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Axons and dendrites originate from neuroepithelial-like processes of retinal bipolar cells

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The cellular mechanisms underlying axogenesis and dendritogenesis are not completely understood. The axons and dendrites of retinal bipolar cells, which contact their synaptic partners within specific laminae in the inner and outer retina, provide a good system for exploring these issues. Using transgenic mice expressing enhanced green fluorescent protein (GFP) in a subset of bipolar cells, we determined that axonal and dendritic arbors of these interneurons develop directly from apical and basal processes attached to the outer and inner limiting membranes, respectively. Selective stabilization of processes contributed to stratification of axonal and dendritic arbors within the appropriate synaptic layer. This unusual mode of axogenesis and dendritogenesis from neuroepithelial-like processes may act to preserve neighbor-neighbor relationships in synaptic wiring between the outer and inner retina.

Studies of neurons in dissociated cell culture drive the prevailing view of how axons and dendrites are specified. These studies indicate that neurons initially extend a number of simple neurites, one of which eventually becomes the axon whereas the others differentiate into dendrites[1–3]. However, in some parts of the CNS, axons and dendrites do not emerge *de novo* from cell bodies; rather, these processes originate from the leading and trailing processes of migrating neurons[4–6]. Here we show that in the mouse retina, axonal and dendritic processes of bipolar cells originated from neuroepithelial-like processes that were attached to the ventricular surface and basal lamina during the period when bipolar cell somata still demonstrated interkinetic movements.

Retinal bipolar cells are interneurons that receive inputs from photoreceptors in the outer retina and synapse onto retinal ganglion cells (RGCs) and amacrine cells in the inner retina. Bipolar cells are separated into two major functional classes: ON-center and OFF-center cells (**Fig. 1a**). The dendrites of ON bipolar cells express metabotropic glutamate receptor 6 (mGluR6)[7–9] and depolarize in response to increases in illumination, whereas OFF bipolar cells express AMPA/kainate receptors[10] and are hyperpolarized. Axon terminals of OFF bipolar cells stratify in the outer half of the inner plexiform layer (IPL) whereas those of ON bipolar cells terminate within the inner half of the IPL. Because bipolar cells are the last cells to connect to inner and outer retinal circuits during development, they must extend axons and dendrites to pre-existing synaptic laminae and elaborate arbors accordingly so as to contact their appropriate synaptic partners.

Using a transgenic mouse line (*Grm6-GFP*; A.D. and N.V., unpublished data) in which ON bipolar cells express GFP under the control of the promoter of the gene *Grm6*, which encodes mGluR6, we followed bipolar cell development during the period of axonal and dendritic growth.

RESULTS

GFP expression is restricted to ON bipolar cells

In adult retinas of *Grm6-GFP* transgenic mice, strong GFP expression was found only in the population of interneurons identified as ON bipolar cells by their somal location and axonal arborization in the inner half of the IPL (**Fig. 1b**). By immunolabeling the ON and OFF cholinergic sublaminae with an antibody to vesicular acetylcholine transporter (VAChT), we confirmed that axon terminals of GFP-positive cells terminated within the inner half of the IPL, below the OFF cholinergic sublamina (**Fig. 1b**). This distribution pattern is consistent with axonal terminal arbors of rod bipolar cells and ON cone bipolar cells. Immunostaining for protein kinase C (PKC)[11] and calcium-binding protein-5 (CaBP5)[12] demonstrated that all immunolabeled rod bipolar cells and ON cone bipolar cells express GFP in this transgenic mouse line (**Fig. 1c–d**).

There was also transient expression of GFP in some amacrine cells and RGCs during early neonatal development. This may not be surprising given that *Grm6* mRNA is present in some developing RGCs[13]. However, RGC and amacrine cell processes were often much dimmer than the axonal branches of bipolar cells, enabling us to separate the processes of these cell types. Furthermore, it was possible to determine which processes were part of bipolar axons by examining confocal stacks plane by plane and by rotating the stacks in three dimensions (**Supplementary Video 1** online).

GFP expression in neuroepithelial-like cells

The expression of GFP followed a developmental gradient from central to peripheral retina (**Fig. 2a**). At postnatal day 7 (P7), when the majority of bipolar cells have differentiated[14], we observed numerous GFP-positive cells with somata in the inner nuclear layer (INL). In

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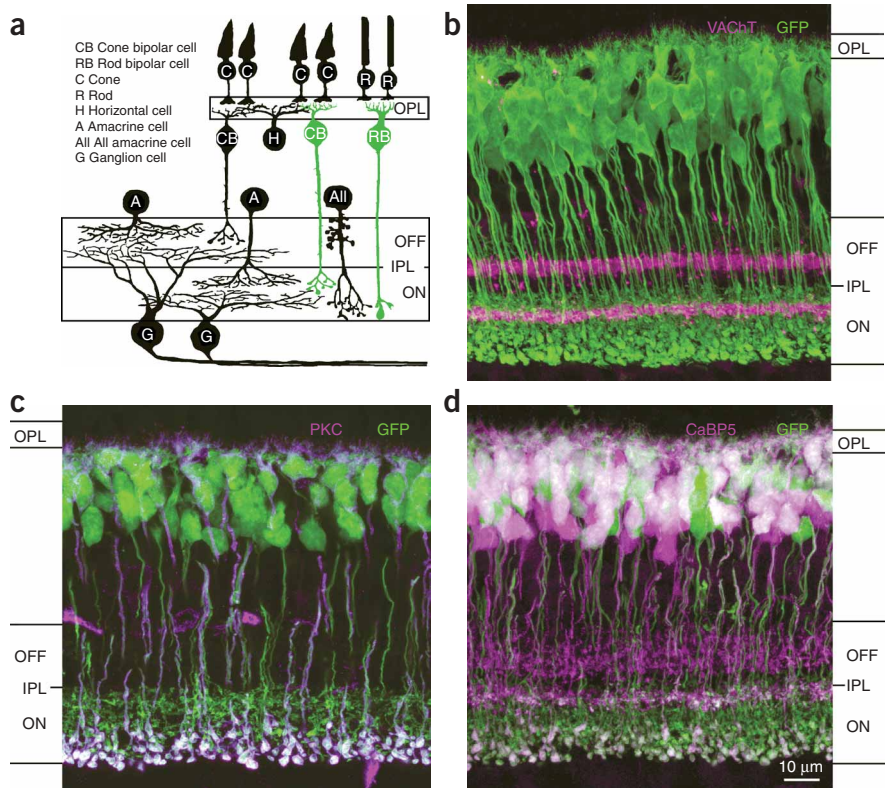

Figure 1 GFP is expressed by ON cone bipolar cells and rod bipolar cells in the *Grm6-GFP* transgenic mouse. (**a**) Schematic showing the basic synaptic organization of the retina. (**b**) Immunostaining of adult retinas from *Grm6-GFP* mice with anti-VAChT demonstrated the spatial relationship between the axonal arbors of GFP-expressing bipolar cells and the ON and OFF cholinergic sublaminae. (**c**) Rod bipolar cells that were immunoreactive for PKC also expressed GFP. (**d**) CaBP5 immunostaining showed that ON cone bipolar cells also expressed GFP.

more central regions, immature axonal and dendritic arbors were evident in the IPL and outer plexiform layer (OPL), respectively (**Fig. 2b–d**). Notably, many cells had processes that extended beyond the IPL and OPL, apparently reaching the inner (ILM) and outer (OLM) limiting membrane (**Fig. 2d,e**). To determine whether processes outside the synaptic layers represent exuberant growth or a vestigial developmental structure, we examined retinas at the earliest age at which GFP expression was detected.

At P3, we observed GFP expression in neuroepithelial-like cells with cell bodies in the INL and processes that spanned the depth of the retina (**Fig. 2e**). At this early age, every GFP-positive cell had an apical process that terminated at the OLM and a basal process that extended at least into the IPL. The basal processes of the majority of cells extended beyond the IPL, terminating either at the fiber layer (FL) or at the ILM. Of the 37 cells reconstructed at P3, 13 (35%) basal processes terminated in the ILM, 7 (19%) in the FL, 11 (30%) in the ganglion cell layer (GCL) and 6 (16%) within the IPL.

The fact that these apical and basal processes are maintained even after the elaboration of axonal and dendritic processes raises the possibility that these radial extensions are still playing a role in somal migration[6]. Indeed, distances between GFP-positive cell bodies and the OLM decreased with age, from (mean ± s.e.m.) 110 ± 2 μm at P3 to 78 ± 6 μm at maturity. Also, distances between bipolar cell somata and the OPL decreased from 55 ± 5 μm to 16 ± 1 μm. Time-lapse two-photon imaging of *Grm6-GFP* retinal explants at P5 confirmed that individual cell bodies translocated more than 10 μm toward the OPL in less than 2 h (**Fig. 2f**). Because not all bipolar cell bodies in a single field of view translocated simultaneously or in the same direction during the recording period, it was unlikely that the somal movements were due to uniform changes in INL thickness (data not shown).

The basal process differentiates into an axonal arbor

A comparison of fixed *Grm6-GFP* retinas across ages suggested that the axonal arbor was derived from lateral extensions from the basal process (**Fig. 3a,b**). At P3, short processes emerged throughout the length of the basal processes (**Fig. 3a,b**). These lateral extensions were concentrated in the IPL but were also found in the INL and GCL. By P5, most basal processes had retracted back into the GCL and IPL, terminating in a round swelling resembling a retraction bulb (**Fig. 3a–c**). Although the majority of lateral processes (56% or 167 of 294) at this age were found in the inner half of the IPL, there was still a significant number in the outer half of the IPL (15% or 45 of 294) and in the GCL (21% or 61 of 294). However, even the simplest P5 arbors appeared somewhat stratified. We determined the distributions of P5 bipolar axonal arbors relative to ON (inner) and OFF (outer) amacrine cell strata using calbindin immunolabeling (**Fig. 3c**). Although the extent of branching differed between these two cells, the lateral processes in both cases were concentrated at or below the calbindin-positive ON sublamina.

With increasing age (P7–P21), the remaining basal processes retracted to the IPL as elaborate axonal arbors were established and confined to the ON sublamina (**Fig. 3a,b**). For older ages (> P9), it was not possible to compare individual axon terminal arbors in the *Grm6-GFP* line because of the high density of labeling. Instead, we illustrate here an example of an ON bipolar cell at P21 in the *GUS8.4-GFP* transgenic mouse line (in which a subset of ON bipolar cells were labeled[15,16]) to show that at this age, axonal arbors were exclusively confined to the ON sublamina (**Fig. 3b**).

To better understand how axonal arbors become restricted to the ON sublamina, we performed time-lapse confocal imaging of individual axons at P5 to determine their dynamic behavior during the period of axonal stratification (**Supplementary Video 2** online). These recordings showed that branches extended along the entire length of the axon (**Fig. 4a**). However, for a given period of recording, more processes were formed in the inner (ON) compared to the outer (OFF) half of the IPL ($P = 0.037$; Wilcoxon rank sum test for equal medians). Within a 3-h recording period, an average of 5.8 ± 1.5 branches emerged in the ON sublamina in contrast to 3.5 ± 1.4 branches in the OFF sublamina ($n = 6$ cells, 56 processes). This bias toward process formation in the ON sublamina may have been due to the greater total axonal length in the ON compared to the OFF sublamina at P5. The total axonal length in each sublamina comprised the length of the basal process and all of its branches located within each sublamina. When the rates of formation of new processes (number of new processes per h) were normalized to total axonal lengths (μm) in each sublamina, there was no

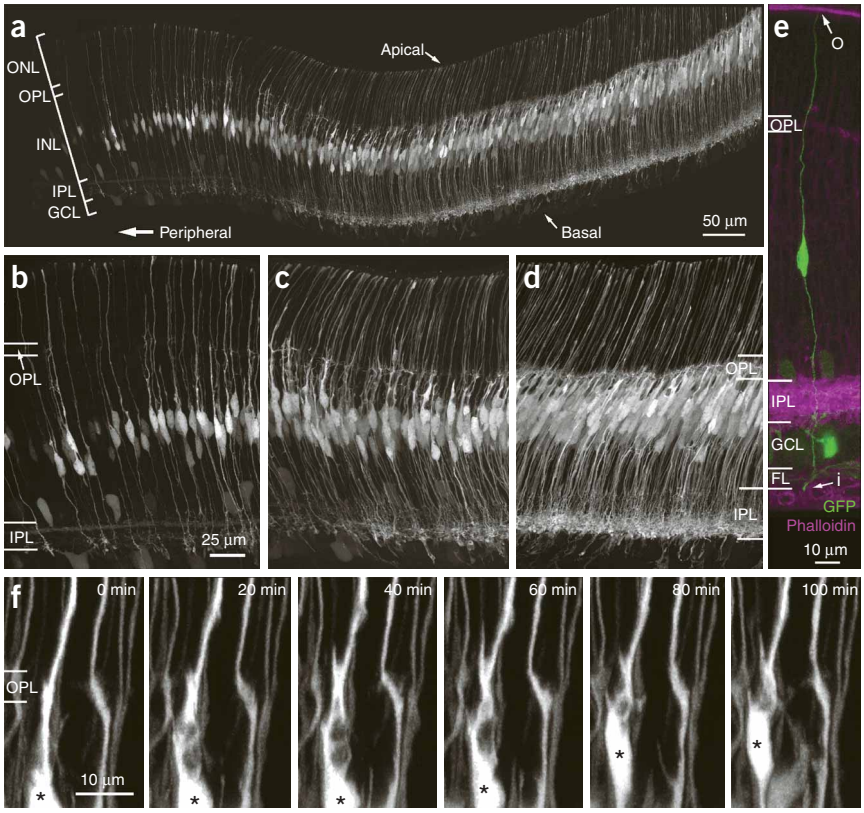

Figure 2 GFP expression reveals bipolar cells with neuroepithelial-like processes during development. (**a**) Cross-section of a P7 *Grm6-GFP* retina, demonstrating a central-to-peripheral gradient in GFP expression and morphological maturation. (**b**–**d**) Higher magnification of three different regions of the section in **a**. (**e**) GFP-expressing cell from a P3 retina with apical and basal processes extending to the OLM ('o') and ILM ('i'). The retinal section was counterstained for phalloidin (purple) to reveal the IPL, the ILM and the OLM. (**f**) Orthogonal projection of time-lapse images (every 20 min) of a P5 *Grm6-GFP* retina showing the translocation of a bipolar cell soma (*).

significant bias toward the formation of new processes in the ON or OFF sublamina ($n = 6$ cells, 56 processes; OFF = 0.015 ± 0.005; ON = 0.013 ± 0.003; Wilcoxon rank sum test, $P = 0.39$). Over 3 h, we did not observe branching from existing processes in the OFF sublamina as we did in the ON sublamina ($n = 6$ cells; number of branches added: ON = 9, OFF = 0). Because of the infrequency of branching within the recording period, it was uncertain whether the lack of secondary branching in the OFF sublamina was due to a relative inability of primary processes in this layer to form branches or simply to their short lifetime. Indeed, a survival curve of process lifetimes (**Fig. 4b**,**c**) demonstrates that axonal branches in the ON sublamina were more stable than those in the OFF sublamina (number of processes: 91 (ON), 24 (OFF); $n = 10$ cells; Wilcoxon rank sum test of pooled lifetimes, $P = 0.02$). Although we cannot rule out differences in branch initiation between sublaminae, preferential stabilization of branches in the ON sublamina of the IPL is likely to play a significant role in the stratification of ON bipolar cells.

Finally, our time-lapse imaging of GFP-positive processes terminating in the GCL revealed that these processes grew and retracted at rates comparable to those observed in the IPL. Processes in the GCL extended

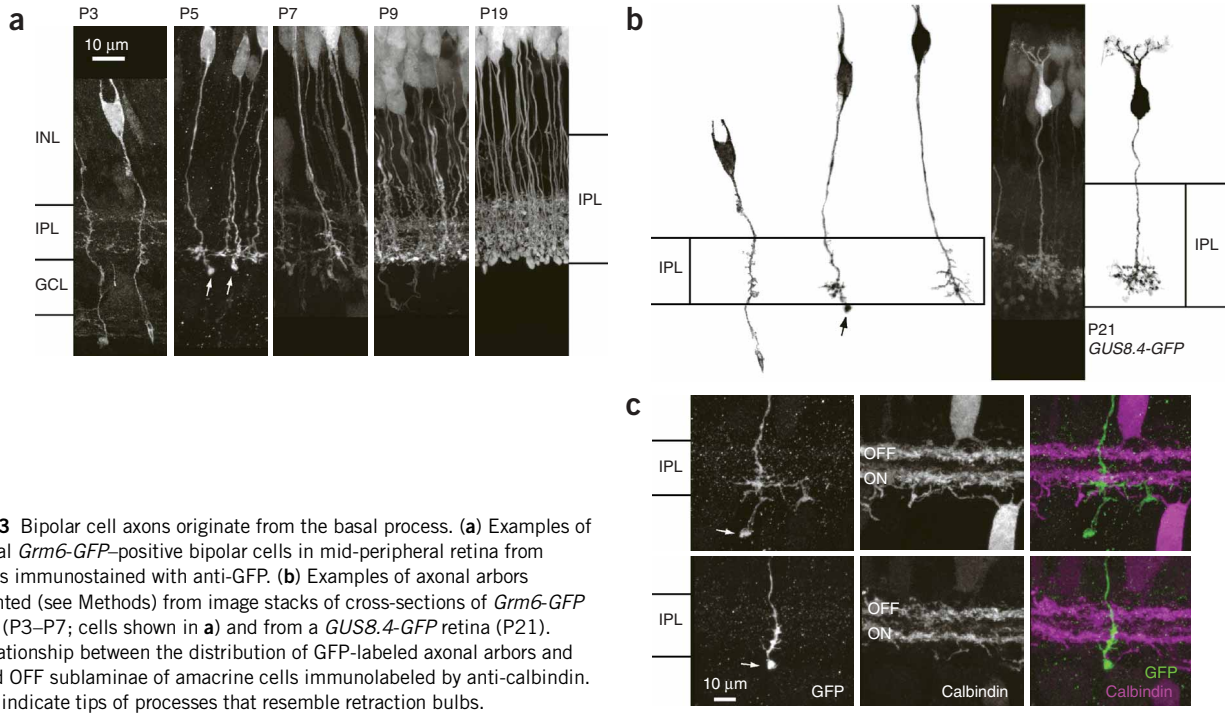

Figure 3 Bipolar cell axons originate from the basal process. (**a**) Examples of neonatal *Grm6-GFP*–positive bipolar cells in mid-peripheral retina from sections immunostained with anti-GFP. (**b**) Examples of axonal arbors segmented (see Methods) from image stacks of cross-sections of *Grm6-GFP* retinas (P3–P7; cells shown in **a**) and from a *GUS8.4-GFP* retina (P21). (**c**) Relationship between the distribution of GFP-labeled axonal arbors and ON and OFF sublaminae of amacrine cells immunolabeled by anti-calbindin. Arrows indicate tips of processes that resemble retraction bulbs.

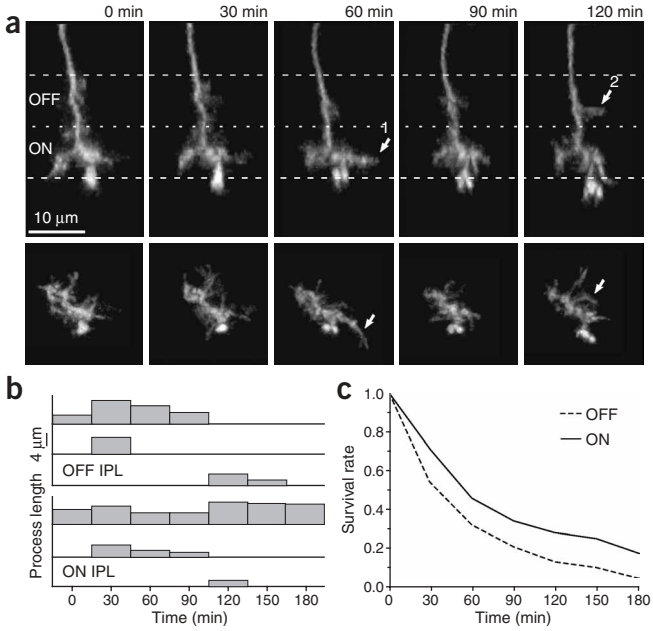

Figure 4 The axons of P5 ON bipolar cells branch throughout their length within the IPL, but branches are preferentially stabilized in the ON sublamina. (**a**) Orthogonal (top row) and axial (bottom row) projections of an axonal arbor imaged every half hour. New processes could be seen forming in the ON ('1') and OFF ('2') sublaminae of the IPL. (**b**) Examples showing changes in process length of three processes each in the ON and OFF sublaminae. (**c**) Comparison of survival curves generated from lifetime measurements of axonal processes in the ON ($n = 91$ processes) and OFF ($n = 24$ processes) sublaminae.

filopodia and branched and extended tens of micrometers, sometimes looping back into the IPL. Many terminals formed large lamellapodia that appeared to cup GCL cell bodies (**Supplementary Video 3** online). Despite these developmental elaborations, however, no GFP-labeled processes were observed in the GCL of mature mouse retina.

The apical process differentiates into a dendritic arbor

Cross-sections of *Grm6-GFP* retinae at different ages demonstrated that dendrites extended from apical processes before their detachment from the OLM (**Fig. 5a**). At P3, apical processes were generally smooth and unbranched. However, by P5, laterally directed processes were seen extending from the shaft between the somata and the location of the OPL as determined by immunolabeling for photoreceptors and horizontal cells (**Fig. 5b**,**c**). Few dendrites were apparent distal to the OPL (**Fig. 5**). At P5, not all horizontal cell processes, as revealed by calbindin immunolabeling, were confined to a single lamina: although most formed a thick plexus at the location of photoreceptor terminals, some horizontal processes were present outside this main plexus (**Fig. 5c**). Because bipolar cell dendrites at this age appeared to also emerge outside the OPL, we further analyzed the specific spatial relationship between bipolar cell dendritic extensions and the location of horizontal cell neurites. We found that almost half (11 of 24 processes; 6 cells) of these presumed dendritic processes emerged from the apical shaft within 2.5 μm of the main horizontal cell lamina (**Fig. 5d**). Moreover, bipolar cell dendrites not closely associated with the OPL were often found (7 of 13 processes; $n = 7$ cells) within 2.5 μm of the location at which the apical process of the bipolar cell intersected horizontal cell bodies or horizontal processes outside the main plexus (**Fig. 5d**). In both cases, Monte Carlo analysis demonstrated that the association of bipolar cell dendrites with horizontal cell processes was significantly greater than that expected if the bipolar processes were randomly distributed relative to the horizontal cell neurites ($P = 0.018$ at OPL; $P = 0.027$ outside the OPL).

To better understand how dendritic arbors are established from the apical processes, we carried out two-photon time-lapse imaging on P5 retinas and monitored the dynamics of the initial dendritic outgrowth (**Supplementary Video 4** online). Two-photon imaging allowed us to visualize entire bipolar cells (**Fig. 6a**), but we imaged only the region from the OLM to the cell body so as to minimize acquisition time and to reduce photodamage. At the depth of the presumed OPL, we typically found clusters of a few short processes (≤ 5 μm) that extended and retracted during the recording period of almost 2 h (**Fig. 6b**; process 1). Two types of processes were observed emerging from the apical processes proximal to the OPL: short processes similar in behavior to those extending within the OPL (**Fig. 6b**; process 2) and, at times, long, vertically oriented collaterals (**Fig. 6b**; process 3) that in some cells reached as far as the OLM (data not shown). These processes showed a range of stabilities (**Fig. 6c**,**d**).

Retraction of the apical process began after substantial dendritic elaboration within the OPL. This event occurred over several hours, beginning with a narrowing of the distal tip as the distal swelling moved back toward the OPL (**Fig. 6e**). In some cases, the narrowed tip seemed to detach from the OLM, following the apical swelling back to the OPL (**Fig. 6e**). In other cases, the tip remained attached while the bulk of the cytoplasm appeared to move toward the cell body (**Fig. 6e**). In these cases, the apical process distal to the swelling narrowed and eventually disappeared (**Fig. 6e**), leaving behind small fluorescent structures similar to the axosomes described at the neuromuscular junction[17].

Collectively, our observations suggest that bipolar cell dendrites are largely generated by extensions from apical processes in the region of the OPL, with virtually no neurite formation distal to this outer synaptic layer. Also, early neurite growth from apical processes undergoes dynamic remodeling within the OPL region, indicating that bipolar cell dendrites may not necessarily target photoreceptor terminals immediately. Dendritic elaboration of bipolar cells thus does not follow the 'conventional' pathway whereby dendrites typically elaborate from the cell body. Rather, dendrites of these interneurons emerge from a neuroepithelial-like apical process.

DISCUSSION

Hippocampal neurons in culture undergo a phase of development whereby several short processes extend and retract from the cell body before one process becomes the axon[18,19]. Which of the early processes becomes the axon seems to be random, as all minor processes seem capable of turning into an axon[1]. However, recent observations suggest that axogenesis and dendritogenesis may not always be random, as some processes are specified early to become axons and others to become dendrites[4–6,20,21]. Although processes associated with migration are known to sometimes give rise to axons and dendrites, axogenesis and dendritogenesis from apical and basal processes while these are both still attached to the neuroepithelium have not been observed before. Our time-lapse observations of GFP-expressing developing mouse ON bipolar cells thus provide the first real-time view of this new mode of axogenesis and dendritogenesis in the CNS.

The possibility that the apical epithelial-like processes are the source of the dendritic arbors of bipolar cells has previously been suggested by Golgi studies of Landolt clubs[22,23], processes that contact the OLM. Only a subset of bipolar cells in adult chick retinas have Landolt clubs. However, in the immature chick retina, all presumed bipolar cells are

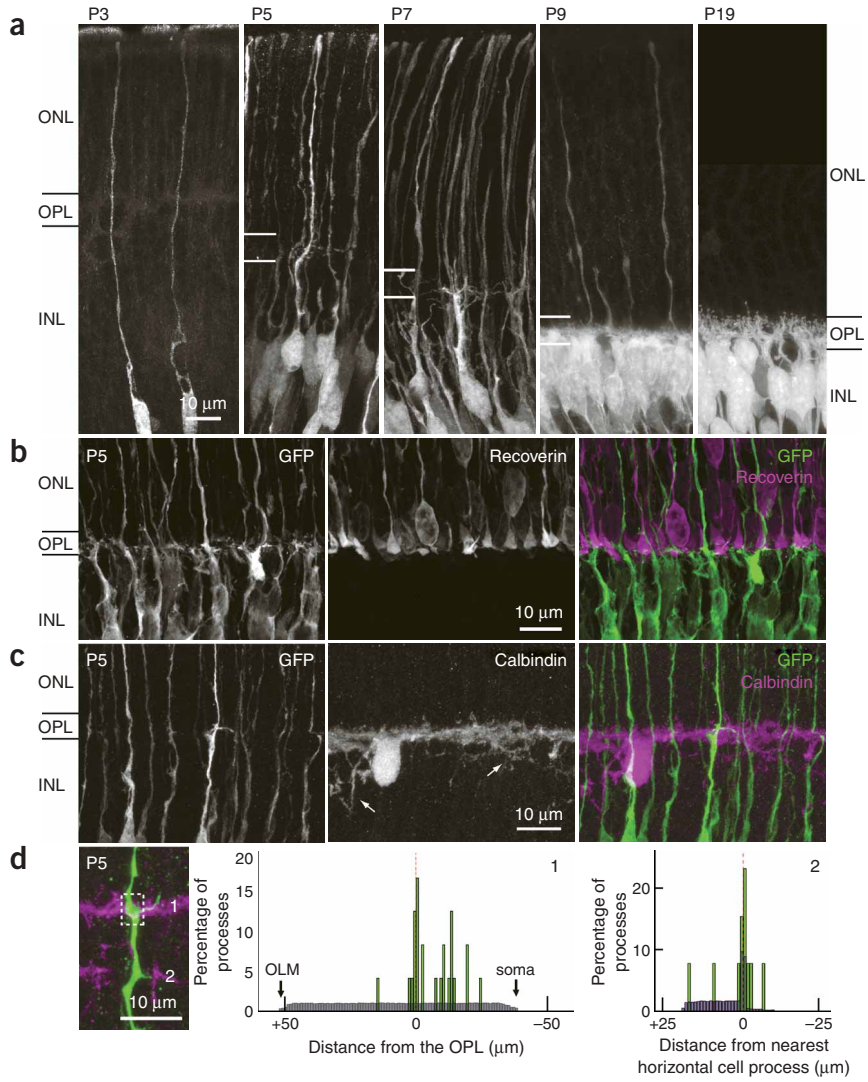

Figure 5 Dendrites originate from the apical process, at or proximal to the OPL. (**a**) Cross-sections of *Grm6-GFP* retinas at various ages labeled with anti-GFP. In P5–P9, OPL location is marked by white bars. (**b**) Dendritic differentiation of GFP-expressing bipolar cells relative to the location of the OPL, defined by anti-recoverin labeling of photoreceptor terminals. (**c**) Extension of individual dendritic processes from GFP-labeled bipolar cells relative to calbindin-labeled horizontal cell processes. Arrows indicate stray horizontal processes. (**d**) Quantitative relationship between the sites of dendritic extension and sites of contact with horizontal cells. Left, example of a bipolar cell apical process used for analysis (see Methods). 1, main lamination of horizontal cells processes defined the location of the OPL. 2, horizontal cell processes could also be found below the OPL, where they sometimes intersected with bipolar cell apical shafts. Dendrites emanating within the white box (2.5 µm above and below the mean depth of the main horizontal cell lamina) are considered as being associated with the OPL. Middle, distribution (green histogram) of dendrites emerging from the apical process as a function of depth, relative to the main horizontal cell plexus (area labeled '1' in left panel indicates the OPL). Right: distribution (green) of dendrites outside the OPL ('2' in left panel) relative to the nearest contact (0 on horizontal axis) between the apical process and a horizontal cell process. Gray distributions in middle and right panels show expected distributions obtained from Monte Carlo simulations (see Methods).

attached to the OLM by apical processes, suggesting a neuroepithelial origin for Landolt clubs. Retraction of apical processes seems to occur only after the dendritic arbors form, implicating a role for apical processes in dendritogenesis[22]. Recent analysis of the *Chx10* bacterial artificial chromosome (BAC) reporter transgenic line also suggests that mouse bipolar cells possess ascending or apical processes during development[24]. As in chicks, only some mouse bipolar cells seem to maintain these processes at maturity[24]. In the *Grm6-GFP* transgenic mouse, all GFP-positive ON bipolar cells have an apical process during development; such processes were not observed in adults. However, because OFF bipolar cells are not labeled in this GFP transgenic line, it may be that the bipolar cells maintaining ascending processes into adulthood[24] represent OFF bipolar cells. Our high-resolution and time-lapse studies of bipolar cell development in the *Grm6-GFP* mouse confirmed that the dendritic arbor originates from the apical process. Bipolar cells across species may thus share a common mechanism of dendritogenesis.

Axogenesis from basal processes in the CNS has also been demonstrated in RGCs. While its apical process retracts as the RGC cell body translocates toward the ILM, the basal process, still attached to the ILM, sprouts a growth cone to form the axon that continues extending toward the optic nerve head[20,25,26]. Similarly, we showed here that basal processes of mouse bipolar cells gave rise to their axonal arbors. However, in contrast to the RGCs, the basal process of the bipolar cell essentially became the cell's primary axon. It is possible that as we gain more insight into the early development of other locally projecting neurons, we will find a more general role for the participation of neuroepithelial-like processes in axogenesis and dendritogenesis.

We were not able to image bipolar cells early enough in development, before their final division, to be certain that their apical and basal processes are derived from conventional neuroepithelial processes. It is possible that processes reaching the ILM and OLM are lost after the initial migratory phase but then regrow, as is observed in some early differentiating cortical neurons[27]. However, this seems unlikely for bipolar cells because the apical and basal processes were present when bipolar cell somal translocation was still occurring and when dendritic and axonal differentiation had not yet taken place. Thus, we believe that the apical and basal extensions of GFP-positive bipolar cells have a neuroepithelial origin.

Is there a developmental advantage for bipolar cells to generate their axonal and dendritic arbors from neuroepithelial-like processes? Bipolar cells are the last neurons in the retina to differentiate[14]. Because their apical and basal processes already span the entire retina, targeting of synaptic partners that are already in place could occur simply by sampling the local environment. Also, the parallel arrangement of the neuroepithelial processes ensures that axonal and dendritic arbors of bipolar cells maintain a consistent neighbor-neighbor relationship in

the inner and outer retina. This continuity is important for preserving spatial information along the vertical pathway of the retina[28–30].

In addition to simply reaching the IPL, bipolar cells axons also need to target specific sublaminae within the IPL to form appropriate connections. Our current study of ON bipolar cell axonal development revealed that early in axogenesis, the entire basal process was competent to produce side branches. Our observations suggest that the stratification of ON bipolar cell axons then depends on the selective maintenance of processes in the ON sublaminae, although it is not possible to rule out some role for selective growth. Upon stabilization, processes in the ON sublamina could provide the substrate for further branching. Iterative rounds of branching would eventually lead to a greater distribution of axonal terminals in the ON compared to the OFF sublamina, in a manner similar to that proposed for zebrafish tectal dendrites[31]. This mode of arbor remodeling in bipolar cells is in contrast to that previously implicated for their major postsynaptic targets, the RGCs. Dendrites of RGCs that are monostratified at maturity initially ramify substantially in both the ON and OFF sublaminae, showing no apparent bias in dendritic elaboration early in development[32]. Unlike RGCs, the ability of bipolar cells to rapidly establish a biased arbor within their appropriate sublamina may exist because, at the stage when bipolar axonal outgrowth occurs, lamination cues are probably in place in the IPL. These cues may come from the arbors of amacrine cells and RGCs which have stratified arbors before bipolar axonal branching begins[32]. Because axonal stratification of bipolar cells is normal in the absence of RGCs[33,34] but not when amacrine stratification is perturbed[33,35,36], it may be that amacrine cells, rather than RGCs, provide cues for bipolar cells stratification.

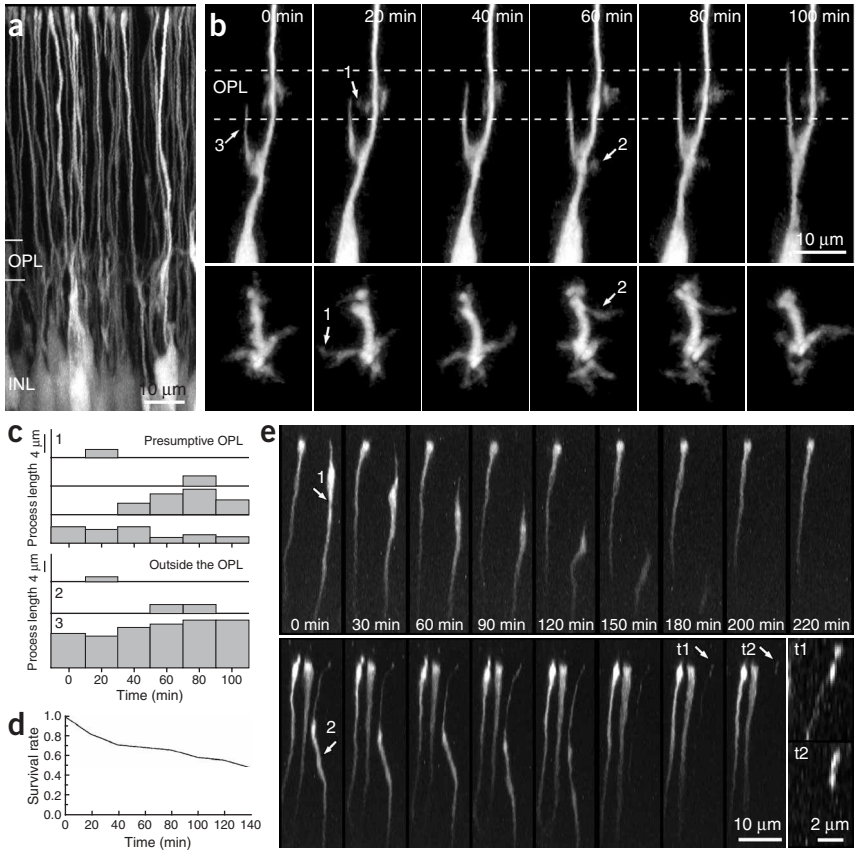

Figure 6 Time-lapse imaging reveals dynamics of dendritic outgrowth and apical process retraction. (**a**) Orthogonal projection of the outer half of a P5 *Grm6-GFP* retina imaged with two-photon microscopy. (**b**) Orthogonal projection (top) and axial projection (bottom) of a bipolar cell apical process imaged every 20 min. Process formation was observed inside ('1') and outside ('2') the OPL. An apical process collateral was also seen reaching up toward the OPL ('3'). Image segmentation (Amira) was used to display a single bipolar cell. (**c**) Plots of process length over time. (**d**) Survival curve of dendritic processes ($n = 38$) imaged every 20 min. (**e**) Orthogonal projections of apical processes showing their retraction in a P9 transgenic retina, imaged every 20–30 min. Process 1 appeared to fully detach from the OLM, whereas process 2 appeared to leave behind some cytoplasm (t1, t2).

We also found that most dendritic processes were localized within the outer synaptic lamina at any given time. Our time-lapse observations demonstrated that the formation of the dendritic arbor of the ON bipolar cell began with an exploratory behavior. However, in contrast to axonal outgrowth, which occurred throughout the length of the basal process, dendritic outgrowth seemed to be largely confined to the apical process at or proximal to the OPL. The cause of the overall lack of dendritic elaboration within the outer nuclear layer (ONL) may be that apical processes themselves are primarily competent to extend dendrites proximal to the OPL, or it may be that the ONL environment does not allow bipolar cell dendritic growth.

As in the IPL, synaptic partners of bipolar cell dendrites are likely to provide positional information to guide the formation of bipolar cell dendritic arbors. Our immunolabeling studies showed that, when ON bipolar cells began extending dendritic branches, the photoreceptor terminals were already tightly laminated and horizontal cell processes were stratified into the OPL, except for a few stray processes. Notably, we observed that bipolar cell dendrites found outside the developing OPL were more likely to be associated with stray horizontal cell processes than would be predicted by chance. This result is consistent with the hypothesis that bipolar cell dendritic growth or maintenance is encouraged locally by horizontal cells.

Regardless of the exact cellular mechanisms that target the axons and dendrites of bipolar cells to their correct synaptic regions, our current findings underscore the unconventional manner in which these interneurons elaborate their presynaptic and postsynaptic neurites to begin this process.

METHODS

Generation of *Grm6-GFP* transgenic mice. In the retina, mGluR6 is expressed exclusively by ON bipolar cells[37]. Using the *Grm6* (*mGluR6*) promoter, a transgenic mouse was generated in which GFP expression is driven selectively in ON bipolar cells. We constructed a transgene vector containing the *Grm6* promoter (in *pN2N*; gift of Y. Nakajima and S. Nakanishi, Kyoto University, Kyoto, Japan) fused to the GFP coding sequence. The linearized targeting construct was injected into the male pronucleus of fertilized eggs (from the B6SJLF1 strain) and transferred into foster females. The pups were screened by genomic polymerase chain reaction (PCR) for the presence of the transgene. The founder mouse lines were mated with C57BL/6J for more than six

generations. This study used the *Tg(Grm6-EGFP)5Var*, in which all ON bipolar cells (and no other cell types) express GFP at maturity. We refer to this line as *Grm6-GFP*.

Tissue preparation. All procedures were performed in accordance with Washington University Institutional Animal Care and Use Committee protocols. For fixed tissue, mice were anesthetized with 5% halothane and killed. The eyes were enucleated and placed in cold, oxygenated mouse artificial cerebral spinal fluid (ACSF)[38,39]. Retinas were removed and fixed in 4% paraformaldehyde for 30 min, and then washed and stored in 0.01 M phosphate-buffered saline. We obtained vibratome sections (60 μm) or frozen sections (30 μm).

Immunolabeling was performed using antibodies to calbindin (anti-calbindin, 1:1,500, Sigma), PKC (anti-PKC,1:1,500, Sigma), CaBP5 (anti-CaBP5, 1:1,500, gift of F. Haeseleer and K. Palczewski, University of Washington, Seattle), VAChT (anti-VAChT, 1:1,500, Promega), GFP (anti-GFP, 1:1,500, Molecular Probes) and recoverin (anti-recoverin, 1:1,000, Chemicon). The secondary antibodies were either Alexa 488 or Alexa 568 conjugates (1:1,000, Molecular Probes).

For live imaging, the retinas were isolated in ACSF, flat-mounted on filter paper and placed in a recording chamber. Alternatively, whole retinas were suspended in a small amount of 1% low-melting-point agarose that was then allowed to polymerize directly within the recording chamber. The tissue was maintained at 30 °C in oxygenated mouse ACSF.

Imaging. Fixed tissue was imaged on a FV-300 Olympus laser scanning microscope (LSM) with an Olympus 60× (1.4 NA) objective. Live confocal imaging was performed using a 60× (1.1 NA) or a 100× (1.0 NA) objective. Two-photon imaging was carried out with a Zeiss LSM 510 microscope and 60× (0.9 NA) objective at 890 nm.

Image analysis was performed using Metamorph (Universal Imaging) and Amira (Mercury Computer Systems, TGS line). All measurements of process length, position and lifetime were performed in Amira so that measurements could be readily made in three dimensions. Image segmentation was used to view individual arbors when surrounding cells obscured the view. Time-lapse images were median filtered to reduce the effects of photomultiplier tube (PMT) noise.

Statistics. For the analysis of axon stratification and dynamics, processes were assigned to the ON or OFF sublamina according to which half of the IPL they emerged in. Statistical analysis was performed using the Wilcoxon rank sum test for equal medians in Matlab (MathWorks).

To investigate whether dendritic outgrowth from bipolar cell apical processes is biased toward the OPL, we first identified the point at which each apical process intersected the middle of the OPL (0 in plots shown in **Fig. 5d**). We focused our analysis on P5 retinas when dendritic outgrowth began. For each cell, we determined the location on the apical process at which point each dendritic process emerged (dendritic initiation point). We then plotted these locations as a function of distance from the OPL (0 on the horizontal axis) for the population of dendrites that were analyzed (7 cells; 24 processes). To determine whether the measured distributions were different from a random distribution, we performed a Monte Carlo simulation (10,000 simulations; Matlab) based on the same data set. In this simulation, the observed intersections between bipolar cells and the OPL were assigned as a template upon which the location of dendrites could be randomly generated. Because some horizontal cell processes resided outside the OPL at P5, we also asked whether bipolar cell dendritic outgrowth from the apical shaft was spatially associated with these processes. Thus, we calculated the distance of dendritic initiation points from the nearest intersection point between the apical process and the 'stray' horizontal cell processes. Comparison with a random distribution was obtained as before using Monte Carlo simulations, except that dendrite positions were randomly assigned only along the apical shaft proximal to the OPL. This was because the vast majority of dendritic initiations and appositions with horizontal cells were observed proximal to the OPL.

Note: Supplementary information is available on the Nature Neuroscience website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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