Somatic tetraploidy in specific chick retinal ganglion cells induced by nerve growth factor

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Abstract

In contrast, retinal neurons lacking Islet1 were consistently diploid (Fig. S1). The presence of tetraploid RGCs was confirmed this finding (Fig. S2B). I-FISH was performed on neurons derived from partially trypsinized P1 retinas to enrich our preparations in RGCs (38.36 ± 0.92% of the cells were positive for the RGC-specific marker βIII tubulin). We observed that 6.19% of the nuclei (n = 210) contained four copies of a partially inverted tandem repeat previously identified in the pericentric region of chicken chromosome 8 (8).

The Mouse Retina Contains Tetraploid Neurons. As a proof that somatic tetraploidization in a subpopulation of retinal neurons also takes place in other higher vertebrates, dissociated retinal cells from adult mice were immunostained for Islet1, a marker expressed in this species by RGCs and a population of neurons from the inner nuclear layer (9), and analyzed by flow cytometry. This analysis demonstrated that 6.82 ± 2.22% (n = 4) of the murine Islet1-positive cells were tetraploid (Fig. ID).

p75NTR Is Expressed by Early Differentiating RGCs. To understand the mechanism responsible for the generation of tetraploid RGCs in the chick retina we focused on the expression pattern of p75NTR at E5, a stage when RGCs are being born and NGF induces cell cycle reentry through p75NTR in vitro (6). As in other neuroepithelia, retinal precursors undergo S-phase at basal positions (i.e., vitreal surface) and mitosis at the apical region, close to the pigment epithelium (10). The first neurons to differentiate in the retina are the RGCs (11), which express the RA4 marker less than 15 min after the end of mitosis (12) and acquire a differentiated morphology as they displace to the basally located GCL (13). In the E5 chick retina, p75NTR is present in migrating and layered RGCs, colocalizing with the early RGC-markers RA4 (12) and βIII tubulin (14), as well as with G4, a chicken glycoprotein mostly expressed by differentiated RGCs in this tissue (15) (Fig. S3). Therefore, p75NTR can be considered as an extremely early RGC marker.

Migrating RGCs Lacking Rb Can Reenter the Cell Cycle. The transcription factor E2F1 is crucial for G1/S-phase progression and Rb is known to prevent E2F1 activity during G1, being the expression of these two proteins tightly controlled during neurogenesis (16). Double labeling with the anti-p75NTR antiserum and an Rb-specific antibody revealed a population of cells containing p75NTR but that lacked Rb expression, representing 25.60 ± 0.86% (n = 4) of all of the p75NTR-positive cells (Fig. S4A). Double labeling for RA4 and Rb confirmed the presence of differentiating RGCs

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This article contains supporting information online at www.pnas.org/cgi/content/full/0906121107/DCSupplemental.

Author contributions: J.M.F. designed research; S.M.M., P.E., and J.M.F. performed research; S.M.M., P.E., and J.M.F. analyzed data; A.d.l.H. contributed new reagents/analytic tools; and A.d.l.H. and J.M.F. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.
lacking Rb expression (Fig. 2C). By contrast, the transcription factor E2F1 was detected in all of the migrating RGCs as revealed by double immunolabeling with RA4- and E2F1-specific antibodies (Fig. S4B). Double labeling for Rb and E2F1 further demonstrated the existence of retinal cells expressing E2F1 but lacking Rb (Fig. S4C). To study whether migrating RGCs which lack the expression of Rb could reenter the cell cycle in vivo, a 1 h pulse of BrdU was given to E5 chicken embryos and BrdU incorporation in retinal cryosections was studied. Besides the expected incorporation of BrdU into nuclei situated basally, there was also a population of retinal cells in S-phase within the apical half of the neuroepithelium (Fig. 2B). Most of these cells lack Rb (78.29 ± 2.85%; n = 3; Fig. 2B), and express the early RGC marker βIII tubulin (80.45 ± 6.33%; n = 3). These data suggest that some of the cells incorporating BrdU at the apical retina are migrating, postmitotic neurons undergoing DNA replication in vivo. This notion was confirmed by BrdU labeling of both RA4 and βIII tubulin-positive cells with extremely short (15 min) BrdU pulses (Fig. 2C and D; Table S2), thus supporting the hypothesis that the presence of BrdU in these cells is not a consequence of rapid differentiation (12).

**NGF Induces Cell Cycle Reentry in Migrating RGCs.** To test whether endogenous NGF (17) might be responsible for the cell cycle reentry of migrating RGCs observed in vivo, hybridoma cells secreting an anti-NGF mAb or control hybridoma cells secreting an irrelevant mAb were applied onto the chorioallantoic membrane of E3 chicken embryos, and they were administered a short pulse of BrdU 1 h before sacrificing at E5. The anti-NGF antibody was previously shown to prevent p75NTR-dependent retinal apoptosis in vivo (17). A significant reduction in the incorporation of BrdU was observed in the RA4-positive RGCs of embryos treated with anti-NGF antibodies when compared with control embryos (Fig. 2E). Similarly, injection of the anti-p75NTR ChEX blocking antibody into the eye also significantly reduced the proportion of βIII tubulin-positive cells that incorporated BrdU when compared to the retinas of embryos injected with normal rabbit serum (Fig. 2F). This effect was specific of differentiating RGCs as anti-NGF treatment did not affect the proportion of RA4-negative cells incorporating BrdU (control: 37.93 ± 0.04%, n = 3; anti-NGF: 37.83 ± 0.13%, n = 3; non significant), and ChEX injection did not affect the proportion of βIII tubulin-negative cells incorporating BrdU (control: 38.68 ± 0.50%, n = 3; ChEX: 38.31 ± 0.46%, n = 3; nonsignificant). Because NGF does not induce the expression of neuronal markers in the retinal precursors (6), we conclude that this neurotrophin exerts its proliferative effect only in differentiating RGCs. This view was confirmed in vitro by using dissociated E5 retinal cells cultured under neurogenic conditions for 22 h (see Materials and Methods). As expected, a subpopulation of these cells reduced the levels of Rb in response to differentiation (Fig. S4D), while still maintaining strong E2F1 expression (Fig. S4E). This effect was largely specific of RGCs because 40.96 ± 2.25% (n = 3) of the G4-positive cells were observed to express Rb at low levels, whereas only 4.44 ± 0.08% (n = 3) of the G4-negative cells showed low levels of Rb expression (Fig. S4G). On the contrary, virtually all G4-positive cells contained high levels of E2F1 (Fig. S4F). As expected, BrdU incorporation (1 h pulse) was increased in response to NGF, mostly in differentiating RGCs expressing βIII tubulin (Fig. S4J). Accordingly, electroporation of the luciferase reporter gene under the control of the murine c-Myc core promoter, known to respond to E2F1 (18), demonstrated that NGF can induce E2F1 activity in the retinal

![Fig. 1. Somatic tetraploid RGCs in the P1 chick and adult mouse retina. P1 chick retina (A and B) and adult mouse retina (C and D). Flow cytometric analysis demonstrates that a subset of Islet1-positive neurons (B and D) are tetraploid (4C) whereas all Islet1-negative (A and C) neurons show a 2C DNA content.](image1)

![Fig. 2. During development endogenous NGF induces cell cycle reentry and somatic tetraploidy in RGCs. Cryostat sections (A–D) or flow cytometry (E–J) from E5 chick retinas. A. The arrow points to a migrating RGC expressing RA4 (red) and lacking Rb immunoreactivity (green). The arrowhead indicates an RA4/BrdU-double labeled cell. B. After a 1 h pulse, BrdU (green) was observed in apically-located cells lacking Rb expression (red, see arrows). C. After a 15 min pulse, most apically-located nuclei positive for BrdU (green) are RA4-positive (red) RGCs (arrows). (D) Arrows indicate the presence of BrdU (15 min treatment; green) in jilhil tubulin-positive neurons (red) located at the apical half of the retina. (E) Anti-NGF antibodies (α-NGF) reduce the proportion of RA4-positive cells incorporating BrdU, when compared to irrelevant antibodies (Control). (F) The anti-p75NTR antiserum ChEX reduces the percentage of jilhil tubulin-positive cells incorporating BrdU, when compared to a control antiserum (Control). G. Islet1-negative precursors show a profile of cycling cells in G1, S and G2/M. (H) A subset of Islet1-positive RGCs are tetraploid (4C). (I) Percentage of Islet1-positive neurons with 4C DNA content as detected by flow cytometry in dissociated retinal cells from embryos treated with hybridoma cells secreting either an irrelevant mAb (Control) or anti-NGF producing hybridoma cells (α-NGF). (J) Percentage of Islet1-negative neurons with 4C DNA content, as detected by flow cytometry in dissociated E5 retinal cells from eyes injected at E4 with normal rabbit serum (Control) or the anti-p75NTR antiserum ChEX (ChEX). RGCs begin to be produced one day before antibody injection, thus explaining the modest reduction of cells with 4C DNA content. PE: pigment epithelium; v: vitreous body. (Scale bars: A, 15 μm; B–D, 12 μm.) *P < 0.05, **P < 0.01, ***P < 0.005.](image2)
cells (Fig. S4H). Although this effect might derive from a putative NGF-dependent decrease of Rb expression, this possibility was ruled out because the percentage of retinal cells expressing low levels of Rb was not affected by NGF (Fig. S4J). In accordance with the hypothesis that NGF acts on RGCs that lack Rb, overexpression of the latter protein reduced the proliferative capacity of the retinal cells and prevented the effect of NGF on BrdU incorporation after 1 h pulse (Fig. S4 K and L).

**Most Migrating RGCs that Reenter the Cell Cycle Remain as Tetraploid Neurons.** The fate of the migrating RGCs undergoing cell cycle reentry was assessed in BrdU pulse-chase experiments in E5 chicken embryos followed by immunolabeling of βIII tubulin-positive neurons. When 1 h BrdU pulses in ovo were followed by addition of an excess of dU (Fig. S5 A and B), a subpopulation of βIII tubulin-positive retinal cells undergoing ectopic S-phase could be labeled in vivo, permitting their fate to be followed. BrdU was initially incorporated into about 9% of βIII tubulin-positive retinal cells (Table S2), and this proportion increased to 21.08 ± 1.67% (n = 3) when considering the nonlayered (i.e., migrating) βIII tubulin-positive cells. Two, 4, and 6 h after dU addition, the proportion of βIII tubulin-positive cells that had incorporated BrdU was similar (Table S2), suggesting that this cell subpopulation had still not mixed with precursors in S-phase-1 (Fig. 3G) that had completed G2/mitosis and initiated the process of neuronal differentiation. Many migrating RGCs that replicated their DNA remained as tetraploid cells in the GCL. Accordingly, 6 h after dU addition around 0.4% of the βIII tubulin-positive cells adjacent to the basal surface contained BrdU in their nuclei (Table S2). This percentage rose to around 3.3% 9 h after addition of dU (see arrowhead in Fig. 3F; Table S2), and it augmented even further 15 h after BrdU administration to 5.47% (± 0.28; n = 3). Because there was no evidence of DNA replication (i.e., BrdU incorporation at short time points) in layered RGCs (Table S2), we concluded that the BrdU-positive RGCs observed in the GCL are derived from the βIII tubulin-positive cells that replicated their DNA while migrating. Flow cytometry analysis in dissociated retinal cells isolated from E5 embryos treated with BrdU for 9 h, revealed that 85.77 ± 1.71% (n = 4) of the BrdU-positive cells expressing Islet1 had a 4C DNA content in their nuclei (Fig. S5 C and D). These data indicate that most migrating RGCs that replicated their DNA remained in the GCL with a 4C DNA content. The actual proportion of tetraploid RGCs was measured by flow cytometry in cells expressing Islet1. DNA quantification of dissociated E5 chick retinal cells demonstrated that around 25% of the Islet1-positive cells were tetraploid (Fig. 2H). In contrast, the Islet1-negative cells (i.e., dividing precursors at this developmental stage) showed the typical profile of proliferating cells (Fig. 2G). As expected, the proportion of tetraploid Islet1-positive cells was significantly reduced when endogenous NGF or p75NTR were inactivated with anti-NGF or anti-p75NTR blocking antibodies (Fig. 2 I and J), demonstrating that RGCs depend on the presence of NGF/p75NTR to become tetraploid. The addition of NGF to dissociated E5 retinal precursors, cultured under neurogenic conditions and in the presence of BDNF to prevent G2/M transition (6), supported this conclusion. NGF increased the proportion of cells with a 4C DNA content, as evidenced by SBC (Fig. S4M), an effect that was prevented by the overexpression of Rb (Fig. S4 M–O). Flow cytometric analysis indicated that 20.87 ± 2.14% (n = 3) of the Islet1-positive cells remain tetraploid in the retina at E9, a stage when RGC neurogenesis has been completed (11), indicating that tetraploid neurons survive throughout development.

**A Minority of RGCs That Attempt Cell Cycle Reentry Undergo Mitosis Followed by Apoptosis.** In vivo, some of the migrating RGCs were observed to undergo mitosis, as evidenced by double labeling of RA4 and the mitotic marker pH3 (Fig. 3). Most pH3-labeled RGCs were located basally (Fig. 3 A and B), as opposed to the apically located mitotic nuclei in neuroepithelial cells (arrowheads in Fig. 3 A and B), suggesting that these neurons have completed G2 as they migrate to the GCL. In quantitative terms, the pH3-positive RGCs constitute only a small subset of the RGCs that undergo cell cycle reentry. Thus, although 9.33 ± 0.73% of βIII tubulin-positive cells were observed to incorporate BrdU (15 min pulse; Table S2), only 0.12 ± 0.01% (n = 3) of these cells underwent basally-located mitosis. These results are consistent with the observation that many RGCs that attempt cell cycle reentry remain in a G2-like state (see above). As expected, pH3-labeled RGCs were always positive for the RA4 marker (n = 131 nuclei analyzed; Fig. 3 A) and always lacked Rb expression (n = 27.
nuclei observed, Fig. 3B). Application of hybridoma cells secreting an anti-NGF mAb or injection of the anti-p75NTR ChEX antibody into the eye significantly reduced the proportion of RA4-positive cells with mitotic figures in their nuclei when compared to control retinas (Fig. 3 C and D). These results further indicate that some RGCs that reenter the cell cycle in response to endogenous NGF can further proceed throughout the cell cycle and undergo mitosis. BrdU pulse-chase experiments in E5 chicken embryos followed by immunolabeling of βIII tubulin-positive neurons confirmed this hypothesis. Thus, 6 h after administration of dU, BrdU was present in around 90% of the βIII tubulin-positive cells that underwent basally-located mitosis (Fig. 3 E and H). In vitro, a fraction of differentiating E5 retinal neurons undergoes mitosis followed by apoptosis when treated with NGF (6). BrdU pulse and chase experiments confirmed these results in vivo. Thus, although 6 h after dU treatment, when most pH3-positive RGCs are labeled with BrdU (see above), BrdU was only evident in around 20% of the βIII tubulin-positive cells showing pyknotic nuclei (Fig. 3H). 3 h later BrdU was already observed in most of the βIII tubulin-positive, pyknotic cells (Fig. 3 F and H). These data indicate that ectopic mitosis was rapidly followed by apoptosis in vivo in the βIII tubulin-positive retinal cells. The reduced amount of RGCs that undergo mitosis (see above) followed by apoptosis agrees with previous published data demonstrating that only 2% of Islet1-positive cells suffer cell death in the embryonic chick retina in vivo (19).

**Endogenous BDNF Prevents Ectopic Mitosis.** BDNF is a neurotrophin known to prevent mitosis and apoptosis in differentiating RGCs (6, 20). Accordingly, inhibition of endogenous BDNF in E4 eye explants with TrkB receptor bodies resulted in a dramatic increase of βIII Tubulin-positive cells undergoing mitosis (Fig. 4B). In contrast, manipulation of BDNF levels did not affect BrdU incorporation by these neurons (Fig. 4A). These results indicate that BDNF is crucial for the maintenance in a G2-like state of RGCs that have reentered into the cell cycle in response to NGF.

**Tetraploidization Correlates with Increased Size and Extensive Dendritic Trees in RGCs.** To define the phenotype of tetraploid RGCs, we focused in the posthatching chick retina. Flow cytometry forward scattering analysis of Islet1-positive cells isolated from P1 chick retinas indicated that, on average, tetraploid RGCs were larger than diploid RGCs (Fig. S6 A–C). A similar conclusion was obtained from the analysis of the soma size of Dil-labeled RGCs, performed by means of SBC in random retinal areas (Fig. 5A; Fig. S2 C and D). In all analyzed areas, tetraploid RGC somas were significantly larger on average than those of the diploid RGCs (Table S1), indicating that the presence of double amount of DNA is translated into increased soma size. The average size of diploid RGC somas ranged from 60 to 110 μm² (mean: 75.40 ± 3.44 μm²; n = 15), whereas the soma size averages of the tetraploid RGCs were significantly higher and ranged between 70 and 230 μm² (mean: 127.73 ± 10.47 μm²; n = 15; ***P < 0.005) (Fig. 5A). These values resemble the bimodal distribution for soma sizes for chick RGCs previously shown by (21). A positive correlation (r = 0.65) between soma sizes of diploid and tetraploid RGCs within the analyzed retinal areas was observed (Fig. 5B), suggesting that tetraploidization results in increased soma size regardless of the initial size of the diploid neurons in which it takes place. The area occupied by the dendritic trees, as evidenced by retrograde DiI labeling,
of both diploid and tetraploid RGCs was estimated by SBC in randomly selected retinal areas. This analysis demonstrated that tetraploid RGCs showed larger dendritic trees than those observed in the diploid RGCs (Fig. 5C; see also Fig. S2 C and D). Dendritic fields of diploid RGCs ranged from 136 to 8,904 μm² (n = 37) whereas dendritic fields of tetraploid RGCs were higher and ranged between 407 and 76,509 μm² (n = 42). These values are in a similar range as those previously described (21).

**Tetraploid RGCs Comprise a Specific Population of Retinal Projection Neurons in the Chick.** A subpopulation of less than 10% of RGCs expresses nicotinic AChR β2 subunit (β2AChR) and innervates SGFS-F in the tectal cortex (22). Analysis by flow cytometry in dissociated P1 chick retinal neurons demonstrated that 83.64 ± 2.45% (n = 4) of tetraploid cells are labeled with the anti-β2AChR antibody mAb 270 (22) and that most Islet1/β2AChR-double labeled cells (85.37 ± 1.96%; n = 7) are tetraploid (Fig. 5D); indicating that the majority of tetraploid RGCs belong to the neuronal population that innervate SGFS-F. Analysis by SBC confirmed that most β2AChR-positive cells present in the GCL (Fig. S2E) showed a 4C DNA content (Fig. 5E). In contrast, the entire population of Islet1-positive cells located in the GCL showed a DNA content profile (Fig. S7) equivalent to that previously observed by flow cytometry in cells expressing Islet1 (Fig. 1B); thus indicating that the SBC analysis performed with the anti-β2AChR antibody was specific. The size of the β2AChR-positive somas, as evidenced by SBC (Fig. 5F), was in the same range as that of the tetraploid RGCs (Fig. 5A). Flow scattering analysis confirmed that, on average, the size of Islet1/β2AChR-double labeled RGCs (Fig. S6 E and F) was in the same range as that of tetraploid RGCs (Fig. S6 B and C). This contrasts with the small size of Substance P-positive RGCs, a population of neurons with somas of areas 28–63 μm² (23) that innervate SGFS-B (22, 23). Therefore, the innervation of specific laminae in the chick optic tectum seems to be associated with the size of the innervating RGCs. To verify whether the expression of β2AChR in tetraploid RGCs is conserved between the chick and mammals we focused in the mouse retina because RGCs in this species are known to express β2AChR (24). By means of flow cytometry we found that only 4.76 ± 0.96% (n = 3) of the tetraploid cells derived from adult mouse retinas expressed β2AChR. Furthermore, only 28.95 ± 0.97% (n = 4) of the β2AChR-positive cells obtained from these retinas showed a 4C DNA content. Therefore, we conclude that β2AChR cannot be considered a marker for tetraploid neurons in the mouse retina.

**Discussion**

Overall, these results demonstrate that endogenous NGF acting through p75NTR forces cell cycle reentry of Rb-negative differentiating retinal neurons, thereby generating tetraploid RGCs. These neurons have large somas and extensive dendritic trees, and most of them express a marker known to be specific of RGCs innervating SGFS-F in the optic tectum. This represents an example of growth-factor-induced somatic polyplody in vertebrates.

We have shown that most RGCs that reactivate the cell cycle can survive in vivo as a result of a developmental program aimed to induce neuronal somatic tetraploidy, which results in morphological diversity among retinal projection neurons. Tetraploid RGCs in the chick show features of primate parasol cells; a population of RGCs equivalent to α-Y cells in the cat (25) with large somas and wide receptive fields, which are involved in motion processing. Parasol cells make up about 10% of the RGCs, they establish contacts with cholinergic amacrine cells, and they project to specific layers of the lateral geniculate nucleus, the major retinoreceptive tissue in mammals (26, 27). Whether or not tetraploid RGCs in the chick are the counterpart of parasol cells requires further analysis. Although in mammals the β2AChR subunit is not specifically expressed by tetraploid RGCs, it may have been reprogrammed with other nicotinic AChR subunits in these organisms. In this regard, medium- to large-sized RGCs have been shown to express β4AChR subunits in the ground squirrel (28).

We show that a minority of postmitotic RGCs that reenter cell cycle in vivo, driven by NGF, undergo mitosis followed by apoptosis, which is consistent with prior reports (6, 19). Notably, the data reported here supports the notion that a major fate for postmitotic RGCs that reenter cell cycle in vivo is replication without cell division, with survival instead of apoptosis.

Our results are consistent with previous observations in vitro that neurite-bearing PC12 cells (29) and sympathetic neurons (30) continue to synthesize DNA in the presence of NGF, resulting in the appearance of cells with high DNA content in culture. To date, the mechanisms used by NGF to trigger cell cycle reentry remain unknown. Our results show that cycle reentry is restricted to the Rb-negative subpopulation of RGCs. It is possible that p75NTR-dependent inhibition of CMAGE in these migrating RGCs could release E2F1 activity and force them to reenter the cell cycle (31). BDNF is a neurotrophin known to prevent mitosis and apoptosis in differentiating RGCs (6, 20), which acts in our system before the classical period of target-dependent neuronal survival. Our results indicate that blockade of BDNF favors mitosis in migrating RGCs, whereas DNA synthesis in these neurons is not affected by this neurotrophin. Therefore, cooperation between NGF and BDNF seems to be required for the induction of tetraploid RGCs and their maintenance in a G2-like state.

Reactivation of the cell cycle triggers neuronal death during both development and disease (32). Our work extends this view because cell cycle reentry can also be associated to an alternative fate—neuron survival as large tetraploid cells, as occurs in the differentiating neurons we describe. Our results demonstrate that induction of neuronal tetraploidy in response to NGF and p75NTR occurs in the normal developing nervous system, correlating with changes in neuronal morphology. Whether induction of large tetraploid neurons in the adult brain in response to endogenous NGF/proNGF and p75NTR (33, 34) may lead to altered neuronal function and changes in neuronal circuits that could trigger neurodegeneration remains unexplored. In this regard, neurons from Alzheimer’s disease patients have been shown to become tetraploid as the disease progresses, in association with a decrease of neuronal numbers (2).
Eye Injections of Anti-p75NTR. The anti-p75NTR antisemur or normal rabbit sereum were injected (1 mL diluted 1:10 in PBS) into the vitreal space of E4-E5 chick eyes. Embryonic retinas were isolated until E5 and then their retinases were subjected to immunostaining or flow cytometry (for details, see SI Materials and Methods).

Electroporation. Small fragments (approximately 10 mm3) of E5-6 chick retinas were electroporated with different plasmid combinations using four 50 millisecond pulses of 25 V at a 500 milliseconds frequency (see SI Materials and Methods for details).

Cell Cultures. Dissociated E5 chick retinal cells were plated on 10-mm round glass coverslips coated with 500 μg/mL poly(D-L)ornithine and 10 μg/mL natural mouse laminin, and cultured at 37 °C in DMEM/Nutrient Mixture F12 HAM containing N2 supplement (DMEM/F12/N2). For details, see SI Materials and Methods.

Explant Cultures. E4 eye explants were cultured at 37 °C in DMEM/F12/N2 containing either 2 ng/mL BDNF, 1 μg/mL TrkB receptor bodies, or vehicle (see SI Materials and Methods for details). Finally, a 1 h pulse of 0.5 μg/mL BrdU was given to label cells in S-phase and then the explants were fixed with 4% PFA for 1 h.

Flow Cytometry. E5 or P1 chick retinas or adult mouse retinas, placed in 1 mL Ca2+-Mg2+-free PBS containing 3 mg/mL BSA, were treated with 0.5 mg/mL trypsin for 5 min (E5 chick retina) or for 8 min (P1 chick retina); or with 0.25 mg/mL trypsin for 5 min (adult mouse retina) at 37 °C. Reactions were stopped by adding 50 μL of a solution containing 10 mg/mL trypsin inhibitor prepared in PBS. 10 μL of DNase I prepared at 1 μg/mL in PBS, and 5 μL of 500 mM EDTA pH 8.0 were then added; and the cells were subsequently dissociated by gentle trituration and fixed on 4 °C in 70% Ethanol/PBS. Flow cytometry analysis of these cells was performed as indicated in SI Materials and Methods.

DiI Retrograde Labeling of RGCs and Nuclear Staining. Small crystals of DiI were impregnated into the optic nerve stumps of P1 chick eyes (see SI Materials and Methods for details). The eyeballs lacking the lens and vitreous body were then incubated at 37 °C in 1% PFA in PBS for 4-6 weeks (soma size analysis) and for 6 months ( dendritic arbor analysis). The retinas were dissected, incubated for 30 min with 100 ng/mL DAPI in PBS/0.1% Tween, washed with PBS/0.1% Tween 20 and PBS (30 min each), and flat mounted with PBS/glycerol (1:1) on glass slides.

SBC. The relative DNA content of the DiI-labeled, j2ACHr-positive, or Islet1-positive RGCs in flat-mounts of fixed P1 chick retinas, or dissociated E5 retinal cells transfected with RFP and cultured under neurogenic conditions was determined by the integral DIAP1 fluorescence values obtained by means of SBC. SBC analysis was performed with an automated Olympus IX81 ScanIR fluorescence microscope-based system equipped with an ORCA-ER CB384-05G01 digital camera, from Hamamatsu Photonics, using 20× magnification objectives (for details, see SI Materials and Methods).

I-FISH. Suspensions of dissociated P1 chick retinas, enriched in RGCs, were obtained by limited trypsinization (0.5 mg/mL trypsin, for 5 min). These cells were then plated onto coverslips previously coated with PLO, maintained in DMEM/F12/N2 for 1 h to allow their attachment to the substrate, and then fixed for 10 min with either 4% PFA (for jill tubulin immunostaining), or ethanol/glacial acetic acid (3:1) (for I-FISH). I-FISH was performed as previously described (8). The probe, derived from the pG6416 IRR sequence (see above), was labeled with biotin using the Biotin-Nick Translation Kit from Roche following the protocol recommended by the supplier. See SI Materials and Methods for details.

Immunohistochemical Data Analysis and Statistics. Dorsoventral sections containing both the lens and optic nerve exit were examined using a Leica TCS SPS confocal set-up, and around 100 labeled cells were analyzed per embryo. Quantitative data are shown as mean ± SEM obtained from at least three different embryos or experimental conditions. The statistical differences of the means were analyzed using the Student’s t-test (one-tailed).

Acknowledgments. We thank R. Diez del Corral, P. de la Villa, and N. Lopez–Sánchez for scientific comments; P. Lastres, G. Gómez-Mariano, and E. Abanto for technical assistance; P. M. Chao, L. F. Reichardt, S. C. McLoon, A. Rodríguez–Tébar, and E. J. de la Rosa for antibodies, and K. Yashikawa, M. Campanero, and S. Wilson for plasmids. The mAbs G343 (K. Kaufman), 40.D2 (T. M. Jessell), AMV–3C2 (D. Boettiger), and mAb 270 (J. Lindstrom) were obtained from the Developmental Studies Hybridoma Bank (University of Iowa). This work was supported by the Ministerio de Ciencia e Innovación, La Caixa Foundation, FUNDALUCE (J.M.F.), and Programa de Biocriticas Medicina Individualizada Translacional en Inflamación y Cáncer-Comunidad de Madrid (A.d.I.H.).

Supporting Information

Morillo et al. 10.1073/pnas.0906121107

SI Materials and Methods

Chicken Embryos and Mice. Fertilized chicken eggs were obtained from Granja Santa Isabel (Córdoba, Spain) and were incubated at 38.5 °C. The embryos were staged according to ref. 1. P1 chicks were killed by decapitation. Adult C57BL/6J mice were also used in this study. All experiments were performed in accordance with the European Union guidelines and were previously approved by the Consejo Superior de Investigaciones Científicas animal ethics committee.

Primary Antibodies. The rabbit polyclonal antiserum [992] against the intracellular domain of human p75 NTR was a kind gift of Moses V. Chao (New York University, New York, NY), and it was diluted 1/1,000 for immunohistochemistry. The rabbit polyclonal antiserum against the extracellular domain of chick p75 NTR, ChEx, was a generous gift from Louis F. Reichardt (University of California, San Francisco, CA), and it was used to block p75 NTR in vivo. The affinity purified rabbit anti-E2F1 polyclonal antiserum C-20 (Santa Cruz Biotechnology) was used at 2 μg/mL for immunohistochemistry. The anti-Rb mAb G3-245 (BD BiosciencesPharringen) was used at 1/400 dilution for immunohistochemistry. This antibody recognizes an epitope between amino acids 332–344 of the human Rb protein, which is conserved in avian Rb. The rabbit anti-Rb polyclonal antibody raised against a peptide derived from the human Rb sequence around Ser780 (Abcam) was used at 1/50 dilution for immunohistochemistry. The mAb G4 labels a glycoprotein located on neurites of a subset of differentiated neurons (2). In the chick retina, it stains the RGCs and a small population of neurons in the inner nuclear layer (Fig. S3) (2). Ascitic fluid containing this mAb was used at 1/400 for immunohistochemistry. The polyclonal antiserum against G4 was a kind gift of Enrique J. de la Rosa (Centro de Investigaciones Biológicas Madrid, Spain), and it was used at a 1/1,000 dilution for immunohistochemistry. The mAb against G4 was a kind gift of Enrique J. de la Rosa (Centro de Investigaciones Biológicas) and it was diluted 1/400 for immunohistochemistry. The mAb anti-RA4, a generous gift from Stephen C. McLoon (University of Minnesota, Minneapolis, MN), recognizes an epitope expressed by RGCs soon after their final mitosis (3, 4), and it was diluted 1/400 for immunohistochemistry. The anti-phospho-Histone H3 rabbit polyclonal antiserum (Upstate Biotechnology) is a well-characterized marker of mitosis and it was diluted 1/400 for immunohistochemistry. BrdU was visualized by immunohistochemistry or immunocytochemistry with a 1/4,000 dilution of the mouse mAb G3G4 (Developmental Studies Hybridoma Bank) or a 1/200 dilution of the rat mAb BU1/75 (ICR1, Serotec). For flow cytometry, BrdU was detected with the rat mAb BU1/75 at a 1/200 dilution. The rat mAb to the β2 subunit of the neuronal nicotinic acetylcholine receptor (mAb 270; Developmental Studies Hybridoma Bank), previously described by ref. 5, was used at 1/1,000 dilution for immunohistochemistry and 1/500 dilution for flow cytometry. The mouse mAb against neuron-specific βIII tubulin (clone 5G8; MILLIPORE/Chemicon) specifically recognizes RGCs from early stages of differentiation (6) and it was diluted 1/1,000 for immunohistochemistry. In the rat retina betaIII tubulin is coexpressed with the differentiating marker doublecortin (7), a marker found in migrating neuroblasts (8). The mAb 40.2D6 (Developmental Studies Hybridoma Bank) recognizes the C-terminal portion of rat Islet-1, a transcription factor from the LIM family of homeodomain containing-proteins expressed by the layered RGCs (9), which remains in a subpopulation of RGCs during adulthood (10). This antibody was diluted 1/200 for immunohistochemistry and 1/200 for flow cytometry. The anti-NGF mAb 27/21 (11), a generous gift of Alfredo Rodriguez-Tébar (Centro Andaluz de Biología Molecular y Medicina Regenerativa, Seville, Spain), was used here to specifically block the biological activity of NGF (12). The AMV-3C2 anti-gag mouse mAb was obtained from the Developmental Studies Hybridoma Bank, and it was used as a control for the anti-NGF mAb 27/21 blocking effect.

Plasmids. The luciferase reporter PGV-B control vector, the luciferase reporter PGV-B vector carrying the mouse c-Myc core promoter (c-Myc-PGV-B), previously described in ref. 13, were a generous gift from Kazuaki Yoshikawa (Osaka University, Osaka, Japan). The pcDNA3-pRb expression vector was provided by Miguel Campanero (IIB-CSIC, Madrid), and described by ref. 14. The pcDNA3 control vector was purchased from Invitrogen. The rRFPRNAiC vector expressing RFP was provided by S. Wilson (University of Sheffield, Sheffield, U.K.). The pGEM-pG6416 plasmid was obtained by inserting the sequence corresponding to chick pg6416 inverted repeat region (pg6416 IRR; 15) into the pGEM-T Easy vector (Promega). The pg6416 IRR sequence was amplified by RT-PCR from chicken genomic DNA using the primers: 5′-CCCTTCTTGCCGCCTCTCT-3′, which contains the sequence corresponding to bp 1–20 and the complementary sequence to bp 1,303–1,322 from pg6416 IRR (National Center for Biotechnology Information accession number AF124927).

Immunostaining. For immunohistochemistry, embryos were fixed for 8 h at 4 °C with 4% paraformaldehyde (PFA; Merck), cryopreserved overnight (ON) at 4 °C in PBS containing 30% sucrose (Merek), and embedded in the OCT compound Tissue-Tek (Sakura). Cryosections (12 μm) were permeabilized and blocked for 30 min at room temperature (RT) in PBS containing 0.5% Triton X-100 (Sigma) and 10% FCS (Invitrogen), and they were then incubated ON at 4 °C with the primary antibody in PBS/0.1% Triton X-100 plus 1% FCS. After 5 washes with PBS/0.1% Triton X-100, the sections were incubated for 1 h at RT with an Alexa 594 coupled anti-mouse IgG antibody (Invitrogen), a Cy2-conjugated anti-rabbit IgG (H+L) antibody (Jackson Immunoresearch), or an Alexa 488 coupled anti-rat antibody (Invitrogen) each diluted 1/1,000. The sections were finally washed 5 times in PBS/0.1% Triton X-100, once in PBS, and they were then incubated with 1 μg/mL bisbenzimide (Sigma) in PBS before mounting in glycerol/PBS (1:1). Cryosections used for BrdU immunostaining were permeabilized for 30 min at room temperature (RT) in the presence of 0.5% Triton X-100 in PBS. The DNA in these sections was denatured by incubating for 30 min with 2N HCl/0.33X PBS at RT, and the sections were then neutralized by three 15 min washes with 0.1 M sodium borate pH 8.9 and two 5 min washes with PBS/0.5% Triton X-100. Sections were then subjected to immunohistochemistry as described above. For whole-mount immunostaining, P1 chick retinas were fixed for 1 h at RT with 3.5% PFA. Retinas were subsequently permeabilized with PBS/1% Triton X-100 and 10% FCS, and they were then incubated for 3–4 h at RT with the primary antibody in PBS/0.1% Triton X-100 containing 1% FCS. After 5 washes with 0.1% Triton X-100 in PBS (30 min each), the retinas were incubated ON at 4 °C with an Alexa 594 coupled anti-mouse antibody (Invitrogen) or Fluorolink Cy3 labeled goat anti Rat IgG (GE Healthcare) each diluted 1/1,000. The retinas were finally washed 5 times (30 min each) in PBS/0.1% Triton X-100, and they were then incubated with 100 ng/mL DAPI (Sigma) for 30 min in 1%...
In vitro electroporation of retinal explants was achieved with a rabbit antiserum that gave endogenous signal. These experiments were performed as described previously (17). Dissociated cells were cultured in DMEM/F-12/N2 containing either 2 ng/mL recombinant human NGF (MILLIPORE/Chemicon) or 2 ng/mL recombinant human BDNF (Sigma).

Explant Cultures. E4 chicken embryos were killed and their eyes were cultured in DMEM/F-12/N2 containing either 2 ng/mL human recombinant BDNF, 1 μg/mL TrkB receptor antibodies (recombinant human TrkB/Fc chimera; R&D Systems) (23), or vehicle. The TrkB/Fc chimera is a BDNF-specific inhibitor that contains the extracellular domain of human TrkB fused to the carboxyl-terminal Fc region of human IgG1 via a polypeptide linker. Cultures were maintained ON at 37 °C in a water-saturated atmosphere containing 5% CO2. Finally, a 1 h pulse of 0.5 μg/mL BrdU was given to label cells in S-phase and then the explants were fixed with 4% PFA for 1 h. Eye explants were then incubated ON at 4 °C in PBS containing 30% Sucrose (Merck), embedded in OCT compound (Sakura), and sectioned at 12 μm using a cryostat. Sections were collected on 3-aminopropyl-trimethoxysilane-coated slides (Fluka), and double immunostained for either BrdU and βIII tubulin or phosphohistone H3 and βIII tubulin.

Flow Cytometry. E5 or P1 chick retinas or adult mouse retinas, placed in 1 ml Ca2+/Mg2+-free PBS containing 3 mg/mL BSA (Sigma), were treated with 0.5 mg/mL trypsin (Worthington) for 5 min (E5 chick retina) or for 8 min (P1 chick retina); or with 0.25 mg/mL trypsin for 5 min (adult mouse retina) at 37 °C. Reactions were stopped by adding 50 μL of a solution containing 10 mg/mL trypsin inhibitor (Sigma) prepared in PBS; 10 μL of DNase I (Roche) prepared at 1 μg/μL in PBS and 5 μL of 500 mM EDTA (Merck) pH 8.0 were then added; and the cells were subsequently dissociated by gentle trituration and fixed ON at 4 °C in 70% Ethanol/PBS. Dissociated cells were incubated for 30 min at RT in PBS/0.1% Triton X-100 and 10% FCS, transferred to 0.1% Ethanol/PBS, and 100 ng/mL recombinant human BDNF, 1 μg/mL trypsin inhibitor (Sigma) prepared in PBS; 10 μL of DNase I (Roche) prepared at 1 μg/μL in PBS and 5 μL of 500 mM EDTA (Merck) pH 8.0 were then added; and the cells were subsequently dissociated by gentle trituration and fixed ON at 4 °C in 70% Ethanol/PBS. Dissociated cells were incubated for 30 min at RT in PBS/0.1% Triton X-100 and 10% FCS, transferred to 0.1% Triton X-100 in PBS containing the anti-Ilet-1 mouse mAb or anti-β2AChR rat mAb, and incubated for 1 h at RT with gentle agitation. After four rounds of washing with PBS/0.1% Triton X-100, the cells were then incubated with an Alexa 488 coupled anti-mouse antibody (Invitrogen) or an Alexa 488 coupled anti-rat IgG antibody (Invitrogen), respectively; diluted 1/1,000 in PBS/0.1% Triton X-100. Finally, the cells were washed four times in PBS/0.1% Triton X-100 and once in PBS, filtered through a 30 μm Nylon filter (SAATIPrint) before treated with 25 μg/mL ribonuclease A (Sigma) for 30 min at room temperature, and stained with 25 μg/mL propidium iodide (PI, Sigma). Negative controls were prepared by incubating the cells with an irrelevant mAb. Flow cytometry experiments were carried out with an Epics XL Analyzer (Beckman Coulter) equipped with an argon-ion laser tuned at 488 nm. Fluorescence signals corresponding to Alexa 488 and PI were collected through 525 nm band pass (bp) and 620 nm bp filters, respectively, counting 5,000 cells specifically labeled for Ilet-1. As an internal reference, 2C and 4C DNA values were estimated from the Ilet-1-negative cell fraction (i.e., precursor cells). Statistical analyses were performed using EXPO32 software (Beckman Coulter). The whole range of cell sizes was represented in the cytometric analysis. Cells were initially collected in a double logarithmic scale in base of their forward scatter and side scatter properties. The dynamic range of the instrument was 4 decades on all parameters with a measurement resolution of 1.024 channels. The threshold level was set in the forward scatter on channel 256. Aggregates were discarded using auxiliary parameters area versus peak signals. Values from primary antibody-positive and primary antibody-negative cells were gated using as a threshold the maximal intensity level of cells incubated with the secondary antibody alone.
Triple labeled retinal cells were analyzed by flow cytometry as follows. Dissociated E5 retinal cells obtained from embryos previously treated for 9 h with BrdU were triple labeled with an anti-Islet-1 mouse mAb, an anti-BrdU rat mAb antibody, and 2 μg/mL DAPI, performing 4 washes with PBS/0.1% Triton X-100 between the different incubation steps. Briefly, dissociated retinal cells were fixed ON at 4 °C in 70% Ethanol/PBS and the cells were then transferred to PBS. Their DNA was denatured by incubating the cells for 10 min at 4 °C in 0.1 N HCl prepared in PBS/0.1% Triton X-100, and the cells were then incubated in PBS/0.1% Triton X-100 for 15 min at 97 °C and chilled in ice-water for 15 min. Finally, the cells were transferred to PBS/0.5% Triton X-100 and 10% FCS and incubated for 30 min at RT. They were then incubated with the anti-Islet-1 mAb as described above, followed by incubation with a donkey anti-mouse IgGs antibody coupled to Alexa 647 (Invitrogen) diluted 1/200 in PBS/0.1% Triton X-100. Cells were then incubated with 10% normal rat serum (NRS) in PBS/0.1% Triton X-100 for 30 min, and BrdU was then immunostained for 1 h with the anti-BrdU rat mAb BU1/75 (ICR1) diluted in PBS/0.1% Triton X-100 containing 1% NRS, followed by incubation with the Alexa 488 coupled anti-rat IgG antibody diluted 1/1,000 in PBS/0.1% Triton X-100. Finally, the cells were washed four times in PBS/0.1% Triton X-100 and once in PBS, filtered through a 30 μm Nylon filter before treated with ribonuclease A as above, and stained with β-2 AChR antibody coupled to Alexa 647 (Invitrogen) diluted 1/200 in PBS/0.1% Triton X-100. β2AChR was then immunostained for 1 h with the rat mAb 270 diluted in PBS/0.1% Triton X-100 containing 10% NRS, followed by incubation with the Alexa 488 coupled anti-rat IgG antibody diluted 1/1,000 in PBS/0.1% Triton X-100. Finally, the cells were washed four times in PBS/0.1% Triton X-100 and once in PBS, filtered through a 30 μm Nylon filter before treated with ribonuclease A as above, and stained with 2 μg/mL DAPI. Negative controls were prepared by incubating the cells with the secondary antibodies alone. Dissociated P1 chick retinal cells were also triple labeled with an anti-Islet-1 mouse mAb, an anti-β2AChR rat mAb antibody, and 2 μg/mL DAPI, performing 4 washes with PBS/0.1% Triton X-100 between the different incubation steps. Briefly, dissociated retinal cells were fixed ON at 4 °C in 70% Ethanol/PBS and the cells were then transferred to PBS/0.5% Triton X-100 and 10% FCS and incubated for 30 min at RT. They were then incubated with the anti-Islet-1 mAb as described above, followed by incubation with a donkey anti-mouse IgGs antibody coupled to Alexa 647 (Invitrogen) diluted 1/200 in PBS/0.1% Triton X-100. β2AChR was then immunostained for 1 h with the rat mAb 270 diluted in PBS/0.1% Triton X-100 containing 10% NRS, followed by incubation with the Alexa 488 coupled anti-rat IgG antibody diluted 1/1,000 in PBS/0.1% Triton X-100. Finally, the cells were washed four times in PBS/0.1% Triton X-100 and once in PBS, filtered through a 30 μm Nylon filter before treated with ribonuclease A as above, and stained with β-2 AChR antibody coupled to Alexa 647 (Invitrogen) diluted 1/200 in PBS/0.1% Triton X-100. β2AChR was then immunostained for 1 h with the rat mAb 270 diluted in PBS/0.1% Triton X-100 containing 10% NRS, followed by incubation with the Alexa 488 coupled anti-rat IgG antibody diluted 1/1,000 in PBS/0.1% Triton X-100. Finally, the cells were washed four times in PBS/0.1% Triton X-100 and once in PBS, filtered through a 30 μm Nylon filter before treated with ribonuclease A as above, and stained with 2 μg/mL DAPI. Negative controls were prepared by incubating the cells with the secondary antibodies alone. In these cases, flow cytometry experiments were carried out with a FACS Vantage Analyzer (Becton Dickinson) equipped with an argon ion laser emitting at wavelengths of 360 nm and 488 nm, and a He-Ne laser tuned at 635 nm. Specific fluorescence signals corresponding to DAPI, Alexa 488, and Alexa 647 were collected through 424 nm, 530 nm, and 660 nm band filters, respectively, counting 5,000 cells specifically labeled for Islet-1. As an internal reference, 2C and 4C DNA values from E5 chick cells were estimated from the Islet-1-negative cell fraction (i.e., precursor cells), and statistical analyses were performed using Cell Quest software (Becton Dickinson). Relative cell size was estimated by means of forward scatter signals.

**Dil Retrograde Labeling of Retinal Ganglion Cells and Nuclear Staining.** Retina explants were processed as previously described (24). Briefly, P1 chicks were killed by decapitation and their eyeballs were dissected out from the orbit by cutting the optic nerve very close to the optic chiasm. One small crystal of the carbocyanine dye Dil (Invitrogen) was implanted into a section of the optic nerve stump. The eyeballs were then cut circularly along the sclera-cornea junction, and the lens and vitreous body were removed. The eyeballs were then incubated at 37 °C in 1% paraformaldehyde in PBS for 4–6 weeks (for analysis of soma sizes) and for 6 months (for analysis of dendritic arbors). Next, the retinas were dissected out from the eyeball, and incubated for 30 min with 100 ng/mL DAPI in PBS/0.1% Tween 20 to label the nuclei of the retinal cells. Retinas were then washed for 30 min with PBS/0.1% Tween 20, followed by four washing steps (30 min each) with PBS, and flat mounted with PBS/glycerol (1:1) on glass slides.

**Slide-Based Cytometry (SBC).** The relative DNA content of the Dil-labeled, β2AChR-positive, or Islet1-positive retinal ganglion cells in flat-mounts of fixed P1 chick retinas was determined by the integral DAPI fluorescence values (i.e., integration of total pixel intensity assigned to the visualized nuclei) obtained by means of SBC. This technique was also used to measure the relative DNA content of dissociated E5 retinal cells transfected with RFP and cultured under neurogenic conditions. SBC is a well standardized method for DNA amount quantification. Mosch et al. (2007) (25) have shown a close correlation between this procedure and FISH or DNA quantification by real time PCR. SBC analysis was performed with an automated Olympus IX81 Scan R fluorescence microscope-based imaging platform (Olympus Europa and European Molecular Biology Laboratory, Heidelberg, Germany) equipped with a digital camera (ORCA-AG C8484-05G01, Hamamatsu Photonics) using 20x magnification objectives. A minimum of 25 fields were acquired in each retina or culture. For analysis of dendritic arbor sizes a series of 19 images along the z axis (Z-stack) with a thickness of 5 μm were acquired for each field comprising the ganglion cell layer and inner plexiform layer. Analysis of acquired images was performed using Scan ^ R Analysis software (Olympus Europa and European Molecular Biology Laboratory, Heidelberg, Germany). Intensity modules were used for setting the main object “cell soma” (Dil staining or β2AChR, Islet1 immunostaining, or RFP) and the secondary object “cell nucleus” (DAPI staining). Basically, cytometry-orientated data analysis was used, setting a first gate of single cells, excluding small particles (less than 30 μm) or doublets with a dot-plot of area vs. circularity. Then, single cells were classified into 2C or 4C DNA content using a histogram of DAPI intensity from the single cells gate. The soma area (μm²) of the 2C and 4C cells was then analyzed. Projections of the planes along the z axis from isolated 2C or 4C RGCs were used to analyze dendritic tree areas in Dil-labeled RGCs. To this aim polygonal lines were drawn using as a reference the tips of the longest dendrites and the area inside the polygon was estimated with Cell ^ R analysis software (Olympus Europa). Figures derived from the original Scan ^ R images, including those for the density plots, were constructed from screen pictures captured with the “Print Screen” key of Windows.

**Interphase-FISH (I-FISH).** Suspensions of dissociated P1 chick retinas, enriched in RGCs, were obtained by limited trypsinization (0.5 mg/mL trypsin, Worthington, for 5 min). These cells were plated onto coverslips previously coated with PLO, maintained in DMEM/F12/N2 for 1 h to allow their attachment to the substrate and then fixed for 10 min with either 4% PFA (for βIII tubulin immunostaining) or ethanol (Mercer)/glacial acetic acid (Merck) (3:1) (for I-FISH). I-FISH was performed as previously described (15). The probe, derived from the pG6416 IRR sequence (see above), was labeled with biotin using the Biotin-Nick Translation Kit (Roche) following the protocol recommended by the supplier. The labeled probe (200 ng) was resuspended in 20 μL hybridization buffer, containing 2x SSC, 10% dextran sulfate (MILLIPORE/Chemicon), 50% deionized formamide (Ambion), and 10 μg human Cot-1 DNA (Invitrogen). After denaturation of the probe for 5 min at 80 °C, the labeled DNA was preassociated with Cot-1 DNA for 1 h at 37 °C. Coverslips containing fixed neurons from P1 chick retinas (see above) were heated at 80 °C for 2 min in 70% formamide (deionized)/2x SSC.
and dehydrated in 70%, 90%, and 100% ethanol (Merck) at 4 °C, and air dried. After ON incubation with the probe at 37 °C, the coverslips were washed in 2× SSC for 5 min at room temperature, in 50% formamide/2× SSC for 20 min at 41 °C, and in 2× SSC for 5 min at 41 °C. Coverslips were then washed with 4× SSC/0.1% Tween 20, and incubated with 5% BSA (Sigma)/4× SSC/0.1% Tween 20 for 30 min at 37 °C. The presence of the biotin-labeled DNA was then detected by incubating coverslips with Streptavidin, Alexa Fluor 594 conjugated (Invitrogen), 1:1000 diluted in 1% BSA/4× SSC/0.1% Tween 20, for 45 min at 37 °C. Coverslips were then washed four times (5 min each at room temperature) with 4× SSC/0.1% Tween 20, and stained with 100 ng/ml DAPI for 2 min. After dehydration with 70% ethanol, 90% ethanol, and 100% ethanol, coverslips were air dried and mounted with ProLong Gold Antifade Reagent (Invitrogen).

**Fig. S1.** Islet-1 colocalizes with the retinal marker G4 at E5 and P1. Cryostat sections from retinas of E5 chicken embryos (A) or P1 chickens (B) were double labeled with an Islet-1-positive mAb (red) and an anti-G4 rabbit polyclonal antiserum (green). Most Islet-1-positive cells colocalized with the RGC marker G4 at both stages (arrows). (B inset) merged image of nuclear staining (bisbenzimide; blue), G4 (green), and Islet-1 (red). PE: pigment epithelium; INL: inner nuclear layer; IPL: inner plexiform layer; GCL: ganglion cell layer; v: vitreous body. (Scale bars: A, 50 μm; B, 30 μm.)
Fig. S2. Somatic tetraploid RGCs in the P1 chick retina. (A) Slide-based cytometry demonstrates that a subset of RGCs retrogradely labeled with Dil showed nuclei with a 4C DNA content. (B) Analysis by I-FISH of dissociated neurons from P1 chick retinas, enriched in RGCs by partial trypsinization (see SI Materials and Methods) and counterstained with 100 ng/mL DAPI (Left, blue). A nucleus with four copies (Right, red spots, see arrows) of a partially inverted tandem repeat from the pericentric region of chicken chromosome 8 (Left, arrow) is shown. This nucleus likely contains a 4C DNA amount, as compared to diploid nuclei showing only two copies of this genomic region (Left, arrowhead). (C) Example of a P1 chick retina retrograde-labeled with Dil (left, red) and counterstained with 100 ng/mL DAPI to quantitatively label nuclei (Middle, blue). A merged image is shown (Right). (D) High magnification of the squares indicated in (B) where labeled RGCs and their dendritic arbors can be observed. Arrow points to a tetraploid RGC, arrowheads point to individual RGCs with 2C DNA content. A number of small nuclei with strong DAPI labeling can be observed (yellow arrowhead). These nuclei are diploid. (E) Example of a P1 chick retina at the level of the GCL, labeled with the β2AChR-specific antibody mAb 270 (Left, green) and counterstained with 100 ng/mL DAPI to quantitatively label nuclei (Middle, blue). A merged image is also shown (Right). Note that the nuclei of the β2AChR-positive neurons (arrows) are similar to those of large RGCs shown in (D). In contrast, the nuclei of the β2AChR-negative neurons (arrowhead) are similar to those with 2C DNA content shown in (D). (Scale bars: C, 30 μm, D and E, 12 μm.)
Fig. S3. \textit{p75}^{NTR}\textsuperscript{-}immunoreactivity colocalizes with known RGC and neuronal markers. Cryostat sections from the retina of E5 chicken embryos were double labeled with a \textit{p75}^{NTR}\textsuperscript{-}specific rabbit antibody (green), and the RGC markers RA4 (red in A), G4 (red in B), or \textit{β}III tubulin (red in C). Nuclei were counterstained with bisbenzimide (blue). All these markers colocalized with \textit{p75}^{NTR}\textsuperscript{-} (arrows). Note that RA4 is unevenly distributed through the cytoplasm of RGCs. PE: pigment epithelium; v: vitreous body. (Scale bar: 25 μm.)
Expression and regulation of the transcription factor E2F1 and Rb by E5 chick RGCs. Cryostat sections (A–C) or differentiating cultures (D–O) from the retina of E5 chicken embryos are shown. (A) Arrows indicate migrating RGCs expressing p75NTR (green) and lacking Rb immunoreactivity (red). p75NTR/Rb-double labeled cells (arrowheads). (B) All RA4-positive, migrating RGCs (red) show E2F1-immunoreactivity (green, see arrows). (C) E2F1 (green) is expressed in the nuclei of some retinal cells lacking Rb (red, see arrows). (D) Dissociated cells from E5 chick retinas were cultured under neurogenic conditions (see SI Materials and Methods) for 4 h (Upper) or 22 h (Lower), and then Rb-specific immunostaining (red) was performed in these cultures, followed by nuclear staining (blue) with bisbenzimide (Bisb.). Four hours after plating most cells expressed Rb at higher levels, in accordance with the proliferative capacity of these cells (1), but 22 h later a conspicuous population showed low Rb expression levels (arrows), in agreement with the neurogenic potential of the culture conditions. (E) Double labeling with anti-E2F1 (green) and anti-Rb (red) antibodies demonstrated that the subpopulation lacking Rb (arrows) was able to express E2F1. An arrowhead indicates an E2F1/Rb double labeled nucleus. (F) Double labeling with anti-E2F1 (green) and anti-G4 (red) antibodies reveals that RGCs express E2F1. (G) Double labeling with anti-Rb (red) and anti-G4 (green) antibodies demonstrates that a subpopulation of RGCs lack Rb expression (arrows). Rb-positive RGCs (arrowheads). (H) 100 ng/mL NGF (NGF), as compared to vehicle (–), induces luciferase activity in retinal differentiating neurons previously electroporated with c-myc-Luciferase (cMyc-Luc.). In contrast, NGF (NGF) did not induce luciferase activity in differentiating neurons previously electroporated with the empty vector (Control). (I) BrdU incorporation in total cells (open bars) or IIII tubulin-positive cells (filled bars) cultured for 18 h and then BrdU pulsed for 1 h; 100 ng/mL NGF (NGF) induces a significant increase of cells incorporating BrdU as compared to vehicle (–). This increase is exacerbated in cells expressing IIII tubulin. (J) 100 ng/mL NGF (NGF) did not affect to the proportion of E5 retinal cells expressing low levels of Rb, as compared to vehicle (–). (K) Dissociated cells from E6 chick retinas electroporated with a RFP-expressing vector (red), along with an Rb expression vector (Rb) or an empty expression vector (Control), cultured for 18 h under neurogenic conditions (see SI Materials and Methods), and immunostained with an anti-Rb specific antibody (green, αRb). Rb-specific immunostaining was enhanced in cells transfected with the Rb expression vector (arrow). (L) Dissociated cells from E6 chick retinas coelectroporated and cultured as described in (K), subjected to a 1 h pulse with BrdU after 18 h in vitro. Quantifications were performed only in cells labeled with RFP. Overexpression of Rb (Rb) reduced the proportion of cells incorporating BrdU, as compared to those cells overexpressing an empty vector (Control). The effect of 100 ng/mL NGF (NGF), as compared to vehicle (–), on BrdU incorporation was also prevented by the overexpression of Rb. (M) Dissociated cells from E6 chick retinas coelectroporated and cultured for 18 h under neurogenic conditions (see SI Materials and Methods) in the presence of 2 ng/mL BDNF and then labeled with 100 ng/mL DAPI and subjected to SBC analysis to quantify the proportion of cells with a 4C DNA amount. Quantifications were performed only in cells labeled with RFP. The presence of BDNF in the culture medium prevents G2/M transition (2), thus facilitating the analysis of the proportion of tetraploid cells; 100 ng/mL NGF (NGF) induced a significant increase of differentiating neurons with 4C DNA content when compared to vehicle (–). This effect was prevented in cells overexpressing Rb (Rb). (N) Dissociated cells from E6 chick retinas coelectroporated with a RFP-expressing vector (in red) and an empty expression vector (Control), cultured for 18 h under neurogenic conditions (see SI Materials and Methods) in the presence of 100 ng/mL NGF and 2 ng/mL BDNF, and labeled with 100 ng/mL DAPI (blue), DAPI. The 2C (arrowhead) and 4C (arrow) nuclei were readily observed in differentiating neurons expressing RFP. For a quantification see (M). (O) Dissociated cells from E6 chick retinas coelectroporated with a RFP-expressing vector (in red) and Rb-expressing vector (Rb), cultured for 18 h under neurogenic conditions (see SI Materials and Methods) in the presence of 100 ng/mL NGF and 2 ng/mL BDNF, and labeled with 100 ng/mL DAPI (blue), DAPI. The 2C (arrowhead) and 4C (arrow) nuclei were readily observed in differentiating neurons expressing RFP. For a quantification see (M).
ng/mL NGF and 2 ng/mL BDNF, and labeled with 100 ng/mL DAPI (blue, DAPI). The overexpression of Rb prevented the presence of 4C nuclei in the transfected cells (arrowheads: nuclei with a 2C DNA content). In contrast, 4C (arrow) nuclei were readily visible in the nontransfected cells (arrows). For a quantification see M. N.S., non significant; **P < 0.01; ***P < 0.005. (Scale bars: A, 30 μm; B, E–G, K, N, O, 20 μm; C and D 25 μm.)


Fig. S5. Labeling of a subpopulation of retinal cells by BrdU. A 1 h pulse of BrdU was given in ovo to E5 chicken embryos, followed by addition of a 100 times amount of dU. (A) This procedure labels the nuclei of a subpopulation of retinal cells (green) located at the basal neuroepithelium (i.e., cells in S-phase; yellow bar) when the embryos are killed 0 h after addition of dU. Some nuclei are also apically located (arrowheads). (B) Due to the interkinetic nuclear migration the BrDU-positive nuclei have moved apically 6 h after addition of deoxyuridine (yellow bar), and mitotic figures are labeled with BrdU as well (arrows). Most basally located nuclei are not BrdU labeled, thus demonstrating that the addition of an excess of deoxyuridine prevented the incorporation of BrdU in neuroepithelial cells undergoing S-phase. This pattern is consistent with the previous description by Waid and McLoon (4) that retinal precursors displace their nuclei to the apical surface and undergo mitosis within 3 h. (C) Analysis of DNA content (DAPI) in dissociated retinal cells, from embryos treated with BrdU for 9 h in ovo, double-immunostained with anti-Islet1 and anti-BrdU antibodies. Most Islet1-positive/BrdU-negative cells were observed to be diploid (2C). (D) Analysis of DNA content (DAPI) in dissociated retinal cells, from embryos treated with BrdU for 9 h in ovo, double-immunostained with anti-islet1 and anti-BrdU antibodies. Most Islet1/BrdU-double labeled cells were observed to be tetraploid (4C). Note that DNA denaturation was necessary for analyses shown in (C and D); which resulted in thickening of the peaks. PE: pigment epithelium, v: vitreous body, Bisb.: bisbenzimide. (Scale bar: 30 μm.)
Fig. S6. Tetraploidization generates a subpopulation of large neurons with size similar to that of β2AChR-positive cells. (A) A representative diagram showing the relative sizes of diploid (2C) Islet1-positive neurons isolated from P1 chick retinas, as indicated by flow cytometric forward scattering (FSC) analysis (in arbitrary units). (B) A representative diagram showing the relative sizes of tetraploid (4C) Islet1-positive neurons isolated from P1 chick retinas, as indicated by FSC analysis (in arbitrary units). (C) FSC average values for diploid vs. tetraploid Islet1-positive neurons obtained from three independent retinas (mean ± SEM are shown). (D) A representative diagram showing the relative sizes of Islet1-positive/β2AChR-negative neurons isolated from P1 chick retinas, as indicated by FSC analysis (in arbitrary units). (E) A representative diagram showing the relative sizes of Islet1-positive/β2AChR-positive neurons isolated from P1 chick retinas, as indicated by FSC analysis (in arbitrary units). (F) FSC average values for Islet1-positive/β2AChR-negative neurons vs. Islet1-positive/β2AChR-positive neurons obtained from three independent retinas (mean ± SEM are shown). ***$P < 0.005$. 

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Fig. S7. DNA content analysis in Islet1 positive cells located in the GCL of the P1 chick retina. Simultaneous analysis of DNA content by SBC in either total or Islet1-positive cells from the GCL show a pattern compatible with Fig. 1B, and demonstrate the specificity of the analysis performed with β2AChR immunolabeling (Fig. 4E).
Table S1. SBC analysis of soma areas from diploid (2C) vs. tetraploid (4C) Dil labeled RGCs present in 12 random regions from four different retinas.

<table>
<thead>
<tr>
<th>Type</th>
<th>Cells counted, %</th>
<th>Area range, μm²</th>
<th>Area mean ± SD, μm²</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 2C</td>
<td>320</td>
<td>33–242</td>
<td>111 ± 63</td>
</tr>
<tr>
<td>4C</td>
<td>40 (11.1)</td>
<td>204–260</td>
<td>230 ± 23***</td>
</tr>
<tr>
<td>2 2C</td>
<td>1,660</td>
<td>34–135</td>
<td>64 ± 21</td>
</tr>
<tr>
<td>4C</td>
<td>210 (11.2)</td>
<td>48–161</td>
<td>90 ± 40***</td>
</tr>
<tr>
<td>3 2C</td>
<td>313</td>
<td>35–91</td>
<td>66 ± 22</td>
</tr>
<tr>
<td>4C</td>
<td>310 (49.8)</td>
<td>73–262</td>
<td>130 ± 50***</td>
</tr>
<tr>
<td>4 2C</td>
<td>489</td>
<td>35–154</td>
<td>66 ± 21</td>
</tr>
<tr>
<td>4C</td>
<td>187 (25.0)</td>
<td>43–156</td>
<td>92 ± 26***</td>
</tr>
<tr>
<td>5 2C</td>
<td>310</td>
<td>37–180</td>
<td>78 ± 31</td>
</tr>
<tr>
<td>4C</td>
<td>124 (27.2)</td>
<td>53–183</td>
<td>100 ± 30***</td>
</tr>
<tr>
<td>6 2C</td>
<td>424</td>
<td>40–113</td>
<td>61 ± 16</td>
</tr>
<tr>
<td>4C</td>
<td>111 (18.1)</td>
<td>53–112</td>
<td>76 ± 18***</td>
</tr>
<tr>
<td>7 2C</td>
<td>195</td>
<td>42–180</td>
<td>74 ± 29</td>
</tr>
<tr>
<td>4C</td>
<td>37 (14.3)</td>
<td>66–186</td>
<td>140 ± 54***</td>
</tr>
<tr>
<td>8 2C</td>
<td>65</td>
<td>42–167</td>
<td>79 ± 32</td>
</tr>
<tr>
<td>4C</td>
<td>10 (13.3)</td>
<td>57–274</td>
<td>150 ± 69***</td>
</tr>
<tr>
<td>9 2C</td>
<td>196</td>
<td>43–98</td>
<td>76 ± 22</td>
</tr>
<tr>
<td>4C</td>
<td>80 (24.3)</td>
<td>66–123</td>
<td>99 ± 23***</td>
</tr>
<tr>
<td>10 2C</td>
<td>2,860</td>
<td>45–191</td>
<td>69 ± 30</td>
</tr>
<tr>
<td>4C</td>
<td>510 (15.1)</td>
<td>71–223</td>
<td>150 ± 65***</td>
</tr>
<tr>
<td>11 2C</td>
<td>1,530</td>
<td>64–225</td>
<td>89 ± 26</td>
</tr>
<tr>
<td>4C</td>
<td>360 (19.0)</td>
<td>76–250</td>
<td>170 ± 56***</td>
</tr>
<tr>
<td>12 2C</td>
<td>640</td>
<td>65–144</td>
<td>92 ± 33</td>
</tr>
<tr>
<td>4C</td>
<td>190 (22.9)</td>
<td>63–205</td>
<td>160 ± 40***</td>
</tr>
</tbody>
</table>

***P < 0.001 (Student’s t test).
Table S2. Estimation of the percentage of βIII tubulin-positive cells that have incorporated BrdU in BrdU pulse-chase experiments performed in E5 chicken embryos (the mean ± SEM from n different retinas are shown)

<table>
<thead>
<tr>
<th>BrdU/dU treatment</th>
<th>βIII tubulin-positive cells incorporating BrdU (%)</th>
<th>Layered* βIII tubulin-positive cells incorporating BrdU (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>15 min BrdU</td>
<td>9.33 ± 0.73 (n = 3)</td>
<td>0.00 ± 0.00 (n = 3)</td>
</tr>
<tr>
<td>1h BrdU + 0h dU</td>
<td>9.34 ± 0.18 (n = 3)</td>
<td>0.00 ± 0.00 (n = 3)</td>
</tr>
<tr>
<td>1h BrdU + 2h dU</td>
<td>9.62 ± 0.15 (n = 3)</td>
<td>0.00 ± 0.00 (n = 3)</td>
</tr>
<tr>
<td>1h BrdU + 4h dU</td>
<td>10.10 ± 0.77 (n = 3)</td>
<td>0.00 ± 0.00 (n = 3)</td>
</tr>
<tr>
<td>1h BrdU + 6h dU</td>
<td>9.25 ± 0.35 (n = 3)</td>
<td>0.38 ± 0.19 (n = 3)</td>
</tr>
<tr>
<td>1h BrdU + 9h dU</td>
<td>8.86 ± 0.50 (n = 3)</td>
<td>3.35 ± 0.14† (n = 3)</td>
</tr>
</tbody>
</table>

*βIII tubulin-positive cells located at the basal (i.e., vitreal) surface of the GCL.
†P < 0.005; compared with 6 h.)