Uncoupling of neurogenesis and differentiation during retinal development

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Abstract

Conventionally, neuronal development is regarded to follow a stereotypic sequence of neurogenesis, migration, and differentiation. We demonstrate that this notion is not a general principle of neuronal development by documenting the timing of mitosis in relation to multiple differentiation events for bipolar cells (BCs) in the zebrafish retina using in vivo imaging. We found that BC progenitors undergo terminal neurogenic divisions while in markedly disparate stages of neuronal differentiation. Remarkably, the differentiation state of individual BC progenitors at mitosis is not arbitrary but matches the differentiation state of post-mitotic BCs in their surround. By experimentally shifting the relative timing of progenitor division and differentiation, we provide evidence that neurogenesis and differentiation can occur independently of each other. We propose that the uncoupling of neurogenesis and differentiation could provide neurogenic programs with flexibility, while allowing for synchronous neuronal development within a continuously expanding cell pool.

Keywords bipolar cells; development; differentiation; neurogenesis; retina
Subject Categories Development & Differentiation; Neuroscience

Introduction

The nervous system is indisputably the most complex structure assembled during vertebrate ontogenesis. Consequently, the developmental processes underlying nervous system assembly must be precisely orchestrated. Neuronal development in the central and peripheral nervous systems (CNS, PNS) is widely accepted to require three major steps: (i) neurogenesis, the birth of neurons by progenitor cell mitosis, (ii) migration, the relocation of post-mitotic neurons from their birthplace in proliferative zones to specific locations, and (iii) neuronal differentiation, the acquisition of molecular and morphological features that permit the integration of newly generated neurons into synaptic circuits. To date, the prevailing view is that these ontogenetic events are discrete steps along a stereotypic sequence, beginning with neurogenesis, followed by migration and concluding with neuronal differentiation. There is however, particularly in the PNS, evidence that some hallmarks of neuronal differentiation might already occur in progenitors (Rothman et al., 1980; Rohrer & Thoenen, 1987; DiCicco-Bloom et al., 1990; Godinho et al., 2007; Attardo et al., 2008). These observations call into question the invariant developmental sequence of neurogenesis, migration, and differentiation, and raise the unresolved question: How stereotypic is the developmental program of a defined progenitor population in vivo?

Here, we examined the developmental fate of a molecularly defined CNS progenitor population (expressing visual homeobox gene 1, vsx1) that gives rise to the vast majority of a specific interneuron cell type (bipolar cells, BCs) in the zebrafish retina by terminal symmetric divisions (He et al., 2012; Weber et al., 2014). The swift development as well as the genetic and optical accessibility of zebrafish permitted us to follow the entire developmental program of vsx1+ progenitors, with single cell precision, in vivo. We used molecular, morphological, and cell biological markers of neuronal differentiation, in conjunction with chronic in vivo time-lapse imaging, to determine the timing of mitosis in relation to a battery of developmental events. We discovered that for BCs, neurogenesis and multiple hallmarks of neuronal differentiation (such as
somal positioning, neuronal marker expression, or neurite elaboration) are timed independently of each other. In other words, rather than dividing at a stereotypic point in their developmental trajectory, vsx1+ progenitors of BCs undergo terminal mitosis at markedly disparate stages of differentiation, suggesting that differentiation is not time-locked to mitosis. However, the state of differentiation of a vsx1+ progenitor at mitosis is not arbitrary, but matches that of the post-mitotic vsx1+ BCs in its vicinity.

**Results**

**Bipolar cell progenitor mitoses occur over an extended time-period and relocate to non-apical sites**

In common with many parts of the developing vertebrate CNS, the retina begins as a pseudostratified neuroepithelium with spindle-shaped progenitors that span its apico-basal extent and undergo interkinetic nuclear migration, an oscillatory nuclear movement linked to specific cell cycle phases (Sauer, 1935; Baye & Link, 2008). At distinct but overlapping times, cells destined for different fates exit the cell cycle. Because mitotic divisions generally occur at the apical surface, newborn cells need to migrate varying distances to occupy their definitive locations within one of the emerging cellular laminae. Thus, while ganglion cells migrate furthest to occupy positions in the basal most part of the neuroepithelium, BCs have a shorter distance to relocate, and photoreceptors remain in situ at the apical surface. BCs, which are ultimately localized to the inner nuclear layer (INL) and confine their dendirctic and axonal processes to the outer and inner plexiform layers (OPL, IPL), respectively, are generated over a protracted period, between 2 and 3 days post-fertilization (dpf) in the zebrafish (He et al., 2012). Thus, early-born cohorts of BCs are generated when the retinal neuroepithelium is not yet laminated, while later-born cohorts are generated when the three cellular laminae are emerging.

To investigate the relationship between BC neurogenesis and differentiation, we examined the expression of vsx1, a transcription factor important for BC development (Passini et al., 1997; Chow et al., 2001; Vitorino et al., 2009; Shi et al., 2011), using a vsx1:GFP transgenic line (Kimura et al., 2008). In the zebrafish retina, vsx1 is expressed at low levels in the majority of committed, terminally dividing BC progenitors, up-regulated during differentiation, and maintained at high levels in mature BCs (Vitorino et al., 2009). This developmental expression profile is faithfully reproduced in the bacterial artificial chromosome (BAC) vsx1:GFP transgenic line (Vitorino et al., 2009), so that we could follow nascent BCs from birth to maturity. Further, we took advantage of the zebrafish retina’s gradient of development (Hu & Easter, 1999), to concurrently visualize immature (unlaminated) and more mature (laminated) parts of the retina and thus compare multiple states of BC differentiation in a single field of view.

At 2 dpf, we found low levels of GFP expression in the unlaminated part of the retina in which vsx1:GFP+ (henceforth referred to as vsx1+) cells span the entire apico-basal axis. These vsx1+ cells represent both BC progenitors and post-mitotic, undifferentiated BCs (Morgan et al., 2006; Randlett et al., 2013). By contrast, vsx1+ cells in the more mature, laminated part of the retina showed high levels of GFP expression and confined their processes to the IPL and OPL, suggestive of post-mitotic, differentiated BCs (Fig 1A). To our surprise, immunostaining of vsx1:GFP retinas for phosphorylated histone H3 (pH3), a late G2/M-phase marker (Hendzel et al., 1997), revealed vsx1+ pH3+ cells not only in the unlaminated retina but also in the INL of the laminated retina. This suggests that not only post-mitotic BCs but also vsx1+ progenitors reside within the INL (Fig 1A–D). Notably, while vsx1+ progenitors in the unlaminated retina undergo mitosis at the apical surface, like “classical” progenitors in many parts of the CNS (Fig 1B), vsx1+ progenitors in the laminated retina undergo mitosis in the INL (Fig 1C), akin to previously described non-apical progenitors (Godinho et al., 2007; Weber et al., 2014). Given the precocious expression of some neuronal characteristics in such non-apical progenitors, we asked how similar vsx1+ progenitors were to the post-mitotic vsx1+ BCs in their immediate surround.

**Marker expression in progenitors matches the surrounding post-mitotic bipolar cells**

GFP levels during mitotic division in vsx1+ pH3+ progenitors showed a striking (3.8-fold) increase between the unlaminated and laminated regions of the developing retina (Fig 1E), which
Figure 1. Uncoupling mitosis and differentiation.
correlated well with the GFP levels in surrounding vsx1+ pH3- cells (Fig 1F; the surrounding vsx1+ cells in laminated regions are expected to be ~90% post-mitotic BCs on average; for an estimate of this number see Appendix Supplementary Materials and Methods). Furthermore, comparison of vsx1:GFP levels in pH3+ progenitors and pH3- surrounding cells across the entire developmental gradient in single retinas revealed a linear increase along the gradient (Fig 1F, inset). Hence, with regard to vsx1 expression, progenitors in the laminated retina are more similar to their BC neighbors than to their early dividing peers and form a continuum with regard to vsx1 promoter activity in lock-step with surrounding BC differentiation. Direct time-lapse observation of vsx1:GFP levels confirmed a parallel increase of fluorescence levels in BC progenitors and surrounding post-mitotic BCs in vivo (Fig EV1). Moreover, based on the decay of GFP in a vsx2:GFP BAC line (Vitorino et al, 2009), vsx2 down-regulation was similarly linked to the progression of differentiation along the retinal gradient independent of mitotic status.

To further establish this similarity in molecular differentiation of vsx1+ progenitors and their post-mitotic neighbors, we examined two additional molecular markers of BC differentiation: cone-rod homeobox (Crx) and Ribeye a. Crx is a transcription factor expressed in mature photoreceptors and BCs (Liu et al, 2001; Shen & Raymond, 2004). In the unlaminated retina, antibody staining for

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**Figure 2. Morphological rearrangement of vsx1+ progenitors matches surrounding BCs.**

A. Confocal in vivo time-lapse recording of a retina from the Q26 transgenic line (crossed to a UAS:memYFP reporter) showing a non-apically dividing vsx1+ progenitor (pseudo-colored cyan) with processes restricted to the IPL and OPL (dashed lines) during mitosis (97'). The last time point at which an apical process (open arrowhead) is detected is 89 min prior to mitosis. Scale bar: 10 μm.

B. Quantification of the distinct morphologies adopted by non-apically dividing vsx1+ progenitors at M-phase entry (122 progenitors, 17 fish). Open arrowheads indicate cytoplasmic processes extending beyond the synaptic layers (OPL and IPL, dashed lines).

C. Quantification of the time interval between retraction of the apical process (triangle) and mitosis (cyan circle) of non-apically dividing vsx1+ progenitors shows a broad range from 18 min to more than 540 min. As only mitosis, but not process retraction was observed for the progenitor depicted with small cyan dots, the movie length of 540 min is an underestimate. 18 progenitors from 11 fish.

D. Schematic of apical process retraction (triangle) in a non-apical vsx1+ progenitor (cyan soma) and the presence or absence of apical processes in the surrounding, post-mitotic BCs.

E. Quantification of the percentage of surrounding post-mitotic BCs without an apical process at the time when pre-mitotic vsx1+ progenitors undergo apical process retraction (triangle, 60.3 ± 6.6%) and at the time when these progenitors undergo mitosis (cyan circle, 95.1 ± 1.7%). Data are presented as mean ± SEM, 17 progenitors, 10 fish.
Crx (combined with pH3) revealed little or no expression in vsx1+ progenitors or the vsx1+ cells in their vicinity, whereas in laminated regions, high levels of Crx expression were found in virtually all vsx1+ progenitors and surrounding post-mitotic vsx1+ BCs (Fig 1G). The levels of Crx antibody staining in mitotic vsx1+ progenitors along the differentiation gradient strongly correlated with that of the vsx1+ cells in their immediate surround (Fig 1H). Moreover, time-lapse imaging of a crx:mCFP transgenic line (Suzuki et al., 2013), in which Crx+ cells are faithfully labeled (Fig EV2A), regularly revealed non-apical crx:mCFP+ mitotic divisions in the laminated retina (Fig EV2B and C). Finally, we analyzed the expression of Ribeye a, a structural protein of ribbon synapses in photoreceptors and BCs (Wan et al., 2005). As expected, using fluorescence in situ hybridization, we found ribeye a mRNA only in the laminated retina, where post-mitotic cells predominate (Fig EV2D and E). Notably, we also observed ribeye a mRNA-containing cells that were pH3+ (Fig EV2F). The fact that these cells were located in the INL suggests they are BC progenitors. Using a transgenic line designed to report ribeye a expression in BCs (ctbp2:mEGFP; Odermatt et al., 2012), we observed ctbp2:mEGFP+ cells dividing at non-apical locations, giving rise to BCs (Fig EV2G). Hence, even with regard to a marker linked to synaptic structures unique to BCs in the inner retina, we find that BC progenitors co-differentiate with post-mitotic BCs in the surround. We next asked whether this similarity extended beyond molecular markers to cellular morphology and dynamics.

**Progenitor morphology and cell biology correspond to the surrounding post-mitotic bipolar cells**

To examine individual cells of the vsx1+ BC lineage, we generated a transgenic Gal4-driver line (referred to as Q26) that was selected to label a sparse subset of vsx1+ cells. As Gal4 expression in Q26 is restricted to vsx1+ BCs and their progenitors in laminated parts of the retinal gradient (Appendix Fig S1), we almost exclusively observed non-apically dividing Q26+ progenitors, which we followed by time-lapse imaging as they terminally divided to produce BCs (Fig 2A). We examined the cleavage plane in dividing Q26+ progenitors (n = 65) and found divisions along the circumferential, apico-basal, and centro-peripheral axes with no preference for a particular orientation, in line with other reports of non-apically dividing progenitors (Kimura et al., 2008; Weber et al., 2014). During mitosis, the vast majority of Q26+ progenitors had limited their processes to the plexiform layers (Fig 2A at 0', Fig 2B), as is characteristic for BCs in the mouse and zebrafish retina (Morgan et al., 2006; Randlett et al., 2013) but not for apically dividing progenitors (Das et al., 2003; Miyata et al., 2004; Noctor et al., 2004). Prior to mitotic division however, the processes of non-apically dividing progenitors extended beyond the OPL or IPL (see Fig 2A at −89') and over time remodeled to become restricted to the synaptic layers. We asked whether this morphological remodeling occurred in a fixed time-window relative to mitotic division and focused our analyses, for technical reasons (see Appendix Supplementary Materials and Methods) on the retraction of processes from the apical surface. We found that apical process retraction occurred over an extended period of time (18 min to >9 h) prior to mitotic division (Fig 2C). In contrast, for apically dividing BC progenitors in the unlaminated retina, apical process retraction to the OPL was a post-mitotic event (as observed in vsx1:GFP), but again occurred over an extended time span following mitotic division (a few min to >8 h). Thus, a single differentiation step, the remodeling of the apical process, occurs both pre- and post-mitotically, and over a time span of more than 17 h relative to mitosis. Notably however, our time-lapse recordings in the Q26 line suggested that apical process remodeling is locally coordinated. When we identified progenitors that had just undergone apical process retraction to the OPL and asked whether post-mitotic BCs in the immediate vicinity had also done the same (Fig 2D), we found that, on the population level, apical process remodeling occurred concurrently (Fig 2E). Moreover, once pruned, the apical and basal processes of pre-mitotic progenitors could form lateral arbors. While these arbors regressed during mitosis, at earlier time points we could not distinguish them from the dendritic and axonal arbors of surrounding post-mitotic BCs (brackets in Fig 3A and E; Fig 4D at −645'), suggesting that the morphological processes of BC differentiation proceed independent of progenitor mitosis. In accordance with observations from other systems and species (Miyata et al., 2001; Das et al., 2003; Saito et al., 2003; Kosodo et al., 2008) the basally directed process of dividing Q26+ progenitors exhibited either splitting or asymmetric inheritance by one daughter cell followed by new outgrowth by the other daughter (Appendix Fig S2). Remarkably, when new process outgrowth was observed, it exhibited directed targeting of the IPL without overshooting beyond it.

Next, we asked whether vsx1+ progenitors undergo interkinetic nuclear migration. We used an mRNA construct encoding a fusion
protein between mOrange2 and proliferating cell nuclear antigen (PCNA; Fig 3A and Appendix Fig S3) that allows determination of progenitor cell cycle phase by distinct nuclear localization patterns (Leonhardt et al., 2000; Leung et al., 2011). Similar to classical progenitors, vsx1+ progenitors in the unlaminated region exhibited rapid apically directed interkinetic nuclear migration during G2.
before mitosis at the apical surface (Fig 3B and Appendix Fig S3). This movement was absent in non-apically dividing \textit{vsx1} \textsuperscript{+} progenitors, making their nuclear movements largely indistinguishable from the post-mitotic BCs in their immediate vicinity (Fig 3A and C). However, the non-apically dividing \textit{vsx1} \textsuperscript{+} progenitors always translocated their nuclei to the INL/OPL interface prior to mitosis. This movement can be explained by the location of the centrosome. By expressing fluorescently tagged centrin4, we found that BC
The post-mitotic differentiation status of late-born bipolar cells is similar to the early-born bipolar cell population in their vicinity

We assessed the rate of differentiation of the BC progeny resulting from non-apically dividing vsx1+ progenitors in the laminated region of the retina. First, we monitored vsx1-driven GFP expression levels in post-mitotic BCs over the course of 10 h after their exit from the cell cycle and found a steady increase in fluorescence intensity (Fig 4A). Ten hours post-division, GFP expression levels in BC sibling pairs were remarkably similar to each other (Fig 4A and B) and remained similar to the cells in their surround, which largely comprised of earlier-born post-mitotic BCs (Fig 4C).

Next, we used an established transgenic line, ctbp2:memYFP (Pelassa et al, 2014), to examine the emergence of ribbon synapses in BC axon terminals. We assessed the time interval between exit from the cell cycle and the appearance of ribeye a clusters (marker of ribbon synapses) as an indication of presynaptic differentiation in vsx1+ BCs (Fig 4D). For late-born BCs derived from non-apically dividing vsx1+ progenitors, the average time interval was 6.5 h. By contrast, for early-born BCs this interval was longer than 13 h on average (Fig 4E). Thus, late-born BCs acquire features of presynaptic differentiation with greater speed than their earlier-born counter-parts. Finally, we measured the lateral extent of the terminal axonal arbors of BCs derived from non-apical vsx1+ progenitors 10 h after they exited the cell cycle, and found this to be remarkably similar to that of the earlier-born post-mitotic BCs in their vicinity (Fig 4F). Taken together, late-born BCs continue to differentiate in lock-step with earlier-born BCs in their vicinity, including the elaboration of markers of axon and synaptic differentiation, thus contributing to synchrony in local neuronal development.

Experimentally delaying cell division does not delay vsx1+ progenitor differentiation

Our experiments have established that along the developmental gradient, BC progenitors blend into the differentiation landscape that surrounds them with regard to their morphological, cell biological, and molecular characteristics. Moreover, the “head-start” gained by pre-mitotic differentiation in late-dividing progenitors continues in their BC progeny, so that the lag in differentiation between late-born and early-born BCs is remarkably low. Two potential scenarios could explain these observations: (i) Multiple, “fixed” vsx1+ progenitors exist, each of which undergoes mitosis at a stereotypic time point in the cell’s differentiation trajectory. (ii) Alternatively, neurogenesis and differentiation could be uncoupled from each other, so that mitotic divisions could occur at various points in any given vsx1+ cell’s differentiation program. To distinguish between these two possibilities, we delayed BC progenitor divisions using hydroxyurea and aphidicolin (HUA). HUA treatment rapidly reduced the number of cells entering the G2/M-phase (Appendix Fig S4), but a small number of progenitors continued to divide, albeit with a prominent delay. We could now ask whether during delayed progenitor mitosis, differentiation stalled (as implied by the existence of “fixed” progenitors) or whether it continued and remained in synchrony with surrounding post-mitotic BC differentiation (as predicted by the “uncoupling” scenario; Fig 5A). We tracked 10 vsx1+ progenitors in HUA-treated embryos from late S-phase until mitosis. Knowing that progenitors, in control experiments, divided on average 142 ± 3.9 min after the onset of late S-phase, we could determine when a HUA-treated cell should have divided (“expected” mitosis) and measured the delay with which the division actually occurred (“observed” mitosis, delay range approximately 3.5–9 h; Figs 5B and EV4). Because we could predict with a high degree of accuracy (86.4%), whether progenitors, in control conditions, would undergo mitosis at the apical surface or in the INL well before the divisions occurred (see...
Figure 5. Neurogenesis and differentiation of vsx1⁺ progenitors are independent of each other.

A Schematic representation of expected outcomes if the immature retina (progenitors, P; neurons, N) is treated with HUA to delay the cell cycle. Upper panel: If there are multiple “fixed” progenitors, a block of cell division should stall progenitors at the differentiation state in which they normally would have undergone mitosis. The result would be a ”salt-and-pepper” pattern of undifferentiated (light green) and differentiating progenitors (dark green). Lower panel: If cell cycle and differentiation are independent, all progenitors should homogenously differentiate. Open arrowheads indicate cytoplasmic processes not confined to the OPL and IPL, filled arrowheads indicate cytoplasmic processes confined to the synaptic layers.

B Confocal images of a 2 dpf retina from a vsx1:GFP embryo injected with a p53 morpholino and mOrange2-PCNA mRNA. A vsx1⁺ progenitor (dashed magenta outline) is shown before HUA treatment (left panel), at the time when it would have been “expected” to undergo mitosis (middle panel, orange diamond) and when it actually underwent mitosis (right panel, cyan circle). The retina and the vsx1⁺ progenitor continue to mature after the “expected” mitosis (retraction of cytoplasmic processes, mitosis at non-apical location, and up-regulation of GFP). Open arrowheads indicate cytoplasmic processes not confined to the OPL and IPL, filled arrowheads indicate cytoplasmic processes confined to the synaptic layers. Dotted lines indicate extent of vsx1⁺ cell somata across retinal thickness. Scale bar: 10 μm.

C Quantification of vsx1:GFP fluorescence intensity of progenitors at the time when they were expected to undergo mitosis (exp, orange) and when they underwent mitotic division (obs, cyan). Data are presented as mean ± SEM, 10 progenitors, four fish. Mann–Whitney U-test, ***P = 0.0002.

D The fluorescence intensity of HUA-treated vsx1:GFP progenitors at the time when they were expected to undergo mitosis (diamonds), and when they were observed to undergo mitosis (circles), plotted against the intensity of the surrounding cells (10 progenitors, four fish). One cell (cyan diamond) was expected to divide non-apically. Green line indicates the fluorescence change of the lowest expressing progenitor for clarity.

E Surrounding cells fluorescence (norm.)
Appendix Supplementary Materials and Methods), we could ask whether the HUA-induced delay of mitosis would shift divisions from apical to non-apical locations (as an “uncoupling” scenario would imply). Nine of the 10 HUA-treated cells fulfilled criteria that identified them to be destined to divide apically. However, all 10 cells instead underwent mitosis in the INL after process remodeling, suggesting that they had been shifted from an apical to a non-apical phenotype simply by delaying mitosis (Fig 5C). Furthermore, vsx1::GFP expression levels in the 10 delayed progenitors increased from the point of expected mitosis to observed mitosis (Fig 5D). Notably, even hours after the delayed mitosis occurred, progenitors still matched the fluorescence levels of cells in their immediate surround (Fig 5E), suggesting uncoupling of cell division and differentiation. Together, these findings support the “uncoupling” scenario laid out above and hence argue for an independence of neurogenesis and differentiation programs during BC development.

Discussion

Our study is the first, to our knowledge, to address the relative timing of neurogenesis, migration, and differentiation for a molecularly defined CNS population in vivo and to elucidate the effects of such timing on progenitor characteristics. In contrast to the widely held view, we found that developing neurons did not adopt a stereotypic sequence of neurogenesis followed by migration and subsequent differentiation. Rather, to our surprise, the developmental trajectories that progenitors adopted were variable and accommodated remarkable flexibility. This resulted in vsx1+ BC progenitors with a wide variety of molecular, morphological, and cell biological characteristics. Importantly, rather than representing many distinct populations, the differentiation status of these progenitors formed a continuum in lock-step with the differentiation of surrounding post-mitotic BCs along the developmental gradient of the retina. Accordingly, cells dividing in the laminated parts of the retina were more BC-like than their early dividing counter-parts. Indeed, without the aid of time-lapse imaging or cell cycle markers it would have been impossible to distinguish between pre-mitotic progenitors and post-mitotic cells. Our results support the conclusion that a stereotypical and fixed sequence of ontogenetic events is not essential during neuronal development. Thus, at least for terminally dividing progenitors, which generate a substantial part of the CNS neuronal population (Nakashima et al., 2015), mitosis does not have to occur before neuronal differentiation is initiated (or at any specific step thereafter), but rather can be flexibly intercalated between other developmental steps.

Our findings could explain two previous intriguing observations: First, that neuronal progenitors in different parts of the nervous system show precocious signs of differentiation prior to cell cycle exit (Rothman et al., 1980; Rohrer & Thoenen, 1987; DiCicco-Bloom et al., 1990; Miyata et al., 2004; Godinho et al., 2007; Attardo et al., 2008; Prasov & Glaser, 2012); and second, that blocking mitosis does not halt neuronal differentiation in many parts of the Xenopus CNS including the retina, spinal cord, and brain stem (Harris & Hartenstein, 1991). Our results now offer a unifying explanation for these previous observations, suggesting that they might simply result from a fundamental uncoupling of cell cycle and neuronal differentiation during normal development. Indeed, the increasingly recognized prevalence of precocious progenitors in many parts of the nervous system of a range of species [e.g., basal progenitors in the neocortex (Haubensak et al., 2004; Miyata et al., 2004; Noctor et al., 2004); neural crest-derived PNS progenitors (Rothman et al., 1980; Rohrer & Thoenen, 1987; DiCicco-Bloom et al., 1990); retinal progenitors (Godinho et al., 2007; Prasov & Glaser, 2012)], implies that such uncoupling may be a general principle of neural development.

What would be the advantages of uncoupling neurogenesis and neuronal differentiation? By comparison with the orderly sequence of developmental events in the classical model, we suggest that the uncoupled model provides two advantages, namely speed and synchrony (Fig EV5). In the classical model, because differentiation can only be initiated following mitosis, the time required for the majority of cells in a defined population to differentiate is dictated by the delay in their birth dates. By contrast, in the uncoupled model, differentiation steps can already occur at the progenitor stage, permitting differentiation across the population to be faster. Furthermore, despite the extended time span over which mitotic divisions can occur, cells in a given population differentiate in relative synchrony. The uncoupling of mitosis and differentiation could thus be particularly pertinent for the assembly of essential neural circuits where swift maturation is paramount for survival (Nikolaou & Meyer, 2015).

Materials and Methods

Animals

All experiments were performed according to regulations as approved by the local regulatory bodies. Zebrafish were maintained, mated, and raised as described in Mullins et al. (1994). Embryos were kept in 0.3× Danieu’s solution at 28.5°C and staged as previously described (Kimmel et al., 1995). Fish were either in an AB wild-type or roy orbison (Ren et al., 2002) background. The transgenic lines used are listed in Appendix Table S1. We generated Tg(vsx1:Gal4)Q26 (Q26) and Tg(14xUAS:memTagRFP-T) by Tol2 mediated insertion (Kawakami, 2004). For details of constructs used to make transgenic fish and for transient injections see Appendix Supplementary Materials and Methods.

mRNA synthesis and injection

Plasmids were linearized (PCNA: NotI, centrin4: Apal). Capped mRNA was produced using the Ambion mMESSAGE mMACHINE kit (Applied Biosystems) according to the manufacturer’s instructions. mRNA was injected at 100 ng/µl into one- or two-cell stage embryos.

Immunohistochemistry and in situ hybridization

Immunostaining to detect pH3, Crx, and GFP was performed with adaptations of previously published protocols either on cryosections (Williams et al., 2010) or on whole-mount embryos (Hunter et al., 2011). In situ hybridization to visualize ribeye a mRNA was performed on whole-mount embryos using a digoxigenin-labeled riboprobe and Fast Red TR/naphthol AS-MX (Sigma) to detect alkaline phosphatase activity. For a detailed description see Appendix Supplementary Materials and Methods.
In vivo imaging

Embryos were prepared for imaging as described previously (Godinho, 2011; Engerer et al., 2016). Between 10 and 18 h post-fertilization (hpf), embryos were transferred to 0.3× Danieau’s solution containing 0.003% 1-phenyl-2-thiourea (PTU, Sigma) to inhibit melanin formation. At 2.25 days post-fertilization (dpf), manually dechorionated embryos were anesthetized using 0.02% tricaine (Pharmacom) in medium containing PTU and embedded laying on their side in low-melting agarose (0.7–0.8%, Sigma).

Fish were imaged starting at 2 dpf on an Olympus FV1000 confocal/2-photon and an Olympus FVMPE-RS 2-photon microscope using water-immersion objectives (Olympus 20×/NA 0.95, Olympus 25×/NA 1.05, Zeiss 40×/NA 1.0, Nikon 25×/NA 1.1, and Nikon 40×/NA 0.8) or a silicon-immersion objective (Olympus 30×/NA 1.05). Embryos were maintained at 28.5°C during all in vivo recordings. At each time point z-stacks were acquired of the peripheral retina, encompassing its entire circumference.

Image processing

Images were viewed and processed using open-source ImageJ/Fiji software (http://fiji.sc). The StackReg function was used for drift compensation in xy. Image panels were assembled in Photoshop CS5 (Adobe) and combined into figures using Illustrator CS5 (Adobe). The “Gaussian blur” function was used to filter noise for clarity. Gamma was not adjusted.

Hydroxyurea-aphidicolin treatment

Hydroxyurea (Sigma) and aphidicolin (BioVitrioc) were used at a final concentration of 20 mM and 150 μM, respectively, in 0.3× Danieau’s containing 1.0–1.7% DMSO. Embryos were injected with a p53 morpholino (0.5–1 mM, Gene Tools) at the one- or two-cell stage to ameliorate HUA-induced apoptosis (Girdler et al., 2013). At 2 dpf embryos were mounted in agarose as described for in vivo imaging above, but leaving the tail fin un-embedded for better drug access. Retinas were imaged for one time point prior to HUA administration. The 0.3× Danieau’s medium was replaced with HUA-containing medium, and time-lapse recording was immediately resumed. Recordings were generally limited to < 16 h after HUA addition as high levels of cell death were observed thereafter.

Data analysis

For details, see Appendix Supplementary Materials and Methods.

Statistics

Mean values and standard error of the mean (SEM) were calculated using Microsoft Excel. We used the Mann–Whitney U-test to compare datasets with GraphPad Prism 5. Data are presented as mean ± SEM unless indicated otherwise. P-values < 0.05 are denoted with “*, P < 0.01 with “**“, and P < 0.001 with “***“.

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Author contributions

PE, PRW, TM, and LG conceived of the project and designed experiments. PE, SCS, TY, NO, and BO generated new constructs and transgenic lines for this research. PE, PRW, TM, and LG wrote the paper with input from all authors.

Conflict of interest

The authors declare that they have no conflict of interest.

References

Attardo A, Calegari F, Haubensak W, Wilsch-Brauninger M, Huttner WB (2008) Live imaging at the onset of cortical neurogenesis reveals differential appearance of the neuronal phenotype in apical versus basal progenitor progeny. PloS One 3: e2388

Baye LM, Link BA (2008) Nuclear migration during retinal development. Brain Res 1192: 29–36

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Uncoupling mitosis and differentiation

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Chow RL, Snow B, Novak J, Looser J, Freund C, Vidgen D, Ploder L, McInnes RR (2001) Vx2, a rapidly evolving paired-like homeobox gene expressed in cone bipolar cells. Mech Dev 109: 315 – 322

Das T, Payer B, Cayouette M, Harris WA (2003) In vivo time-lapse imaging of cell divisions during neurogenesis in the developing zebrafish retina. Neuron 37: 597 – 609

DiCicco-Bloom E, Townes-Anderson E, Black IB (1990) Neuroblast mitosis in dissociated culture: regulation and relationship to differentiation. J Cell Biol 110: 2073 – 2086

Engerer P, Plucinska G, Thong R, Trovo L, Paquet D, Godinho L (2016) Imaging subcellular structures in the living zebrafish embryo. J Vis Exp 110: e53456

Girdler GC, Araya C, Ren X, Clarke JD (2013) Developmental time rather than local environment regulates the schedule of epithelial polarization in the zebrafish neural rod. Neural Dev 8: 5

Godinho L, Williams PR, Claassen Y, Provost E, Leach SD, Kamermans M, Wong RO (2007) Nonapical symmetric divisions underlie horizontal cell layer formation in the developing retina in vivo. Neuron 56: 597 – 603

Godinho L (2011) Live imaging of zebrafish development. Cold Spring Harb Protoc 2011: 770 – 777

Harris WA, Hartenstein V (1991) Neuronal determination without cell division in Xenopus embryos. Neuron 6: 499 – 515

Haubensak W, Attardo A, Denk W, Huttner WB (2004) Neurons arise in the basal neuroepithelium of the early mammalian telencephalon: a major site of neurogenesis. Proc Natl Acad Sci USA 101: 3196 – 3201

He J, Zhang G, Almeida AD, Cayouette M, Simons BD, Harris WA (2012) How variable clones build an invariant retina. Neuron 75: 786 – 798

Hendzel MJ, Wei Y, Mancini MA, Van Hooser A, Ranalli T, Brinkley BR, Bazett-Jones DP, Allis CD (1997) Mitosis-specific phosphorylation of histone H3 initiates primarily within pericentromeric heterochromatin during G2 and spreads in an ordered fashion coincident with mitotic chromosome condensation. Chromosoma 106: 348 – 360

Hu M, Easter SS (1999) Retinal neurogenesis: the formation of the initial central patch of postmitotic cells. Dev Biol 207: 309 – 321

Hunter PR, Nikolaou N, Odermatt B, Williams PR, Drescher U, Meyer MP (2011) Localization of Cadm2a and Cadm3 proteins during development of the zebrafish nervous system. J Comp Neurol 519: 2252 – 2270

Kawakami K (2004) Transgenesis and gene trap methods in zebrafish by using the Tol2 transposable element. Methods Cell Biol 77: 201 – 222

Kimmel CB, Ballard WW, Kimmel SR, Ullmann B, Schilling TF (1995) Stages of embryonic development of the zebrafish. Dev Dyn 203: 253 – 310

Kimura Y, Satou C, Higashijima S (2008) VZa and VZb neurons are generated by the final divisions of pair-producing progenitors in the zebrafish spinal cord. Development 135: 3001 – 3005

Kosodo Y, Toida K, Dubreuil V, Alexandre P, Schenk J, Kiyokage E, Attardo A, Mora-Bermudez F, Ari T, Clarke JD, Huttner WB (2008) Cytokinesis of neuroepithelial cells can divide their basal process before anaphase. EMBO J 27: 3151 – 3163

Leonhardt H, Rahn HP, Weinzirl P, Sporbert A, Cremer T, Zink D, Cardoso MC (2000) Dynamics of DNA replication factories in living cells. J Cell Biol 149: 271 – 280

Leung L, Klopper AV, Grill SW, Harris WA, Norden C (2011) Apical migration of nuclei during G2 is a prerequisite for all nuclear motion in zebrafish neuroepithelia. Development 138: 5003 – 5013

Liu Y, Shen Y, Rest JS, Raymond PA, Zack DJ (2001) Isolation and characterization of a zebrafish homologue of the cone rod homeobox gene. Invest Ophthalmol Vis Sci 42: 481 – 487

Miyata T, Kawaguchi A, Okano H, Ogawa M (2001) Asymmetric inheritance of radial glial fibers by cortical neurons. Neuron 31: 727 – 741

Miyata T, Kawaguchi A, Saito K, Kawano M, Muto T, Ogawa M (2004) Asymmetric production of surface-dividing and non-surface-dividing cortical progenitor cells. Development 131: 3133 – 3145

Morgan JL, Dhingra A, Vardi N, Wong RO (2006) Axons and dendrites originate from neuroepithelial-like processes of retinal bipolar cells. Nat Neurosci 9: 85 – 92

Mullins MC, Hammerschmidt M, Haffter P, Nusslein-Volhard C (1994) Large-scale mutagenesis in the zebrafish: in search of genes controlling development in a vertebrate. Curr Biol 4: 189 – 202

Nakashima K, Umeshima H, Kengaku M (2015) Cerbellar granule cells are predominantly generated by terminal symmetric divisions of granule cell precursors. Dev Dyn 244: 748 – 758

Nikolaou N, Meyer MP (2013) Lamination speeds the functional development of visual circuits. Neuron 88: 999 – 1013

Noctor SC, Martinez-Cerdeno V, Ivic L, Kriegstein AR (2004) Cortical neurons arise in symmetric and asymmetric division zones and migrate through specific phases. Nat Neurosci 7: 136 – 144

Odermatt B, Nikolaev A, Lagnado L (2012) Encoding of luminance and contrast by linear and nonlinear synapses in the retina. Nat Neurosci 73: 758 – 773

Passini MA, Levine EM, Canger AK, Raymond PA, Schechter N (1997) Vsx-1 and Vsx-2: differential expression of two paired-like homeobox genes during zebrafish and goldfish retinogenesis. J Comp Neurol 388: 495 – 505

Pelassa I, Zhao C, Pasche M, Odermatt B, Lagnado L (2014) Synaptic vesicles are “primed” for fast clathrin-mediated endocytosis at the ribbon synapse. Front Mol Neurosci 7: 91

Prasov L, Glaser T (2012) Dynamic expression of ganglion cell markers in retinal progenitors during the terminal cell cycle. Mol Cell Neurosci 50: 160 – 168

Randlett O, MacDonald RB, Yoshimatsu T, Almeida AD, Suzuki SC, Wong RO, Harris WA (2013) Cellular requirements for building a retinal neuropil. Cell Rep 3: 282 – 290

Ren JJ, McCarthy WR, Zhang H, Adolph AR, Li L (2002) Behavioral visual responses of wild-type and hypopigmented zebrafish. Vision Res 42: 293 – 299

Rohrer H, Thoenen H (1987) Relationship between differentiation and terminal mitosis: chick sensory and ciliary neurons differentiate after terminal mitosis of precursor cells, whereas sympathetic neurons continue to divide after differentiation. J Neurosci 7: 3739 – 3748

Rothman TP, Specht LA, Cershon MD, Joo TH, Teitelman G, Pickel VM, Reis DJ (1980) Catecholamine biosynthetic enzymes are expressed in replicating cells of the peripheral but not the central nervous system. Proc Natl Acad Sci USA 77: 6221 – 6225

Saito K, Kawaguchi A, Kashiwagi S, Yasugi S, Ogawa M, Miyata T (2003) Morphological asymmetry in dividing retinal progenitor cells. Dev Growth Differ 45: 219 – 229

Sauer FC (1935) Mitosis in the neural tube. J Comp Neurol 62: 377 – 405

Shen YC, Raymond PA (2004) Zebrafish cone-rod (crx) homeobox gene promotes retinogenesis. Deu Biol 269: 237 – 251

Shi Z, Trenholm S, Zhu M, Buddingh S, Star EN, Awatramani GB, Chow RL (2011) Vxs1 regulates terminal differentiation of type 7 ON bipolar cells. J Neurosci 31: 13118 – 13127

Suzuki SC, Bleckert A, Williams PR, Takechi M, Kawamura S, Wong RO (2013) Cone photoreceptor types in zebrafish are generated by symmetric terminal divisions of dedicated precursors. Proc Natl Acad Sci USA 110: 15109 – 15114
Vitorino M, Jusuf PR, Maurus D, Kimura Y, Higashijima S, Harris WA (2009) Vsx2 in the zebrafish retina: restricted lineages through derepression. Neural Dev 4: 14

Wan L, Almers W, Chen W (2005) Two ribeye genes in teleosts: the role of Ribeye in ribbon formation and bipolar cell development. J Neurosci 25: 941 – 949

Weber IP, Ramos AP, Strzyz PJ, Leung LC, Young S, Norden C (2014) Mitotic position and morphology of committed precursor cells in the zebrafish retina adapt to architectural changes upon tissue maturation. Cell Rep 7: 386 – 397

Williams PR, Suzuki SC, Yoshimatsu T, Lawrence OT, Waldron SJ, Parsons MJ, Nonet ML, Wong RO (2010) In vivo development of outer retinal synapses in the absence of glial contact. J Neurosci 30: 11951 – 11961

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