Exogenous growth factors induce the production of ganglion cells at the retinal margin

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Accepted 31 January 2002

SUMMARY

Neural progenitors at the retinal margin of the post-hatch chicken normally produce amacrine and bipolar cells, but not photoreceptor or ganglion cells. The purpose of this study was to test whether exogenous growth factors influence the types of cells produced by progenitors at the retinal margin. We injected insulin, FGF2 or a combination of insulin and FGF2 into the vitreous chamber of post-hatch chickens. To assay for growth factor-induced changes at the retinal margin, we used in situ hybridization and immunocytochemistry on cryosections. One day after the final injection, we found that insulin alone stimulated the addition of cells to the retinal margin, but this was not further increased when FGF2 was applied with insulin. Insulin alone increased the number of cells in the progenitor zone that expressed neurofilament, and this was further increased when FGF2 was applied with insulin. These neurofilament-expressing cells in the progenitor zone included differentiating neurons that expressed Islet1 or Hu. Four days after the final dose of growth factor, we found that the production of ganglion cells was induced by co-injection of insulin and FGF2, but not by either insulin or FGF2 alone. We conclude that the types of cells produced by progenitors at the retinal margin can be altered by exogenous growth factors and that normally the microenvironment imposes limitations on the types of neurons produced.

Key words: Growth factors, Chick, FGF2, Insulin

INTRODUCTION

While the majority of neurons and glia of the vertebrate central nervous system (CNS) are generated by neural stem cells during embryonic development, the persistence of neural stem cells in some regions of the adult CNS is a well-documented phenomenon. A considerable amount of effort has been made to better understand neural stem cells and to characterize their properties (for a review, see Gage, 2000). For neural stem cells derived from different regions of the CNS, attempts have been made to define the potential of these cells to generate different types of cells. Many of these studies have relied on in vitro manipulations, including expansion of numbers of the cells prior to transplantation to assess their potential. For example, mitotically active cells from the adult mouse subventricular zone (SVZ) can be grown in culture, stimulated to proliferate by growth factors, and induced to differentiate into various types of neurons (reviewed by Cameron et al., 1998; Goldman, 1998). Moreover, transplantation of stem cells derived from the hippocampus into the olfactory bulb has shown that these cells can give rise to neurons with characteristics not normally found in hippocampal cells (Yang et al., 2000). More recently, transplantation of neural stem cells derived from the adult brain into mammalian or avian embryos has demonstrated that adult neural stem cells are nearly totipotent: able to generate cell types derived from all germ layers, including muscle and blood cells, when placed in the embryonic environment (Clarke et al., 2000; Galli et al., 2000).

Despite these clear demonstrations of the remarkable plasticity of neural stem cells, evidence from in vivo studies has demonstrated restrictions in the types of neurons generated by neural stem cells. In the brain of the adult songbird, for example, neurons that project from the higher vocal center (HVC) to the robust nucleus of the archistriatum (RA) are produced throughout life, while other types of HVC neurons are not (reviewed by Goldman, 1998; Scharff et al., 2000). Similarly, in the subventricular zone of the adult rodent, stem cells produce only glia and granule neurons in the olfactory bulbs (Luskin, 1993; Luskin, 1998; Goldman, 1995; Lois and Alvarez-Buylla, 1993; Lois et al., 1996). Other types of neurons present in the olfactory bulbs are not (reviewed by Goldman, 1998; Scharff et al., 2000). Similarly, in the subventricular zone of the adult rodent, stem cells produce only glia and granule neurons in the olfactory bulbs (Luskin, 1993; Luskin, 1998; Goldman, 1995; Lois and Alvarez-Buylla, 1993; Lois et al., 1996). Other types of neurons present in the olfactory bulbs are not (reviewed by Goldman, 1998; Scharff et al., 2000). Similarly, in the subventricular zone of the adult rodent, stem cells produce only glia and granule neurons in the olfactory bulbs (Luskin, 1993; Luskin, 1998; Goldman, 1995; Lois and Alvarez-Buylla, 1993; Lois et al., 1996). Other types of neurons present in the olfactory bulbs are not (reviewed by Goldman, 1998; Scharff et al., 2000). Similarly, in the subventricular zone of the adult rodent, stem cells produce only glia and granule neurons in the olfactory bulbs (Luskin, 1993; Luskin, 1998; Goldman, 1995; Lois and Alvarez-Buylla, 1993; Lois et al., 1996). Other types of neurons present in the olfactory bulbs are not (reviewed by Goldman, 1998; Scharff et al., 2000). Similarly, in the subventricular zone of the adult rodent, stem cells produce only glia and granule neurons in the olfactory bulbs (Luskin, 1993; Luskin, 1998; Goldman, 1995; Lois and Alvarez-Buylla, 1993; Lois et al., 1996). Other types of neurons present in the olfactory bulbs are not (reviewed by Goldman, 1998; Scharff et al., 2000). Similarly, in the subventricular zone of the adult rodent, stem cells produce only glia and granule neurons in the olfactory bulbs (Luskin, 1993; Luskin, 1998; Goldman, 1995; Lois and Alvarez-Buylla, 1993; Lois et al., 1996). Other types of neurons present in the olfactory bulbs are not (reviewed by Goldman, 1998; Scharff et al., 2000). Similarly, in the subventricular zone of the adult rodent, stem cells produce only glia and granule neurons in the olfactory bulbs (Luskin, 1993; Luskin, 1998; Goldman, 1995; Lois and Alvarez-Buylla, 1993; Lois et al., 1996). Other types of neurons present in the olfactory bulbs are not (reviewed by Goldman, 1998; Scharff et al., 2000). Similarly, in the subventricular zone of the adult rodent, stem cells produce only glia and granule neurons in the olfactory bulbs (Luskin, 1993; Luskin, 1998; Goldman, 1995; Lois and Alvarez-Buylla, 1993; Lois et al., 1996). Other types of neurons present in the olfactory bulbs are not (reviewed by Goldman, 1998; Scharff et al., 2000). Similarly, in the subventricular zone of the adult rodent, stem cells produce only glia and granule neurons in the olfactory bulbs (Luskin, 1993; Luskin, 1998; Goldman, 1995; Lois and Alvarez-Buylla, 1993; Lois et al., 1996). Other types of neurons present in the olfactory bulbs are not (reviewed by Goldman, 1998; Scharff et al., 2000).
not clear whether the same factors that regulate neuronal phenotype in the developing CNS will continue to be active in controlling the cell fate decisions of the stem cells in the mature CNS.

Recent studies have demonstrated that the eyes of birds and mammals contain neural stem cells (Fischer and Reh, 2000; Tropepe et al., 2000; Ahmad et al., 2000). In the retinal margin of the post-hatch chicken, there is a population of neural stem cells that proliferate and generate neurons that are integrated into the peripheral edge of the retina (Fischer and Reh, 2000). These cells are reminiscent of the well-described stem cells of the ciliary marginal zone (CMZ) of fish and amphibians (reviewed by Reh and Levine, 1998). However, under normal conditions, the retinal CMZ cells of the chick produce only amacrine and bipolar neurons, while all types of retinal neurons are produced by stem cells in the CMZ of fish and amphibians. Amacrine and bipolar cells are generated late relative to other retinal cell types during embryonic histogenesis (Prada et al., 1991), and it has been proposed that retinal progenitors become progressively restricted to producing specific cell types as development proceeds (Jasoni et al., 1994). Retinal stem cells in the adult rodent eye may be similar to late-stage embryonic progenitors and the CMZ cells of the post-hatch chick eye in that they are restricted to producing late-generated cell types in vitro (Tropepe et al., 2000). The projection neurons of the retina, the ganglion cells, are not generated in vitro or in vivo by retinal stem cells of mature birds or mammals.

Neural stem cells in the eyes of adult birds and mammals appear to be restricted in the types of neurons that they are capable of producing. The purpose of this study was to test whether exogenous growth factors are capable of inducing the production of ganglion cells from the CMZ cells at the retinal margin of the chicken. We show that intraocular injections of insulin and fibroblast growth factor 2 (FGF2) stimulated the proliferation of CMZ cells and production of ganglion cells at the retinal margin. These results show that the same microenvironmental factors that are known to control the cell fate choices in the developing retina continue to act in the mature retina, and further suggest that the potential of neural stem cells in the mature CNS can be expanded by experimental manipulations.

MATERIALS AND METHODS

Animals

The use of animals in these experiments was in accordance with the guidelines established by the National Institutes of Health and the University of Washington. Newly hatched leghorn chickens (Gallus gallus domesticus) were obtained from H&N Highline International (Seattle, WA) and kept on a cycle of 16 hours light, 8 hours dark (lights on at 6:00 am). Chicks were housed in clear Nalgene® cages at about 25°C and received water and Purina® chick starter ad libitum.

Injections

Chicks were anesthetized and injected as described elsewhere (Fischer et al., 1998; Fischer et al., 1999; Fischer and Reh, 2000). The left eye (control) was injected with 20 μl of vehicle (sterile saline plus 0.1 mg/ml bovine serum albumin) and the right eye (treated) was injected with growth factors. Growth factors used in these experiments included purified bovine insulin (2 μg per injection) and purified bovine fibroblast growth factor 2 (FGF2; 100 ng per injection). All growth factors were obtained from R & D Systems and were dissolved in saline plus 0.1 mg/ml BSA and 100 μg/ml 5-bromo-2-deoxyuridine (BrdU; Sigma). We made two or three consecutive daily injections of BrdU plus growth factors starting at post-hatch day 8 (P8) and harvested tissues at 1, 4 or 10 days after the final injection.

Fixation and sectioning

Dissection, fixation and sectioned where performed as described elsewhere (Fischer et al., 1998; Fischer et al., 1999; Fischer and Reh, 2000).

Immunocytochemistry

Standard immunocytochemical techniques were applied to sections as described elsewhere (Fischer et al., 1998; Fischer et al., 1999; Fischer and Reh, 2000). To obtain adequate immunolabeling in wholemounts of the peripheral retina, the following procedures were used to overcome barriers to antibody penetration. Twenty-four hours before dissection, eyes were injected with 300 units of hyaluronidase (Sigma). Eyes were enucleated, transected equatorially and the anterior hemi-segment immersed in fixative (4% paraformaldehyde (PFA) in 0.1 M dibasic phosphate buffer (PB) plus 3% sucrose pH 7.4) for 30 minutes at room temperature. All washes and incubations were done on free-floating samples at room temperature and on a nutator, with washes lasting 15 minutes and incubations lasting 24 hours. Tissues were washed three times in phosphate-buffered saline (PBS), equilibrated in 20% sucrose in PBS and subjected to three freeze/thaw cycles. The retina, pars plana and adherent pigmented epithelium was dissected away from the choroid, sclera and lens, cut into four radial quadrants, and each piece of tissue placed individually into the wells of a 24-well plate. This was followed by consecutive washes in PBS, deionized H2O, 30% dimethylsulfoxide (DMSO) in deionized H2O, 70% DMSO, 30% DMSO, deionized H2O and PBS. Tissues were incubated in 250 μl of antibody solution (antibody diluted in PBS added with 5% goat serum, 0.3% Triton X-100 and 0.01% NaN3). Tissues were washed three times in PBS and incubated with secondary antibody. After incubation in the secondary, tissues were washed three times in PBS, fixed for 30 minutes in 2% paraformaldehyde in 0.1M PB plus 3% sucrose. This was followed by a series of washes in PBS, 4 N HCl for 7 minutes, PBS, and incubation with BrdU antibodies. After incubation with antibodies to BrdU, tissues were washed twice in PBS, incubated with secondary antibodies, and washed twice in PBS. Tissues were mounted in 4:1 glycerol to water for observation under an epifluorescence microscope.

Working dilutions and sources of antibodies used in this study included the following: mouse anti-Pax6 at 1:50 (Developmental Studies Hybridoma Bank; DSHB); rabbit anti-Chx-10 at 1:4000 (Dr T. Jessell, Columbia University); mouse anti-Isl-1 at 1:50 (3rd45; DSHB); mouse anti-Hu at 1:200 (Monoclonal Antibody Facility, University of Oregon); rabbit anti-Brn3.0 at 1:1000 (Dr E. Turner, University of California, San Diego); mouse anti-neurofilament at 1:2000 (recognizes the 160 kDa subunit of neurofilament; RMO270; Zymed); rabbit anti-neurofilament at 1:1000 (recognizes the 145 kDa subunit of neurofilament; Chemicon); rabbit anti-glutamine synthetase at 1:2000 (Dr P. Linser, University of Florida); rat anti-BrdU at 1:80 (Accurate Chemicals); and mouse anti-BrdU at 1:80 (G3C4; DSHB). Secondary antibodies included goat-anti-rabbit-Alexa568, goat-anti-mouse-Alexa568, goat-anti-mouse-Alexa488 and goat-anti-rat-Alexa488 (Molecular Probes, Eugene, OR) diluted to 1:500 in PBS plus 0.3% Triton X-100.

In situ hybridization

Tissues were dissected and immediately embedded and frozen in OCT medium (Tissue-Tek). Sections (14 μm) were cut in the naso-temporal plane, thaw mounted onto Super-Frost™ slides (Fischer Scientific), and stored desiccated at –80°C until use. Upon thawing, slides were immediately fixed for 10 minutes in 4% paraformaldehyde in DEPC-
treated PBS, followed by two 15 minutes washes in 0.1% active DEPC in PBS, and a 15 minute wash in DEPC-treated 5×SSC. Sections were prehybridized for 2 hours at 60°C in 50% formamide, 5× SSC, 5× Denhardt's, 250 µg/ml yeast RNA and 500 µg/ml herring sperm DNA. This solution was replaced with fresh hybridization buffer with 1 µg/ml DIG-labeled riboprobe and sections were incubated overnight at 60°C in a humidified chamber. Sections were rinsed with 2× SSC at 65°C and washed for 1 hour in 0.2× SSC at 72°C. Sections were processed for DIG-immunolabeling as described elsewhere (Jasoni et al., 1994). Riboprobes to Cath5 were made from base pairs 11-501 by· at 65°C and washed for 1 hour in 0.2× SSC at 72°C. Sections were processed for DIG-immunolabeling as described elsewhere (Jasoni et al., 1994). Riboprobes to Cath5 were made from base pairs 11-501 by

**Measurements, cell counts, and statistical analyses**

Errors were calculated as the standard deviation of each sample that was comprised of at least five individuals per group. To compare data from treated and control eyes, statistical significance was assessed by using a two-tailed Student t-test. All measurements were made from digital micrographs of the retinal margin, while all cell-counts were made under the microscope on at least eight different sections per individual.

**RESULTS**

**Growth factors stimulate the proliferation of CMZ cells**

To test whether exogenous growth factors stimulated the production of ganglion cells in the retinal margin, we made intraocular injections of two proteins, FGF and insulin. We have previously shown that intraocular injections of insulin alone can stimulate the overall amount of proliferation at the retinal margin, while FGF2 alone had no such effect (Fischer and Reh, 2000). There is considerable evidence to indicate that FGF promotes ganglion cell development from embryonic retinal progenitor cells (Pittack et al., 1991; Pittack et al., 1997; Guillemot and Cepko, 1992; McCabe et al., 1999). Therefore, we reasoned that a combination of these growth factors could promote the production of ganglion cells from the CMZ cells. Consistent with previous findings (Fischer and Reh, 2000), we found that insulin alone caused a marked increase in the number of BrdU-labeled cells at the retinal margin (compare Fig. 1A with Fig. 1B). By comparison, intraocular injections of either FGF2 alone (Fig. 1C) or saline failed to increase the number of BrdU-labeled cells. When FGF2 was applied with insulin, the number of BrdU labeled cells was not significantly different from that obtained with insulin alone (Fig. 1D,E).

Thus, these factors do not appear to be a synergistic interaction of these growth factors on the proliferation of CMZ cells. Although the combination of these growth factors did not increase the number of BrdU-labeled cells over that observed with insulin alone, the distribution of labeled cells differed between the two conditions. We noted many more BrdU-labeled cells in the ganglion cell layer (GCL) of retinas treated with both factors compared with the distribution of BrdU-labeled cells in retinas treated with either factor alone (compare Fig. 1B,D). Using the TUNEL method, we did not observe apoptotic nuclei within retinas that were treated with insulin alone, FGF2 alone, or insulin and FGF2. Thus, insulin alone caused an upregulation of proliferation of the CMZ cells, while insulin and FGF caused a change in the distribution of BrdU-labeled cells that may represent a change in the types of cells generated from the CMZ.

Because insulin, but not FGF2 alone or in combination with insulin, stimulated the accumulation of BrdU-labeled cells within the retinal margin, this treatment might also induce the accumulation of CMZ cells within the retinal margin.

**Insulin and FGF2 induce the production of ganglion cells at the retinal margin**

To determine whether the intraocular injections of insulin and FGF2 caused a change in the types of neurons generated by the cells of the CMZ, we used double-labeling with BrdU and several markers of retinal neurons. We concentrated our analysis on retinal ganglion cells, as they are not normally generated by the CMZ cells (Fischer and Reh, 2000). Neurofilament protein expression is normally confined to orthotopic and ectopic ganglion cells in the chick retina (Fig. 2A). Thus, we labeled sections of the retinal margin with antibodies to the 160 kDa subunit of neurofilament. At the retinal margin of the untreated post-hatch chick eye, we found a few neurofilament immunoreactive cells (Fig. 2B). These neurofilament-positive cells in the CMZ at the retinal margin were vertically oriented and appeared to produce processes that spanned the depth of the retinal margin. The morphology of these cells is similar to that of differentiating ganglion cells in the embryonic retina (McLoon and Barnes, 1989; Brittis et al., 1995; McCabe et al., 1999). Three consecutive daily injections
of FGF2 alone did not cause an increase in the number of neurofilament-positive cells in the CMZ (Fig. 2F), while three injections of insulin alone caused an increase in the number of neurofilament-positive cells in the CMZ (Fig. 2C,F). This effect was further increased when FGF2 was applied with insulin (Fig. 2D,F). The effect of these factors on neurofilament expression in the CMZ was transient because at 4 days after the final dose either factor, the number of neurofilament-positive cells in the CMZ at the retinal margin was reduced to levels seen in saline-treated eyes (Fig. 2E,F). We found many neurofilament-immunoreactive cells in the CMZ that were labeled for BrdU (Fig. 3A-C), indicating that these cells were newly generated.

To determine whether further ganglion cell development occurs in the growth factor treated retinas, we used additional markers known to be expressed in ganglion cells: the neuron-specific markers Hu or Islet1. In the chick retina, Hu is expressed by most if not all amacrine and ganglion cells (Fischer and Reh, 2000; Fischer and Reh, 2001), and Hu and neurofilament are co-expressed by all ganglion cells (Fig. 3D-F). At the retinal margin of eyes that received three injections of insulin, we found that a few neurofilament-immunoreactive cells in the CMZ expressed Hu (Fig. 3G-I). In the post-hatch chick retina, Islet1 was expressed by cholinergic amacrine cells, many bipolar cells and most ganglion cells (A. J. F. and T. A. R., unpublished). Islet-1 is expressed by 77.5±9.5% (100 of 129 cells counted from eight eyes) of neurofilament-positive cells in the GCL (Fig. 3J-L) and all displaced ganglion cells (68 cells counted from four eyes). In the CMZ of retinas treated with three doses of insulin, we found that 9.6% (10 of 104 cells counted from three individuals) of the neurofilament-expressing cells were immunoreactive for Islet1 (Fig. 3M-O). Within the CMZ, all cells that were immunoreactive for Hu or Islet1 were also immunoreactive for neurofilament.

As noted above, intraocular injections of insulin and FGF caused many of the BrdU-labeled cells from the CMZ to migrate to the ganglion cell layer. To test whether BrdU-labeled nuclei in the GCL were those of ganglion cells, we double labeled sections for BrdU and either Islet1 or Brn3.0. One day after the final injection of three consecutive daily injections of insulin alone, we found few cells that were double-labeled for Islet1 and BrdU (Fig. 4A). By comparison, 4 days after the final injection of insulin alone we found increased numbers of cells that were double labeled for Islet1 and BrdU, and most of the these cells were in the INL (Fig. 4A). Four days after the final injection of insulin and FGF2, we found many cells in the GCL that were labeled for BrdU and Islet1 (Fig. 4B-E). In eyes treated with saline, we did not find any cells in the GCL that were labeled for BrdU and Islet1 (Fig. 4B). By comparison, we did find a few cells in the GCL (<1 cell per section of the retinal margin) that were labeled for
Brdu and Islet1 in retinas that were treated with insulin alone (Fig. 4B). Compared with retinas treated with insulin alone, retinas treated with insulin and FGF2 had about 10 times as many cells in the GCL that were double labeled for BrdU and Islet1 in the far peripheral retina (Fig. 4B).

To corroborate these findings, we probed for newly generated, Brn3.0-expressing ganglion cells at the retinal margin. Brn3.0 is a POU-domain homeotic transcription factor that is known to be required for the differentiation and survival of retinal ganglion cells (Xiang et al., 1993; Gan et al., 1996; Liu et al., 2000). In the chicken retina, immunoreactivity for Brn3.0 was detected in 92.8±5.2% (118 cells counted from eight eyes) of the neurofilament-positive cells in the GCL, but did not label ganglion cells displaced to the proximal INL (Fig. 5A-C), indicating that Brn3.0-immunolabeling is exclusive to most orthotopic ganglion cells in the chick retina. At the retinal margin of eyes treated with three doses of saline, insulin alone or FGF2 alone, Brn3.0-immunoreactive cells were never observed near the peripheral edge of the retina. Instead, Brn3.0-immunoreactive cells were observed in the GCL at least 100 μm away from...
the peripheral edge of the retina (Fig. 5D-F). In untreated retinas, we never observed cells double-labeled for Brn3.0 and BrdU. However, in eyes treated with three doses of insulin and FGF2, some of the BrdU-labeled cells at the retinal margin were also immunoreactive for Brn3.0 (Fig. 5G-I). These cells were often located near the CMZ at the retinal margin (upper cell in Fig. 5I).

The results from our analysis of retinal sections from eyes injected with a combination of FGF2 and insulin indicate that retinal ganglion cells can be generated from the cells of the CMZ. To gain a better appreciation of the morphology of these newly generated ganglion cells, we used whole-mount preparations of the retinal margin to probe for cells that were double labeled with BrdU and neurofilament. BrdU/neurofilament-labeled cells were observed only in tissue treated with three doses of insulin alone, Cath5 expression was never observed in cells at the retinal margin (Fig. 7B,D). By contrast, Cath5 was expressed by cells at the retinal margin of eyes treated with both insulin and FGF2 (Fig. 7C,D). In eyes treated with insulin and FGF2, Cath5 expression was observed in every section of the retinal margin from four individuals. The labeled

These observations were made 10 days after the final injection of insulin and FGF2; at a time when the growth factors should have been cleared from the eye (Lewis et al., 1996) and the phenotype of growth factor-affected cells should have stabilized.

Taken together, these data are consistent with the hypothesis that the combination of growth factors insulin and FGF2 promote the development of ganglion cells in the CMZ of the post-hatch chicken. We further tested this hypothesis by probing for the expression of Cath5 (chick atonal homolog 5). Cath5 is a basic helix-loop-helix (bHLH) transcription factor that is transiently expressed by developing ganglion cells and is required for their normal development (Brown et al., 1998; Wang et al., 2001; Liu et al., 2001). In central regions of the retina of post-hatch chickens, we did not detect Cath5 transcripts (Fig. 7A), consistent with previous reports that the gene is only transiently expressed in developing ganglion cells. In eyes treated with three doses of insulin alone, Cath5 expression was never observed in cells at the retinal margin (Fig. 7B,D). By contrast, Cath5 was expressed by cells at the retinal margin of eyes treated with both insulin and FGF2 (Fig. 7C,D). In eyes treated with insulin and FGF2, Cath5 expression was observed in every section of the retinal margin from four individuals. The labeled
cells were typically located within the CMZ or the adjacent retina, and are likely to be immature ganglion cells migrating to the ganglion cell layer.

DISCUSSION

We report that the types of neurons produced by CMZ cells at the retinal margin of the post-hatch chick can be influenced by exogenous growth factors. In untreated eyes, the CMZ of the chicken retina generates only a few types of neurons: bipolar and amacrine cells. By contrast, intraocular injections of insulin and FGF2 induced the production of cells at the retinal margin that were labeled for BrdU and Islet1, BrdU and Brn3.0, BrdU and neurofilament, and Cath5. These findings indicate that exogenous insulin and FGF2 induced the addition of ganglion cells to the peripheral edge of the retina. Taken together, these findings indicate that the CMZ cells at the margin of the chicken retina can be induced to produce a neuronal type not normally generated. These findings demonstrate that CMZ cells at the retinal margin are not intrinsically limited to producing only bipolar and amacrine neurons. This effect was not caused by an overall increase in histogenesis, as exogenous insulin causes the same degree of increase in the proliferation of CMZ cells without inducing the production of ganglion cells.

The expression of the ganglion cell marker neurofilament in the CMZ was stimulated by insulin alone, but insulin alone did not induce the production of ganglion cells. This finding suggests that the expression of neurofilament may not represent a commitment to a ganglion cell phenotype. We found that numbers of neurofilament-expressing cells in the CMZ were related to the proliferation of CMZ cells, but not the production of ganglion cells. For example, we found that insulin alone increased the number of BrdU-labeled cells added to the retina, increased the number of Pax6/Chx10-expressing cells within the retinal margin and increased the number of neurofilament-expressing cells in the CMZ, but did not increase the number ganglion cells that were produced (i.e. cells that were labeled for BrdU and Islet1, Brn3.0 or neurofilament at 4 days after the final injection of growth factor). By contrast, the production of ganglion cells was induced when FGF2 was applied with insulin without further stimulating the proliferation of CMZ cells. The fate of neurofilament-expressing cells observed in the CMZ of eyes treated with insulin alone remains uncertain. It is possible that the expression of neurofilament is transient in some CMZ cells or in some types of differentiating neurons. Consistent with this hypothesis, Bennett and DiLullo (Bennett and DiLullo, 1985a; Bennett and DiLullo, 1985b) reported that neuroepithelial and neuroblastic cells of the chick central nervous system express neurofilament proteins and that some

Fig. 6. Insulin and FGF2 induce the production of neurofilament-expressing ganglion cells at the retinal margin. Whole-mount preparations of the retinal margin were obtained from eyes 4 days after three consecutive daily injections of insulin and FGF2. Tissues were labeled with antibodies to (A,C,D,F; green) BrdU and (B,C,E,F; red) neurofilament. Large arrows indicate the retinal margin and small arrows indicate double-labeled cells. Scale bar: 50 μm.

Fig. 7. Cath5 is expressed at the retinal margin of eyes treated with insulin and FGF2. Vertical sections of the retinal margin were labeled for Cath5 transcripts. In situ hybridization was used to detect Cath5 mRNA in tissues treated with (B) insulin alone or (A,C) insulin and FGF2. Tissues were processed for in situ hybridization 24 hours after the final dose of three consecutive daily injections of growth factor. Large arrows in B.C indicate the retinal margin and the small arrows indicate Cath5-expressing cells. The boxed area in C is increased 2.5-fold below to better demonstrate Cath5-expressing cells. ONL, outer nuclear layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. Scale bar: 50 μm. (D) Histogram demonstrating the numbers of Cath5-expressing cells observed at the retinal margin of eyes treated with insulin alone, FGF2 alone, and a combination of insulin and FGF2.
of this expression is transient. In addition, others have shown that neurofilament expression is transient during the differentiation of hair cells in the mouse cochlea (Hasko et al., 1990), cerebellar granule cells in the rat (Cambray-Deakin and Burgoyne, 1986) and cholinergic amacrine cells in the chick retina (A. J. F. and T. A. R., unpublished).

Our data suggest that the differentiation of neurons at the retinal margin begins in scleral layers of the CMZ. Neurofilament-positive cells in the CMZ expressed Isl1 or Hu, and many of these cells were located towards the scleral surface of the CMZ. Coincident with the location of these cells, we found cells that expressed Cath5 or Brn3.0. In the embryonic chick retina, both Brn3 and Cath5 are expressed by cells distal to the developing GCL; the expression of these factors ectopic to the GCL is presumed to be produced by differentiating ganglion cells that are migrating toward the GCL (Liu et al., 2000; Matter-Sadzinski et al., 2001). By comparison, others have found that the differentiation of ganglion cells, as detected by the expression of RA4, neurofilament or Isl1, in the embryonic retina begins near the ventricular surface (McLoon and Barnes, 1989; Brittis et al., 1995; McCabe et al., 1999; Zhang and Yang, 2001), which is equivalent to the scleral surface of the post-hatch chick CMZ.

The notion of FGF-induced production of ganglion cells is consistent with observations made in the developing retina. Previous studies have shown that exogenous FGFs can promote the production of ganglion cells in cultures of embryonic retinas from chicks and rodents (Pittack et al., 1991; Pittack et al., 1997; Guillemot and Cepko, 1992; Zhao and Barnstable, 1996; McCabe et al., 1999). In addition, overexpression of FGF2 in Xenopus retina promotes the production of ganglion cell (Patel and McFarlane, 2000). Consistent with the findings of these gain-of-function studies, the differentiation of ganglion cells can be inhibited by antisense-mediated suppression of FGF expression in embryonic chick retinas in vitro and in vivo (Desire et al., 1998), and by the FGF-receptor inhibitor SU5402 in explant cultures of the embryonic chick retina (McCabe et al., 1999). Thus, it appears that CMZ cells at the margin of the chicken eye resemble embryonic chick retinal precursors in their response to FGFs.

Our results suggest that adult neural stem cells may be restricted in the types of neurons they generate because of limitations imposed by the secreted factors in the local microenvironment. As noted in the Introduction, a variety of different approaches have been used to address this question. In vitro studies have generally emphasized the plasticity of neural stem cells, and some amount of cell culture may even induce this plasticity. For example, studies showing that the neural stem cells can generate diverse cell types appear to require that neural stem cells proliferate in culture prior to differentiation (for a review, see Anderson, 2001). It is possible that the growth factors used to stimulate the proliferation of neural stem cells and, subsequently, to induce differentiation into different cell types are required to bestow plasticity upon these cells. The data from in vivo studies has generally shown that neural stem cells have a more limited potential. For example, targeted photolysis of projections neurons in the HVC of adult songbirds results in a compensatory replacement of neurons that are normally turned over, but replacement of neuronal types that are not normally generated does not occur (Scharff et al., 2000). Despite the evidence for some intrinsic limits to the types of progeny derived from neural stem cells in vivo, recent studies by Magavi and colleagues (Magavi et al., 2000), indicate that selective ablation of projection neurons stimulates the regeneration of the ablated cell type in the cerebral cortex of adult mice. It is possible that the highly selective loss of a cell type via photolysis induces changes in the microenvironment that allow or promote the differentiation of cells derived from adult neural stem cells.

In conclusion, the findings presented demonstrate that the same factors that control neuronal cell fate decisions in the embryonic CNS are also active in stem cell zones such as the CMZ to direct precursors to specific fates. Moreover, our results are consistent with the hypothesis that part of the restriction in cell fates generated by the cells of the CMZ in the post-hatch chicken is due to limiting amounts of FGF in the microenvironment. Thus, these findings suggest that exogenous growth factors can be used in vivo to influence the types of neurons produced by stem cells and possibly stimulate the replacement of particular types of neurons.

We thank Josh Friedland-Little for providing expert technical assistance. We also thank Dr M. Roberts, R. Kubota and P. Horner for their comments that helped to contribute to the final form of this paper. We thank Dr Olivia Bermingham-McDonogh for the Cath5 clone. The BrdU, Pax6 and Isl1 antibodies developed by Drs S. J. Kaufman, A. Kawakami and T. Jessell, respectively, were obtained from the Developmental Studies Hybridoma Bank developed under auspices of the NICHD and maintained by the University of Iowa, Department of Biological Sciences, Iowa City, IA 52242. This work was supported by fellowships from the Alberta Heritage Foundation for Medical Research and the Canadian Institute of Health Research to A. J. F. and by NSF grant IBN-0080197 and NIH grant NS28308 to T. A. R.

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