Ligands of steroid/thyroid receptors induce cone photoreceptors in vertebrate retina

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SUMMARY

The mechanisms by which multipotent progenitor cells are directed to alternative cell identities during the histogenesis of the vertebrate central nervous system are likely to involve several different types of signaling systems. Recent evidence indicates that 9-cis retinoic acid, which acts through members of the steroid/thyroid superfamily of receptors, directs progenitor cells to the rod photoreceptor cell fate. We now report that another effector of this family of receptors, thyroid hormone, induces an increase in the number of cone photoreceptors that develop in embryonic rat retinal cultures, and that combinations of 9-cis retinoic acid and triiodothyronine cause isolated progenitor cells to differentiate as either rods or cones, depending on the relative concentrations of the ligands. These results implicate thyroid hormone in CNS cell fate determination, and suggest that different photoreceptor phenotypes may be modulated through the formation of thyroid/retinoid receptor heterodimers.

Key words: thyroid hormone, retinoic acid, neural retina, neurogenesis, cell fate, rat, development

INTRODUCTION

The mechanisms that direct neuronal progenitor cells to differentiate into various types of neurons in the vertebrate central nervous system are not well understood. Heterochronic coculture studies and in vivo cell deletion studies have demonstrated that factors in the local microenvironment of retinal progenitor cells can influence their ultimate choice of fate (Reh, 1987, 1992a; Raymond et al., 1988). Recently, a number of laboratories have concentrated on the factors that regulate rod photoreceptor identity and several molecules have now been shown to have effects on rod photoreceptor differentiation, including peptide growth factors, taurine and retinoic acid (Anchan et al., 1991; Hicks and Courtois, 1992; Altshuler et al., 1993; Kelley et al., 1994). However, specific cues that promote or influence the differentiation of other types of retinal neurons have not been identified.

Several laboratories have demonstrated that the vitamin A derivative, retinoic acid, may be involved in various aspects of retinal development (McCaffery et al., 1993; Kelley et al., 1994; Marsh-Armstrong et al., 1994). In particular all-trans and 9-cis retinoic acid (RA) induce retinal progenitor cells to differentiate as rod photoreceptors and inhibit progenitor cells from differentiating as amacrine cells in vitro (Kelley et al., 1994). These results suggest that one of the effects of retinoic acid may be to specifically direct progenitor cells towards one cell fate over another. The effects of RA are mediated through the activation of retinoic acid receptors (RARs) and retinoid X receptors (RXRs; Petkovich et al., 1987; Giguere et al., 1987; Mangelsdorf et al., 1990), and at least one member of this family of receptors, RXR-γ, is expressed by the progenitor cells in embryonic retina (Kelley, Williams and Reh, unpublished observations). These receptors are members of the steroid/thyroid receptor superfamily of transcription factors, and are believed to bind to DNA response elements only after the formation of either RXR:RXR homodimers, or heterodimers of RXR with other members of this receptor family, including RAR, thyroid receptors (TR) and vitamin D receptors (Yu et al., 1991; Durand et al., 1992; Kleiwer et al., 1992; Zhang et al., 1992; Marks et al., 1992; Bugge et al., 1992; Leid et al., 1992).

Previous studies have demonstrated that changes in the levels of thyroid hormones during development can have diverse influences on the nervous system and brain. In particular, the rates of cell proliferation and neuronal differentiation in the cerebral cortex and cerebellum are altered in both hyper- and hypothyroid animals (Nicholson and Altman, 1972; Lauder, 1977; Hetzel et al., 1988; Gould and Butcher, 1989; Pickard et al., 1993). In addition, thyroid receptors are expressed throughout the developing rat brain, including the progenitor zones, beginning as early as embryonic day 11.5 (E11.5; Mellstrom et al., 1991; Bradley et al., 1992). Finally, a novel thyroid hormone receptor isoform has recently been shown to be expressed in embryonic chick retina, at stages when progenitor cells are becoming committed to differentiate as photoreceptors (Sjoberg et al., 1992). Based on these results, we hypothesized that activation of TRs might also influence determination of photoreceptor cells in the developing retina.
MATERIALS AND METHODS

Cell culture
Retinal cells were dissected from embryonic day 18 (E18) Long Evans rat fetuses and established in high density cultures, as described previously (Kelley et al., 1994). At this stage in vivo, approximately 10% of newly generated cells will differentiate as cone photoreceptors, 35% as amacrine cells, and 50% as rod photoreceptors (M. LaVail, personal communication). Since progenitor cells are influenced to differentiate as several different types of retinal cells at this point in development, this is an ideal stage to examine the effects of different factors on determination of cell fate. Immediately after plating, a stock solution of 10 mM triiodothyronine (T3; Sigma), the active form of thyroid hormone, in saline was diluted with the culture medium to specific concentrations. Subsequent media changes were made every 48 hours and cultures were maintained for a total of either 6 or 14 days in vitro (DIV). In cultures that were maintained for 14 DIV, increased cell density prevented accurate identification of individual immunolabeled cells. Therefore, 12 hours prior to the completion of an experiment these cultures were redissociated using a previously published technique (Kelley et al., 1995) and then replated at a lower density to allow identification of single-labeled cells. Briefly, the culture medium was removed from individual culture wells and replaced with calcium- and magnesium-free saline with 0.15% trypsin. The culture plates were then incubated with gentle agitation for 10 minutes at 37°C. Cultured retinal cells were collected by aspiration, concentrated by centrifugation, resuspended in medium, dissociated by trituration, and replated at a lower density onto new Matrigel-polylysine-coated coverslips. At the end of the culture period, cells were fixed with 4% paraformaldehyde for 2 hours at room temperature and then washed with phosphate-buffered saline. Overall cell number was determined using a previously described technique (Kelley et al., 1994). Briefly, each well of the 24-well plate contained a coverslip that covered 64% of the total area of the bottom of the well. After the coverslips were removed and fixed for immunohistochemistry, the remaining cells (36% of the total) were dissociated with trypsin and counted using a hemocytometer.

Determination of retinal phenotypes
Determination of specific retinal phenotypes was determined using immunocytochemical markers. The following criteria were used to classify retinal cell types. (1) Newly differentiated photoreceptors were immunoreactive for recoverin, a protein found in both rod and cone photoreceptors (Dizhoor et al., 1991; Milam et al., 1993) but not immunoreactive for other photoreceptor markers. (2) Developing cone photoreceptors were immunoreactive for both recoverin and peanut agglutinin (PNA), a lectin that binds to the matrix sheath surrounding cone photoreceptors (Takumi and Uehara, 1991). Although recoverin and PNA are each expressed by small numbers of other retinal cell types, only cone photoreceptors express both antigens, therefore, double-labeling with both antibodies was used to identify cones. (3) More mature cone photoreceptors were immunoreactive for red/green cone opsins, exclusively expressed by a subclass of cone photoreceptors (Kelley et al., 1995). This antibody was generated against the unique amino-terminal extension of the human red/green cone opsins (Hargrave and McDowell, 1992) and previous studies have demonstrated that antibodies specific for this region of the opsin peptide exclusively label cone photoreceptors (Lerea et al., 1989). (4) More mature rod photoreceptors were immunoreactive for rod opsin, exclusively expressed by rod photoreceptors (Hicks and Barnstable, 1987). (5) Amacrine cells were immunoreactive for cellular retinoic acid binding protein (CRABP; Guarr et al., 1990). (6) Rod bipolar cells were immunoreactive for PCP-2 (Vandaele et al., 1991; Table 1). For each experiment the number of labeled cells was determined by counting 10 random fields along the vertical and horizontal axes of each coverslip. Each experiment was repeated a minimum of three times and a minimum of three coverslips was counted for each experiment.

Labeling of proliferative cells
To identify the direct effects of T3 on cone photoreceptors that were generated in vitro, proliferative cells were labeled by adding the thymidine analog bromodeoxyuridine (BrdU; Sigma) at a concentration of 10^-3 M to the culture medium for some of the experiments. At the end of these experiments, cultures were fixed as described and then double-labeled with antibodies against cone-specific antigens and antibodies against BrdU.

Low density cell culture
To determine the direct effects of T3 and RA on E18 retinal cells in the absence of cell-cell contact, low density, single cell cultures were established using a previously described technique (Kelley et al., 1994). Briefly, E18 retinal cells were dissected and dissociated with trypsin to form a single cell suspension. Single cells were then plated at a density of 10,000 to 50,000 cells per well (24 well plate) onto coverslips that were coated with collagen gels. Collagen gels were prepared using Vitrogen (Celtrix) according to the manufacturer’s protocol. To eliminate the possibility that undefined factors in fetal calf serum might inhibit or augment the effects of T3 or RA, these cultures were maintained in serum free medium. The composition of the culture medium was as previously described except that fetal bovine serum was excluded (Kelley et al., 1994). Different concentrations of T3 and RA (10 nM stock in DMSO) were added to the culture medium by diluting stock solutions immediately after cell plating. 9-cis retinoic acid was a gift from Hoffman-La Roche Pharmaceuticals. Cultures were maintained for 6 DIV and then fixed in 4% paraformaldehyde. Photoreceptor phenotypes were determined using primary antibodies described above. Single isolated cells that were immunoreactive for recoverin only were classified as photoreceptors, while single isolated cells that were immunoreactive for rhodopsin were classified as rod photoreceptors. To determine whether the presence of T3 and/or RA influenced cell survival in these cultures, viability was assessed by Alcian Blue dye exclusion (Yip and Auersperg, 1972). For each experiment the number of labeled cells was quantified by determining the percentage of single cells that were immunoreactive for either recoverin or rhodopsin in six randomly selected fields. Significance was determined using the Student’s t-test and each experiment was repeated a minimum of three separate times except for the dye exclusion experiment which was only done once.

Table 1. Immunocytochemical markers used to identify different retinal cell types in dissociated cultures of embryonic rat retina

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Marker</th>
<th>References</th>
</tr>
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<tr>
<td>Early developing photoreceptors</td>
<td>Recoverin</td>
<td>Dizhoor et al., 1991</td>
</tr>
<tr>
<td>Developing cone photoreceptors</td>
<td>Recoverin and PNA</td>
<td>Takumi and Uehara, 1991</td>
</tr>
<tr>
<td>Late cone photoreceptors</td>
<td>Red/green cone opsin</td>
<td>Lerea et al., 1989</td>
</tr>
<tr>
<td>Developing rod photoreceptors</td>
<td>Rhodopsin</td>
<td>Hicks and Barnstable, 1987</td>
</tr>
<tr>
<td>Amacrine cells</td>
<td>CRABP</td>
<td>Gaur et al., 1990</td>
</tr>
<tr>
<td>Rod bipolar cells</td>
<td>PCP-2</td>
<td>Vandaele et al., 1991</td>
</tr>
<tr>
<td>Proliferating cells</td>
<td>BrdU</td>
<td></td>
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All of the markers represent proteins that are expressed exclusively in specific cell types except for the lectin peanut agglutinin (PNA) which binds to the matrix sheath surrounding cone photoreceptors and the PCP-2 antibody which binds to an undetermined epitope in rod bipolar cells. Abbreviations: cellular retinoic acid binding protein (CRABP); Bromodeoxyuridine (BrdU).
RESULTS

T3 induces the differentiation of cone photoreceptors

Previous studies have demonstrated that embryonic retinal cells continue to survive, proliferate and differentiate in high density cultures for up to 8 days in vitro (Anchan et al., 1991; Reh, 1992a; Kelley et al., 1994). These cultures can be assayed for several different neuronal cell types using a number of different phenotype-specific antibodies (Table 1). As described in previous publications, many of the cultured cells developed a regular rosette-like organization in which photoreceptors were located in radial arrays, while amacrine cells were located outside of the radial portion of the rosettes (Reh, 1992a; Fig. 1A).

Continuous exposure of the embryonic retinal cells to T3 for 6 days in vitro induced a significant, dose dependent, specific increase in the number of cells that differentiated as cone photoreceptors (Figs 1A,B and 2A). At concentrations of 1.6 nM T3, or greater, there was a significant ($P = 0.005$) increase in the number of cells that were immunoreactive for recoverin (Fig. 2A), while at concentrations of 3.3 nM T3, or greater, there was a significant ($P = 0.05$) increase in the number of cells that were labeled with both recoverin and PNA (Figs 1C,D and 2A). The effects of T3 on the number of cells that expressed either recoverin or recoverin and PNA showed a dose dependent response with a peak at a concentration of 6.5 nM (Fig. 2A). Higher concentrations of T3 (up to 160 nM) did not induce significantly greater numbers of cone photoreceptors (data not shown). The effects of T3 appeared to be specific for cone photoreceptors since addition of T3 did not induce an increase in the number of amacrine cells, rod photoreceptors (Fig. 2B), or rod bipolar cells. The values for rod bipolar cells at 6.5 nM T3 were $94\% \pm 20.5\%$ s.e.m. of control. T3 did not influence the rate of cellular proliferation, since the total number of cells was not significantly different between control and T3 cultures (Fig. 2B).

To definitively identify cone photoreceptors in retinal cultures, cells were maintained for 14 DIV, so that developing cones would begin to express cone-specific opsins. To quantify cone opsin immunoreactive cells in these longer term cultures, the cells were redissociated and then re-plated at a lower density. Redissociated cells appeared healthy, readily attached to the Matrigel substrate and began to extend processes within the 12 hour incubation period following the redissociation (Fig. 1E). We quantified the number of cells that expressed red/green cone opsin in the control and T3-treated cultures. In most of the cells that expressed this antigen, labeling was restricted to the short apical process of the cell (Fig. 1F). This distribution is consistent with the restriction of cone opsins to the developing inner and outer segments of cone photoreceptors (Bumsted et al., 1993).

The T3-treated cultures contained more than twice the number of cells that differentiated as red/green cone photoreceptors as compared with controls (Fig. 3). This increase was significant ($P = 0.002$) and was comparable to the increase in the number of recoverin/PNA double-labeled cells in T3-treated cultures after 6 DIV (Figs 2B and 3). The effects of thyroid hormone appeared to be specific for cone photo-

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**Fig. 1.** Differentiation of photoreceptors in high density cultures established from E18 rat retinal cells. (A) Expression of recoverin in a control culture after 6 days in vitro (DIV). (B) Expression of recoverin in a sister culture that was exposed to 6.5 nM T3 for the same period of time. Note that T3 induces an increase in the number of cells that express recoverin. (C,D) A single cell from a high density culture that is immunoreactive for recoverin (arrowhead in C) and also labels with the lectin, peanut agglutinin (PNA; arrowhead in D). (E) Phase contrast micrograph of a group of retinal cells after 14 DIV. (F) Fluorescent image of the same field as in E. A single cell (arrowhead) is labeled with a cone-specific antibody directed against the red/green cone opsin. Scale bar in A (same in B) = 40 μm; in C (same in D) = 10 μm; in E (same in F) = 20 μm.
receptor cells, since neither overall cell number nor the numbers of the other two types of retinal cells that are normally generated at this age in vivo, amacrine cells or rod photoreceptor cells, showed a significant increase in their number in the T3-treated cultures (Fig. 3). In cultures that were exposed to T3 for 14 DIV, the increase in the number of cells that expressed the early photoreceptor marker, recoverin, was not as great as the increase in the number of cells that expressed the late cone photoreceptor marker, red/green cone opsin (155% versus 210%).

T3 directly influences retinal progenitor cells
To determine whether T3 acts directly on progenitor cells to influence them to differentiate as cone photoreceptors, the retinal progenitor cells were labeled with BrdU. Using double-label immunohistochemistry for cone-specific antigens and anti-BrdU it was possible to discriminate cone photoreceptors generated in vitro from those born prior to E18. Results indicate that addition of T3 caused a significant increase in the number of double-labeled cells but did not have a significant effect on the number of cells that were only labeled with the cone antibody (Fig. 4). This result indicates that T3 acts directly on the progenitor cells to influence them to differentiate as cone photoreceptors. In addition, since the number of cone cells that were BrdU negative did not increase in the presence of T3 (Fig. 4), this result suggests that T3 does not have an effect on the expression of cone-specific antigens in previously generated cone photoreceptors.

T3 and RA have antagonistic effects on photoreceptor differentiation
To determine whether T3 and/or RA are sufficient, in the absence of cell contact, to direct cells to photoreceptor cell fates, we examined the effects of these molecules in low density cultures of E18 retinal cells (Fig. 5A). Previous studies have shown that photoreceptors do not differentiate in low density cultures under control conditions (Sparrow et al., 1990; Reh, 1992a; Altshuler and Cepko, 1992; Harris and Messer-
Different combinations of RA and T3 were added to serum-free medium to determine the effects of activation of either RARs/RXRs or TRs, or both on the differentiation of cells as rod and cone photoreceptors. Differentiation of photoreceptors was assessed by expression of recoverin while differentiation of rod photoreceptors was assessed by expression of both recoverin and rhodopsin (Fig. 5B,C). Since recoverin has been reported to be expressed in a subset of bipolar cells (Milam et al., 1993), the differentiation of bipolar cells was determined using the PCP antibody (Vandaele et al., 1991).

Addition of T3 alone induced an increase of approximately 5% in the total number of single cells that expressed recoverin, but did not induce expression of rhodopsin (Fig. 6A,B). The increase in recoverin expression was not dose dependent. Addition of RA alone induced a significant increase in the number of cells that expressed both recoverin and rhodopsin (Fig. 6A,B). At 5 nM RA over 25% of the cells express recoverin and nearly 14% of the cells were immunoreactive for rhodopsin. The effects of RA were highly dose dependent with a peak response at concentrations of 5 to 10 nM. Although more cells were immunoreactive for recoverin than for rhodopsin, this difference is probably a result of the difference in the timing of the expression of these two antigens.

In the next series of experiments, we combined the two ligands to test for the effects of co-activation of RXRs and TRs. The addition of both T3 and RA to the same cultures induced a significant increase in the number of single cells that expressed recoverin (Fig. 6). In the presence of T3 and RA approximately 30 to 35% of all isolated cells expressed recoverin. We suspect that these recoverin immunoreactive cells were cone photoreceptors, since the number of cells that expressed rhodopsin in cultures exposed to T3 and RA was reduced to almost zero. The presence of T3 inhibited retinoic acid induced rhodopsin expression even when the ratio of T3 to RA was as low as 1:50. Labeling of low density cultures with an antibody specific for bipolar cells indicated that no cells differentiated as bipolar cells under any of the conditions tested (data not shown), so the effects of T3 appear to be specific for photoreceptors.

We believe that T3 and RA alter the pathway of differentiation of the retinal progenitor cells; however, it is also possible that T3 and RA act to selectively promote survival of photoreceptors. To control for this possibility, we quantified the total number of viable cells under the different conditions. In control cultures that did not receive any T3 or RA, only approximately 44±5% of the single cells survived (n = 2) and none of the single cells expressed either recoverin or rhodopsin. Addition of either T3 or RA caused survival of single cells to increase to approximately 56%. Addition of higher concentrations of either factor or combinations of the two factors did not result in further increases in cell survival (59±0.5%), even though the number of photoreceptors that differentiated in the cultures was significantly greater in the presence of both ligands.
Fig. 6. Direct effects of T3 and 9-cis RA on expression of recoverin and rhodopsin by single isolated E18 retinal cells after 6 DIV. Data are represented in both graphic (left) and tabular (right) form. (A) Mean percentage of single cells that expressed recoverin in the presence of different concentrations of T3 and 9-cis RA. In cultures that were exposed to control medium only, none of the single isolated cells expressed recoverin. Addition of either T3 or 9-cis RA induced some increase in expression of recoverin as compared with control. However, combinations of T3 and 9-cis RA induced a much greater increase in the number of cells that expressed recoverin. The effects of 9-cis RA alone were highly dose dependent with a peak effect at a concentration of 5 nM. (B) Mean percentage of single cells that expressed rhodopsin in the presence of different concentrations of T3 and 9-cis RA. In cultures that were exposed to control medium only or to medium containing T3 alone, none of the single isolated cells expressed rhodopsin. Addition of 9-cis RA alone induced a significant, dose-dependent increase in the number of cells that expressed rhodopsin as compared with control values. Note that the shape of the dose response curve in response to 9-cis RA alone is similar for both recoverin and rhodopsin. The presence of T3 inhibited rhodopsin expression in all cases even when the concentration of RA was significantly higher than the concentration of T3. Data are from a representative experiment.
DISCUSSION

T3 induces progenitor cells to differentiate as cone photoreceptors

The results of several recent studies have demonstrated that the differentiation of different retinal phenotypes is controlled, at least in part, by factors in the cellular microenvironment (Reh, 1987, 1992a; Altshuler and Cepko, 1992; Altshuler et al., 1993; Watanabe and Raff, 1992; Harris and Messersmith, 1992). Two different specific factors that can induce the differentiation of rod photoreceptors have been identified (Altshuler et al., 1993; Kelley et al., 1994), but specific factors for other retinal phenotypes are not known. The results of these experiments indicate that thyroid hormone can induce the differentiation of cells as cone photoreceptors. Addition of T3, the active form of thyroid hormone, induced an increase in the number of cells that were double-labeled with recoverin and PNA after 6 DIV and a similar increase in the number of cells that expressed red/green cone opsins after 14 DIV. In rat, the effects of T3 were specific for cone photoreceptors since after both 6 and 14 DIV there was no significant increase in the number of amacrine cells, rod bipolar cells, or rod photoreceptors.

It is important to note that exposure to T3 for 6 DIV induced a greater increase in the overall number of photoreceptors than in the overall number of cone photoreceptors (Fig. 2A). This result suggests that T3 may influence the development of rod photoreceptors as well. However, as noted previously, it seems unlikely that T3 has an effect on the development of rod photoreceptors since exposure to T3 did not induce a change in the number of cells that expressed rhodopsin (Fig. 2B). A more likely explanation for the difference between the number of overall photoreceptors and the number of cone photoreceptors is the difference in the timing of the onset of expression of the markers used to identify photoreceptors (recoverin) and cone photoreceptors (PNA; Table 1). Recoverin is expressed in all newly differentiated photoreceptors while PNA is expressed later in cells that will differentiate as cone photoreceptors. Since these experiments were terminated prior to the complete development of all photoreceptors, it seems likely that the increased number of overall photoreceptors in the presence of T3 represents a population of newly differentiated photoreceptors that will probably develop as cone photoreceptors.

T3 acts on proliferating progenitor cells

Previous studies have demonstrated that factors that affect differentiation can influence the commitment of proliferating progenitor cells, the subsequent expression of differentiation in post-mitotic cells or both (reviewed by Reh, 1992b). The BrdU/cone opsins double-labeling experiments show that T3 acts on the population of proliferating progenitor cells to direct more cells to differentiate as cone photoreceptors. Since exposure to T3 did not induce a significant increase in the number of post-mitotic cells that expressed cone markers, it seems unlikely that T3 plays a significant role in the expression of cone-specific markers in post-mitotic cells that are differentiating as cone photoreceptors. These results suggest that T3 acts on progenitor cells to direct their cell fate towards a specific cellular phenotype. Thus T3 may be important in the control of cell fate during retinal development.

Although no decreases were observed in either of the other predominant cell phenotypes that differentiate on E18 (amacrine cells and rod photoreceptors) there are three possible hypotheses to explain this result. First, we did not assay for the number of cells that differentiated as retinal ganglion cells. Although these cells represent a small percentage of the total number of cells that are birthdated on E18, studies indicate that throughout retinal neurogenesis the birthdates of ganglion cells and cone photoreceptors closely overlap (LaVail, unpublished results). Therefore, it seems possible that T3 may act to direct cells towards a cone photoreceptor fate and away from a ganglion cell fate. A similar effect has been demonstrated between rod photoreceptors and amacrine cells in response to retinoic acid (Kelley et al., 1994). A second possible hypothesis to account for the source of new cone photoreceptors is that cells that would differentiate as rod photoreceptors in control conditions, may be induced to differentiate as cone photoreceptors instead. Although there was no significant decrease in the overall number of rod photoreceptors after either 6 or 14 DIV, in two out of three of the experiments that were maintained for 14 DIV there were decreases in the number of rod photoreceptors (Experiment 1: rods in T3-treated cultures equaled 91% of control, Experiment 2: rods equaled 66% of control). These results and the results of the low density experiments, to be discussed below, indicate that T3 inhibits the differentiation of cells as rods by directing some cells that would differentiate as rods to differentiate as cones instead. Finally, it is also possible that T3 may simply influence the pool of progenitor cells, which comprise 44% of an E18 retinal culture after 7 DIV (Kelley et al., 1994).

Thyroid hormone and/or retinoic acid are sufficient to induce photoreceptor differentiation

Single isolated cells from E18 rat retina will not express differentiation markers for either rod or cone photoreceptors in serum free media (Sparrow et al., 1990; Reh, 1992b; Altshuler and Cepko, 1992; Harris and Messersmith, 1992). However, the presence of either T3 or RA in low density cultures was sufficient to induce the expression of either recoverin or recoverin and rhodopsin, respectively. The addition of T3 and retinoic acid are thus sufficient for the initial stages of photoreceptor differentiation to occur in vitro. In addition, the combination of the two ligands had a profound impact on the differentiation of the cells; the addition of 5 nM RA caused a 30% increase in the expression of recoverin in response to T3, while the expression of rhodopsin by single cells was essentially eliminated. Thus the presence of RA enhances the cellular response to T3, while the presence of T3 inhibits the cellular response to RA.

The effects of T3 and RA are mediated through steroid/thyroid receptors

Recent studies have demonstrated that the effects of retinoic acid and thyroid hormone are mediated through binding of these ligands to specific nuclear receptors. These receptors are members of the steroid/thyroid superfamily of transcription factors. The results of the low density experiments described here, as well as previous studies (Kelley et al., 1994), suggest that activation of RXR and/or RAR receptors by retinoic acid is sufficient to induce the differentiation of cells as rod photoreceptors. Although the expression of each of the RXR and RAR genes has not been fully characterized during retinal development, preliminary evidence indicates that several RXRs, RARs and TRs are expressed in cells of the embryonic
and early post-natal retina, including the retinal progenitor cells (Ruberte et al., 1991; Mangelsdorf et al., 1992; Sjoberg et al., 1992; Dolle et al., 1994; Kelley, Williams and Reh, unpublished observations). It is likely that the retinal progenitor cells express both types of receptors, since the presence of RA enhances the cellular response to T3, while the presence of T3 inhibits the cellular response to RA. Moreover, the inhibition of the differentiation of rod photoreceptors in the presence of both T3 and RA suggests that RXR:TR heterodimers may be dominant over RXR:RXR homodimers or RXR:RAR heterodimers in this system.

A role for steroid/thyroid ligands and receptors in retinal neurogenesis

The results presented here indicate that the presence of thyroid hormone is sufficient to induce retinal progenitor cells to specifically differentiate as cone photoreceptors. Taken together with the previously demonstrated action of 9-cis retinoic acid in inducing rod photoreceptor differentiation, we propose that the differentiation of retinal progenitor cells as photoreceptors is controlled through the activation of specific steroid/thyroid receptor family members. Recent work has demonstrated that ligand activated steroid/thyroid receptors may play similar roles in cell fate decisions in other developing systems including hindbrain and hematopoietic system (Studer et al., 1994; Tsai et al., 1992). Studies from our laboratory and others have demonstrated that retinoic acid and its receptors are present in the developing retina (Dolle et al., 1990; McCaffery et al., 1992; Mangelsdorf et al., 1992; Seleiro et al., 1994). In particular, preliminary evidence from our laboratory indicates that RXRα is expressed in the progenitor zone between E15 and P-2 and that 9-cis retinoic acid is also present in the retina on P0 (Kelley, Williams, Kreech-Craft and Reh, unpublished observations).

The expression of thyroid hormone and thyroid receptors in the developing retina has not been completely characterized yet, however, thyroid hormone, T3 and thyroid receptors have been localized to the developing chick retina (Forrest et al., 1990; Sjoberg et al., 1992; Prati et al., 1992). In particular, Sjoberg et al. (1992) demonstrated high levels of expression of TRβ in the embryonic chick retina and demonstrated that the TRβ2 isoform is expressed in the developing cones. More recent studies have shown TRβ to be expressed in the progenitor zone on the developing nervous system (Bradley et al., 1992; Mellstrom et al., 1991), suggesting that this form of the receptor may play a role in cell fate determination. These results also suggest that other members of the steroid/thyroid family, in particular recently identified inhibitors of steroid/thyroid mediated transcription such as the vertebrate homolog of seven-up, COUP-TF (Mlodzik et al., 1990; Kliwer et al., 1992), may also play a role during retinal neurogenesis.

Based on these results we propose the following model for the role of retinoic acid and thyroid hormone during retinal development. During early phases of retinal development, the predominant cell types that are generated are ganglion cells and cone photoreceptors. At this stage, RXRs, TRs, 9-cis RA and T3 would all be present in the developing retina with the levels of activated TRs acting as the limiting factor for cone production. Also, the presence of activated TRs would inhibit the production of rod photoreceptors. As development proceeds and the predominant cell types that are generated switches to amacrine cells and rod photoreceptors, the levels of T3 or TRs would decrease, allowing the formation of RXR-RXR homodimers or RXR-RAR heterodimers and the differentiation of cells as rod photoreceptors. As discussed, at the present time the relative levels of expression of the different receptors and their ligands have not been sufficiently characterized to determine whether this model is feasible.

Finally, these results have important implications for the development of the photoreceptor mosaic and the establishment of the normal ratios of rod and cone photoreceptors. In the retinas of many vertebrates, there are regional specializations in the ratio of rods to cones, such as the cone-rich fovea of primates. Although migration of appropriate photoreceptor cell types into and out of the fovea does occur during development (Hendrickson and Kupfer, 1976; Yuodelis and Hendrickson, 1986; Packer et al., 1990; Hendrickson, 1992; Wikler and Rakic, 1991), previous studies have demonstrated that a greater number of progenitor cells differentiate as cones in the fovea than in other regions of the retina (Hendrickson and Kupfer, 1976; Packer et al., 1990; Hendrickson, 1992; Diaz-Araya and Provis, 1992). Our results predict that a differential distribution of steroid/thyroid ligands, their receptors, or molecules that interact with these ligand-activated transcription factors, regulate the relative densities of rod and cone photoreceptor production during retinal neurogenesis.

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