Development of the Ipsilateral Retinothalamic Projection in the Frog *Xenopus laevis*

III. The Role of Thyroxine¹

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Abstract

The ipsilateral retinothalamic projection in *Xenopus laevis* normally first appears at about stage 54, at a time when a number of other changes known to be dependent on a rise in circulating levels of thyroxine begin to occur. We have investigated the role of thyroxine in the development of the ipsilateral retinothalamic projection by studying retinal projections and patterns of retinal histogenesis in tadpoles whose ability to produce thyroxine was blocked by treatment with propylthiouracil (PTU), and in similar tadpoles in which thyroxine was restored by injection of small amounts of thyroxine into one eye.

PTU-reared tadpoles continue to grow and to add neurons to the retina in a symmetric pattern like that of normal tadpoles at early developmental stages. The PTU-reared tadpoles remained by external criteria at stage 54. Like normal stage 54 tadpoles, the PTU-reared tadpoles either lacked an ipsilateral projection entirely or had an extremely sparse projection.

Injection of thyroxine into one eye of PTU-reared tadpoles resulted in the production of substantial ipsilateral projections from the treated eyes as well as shifts to the asymmetric pattern of retinal cell addition which normally begins after stage 54. Such changes were much more prominent in hormone-treated than in untreated eyes, suggesting that they are caused by local action of thyroxine on the treated eyes. With low doses, thyroxine-induced effects on the development of the ipsilateral projection and on retinal histogenesis were restricted to the treated eye. These results suggest that the presence of thyroxine in one eye alone is sufficient to cause the development of the ipsilateral projection.

The ipsilateral retinothalamic projection in *Xenopus laevis* normally develops during metamorphosis, well after the appearance of the crossed projections to the tectum and thalamus, which are present

in the tadpole. In the preceding paper (Hoskins and Grobstein, 1985b), we showed that the initial development of the projection, as well as the production of the majority of ganglion cells contributing to it, begins at or shortly after tadpole stage 54. The stage is significant in that it is at roughly this time that there is an increase in synthesis of a hormone, thyroxine, known to play an important role in metamorphic change generally (Dodd and Dodd, 1976) and in changes in the nervous system in particular (Kollros, 1981). An involvement of thyroxine in the development of the ipsilateral retinothalamic projection specifically is suggested by the finding that asymmetric retinal growth, which normally begins at about stage 54 (Beach and Jacobson, 1979a) and yields the retinal regions containing ipsilaterally projecting cells in the adult (Hoskins and Grobstein, 1985b), can be induced prematurely by injection of thyroxine into the eye of younger tadpoles (Beach and Jacobson, 1979b).

In this paper we report an analysis of the role of thyroxine in the development of the ipsilateral retinothalamic projection. We have examined retinofugal projections and patterns of retinal histogenesis in tadpoles whose production of thyroxine was blocked by treatment with the goitrogen propylthiouracil (PTU), as well as in similar tadpoles injected intraocularly with thyroxine. The results confirm that thyroxine is involved in producing an ipsilateral projection and suggest that it does so by a direct action on the eye. Robust ipsilateral projections, resembling those of metamorphosing animals, were produced in thyroxine-treated tadpoles which in all other respects remained at premetamorphic stage 54. Our findings provide a striking example of the role of hormones in inducing neuronal projections, and are also significant with regard to the question of how patterns of axonal projection are controlled at the optic chiasm. Preliminary reports of some of these observations have appeared (Hoskins and Grobstein, 1981, 1984).

Materials and Methods

Laboratory-bred and reared *Xenopus laevis* tadpoles were used for all experiments. To determine whether thyroxine was necessary for development of the ipsilateral projection, we raised groups of 100 tadpoles in 40-liter aquaria containing 10% Holtfreter's solution and 0.01% PTU (Sigma) (Doyle and MacLean, 1978). PTU prevents thyroxine synthesis by preventing iodination of tyrosine, an essential step in the production of thyroid hormone (Green, 1971). PTU was added to the aquaria when the tadpoles had reached stages 48 to 50 (Nieuwkoop and Faber, 1967). The PTU had no detectable effect on growth or development of these tadpoles, as compared with animals reared in the absence of PTU, until stage 53 or 54. Having reached this stage of development on a normal time course, the PTU-reared animals continued to increase in size without progressing in morphological stage. The precise stage at which a PTU-reared group ceased progressing morphologically varied slightly among different groups of tadpoles, with some blocked at stage 53/54 and others at stage 54.

To examine the effects of local application of thyroxine, we injected thyroxine into one eye of a group of PTU-reared tadpoles. These experimental animals were housed singly in plastic boxes containing 2 liters of PTU

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medium. Tadpoles were anesthetized briefly in 5×10^{-5} M tricaine methane-sulfonate (MS-222), and small amounts of L-thyroxine (Sigma) in 50 nl of Wesson Oil were pressure-injected into one eye (see Tables I to III for doses). The animals were returned to the boxes for the duration of the experiment and were fed yeast. After 3 to 12 weeks, the optic nerve from either the thyroxine-injected eye or the untreated eye was cut and exposed to crystals of HRP. Twenty-four hours later, the tadpoles were killed and their brains were processed as described previously (Hoskins and Grobstein, 1985a).

As the retina of *X. laevis* grows, new cells are added at the periphery (Hollyfield, 1971; Straznicki and Gaze, 1971; Jacobson, 1976). To analyze patterns of retinal growth during our experiments, we marked the retinal periphery of the stage 54 tadpoles at the beginning of the experiment by making intra-abdominal injections of 5 μ l of [3 H]thymidine (specific activity, 81 to 86 Ci/mmol, 1 mCi/ml; New England Nuclear) just prior to the intraocular injections of thyroxine. At the end of the experimental period, the eyes of the animals were fixed in 1% paraformaldehyde/2% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, embedded in paraffin, and processed for autoradiography as described previously (Hoskins and Grobstein, 1985b). Parasagittal sections which included the optic disc were scanned, and a sample of three alternate central sections through the optic disk was chosen for analysis. Using a modification of the method of Beach and Jacobson (1979a), we quantitated shifts in the pattern of retinal histogenesis by measuring the lengths of retina generated at the extreme dorsal and ventral peripheries of the eye, between the border of heavily [3 H]thymidine-labeled cells and the peripheral germinal zone, during the experimental period. Three sections taken at the level of the optic disc were analyzed for each eye. The symmetry or asymmetry of retinal growth was then determined by computing a ratio of micrometers added in the ventral periphery to micrometers added in the dorsal periphery. To control for the possibility that increases in length were caused by changes in cell size rather than addition of new cells, we also counted the number of cells added in the dorsal and ventral peripheries of the same sections. The extreme edge of the retina, the germinal zone, was not counted, as it was not possible to tell which cells within it were destined for the ganglion cell layer.

Results

Effects of PTU rearing. *X. laevis* tadpoles normally complete metamorphosis in 6 to 8 weeks (Nieuwkoop and Faber, 1967). In PTU-treated tadpoles, development as measured by progression through successive morphological stages stops at about stage 54. The blocked animals remain viable and continue to grow for a year or more (MacLean and Turner, 1976). To determine the effects of PTU rearing on the development of the ipsilateral retinothalamic projection, we assayed retinal projections in tadpoles kept in PTU for between 1 and 3 months past the chronological age at which the projection normally develops. The brains illustrated in Figure 1 represent the range of results observed. In no case was there any sign of a projection to the ipsilateral rostral visual nucleus (RVN), corpus geniculatum thalamicum (CGT), or uncinata field (UF). In some animals there was a very sparse projection to the nucleus of Bellonci (NB); in others this projection also was absent. The range of results seen was identical to that observed in normal stage 54 tadpoles (Hoskins and Grobstein, 1985b). We conclude that, like many other metamorphic events, the development of the ipsilateral retinothalamic projection is blocked by treatment with PTU.

PTU treatment also blocked the onset of the normal asymmetric pattern of cell addition to the retina but did not prevent addition of neurons at the retinal periphery, as shown by our autoradiographic studies of the same animals. Figure 2 documents incorporation of [3 H]thymidine at the retinal periphery in a tadpole maintained in PTU for 11 weeks after the injection of [3 H]thymidine. The displacement of the autoradiographic grain border from the germinal zone at both dorsal and ventral peripheries indicates that neurons were added while the tadpole was reared in PTU. The amount of displacement is equivalent dorsally and ventrally. Such symmetrical growth is characteristic of retinas of *X. laevis* tadpoles through stage 54 (Beach and Jacobson, 1979a), and quite different from the pattern of increased growth in ventral retina and a decreased rate of growth in dorsal retina, which in normal tadpoles begins at stage 55 and which can be induced precociously by treatment with thyroxine at younger stages (Beach and Jacobson, 1979a, b).

The effects of PTU on general morphological changes and on the development of the ipsilateral projection are reversible. When PTU-reared tadpoles are shifted back to normal rearing medium (10% Holtfreter's solution), normal morphological changes resume and the animals complete metamorphosis within a few weeks. In several such frogs, including one which as a tadpole had spent 8 months in PTU, we studied optic nerve projections using methods described previously (Hoskins and Grobstein, 1985b). The projections formed were typical of normal postmetamorphic animals. Contralateral projections to the optic tectum and thalamus were present, and projections had also been made to all of the usual terminal fields in the ipsilateral thalamus. Thus, even a long delay at stage 54 does not prevent the eventual development of normal optic projections, once treatment with PTU is terminated.

Effects of exogenous thyroxine on development of the ipsilateral projection in PTU-reared tadpoles. The results in the preceding section suggest that the development of the ipsilateral retinothalamic projection depends on the increased levels of thyroxine which are normally associated with metamorphosis. However, PTU treatment, in addition to preventing thyroxine synthesis, might have other unknown effects which block development of the projection for reasons unrelated to the block of thyroxine production. To verify the involvement of thyroxine in the development of the projection as well as to investigate the level at which the hormone acts, we studied retinal projections of PTU-reared animals after injecting thyroxine into one eye.

Small amounts (3 to 17.5 ng) of thyroxine in 50 nl of oil were pressure-injected into one eye of a series of PTU-reared tadpoles, which were subsequently returned to and housed in PTU solution throughout the experimental period. After a period of 3 to 12 weeks, we examined the optic projections from either the thyroxine-treated or untreated eye of each tadpole, using anterogradely transported horseradish peroxidase (HRP) as described previously. Optic projections were reconstructed from serial 40- μ m frozen sections, using a Zeiss drawing tube at $\times 25$. Reconstructed optic projections of the experimental tadpoles were assessed by comparison with similarly reconstructed projections from normal animals.

The results of these studies are summarized in Tables I to III, which include details of the amount of thyroxine injected and the time between injection and sacrifice for each case. The optic nerve projections from the thyroxine-treated eye were analyzed in 17 cases (Table I and the first three animals in Table III). In all but one of these (No. 133 of Table I), there was clear evidence that the injected thyroxine promoted development of the ipsilateral projection, in that projections were observed in ipsilateral terminal zones (RVN, CGT, UF) never innervated in PTU-reared animals without thyroxine treatment or in normal stage 54 tadpoles. As is evident in Table I, not every animal showed reaction product in all of the ipsilateral terminal zones. The ipsilateral NB and RVN were most often innervated. These projections are the ones most reliably (and in the case of NB, heavily) labeled in normal metamorphosing tadpoles. The ipsilateral CGT and UF are the last to become innervated during normal development and seem to undergo a good deal of further development after the conclusion of metamorphosis. In five experimental cases, label was present in all four of the normal ipsilateral terminal zones. This, and the intensity of the labeling, suggests a degree of development in these animals comparable to that normally reached in early postmetamorphic stages.

The induced fibers were not distributed randomly on the ipsilateral side of the brain, but instead were found only in the normal terminal zones. We looked in particular for the existence of projections to the ipsilateral tectum. These were lacking in 18 of the cases and present as a few fibers in 8 cases. This is similar to the situation in normal postmetamorphic animals where a sparse ipsilateral retinotectal projection can sometimes be observed (Levine, 1980; S. G. Hoskins and P. Grobstein, unpublished observations). Induced ipsilateral retinothalamic projections were seen at the shortest post-treatment time investigated (3 weeks) and with the smallest thyroxine dosage

Figure 1. Retinothalamic projections of stage 54 PTU-reared tadpoles. A, Transverse section through rostral thalamus of an animal in which one optic nerve was exposed to HRP after more than 2 months of PTU rearing. On the contralateral (CONTRA) side, a substantial projection to the NB is apparent (solid arrow). On the ipsilateral (IPSI) side, only a small amount of reaction product is present (see enlargement of boxed region on the right; small arrows indicate grains of HRP reaction product). This was the only ipsilateral terminal field which showed any evidence of innervation. Scale bars = 100 μ m to left, 20 μ m to right. B, A second example, oriented as in A. Again, a substantial projection to the contralateral (CONTRA) NB is present (solid arrow). In this tadpole, no reaction product was found in the ipsilateral (IPSI) terminal fields (see boxed region and enlargement to the right). A single stained fiber can be seen at the lateral wall of the brain. Scale bars = 100 μ m to left, 20 μ m to right.

used (3 ng). Induced projections were not, however, seen in three additional control tadpoles analyzed 6 weeks after receiving injections of oil without thyroxine. We conclude that the block of development of the ipsilateral retinothalamic projection brought about by PTU treatment can be overcome by exposure to exogenous thyroxine.

Many of the tadpoles studied showed not only an induced ipsilateral projection but also other signs of metamorphic change as defined by external morphological criteria (Nieuwkoop and Faber, 1967). In these cases (Table I), we assessed whether the degree of development of the induced ipsilateral projection was enhanced

relative to that of the animal in general by comparing the ipsilateral projections of the hormone-treated eyes with the ipsilateral projections typical of normal untreated tadpoles at the equivalent stage (as described in our previous study; Hoskins and Grobstein 1985b). Ten of the 14 tadpoles clearly had projections from the thyroxine-treated eye which were morphologically advanced relative to the animal's stage, as judged both by the number of terminal zones labeled and the intensity of the labeling. An example is illustrated in Figure 3, left.

The finding that the induced ipsilateral projections produced by thyroxine-treated eyes were in many cases enhanced relative to the

Figure 2. Retinal growth during PTU rearing. To the left is a camera lucida drawing of a sagittal section through the retina of a PTU-reared tadpole which was injected intra-abdominally with [^3H]thymidine, returned to PTU, and killed 11 weeks later. D, dorsal; V, ventral; pe, pigment epithelium; on, optic nerve; gc, ganglion cell layer. Scale bar = 250 μm . Addition of new cells to the dorsal and ventral retinal peripheries (boxed regions on the left) during continued PTU rearing is indicated by the presence of silver grains over retinal ganglion cells (arrowheads) in the photomicrographs to the right. Grains are also apparent in the other layers of the neural retina. Scale bar = 20 μm .

TABLE I

Assessment of the ipsilateral retinothalamic projections from thyroxine-treated eyes of PTU-reared tadpoles which advanced slightly in developmental stage after injection of thyroxine into one eye

Animals identified by number in the first column received thyroxine doses as indicated in the second column. The third and fourth columns show the developmental stage reached at the time the animals were killed and the number of weeks that had elapsed after the thyroxine injection. The next four columns indicate whether HRP reaction product was detectable in the four major retinothalamic terminal zones ipsilateral to the optic nerve exposed to HRP. The last column indicates whether the observed projections were more advanced than those expected of animals at the developmental stage reached.

Tadpole	Thyroxine (ng)	Final Stage	Weeks Elapsed	RVN	NB	CGT	UF	Enhanced Overall?
128	9.00	57/58	6	(+) ^a	+	(+)	—	No
134	17.50	57	7	—	+	(+)	+	yes
138	9.00	56/57	8	+	+	(+)	+	yes
139	9.00	56/57	9	+	+	—	—	yes
137	9.00	56	8	+	+	—	—	yes
133	17.50	56	7	—	+	—	—	no
160	3.75	56	6	—	+	(+)	—	no
158	3.75	56	6	+	+	+	—	yes
168	3.00	55	12	—	+	+	—	yes
164	3.00	55	11	+	+	—	—	yes
146	3.75	55	3.5	+	+	+	—	yes
143	3.75	55	3	+	+	—	+	yes
162	3.00	54/55	11	+	+	—	—	no
149	3.75	54/55	3.5	+	+	+	+	yes

^a(+), uncertain; +, detectable reaction product; —, no detectable reaction product.

Figure 3. Retinothalamic projections in PTU-reared tadpoles. *Left*, Retinothalamic projection in a PTU-reared tadpole which advanced to stage 56 during the 6 weeks following injection of thyroxine into one eye. The projection from the thyroxine-treated (*Thyrox-treated*) eye was analyzed. A substantial projection to the ipsilateral (*IPSI*) NB is apparent (*open arrow*). Analysis of adjacent sections indicated that reaction product was also present in the ipsilateral RVN, CGT, and UF, which are not innervated until stages 57, 60, and 64, respectively, in normally developing tadpoles. This ipsilateral projection is significantly enhanced compared to the ipsilateral projections typical of normal or PTU-reared tadpoles at stage 56 (see *right*). Scale bar = 200 μ m. *Right*, Retinothalamic projection from the untreated (*Untreated*) eye of a PTU-reared tadpole which advanced to stage 56 following injection of thyroxine into the other eye. The ipsilateral projection to the NB is at the extremely sparse level typical of normally reared tadpoles at stage 56, and not apparent at this low magnification. Scale bar = 200 μ m.

tadpoles' morphological stages suggested that the action of thyroxine was, at least in part, directly on the eye. To further evaluate this possibility we assessed the ipsilateral projections formed by the *untreated* eye of thyroxine-treated tadpoles, reasoning that any secondary or indirect effect of thyroxine should affect *both* eyes. The results for this series of animals, which had survival times and injection dosages comparable to the cases in Table I, are presented in Table II. Of eight tadpoles, only one showed any indication of a projection enhanced relative to what would be expected from the animal's stage according to external morphological criteria. Seven of the eight had the sparse projections typical of normal tadpoles at equivalent stages. An example, treated comparably to that of Figure 3, *left*, is illustrated in Figure 3, *right*. That the seven "unenanced" cases were not judged unenanced because of a failure of anterograde transport of HRP was checked by noting the extent of reaction product in the contralateral tectal lobe of each case. These were labeled satisfactorily in all cases, indicating the optic nerves had been exposed adequately to HRP.

The results indicate that tadpoles prevented from synthesizing thyroxine fail to develop mature ipsilateral projections. Intraocular injections of thyroxine and oil, but not of oil alone, can cause the development of significantly enhanced ipsilateral projections in animals which remain at tadpole stages. The projections which form precociously are specific, in that the retinal afferents run to the usual

thalamic terminal zones and form terminal fields of appropriate morphology. The fact that the induced ipsilateral projections produced by thyroxine-treated eyes are enhanced relative to the tadpoles' morphological stages suggests further that thyroxine acts directly on the injected eye. Overall, the data in Tables I and II indicate that eyes treated directly with thyroxine are much more likely to develop enhanced ipsilateral projections than are the untreated eyes of similar tadpoles. Taken together, the analyses of projections made by thyroxine-treated or untreated eyes suggest that the thyroxine does not exert its effect on retinofugal projections through a general systemic action but instead by a local induction of the population of retinal ganglion cells.

Four animals studied (Table III) provided evidence not only that thyroxine acts locally on the eye but that such action may be sufficient to cause the development of an ipsilateral projection. These tadpoles were injected with 3.75 ng of thyroxine in 50 nl of oil and remained at stage 54 throughout the experimental period. In three cases, the projection from the treated eye was analyzed. In all three, there were projections to each of the four major ipsilateral terminal zones, indicating a degree of development normally seen only in late metamorphic or early postmetamorphic stages. In the fourth tadpole, the projection from the untreated eye was assayed (Fig. 4, *E-H*). The ipsilateral projection was typical of that seen in PTU-reared, non-thyroxine-treated tadpoles, or in normal stage 54 tadpoles.

TABLE II

Assessment of the ipsilateral retinothalamic projections from untreated eyes of PTU-reared tadpoles which advanced slightly in developmental stage after injection of thyroxine into one eye^a

Tadpole	Thyroxine (ng)	Final Stage	Weeks Elapsed	RVN	NB	CGT	UF	Enhanced Overall?
135	17.50	57/58	7	+	+	(+)	—	no
140	9.00	56/57	9	(+)	+	—	—	no
157	3.75	56	6	—	—	—	—	no
151	3.75	56	4	+	+	(+)	+	yes
169	3.00	55/56	12	—	+	—	—	no
165	3.00	55/56	11	—	+	—	—	no
150	3.75	55	4	—	—	—	—	no
163	3.00	54/55	11	—	—	—	—	no

^a Conventions are as in Table I.

TABLE III

Assessment of the ipsilateral retinothalamic projections from treated and untreated eyes of PTU-reared tadpoles which remained at stage 54 following injection of thyroxine into one eye^a

Tadpole	Thyroxine (ng)	Final Stage	Weeks Elapsed	Eye Analyzed	RVN	NB	CGT	UF	Enhanced Overall?
152	3.75	54	4.0	thyroxine treated	+	+	+	+	yes
147	3.75	54	3.5	thyroxine treated	+	+	+	+	yes
145	3.75	54	3.0	thyroxine treated	+	+	+	+	yes
148	3.75	54	3.5	untreated	—	+	—	—	no

^aConventions are as in Tables I and II except for the addition of column 5, indicating which eye was analyzed.

Labeling in the four major ipsilateral terminal zones in one of the animals which remained at stage 54 and developed an enhanced ipsilateral projection is illustrated in Figure 4, A–D. Comparable results were observed in the other two tadpoles. Reaction product was found at the lateral edge of the cellular portion of the ipsilateral RVN, and as in the ipsilateral RVN of normal adults, reaction product was absent from rostral sections of the nucleus. The terminal zone extended continuously into the neuropil caudal to the cellular portion of the ipsilateral RVN, just below the lateral tip of nucleus ventrolateralis (Fig. 4A), as it does from stage 60 on in normal cases.

The NB terminal fields seen ipsilateral to the eyes injected with thyroxine typically contained quite intense, dense deposits of reaction product. The terminal fields were much more heavily filled with reaction product than were the occasional sparse terminal fields seen in normal tadpoles at stage 54. Variations in density of reaction product like those seen in subregions of the ipsilateral NB of metamorphic (stage 60 on) and postmetamorphic *X. laevis* were seen. The body of the NB terminal field was oval shaped and lay in the neuropil between the wall of the diencephalon and the lateral extent of the gray matter (Fig. 4B). More caudally, both the body and appendage of the terminal field showed dense and sparse subregions of reaction product. The terminal zone is, in fact, strikingly similar in distribution and intensity of label to that seen in normal postmetamorphic frogs (Hoskins and Grobstein, 1985b). In both cases, two label-dense areas are found in the NB. The dense areas intersect at their medial extremes and enclose a central label-sparse zone. In the induced terminal field, as in normal postmetamorphic frogs, the regions with dense reaction product converge medially in more caudal sections. The more dorsal of the two strips remains intense in subsequent sections and projects furthest caudomedially in the brain (Fig. 4C). It should be noted explicitly that the ipsilateral projection illustrated was produced in the brain of a stage 54 tadpole. At this premetamorphic stage in normal *Xenopus*, traces of ipsilateral labeling are seen only occasionally and, if present in the ipsilateral NB, are not clustered into dense and sparse subregions. The projection illustrated is most comparable to the 1-month postmetamorphic case described in the preceding paper (Hoskins and Grobstein, 1985b).

The CGT in thyroxine-treated tadpoles was evident as a dense clustering of HRP reaction product lateroventral to and separate from the NB (Fig. 4C). The induced CGT was not wedge shaped, as it is in the adult, but contained a significant amount of reaction product, not seen in normal animals until stage 60 or later.

In normally developing tadpoles, an ipsilateral projection to the UF is not reliably detected until stage 64, at which time labeling is quite faint. In the stage 54 hormone-treated cases, reaction product was found dorsomedially in the brain, just rostral to the optic tectum, and extending ventrolaterally, as illustrated in Figure 4D. The labeling, both in intensity and distribution, was comparable to that seen in normal animals near the completion of metamorphosis.

Patterns of retinal histogenesis. The fact that a projection to the ipsilateral thalamus can be induced in an animal which shows no external signs of advancement in stage suggests that the effects of the injected thyroxine are not widespread. A similar argument comes from the fact that, in the vast majority of hormone-treated tadpoles, the untreated eye does not produce an enhanced ipsilateral projec-

tion (see Table II). A third line of evidence relates to the growth patterns of the untreated eye. As described earlier, cells are added to the retina symmetrically until stage 55, at which time ventral retina begins to grow more rapidly than does dorsal retina (Beach and Jacobson, 1979a). Since the shift in the pattern of retinal histogenesis can be brought about directly and precociously by intraocular injection of thyroxine (Beach and Jacobson, 1979b), the growth dynamics of the ventral retinal periphery provide an additional assay for the presence of elevated levels of thyroxine.

Figure 5, A and B, shows representative sections of both eyes of the previously described animal which remained at stage 54 but developed a substantial ipsilateral projection from the thyroxine-treated eye. Similar results were observed in the other two comparably treated tadpoles of Table III. Ventral retina grew substantially in the thyroxine-treated eye during the experimental period, as indicated by the large displacement of the autoradiographic grain border from the retinal periphery. Counts of neurons confirmed what is evident from the photomicrographs, that the displacement resulted largely from addition of neurons at the retinal periphery and not simply from a stretching of the retina. The untreated eye continued to grow in the symmetrical fashion characteristic of PTU-reared tadpoles (see Fig. 2), as indicated by the small and equivalent displacement of the grain border from the dorsal and ventral retinal peripheries. Similar results were observed in the previously described stage 54 tadpole in which the optic nerve projection from the untreated eye was examined (No. 148 of Table III). Again, the thyroxine-treated eye showed enhanced ventral growth, whereas the untreated eye continued to grow symmetrically (Fig. 5, C and D).

The observations on the case illustrated in Figure 5, C and D, confirm that the failure to observe an induced ipsilateral projection from the untreated eye was not due to failure of the injection of thyroxine, since the injected hormone clearly altered the growth of the eye. The observations in both tadpoles indicate that the effects of thyroxine were largely confined to the injected eye. If any thyroxine had escaped from the site of injection, it must have been present in the circulatory system in concentrations too low to produce a detectable shift in growth pattern in the retina of the untreated eye.

Discussion

The results presented in this paper confirm that thyroxine is required for the development of the ipsilateral retinothalamic projection by showing, first, that the projection fails to develop in tadpoles whose thyroid function is blocked by rearing in PTU and, second, that injections of thyroxine will overcome the effects of PTU and promote both advancement in morphological stage and development of the ipsilateral projection. The present results also indicate that the presence of thyroxine in one eye alone is sufficient to cause the development of an ipsilateral projection in the absence of numerous other metamorphic changes which normally occur during the stages when the ipsilateral projection develops. Thus, it appears that the hormone acts directly on cells of the eye to induce the new pathway (see also Hoskins and Grobstein, 1984).

Thyroxine and the development of the ipsilateral projection. With regard to dependence on thyroxine, the retina can be subdivided into a central region composed of neurons which are born early and

Figure 4

make their contralateral projections in the absence of the hormone, and a peripheral, bilaterally projecting region composed of neurons born beginning at about stage 55, which is not produced if thyroxine is not present. As discussed previously (Hoskins and Grobstein, 1985a), the intermingling of ipsilaterally projecting and contralaterally projecting cells in the latter region of the adult retina puts constraints on the types of mechanisms which can be proposed to control the trajectories of the ipsilaterally projecting axons. If axons of ganglion cells determined for projections to the ipsilateral thalamus followed the axons of their nearest neighboring retinal ganglion cells out of the eye, along the optic nerve, and through the optic chiasm, they would almost without exception be routed to the wrong, contralateral side of the brain, since contralaterally projecting retinal ganglion cells far outnumber ipsilaterally projecting ones (Hoskins and Grobstein, 1985a), and a substantial contralateral projection has formed well before an ipsilateral projection begins to develop (Grant and Ma, 1983; Holt and Harris, 1983). Our earlier work indicated that the absolute timing of development and any normally occurring interactions among the developing fibers, both of which have been shown to play roles in some developing systems (Gottlieb and Cowan, 1972; Lund, 1978; Macagno, 1978), do not seem critical for control of projection laterality for the ipsilateral fibers. The present results, together with previous ones (Hoskins and Grobstein, 1984, 1985a), indicate that a mechanism which relies heavily on "mechanical guidance" of new fibers (Singer et al., 1979; Silver, 1984) or on the following of pathways formed by previous fibers (Bentley and Keshishian, 1982; Ho and Goodman, 1982) is also inadequate to explain the initiation of a new pathway in this system.

In principle, one could imagine that the change in the laterality of optic fiber projections could be due to an alteration at a variety of points in the visual system, for example, at the optic chiasm or within the ipsilateral thalamus itself. Arguing against the former is the observation that the *untreated* eyes of thyroxine-treated tadpoles *fail* to form advanced ipsilateral projections. Were the development of an ipsilateral projection the result of a critical change at the optic chiasm, one would have expected the optic nerves from *both* eyes to have formed ipsilateral projections. A similar argument can be raised against the second possibility, namely, that the injected thyroxine affects the thalamus so that it somehow attracts axons, causing some to project ipsilaterally at the chiasm. It does not seem likely that thyroxine escaping from the eye into the general circulation would alter the thalamus on only one side of the brain. Rather, the results presented above are most consistent with the idea that thyroxine acts locally, at the level of the eye. In general, our findings strongly indicate that the critical change responsible for the development of the ipsilateral retinothalamic projection is a change in the cells of the eye itself, and that the change can be mimicked by the localized action of thyroxine. Both in the present experimental case and in normal development, the appearance of an ipsilateral projection is correlated with a change in the pattern of cell addition to the retina. In the previous paper (Hoskins and Grobstein, 1985b), we showed that ipsilaterally projecting cells are part of the population born after this thyroxine-induced change. It seems likely that the same is true in the experimental situation reported here, although this point was not examined directly.

Mode of action of thyroxine. Since ipsilateral projections are not formed in tadpoles maintained in a premetamorphic state by PTU

rearing, even after many weeks, it might appear that some signal triggered by the increase in thyroxine level, or perhaps the thyroxine itself, is involved, at stage 54, in "labeling" some ganglion cells so that they will subsequently project ipsilaterally. Several lines of existing evidence, however, suggest a different role for the hormone, by showing that the retinal region which will project ipsilaterally is determined quite early in development. These findings, summarized below, indicate that thyroxine probably plays an inductive role upon a previously regionally individuated group of neurons.

In retinas of adult frogs, ipsilaterally projecting ganglion cells are most common ventrally and temporally; that is, in the region of the retina produced by the differential growth normally initiated by thyroxine beginning at stage 55. Eyes rotated 180° at embryonic stages 26 to 36 show increased growth, beginning at stage 55, of the regions of the periphery descended from the portion of the retinal rudiment which was originally situated ventrally. Such a result suggests that regional determination of future proliferative response occurs at least as early as embryonic stage 26 (Beach and Jacobson, 1979c). That is, the ability to undergo increased growth beginning at stage 55 does not appear to be a property conferred upon particular cells based on their position in the retina at stage 55, but rather a property intrinsic to cells of *embryonic* ventral retina, and one which is retained even in the face of environmental perturbation.

Eye rudiments rotated as early as embryonic stage 32 later produce ipsilaterally projecting axons from retinal loci appropriate for the original rather than the rotated axes of the eye (Kennard, 1981), suggesting that the region of the retina which will contain ipsilaterally projecting cells is, similarly, determined long before the cells which actually project ipsilaterally are born. The results of experiments involving compound eyes (Straznicki and Tay, 1977) also suggest early determination of the ipsilaterally projecting region. In general, then, the role of thyroxine seems to be to bring about the expression of new properties in some of the neurons which arise late in development as descendants of a previously committed region of the retina. The fact that tadpoles at stages 48 to 50 will undergo changes in external morphology (Tata, 1968) or retinal growth pattern (Beach and Jacobson, 1979b) if treated with thyroxine supports the idea that cells may be determined for responsiveness to the hormone well before it is normally present in significant amounts. Thus, with regard to the ipsilateral retinothalamic projection, stage 54 may not be the stage at which the ability to project ipsilaterally is *acquired*, but rather the stage at which the ability to project ipsilaterally is *expressed*, due to the presence of increasing titers of thyroxine.

Our previous studies indicated that laterality of axonal projection is a regionally heterogeneous property of the frog retina (Hoskins and Grobstein, 1985a). The present observations suggest further that, in terms of its response to thyroxine, the retina is not only regionally heterogeneous but locally heterogeneous as well. Not all cells in the retinal region generated after stage 54 project ipsilaterally; there are also many contralaterally projecting cells in this region. In principle, thyroxine could produce a heterogeneous population of neurons either by inducing a homogeneous population of cells in the germinal epithelium of the stage 54 retina to begin to divide asymmetrically, or by differentially affecting an already heterogeneous population of precursor cells. Local heterogeneity within the region of the embryonic retina which will give rise to the bilaterally projecting region of the adult retina is suggested by studies of cell

Figure 4. Ipsilateral projections from thyroxine-treated (*T₄-TREATED*; A to D) or untreated (*UNTREATED*; E to H) eyes of stage 54 PTU-reared tadpoles which had 3.75 ng of l-thyroxine in 50 nl of oil injected into one eye. The projections made by the thyroxine-treated eye to the ipsilateral RVN (A), NB (B), CGT (C), and UF (D) are illustrated. With the occasional exception of the NB, none of these terminal zones normally are innervated at stage 54. Reaction product in the RVN (A) is in the usual lateroventral region of central sections of the nucleus (arrow). The projection to the NB (B) is much more dense and extensive than that typical of PTU-reared tadpoles at stage 54 (Fig. 1), and is subdivided into dense and sparse subregions (arrows), as is the ipsilateral NB terminal field in normal metamorphosing animals, beginning at stage 60. Density of innervation in the case illustrated is comparable to that seen normally in early postmetamorphic animals. The projections to the CGT (C, arrowheads) and UF (D, between arrowheads), appear equivalent to that of a normal frog. The ipsilateral projections formed by the *untreated* eye of a similar tadpole, in contrast, are at the sparse level characteristic of some PTU-reared or normal tadpoles at stage 54 (see Fig. 1). The RVN (E), CGT (G), and UF (H) are devoid of reaction product. The NB (F) contains a small amount of reaction product (arrowhead) at the lateral edge of nucleus posterocentralis (PC). Scale bar = 50 μ m.

Figure 5. Retinal growth in thyroxine-treated, PTU-reared tadpoles. A and B, Comparable sagittal sections through the thyroxine-treated (*Thyrox-treated*) and untreated eyes of the tadpole whose optic nerve projections are illustrated in Figure 4, A to D. [^3H]Thymidine labeling was evident at the dorsal periphery (thin arrows) in both retinas. The labeling was also peripherally located in the ventral retina of the untreated eye (boxed region in B and enlargement to the right). In the thyroxine-treated eye, the border between [^3H]thymidine-labeled and unlabeled regions was displaced centrally (boxed region in A and enlargement to left) indicating enhanced ventral growth. C and D, Similar illustrations for the tadpole whose optic nerve projections are illustrated in Figure 4, E to H. Scale bars = 300 μm for retinal sections, 10 μm for enlargements.

lineage in *X. laevis* embryos. When HRP is injected into one of the two blastomeres produced at the first cleavage, and the descendants of the labeled cell are mapped at an early tadpole stage, the vast majority of stained cells are found confined to one half of the brain, on the same side as the labeled blastomere (Jacobson and Hirose, 1978). Dorsal retina similarly appears to be descended from either one blastomere or the other, as it is found to be composed either of labeled or of unlabeled cells in cases where a single blastomere was labeled at the two-cell stage. In the ventral part of such retinas, however, stained and unstained cells are mingled, indicating that they are descended from both blastomeres. Ventral retina is thus more heterogeneous in lineage than is dorsal retina.

The parallel between lineage heterogeneity and projection heterogeneity in ventral retina is suggestive, but further work is necessary to establish its significance. Most of ventral retina projects bilaterally, but so, too, does a substantial amount of dorsal retina (Hoskins and Grobstein, 1985a). Furthermore, ventral retina seems to be distinctive not only in lineage heterogeneity but in other ways as well. The cells which produce the ventral retina migrate into the embryonic eyecup from the optic stalk relatively late (Holt, 1981), and axons from ventral retina reach the brain later than do those of dorsal retina (Rubin and Grant, 1980; Holt and Harris, 1983). Exact determinations of the extent of the distinctive retinal regions were not made in any of the cases described. A finding that the retinal region which receives contributions from both blastomeres in fact corresponds in extent

to that which projects bilaterally would significantly strengthen the evidence for a causal relation between lineage heterogeneity and projection heterogeneity.

Control of the ipsilateral projection in mammals. There are interesting parallels between our findings in the frog and those on mutations which affect both retinal melanin and the laterality of axonal projections in mammals. In melanin-deficient mammals, an abnormally large proportion of retinofugal fibers crosses in the chiasm, and fewer fibers than usual project ipsilaterally (Lund, 1965; Guillery, 1969; Guillery and Kaas, 1971). There is a correlation between the number of misrouted optic fibers and the amount of melanin pigment missing from the retinal pigment epithelium (Sander et al., 1974), suggesting that melanin may be in some way influential in the control of axonal trajectories. Melanin is produced in the embryonic eyecup early in development, and some melanin-containing cells are found in the early eyestalk, raising the possibility that the pigment cells themselves may influence the trajectories of developing axons (Strongin and Guillery, 1981). However, it seems unlikely that melanin is involved in "labeling" retinal ganglion cells directly since, in at least some situations, the accuracy of the trajectory taken at the optic chiasm by an axon from a cell in a particular region of the retina is not affected by the absence of pigment in the immediately adjacent retinal pigment epithelium (Guillery et al., 1973). Rather, the amount of misrouting in the entire population of retinal fibers reflects the overall severity of the melanin

deficit (Sanderson et al., 1974). A further parallel between the frog and many mammals is that, in both, the ipsilaterally projecting region is heterogeneous, containing contralaterally as well as ipsilaterally projecting cells (Guillery, 1982).

In the frog, our evidence suggests that thyroxine causes the production of an ipsilateral projection by triggering the production of a new population of neurons, some of which project ipsilaterally. Intriguingly, there is a suggestion that a similar event may be involved in the development of an ipsilateral projection in mammals since, as in the frog, ipsilaterally projecting cells in the normally developing cat apparently begin to be born later than contralaterally projecting ones (Walsh et al., 1983). A delay in the occurrence of the event which determines ganglion cells for ipsilateral projections in developing cats might result in the reduction of the retinal area which contains such cells and a consequently reduced ipsilateral projection in albinos. Since both thyroxine and melanin are synthesized from the amino acid tyrosine (Garcia et al., 1979; Norris, 1980), the possibility of a common signal, or of a common mechanism which underlies both substances' effects on axonal projection laterality, is raised. In this context, it is also of interest that strabismus, a misalignment of the two eyes which often accompanies albinism in mammals, has also been noted as a symptom of the human hypothyroid syndrome, cretinism (Costa, 1972).

The involvement of hormones in metamorphic change and in development of the nervous system. The present findings are of interest both in understanding the basis of metamorphic changes in the anuran nervous system and in considering the involvement of hormones in nervous system development generally. Both changes in neuronal connectivity during anuran metamorphosis (reviewed in Kollros, 1981) and direct effects of thyroxine on neuronal tissue (Kaltenbach, 1953a, b; Kollros and McMurray, 1956; Kaltenbach and Hobbs, 1972; Gona and Gona, 1977) have been documented previously. The present case, however, is distinctive in that it represents the first in which it has been shown that the local action of thyroxine can result in the production of a new pathway to a target some distance away. Given our previous results on the birth dates of ipsilaterally projecting neurons (Hoskins and Grobstein, 1985b), as well as the fact that induced ipsilateral projections are always accompanied by enhanced asymmetric growth in the hormone-treated eye, it seems likely that the ipsilaterally projecting axons in the thyroxine-treated tadpoles originate from retinal ganglion cells which were produced as a result of the hormone injection. This would suggest that thyroxine is controlling the production of a new population of ganglion cells as well as influencing in some way the axonal projections of some of these cells.

The effect of local thyroxine action in the present case is dramatic in the context of the nervous system, but the mitogenic and morphogenetic effects of thyroxine on some non-neural tissues are well established. For example, a metamorphic transition in hemoglobin type has been shown to result not from changes in the hemoglobin content of existing blood cells but rather from the thyroxine-dependent production of a new, adult-type blood cell population, and the coincident thyroxine-mediated destruction of the larval red blood cell population (Forman and Just, 1981). Similarly, the thyroxine-dependent formation of gland rudiments in the skin of *X. laevis* tadpoles seems to involve a thyroxine-triggered alteration in the mitotic pattern of the skin cell population, rather than direct effects of the hormone on the morphological characteristics of existing skin cells (McGarry and Venable, 1969). In these cases, as in the present one, thyroxine seems to act by altering a proliferative pattern and producing a new kind of cell.

In light of the above evidence that thyroid hormone influences patterns of proliferation and subsequent cell differentiation in systems as diverse as the nervous system, the circulatory system, and the epidermis, it is worth considering the possibility that such effects will be found in other parts of the metamorphosing anuran nervous system as well. At the same time, it is already clear that neither the action of thyroxine on the nervous system nor metamorphic change

in general can be understood solely in terms of altered patterns of proliferation. Thyroxine is known to have a variety of effects on the morphology of post-mitotic neurons. A thorough review of such effects is beyond the scope of the present discussion, but it is worth noting, for example, that the ability of thyroxine to specifically affect microtubule formation (Francon et al., 1977) may underlie its effects on dendritic morphology in the visual cortex (Schapiro et al., 1973) and cerebellum (Faivre et al., 1983). Such effects may also be relevant to the involvement of thyroxine in the production of ipsilaterally projecting axons in the present situation.

The finding that a neural pathway to a distant target can be induced by thyroxine is novel both in the literature on metamorphosis and in the literature on the involvement of other hormones in neuronal development. Since the relevant thyroxine-sensitive cells are restricted in the retina, both geographically (to peripheral and non-nasodorsal retina) and in terms of birth date, it may be possible to isolate them and characterize the mechanism(s) by which the hormone brings about proliferative and morphological changes in the ipsilaterally projecting cells. It will also be interesting to see whether other hormones known to influence the development of the nervous system do so by affecting patterns of proliferation as well as morphological differentiation of their target cells.

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