells profiled earliest in development, only the GABAergic group was represented (Fig. 5*A*). It was not until after p4 that the glycinergic group of amacrine cells emerged. One explanation for this may be that the molecular marker of glycinergic cells, *GlyT1*, is not expressed until this relatively late time point. Alternatively, GABAergic amacrine cells may be specified from retinal progenitor cells during an earlier window of development than glycinergic amacrine cells.

To test this hypothesis, we performed classical birthdating studies of GABAergic and glycinergic amacrine cells (9, 18, 19). Pregnant mice were injected with [^{3}H]-thymidine at 1 of 4 time points during the window of amacrine cell production. At p12, labeled retinae were harvested, and cells were dissociated, immunostained for Gad1 and GlyT1, and then processed for autoradiography. The percentage of the total GABAergic or glycinergic population that was born within 1 cell cycle of the time of injection was plotted over developmental time (Fig. 5*B*). GABAergic amacrine cells were born throughout this window. Glycinergic amacrine cell birth, however, was observed to begin only at e18, indicating that GABAergic and glycinergic amacrine cell production is temporally distinct. These results were extended for cholinergic and AII amacrine cell types by in utero or in vivo electroporation (Fig. S5).

Discussion

High Resolution, Reproducibility, and Robustness of Single Profiling. Microarray expression studies offer a powerful means to refine the concept of the neuronal cell type (20). We have chosen the single cell profiling technique for our studies, in part, to identify previously unknown markers for previously uncharacterized cells and to identify these markers with cellular resolution. As a measure of the reproducibility and robustness of single cell profiling, we were able to evaluate the single profiled amacrine cells according to known molecular markers for this class, as well as by unsupervised clustering. The single cell profiles recapitulated known marker expression very well. In unsupervised clustering, we observed that cells belonging to the same type, according to known cell type-specific markers, were highly correlated and clustered together. Profiling and clustering has also helped to identify candidate molecular markers for uncharacterized amacrine cells. By comparing known and previously unknown markers, as well as the full transcriptional profiles of single cells, we hope to move toward a more complete molecular taxonomy of the amacrine cell class.

Molecular Diversity Within the Amacrine Cell Class. We screened the transcriptional profiles of 32 single profiled amacrine cells and those of 111 other nonamacrine retinal cells to identify 467 marker genes of the amacrine class. Interestingly, no genes were found to be expressed in all amacrine cells and only in amacrine cells. Because amacrine cells are recognized as a distinct class of retinal neurons, one might expect a specific pan-amacrine marker to emerge from this analysis. Such markers may exist and may have been missed in our analysis. Alternatively, the lack of specific, pan-amacrine markers may reflect the underlying heterogeneity of the amacrine cell class and that types of amacrine cells are not necessarily more similar to one another than they are to other classes of neurons in the retina. A previous study has demonstrated that there is a substantial overlap of gene expression between a subset of ganglion cells and a subset of amacrine cells (8). While highlighting heterogeneity within the amacrine cell class, this may also reflect distinct mechanisms for producing different amacrine cell types during development.

Generating Diversity in the Amacrine Cell Class. Diversity across retinal cell classes is established in part by the temporal patterning of multipotent retinal progenitor cells (21). It has also been observed that temporal patterning can give rise to diversity within the bipolar cell class of retinal neurons (22). In this study, we see that GABAergic amacrine cell birth begins early in retinal development, whereas glycinergic amacrine cells do not emerge until e18. This finding suggests a model of amacrine cell diversification (Fig. S6), where at least some aspects of amacrine type fate are directed by the temporal properties of progenitor cells and/or the environment.

Experimental Procedures

Animals. CD1 mice were obtained from Charles River Laboratories. All of the experiments in this study were approved by the Institutional Animal Care and Use Committee at Harvard University.

Genetic Labeling. The *Ndrg4* promoter (5) was cloned upstream of GFP. The *Syn1* promoter was obtained from B. Roska (Friedrich Miescher Institute, Basel, Swit-

zerland) (7) and cloned upstream of GFP. In vivo and ex vivo electroporation was performed exactly as described (5).

Single Cell Collection and PCR Based cDNA Amplification. Single cells were isolated and profiled exactly as described (8). Additional identification steps were made for genetically labeled cells. Individual GFP$^+$ cells were identified, using an Olympus IMT-2 microscope, isolated using a pulled glass pipette, expelled into a wash plate, and transferred into a 100-μL PCR tube containing cold lysis buffer before RT-PCR.

Affymetrix Array Hybridization. Probe reactions and Affymetrix microarrays were prepared using standard Affymetrix protocols (8, 23). To facilitate comparisons among microarrays, global scaling was performed using the Affymetrix Microarray software (MAS 5.0) and the target intensity was set to 500. The resulting signal data were exported, and subsequent analyses were performed using R (24), TreeView (25), or Microsoft Excel. The raw and processed Affymetrix data files have been deposited in the NCBI Gene Expression Omnibus.

Analysis of Microarray Data. To eliminate probesets called marginal or absent and to reduce the false-positive rate, only probesets with a RS $>$2000, as determined by MAS 5.0, were considered in this analysis. Previous reports suggest that this threshold corresponds to transcripts that are present at between 10 and 100 copies per cell (23). To identify amacrine or amacrine type-specific genes, data filters were created in Excel to remove all genes expressed at an RS $<$2000 in all of the cells of interest and to remove all genes expressed $>$2000 in any comparison cells.

Hierarchical Clustering. Pearson's correlation coefficient of $\log_2$ RS values was used as the similarity metric between single cell profiles. Only probesets with maximum expression and variance across single cells above 10.97 were included in the clustering. Ward's method was used to cluster single cells into dendrograms (14), as implemented in the R cluster package.

Birthdating of Amacrine Cell Types. Pregnant mice were injected with [^{3}H]-thymidine (GE Healthcare) at 4 gestational time points to birthdate cells as described previously (18). Dissociated and plated cells were immunostained with 1:200 anti-Gad65/67 (Chemicon AB1511) or 1:5000 anti-GlyT1 (Chemicon AB1770) and processed for autoradiography. For each marker, 100 cells were scored for each retina from 3 separate retinae at each time point.

In Situ Hybridization and Immunofluorescence. In situ hybridization and immunostaining were performed as previously described (5, 8). Specific antibodies used in this study were anti-ChAT (1:30, Chemicon AB144P) for cholinergic amacrine cells, anti-Dab1 (1:500, a gift from B. Howell, National Institute of Neurological Disorders and Stroke, Bethesda, MD) for the AII type, and anti-GFP (1:300, Molecular Probes).

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