

**Title:** Mechanisms of action of PCBs on neurological development of Great Lakes amphibians.

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**Part I. High affinity interactions of polychlorinated biphenyls (PCBs) with bullfrog transthyretin and effects of PCB exposure on thyroid function in tadpoles.**

**Abstract.** Polychlorinated biphenyls (PCBs) are known to disrupt thyroid function in humans and wildlife. At least two sites of action have been identified for thyroid disruption by PCBs in mammals. First, PCBs alter thyroid hormone (TH) excretion/metabolism by altering T<sub>4</sub> conjugation via UDP-glucuronoyl transferases. Second, PCBs disrupt blood thyroxine (T<sub>4</sub>) transport by competing for binding to transthyretin (TTR). In mammals, TTR is a T<sub>4</sub>-specific binding protein. However, in nonmammalian species, TTR binds 3,5,3'-triiodothyronine (T<sub>3</sub>), the biologically active form of TH, with much higher affinity than T<sub>4</sub>. We conducted competitive binding assays with several PCB congeners or hydroxylated PCBs to determine whether they exhibit competitive binding activity with [<sup>125</sup>I]-T<sub>3</sub> for recombinant bullfrog TTR (rbTTR). One goal was to evaluate predictions (based both on empirical studies with human TTR and molecular modeling) of the T<sub>4</sub> or T<sub>3</sub>-like properties of the various PCB congeners. Our results show that 8 of the 12 PCBs that we tested exhibit moderate to high affinity for rbTTR. We treated bullfrog tadpoles with a subset of these 8 PCBs that bound to rbTTR and measured plasma T<sub>3</sub> binding capacity, plasma T<sub>3</sub> concentration and brain T<sub>3</sub> content.

**Final Report to the MI-DEQ, MGLPF  
PI: Robert J. Denver**

Treatment with the hydroxylated PCB4008, which exhibits both T<sub>3</sub> and T<sub>4</sub>-like properties, consistently decreased plasma [<sup>125</sup>I]-T<sub>3</sub> binding capacity at three developmental stages tested (early prometamorphosis, late prometamorphosis and metamorphic climax). By contrast, PCB 128, which exhibits only T<sub>3</sub>-like properties, tended to increase plasma [<sup>125</sup>I]-T<sub>3</sub> binding capacity. All PCBs tested (128, 153 and 4008) significantly inhibited metamorphosis. Only PCB 4008 decreased plasma T<sub>3</sub> concentration, but all PCBs tested significantly decreased brain T<sub>3</sub> content. There was no mortality in any of the treatment groups throughout the experiment. Our data suggest that PCBs can inhibit development in bullfrog tadpoles, and these effects are likely mediated through disruption of the thyroid axis.

## **Introduction**

Chronic exposure to environmental contaminants poses a threat to wildlife populations through sublethal alterations in physiological and ecological systems. While large-scale, acutely toxic exposure to contaminants still occurs in the form of oil spills and other chemical leaks, the more pervasive threat to wildlife in the modern world is that of continued exposure to low levels of persistent contaminants in the environment. Endocrine disruption has emerged as one of the most serious and pervasive of pollution's sublethal effects on wildlife populations. Animal endocrine systems regulate a vast array of processes, including reproduction, growth, development, metabolism, behavior, and various homeostatic mechanisms. The types of contaminants that disrupt wildlife endocrine systems consist largely of the persistent, organochlorine (OC) pollutants such as polychlorinated biphenyls (PCBs), dichlorodiphenyltrichloroethane (DDT), other pesticides, and dioxin. Their endocrine-disrupting effects stem from their structural similarities to the steroid- and thyroid hormones and their interactions with multiple components of these endocrine axes (1,2).

The mechanisms by which OCs affect reproduction and development in wildlife likely involve disruption of the endocrine system. Hormones orchestrate the processes of growth, development (especially postembryonic development) and reproduction. Current data suggest that most OC effects on the endocrine system occur through disruption of signaling pathways that involve hydrophobic signaling molecules such as steroid hormones, thyroid hormone and retinoids. All of these hormonal compounds exert their effects by binding to receptors that are ligand-activated transcription factors belonging to the steroid receptor superfamily (3). Many studies have documented alterations in the

production and actions of reproductive hormones such as estrogen and androgens (4-7). Numerous studies have shown that most OCs tested result in altered thyroid system function (8). Birds, fish, and mammals from contaminated areas exhibit abnormal plasma thyroid hormone concentrations and/or changes in thyroid morphology (9) (10,11). Exposure to OCs has been reported to decrease circulating thyroid hormone and to produce goiters in both developing and adult animals (8).

While there has been a considerable amount of research done in recent years on the impacts of environmental contamination with organochlorines (OCs) on wildlife, most studies have focused on mammals, birds, reptiles and fishes. There is a striking deficit of information on the sensitivity of amphibians to pollutants despite recent reports of global declines in amphibian populations and deformed frogs (12) (13,14). While both global scale (increased ultraviolet radiation, acid precipitation, climate change) and local scale (habitat fragmentation, introduction of exotic species, pollution) factors have been proposed as being responsible (15), none have been causally linked to amphibian declines. Polychlorinated biphenyls (PCBs) are known to impair development and reproduction, to be neurotoxic and endocrine disruptive in mammals, birds, reptiles and fishes. Little is known of the potential lethal or sublethal effects that these compounds may have on amphibians.

Relatively few studies have examined the effects of OCs on the amphibian endocrine system (16) or on amphibian growth and development. Jung and Walker (17) administered dioxin to three species of anuran larvae and found species-specific changes in timing of metamorphosis, size at metamorphosis, total length, and swimming speed. Gutleb et al. (18) reported developmental abnormalities and altered retinoid

concentrations in two amphibian species (*R. temporaria* and *X. laevis*) exposed to mixtures of PCBs. Rosenshield et al. (19) reported increased mortality in *R. clamitans* and *R. pipiens* exposed to PCB 126. Exposure to the technical PCB mixture Clophen A50 or PCB126 delayed metamorphosis in *Xenopus laevis* (20).

Endocrine disrupting chemicals such as PCBs are known to disrupt function of the thyroid system in mammals and birds. Thyroid hormone plays essential roles in vertebrate development and is especially critical for proper development of the central nervous system (CNS). Thyroid hormone deficiency in humans during fetal and neonatal life results in a severe condition of mental retardation known as cretinism (21). Thus, there is a strong potential for PCBs that alter thyroid function to disrupt brain development (9). Thyroid hormone is the primary morphogen controlling amphibian metamorphosis (22) and thus disruption of the thyroid system in tadpoles could alter development. Plasma thyroid hormone concentrations are low early in tadpole development, increase dramatically at the onset of metamorphosis and reach a peak at metamorphic climax (23). Thus, disruption of thyroid hormone production or action would have major consequences for amphibian development.

Nearly all PCBs or PCB mixtures disrupt thyroid physiology. Because of the structural similarities to thyroid hormone, PCBs may mimic or block the hormone's actions and have been shown to decrease both total and free thyroxine (3,5,3'5' tetraiodothyronine; T<sub>4</sub>) in pregnant rats and their fetuses (8). Such reductions in circulating thyroid hormone concentration by itself could result in impairment of neurological development (24). Current data suggest that a major site for PCB disruption of thyroid function in mammals is through competition with T<sub>4</sub> for binding to

transthyretin (TTR; TTR is a major serum transport protein for thyroid hormone in vertebrates; (25,26)). This may contribute to the reduction in circulating T<sub>4</sub> concentrations observed in PCB-exposed animals since free T<sub>4</sub> is rapidly cleared from the circulation. In mammals TTR exhibits 10 times the affinity for T<sub>4</sub> than for 3,5,3'-triiodothyronine (T<sub>3</sub>; considered the active form of thyroid hormone since it binds to the nuclear receptor with 10-fold greater affinity than T<sub>4</sub>). However, the situation is reversed in frogs where the amphibian TTR exhibits >20 fold greater affinity for T<sub>3</sub> than for T<sub>4</sub> (27). Thus, responses of the amphibian thyroid system to specific PCBs may differ from mammals and may depend on whether they are T<sub>3</sub> or T<sub>4</sub>-like in their structures ((26); see Table 1).

In the present study we evaluated the potential for PCBs to disrupt thyroid physiology in tadpoles of the bullfrog, *Rana catesbeiana*. We first tested whether a subset of PCBs, known to be present at high concentration in contaminated sites throughout Michigan ((28); see Table 1), could compete for binding of [<sup>125</sup>I]-T<sub>3</sub> to recombinant bullfrog TTR. We compared PCBs with predicted T<sub>3</sub> or T<sub>4</sub> like properties. Based on our in vitro studies we chose a subset of PCBs for testing in vivo. We treated tadpoles with individual PCBs and examined effects on growth and development, plasma T<sub>3</sub> concentration, brain T<sub>3</sub> content and plasma T<sub>3</sub> binding capacity.

## Materials and Methods

### Reagents

L-[3',-<sup>125</sup>I]T<sub>3</sub> with a specific activity of 3390 μCi/μg and radiochemical purity of >95% was purchased from NEN Life Science Products (Boston, MA). Unlabeled L-thyroxine and 3,5,3-triiodothyronine were purchased from ICN Biomedicals (Aurora, OH), and the anion exchange resin, AG-1-X2 (75-180 micron wet mesh), was purchased from Bio-Rad (Hercules, CA). The expression vector (pET3a/bTTR) containing the bullfrog *Rana catesbeiana* TTR cDNA was obtained from K. Yamauchi and transformed into *Escherichia coli* BL21 (DE3) cells.

All PCB congeners (purity > 99%) were purchased from Accu-Standard (New Haven, CT). Stock solutions of PCBs were dissolved in 100% DMSO at 25-50 mM and stored at 4C. Working dilutions were made from the stock solutions at 25-50 μM, also in 100% DMSO, and stored at 4C. Aliquots of the working dilutions were added to the incubation mixture to yield the desired final concentrations. DMSO at this concentration did not affect [<sup>125</sup>I]T<sub>3</sub> binding to TTR.

**Choice of PCBs.** Individual PCB congeners were tested for disruption of the amphibian thyroid system. These individual congeners were chosen based on: 1) demonstrated effects on the thyroid axis or neurological development in other vertebrates, 2) structure-function analyses of binding to mammalian thyroid hormone binding proteins, and 3) presence at contaminated sites throughout Michigan (see Table 1). Compounds listed in Table 1 are found at high concentration at contaminated sites in Michigan (28) and have structural similarities to thyroid hormone (see Table 1). Sediment levels of PCB 80 and PCB 127 were not determined (28) but these congeners were included because of their

high potency in the human TTR binding assay (suggesting that they could bind to some forms of amphibian thyroid hormone binding proteins.)

### ***In vitro recombinant bullfrog TTR binding assays***

#### **Preparation of Bacterial Lysate**

Lysate from cells expression recombinant bTTR was prepared following methods described by Yamauchi and colleagues (27) with modifications. *Escherichia coli* BL21 (DE3) cells transformed with the bTTR expression vector were grown (10 ml LB, 2% glucose, 50 µl amp) overnight at 37°C with shaking at 225 rpm. In the morning, five separate cultures (20 ml LB, 2% glucose, and 30 µl amp, with 300 µl of the over night culture) were inoculated and grown until OD<sub>600</sub> reached 1.0 (approximately 3 hrs). Expression of bTTR was induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside for 3 hrs at 28C with shaking at 225 rpm. Bacteria were pelleted by centrifugation (1200g) at 4 C for 15 min. and then resuspended in 2 ml of Tris-HCl (pH 8.0), 2 mM EDTA, 1% Triton X-100, and 1 mg/ml lysozyme. The lysate was then transferred to 12x75 mm polypropylene tubes to incubate for 15 min at 30C. The cells were sonicated on ice with one or two 10 s pulses at a high output setting, and then centrifuged (12,000g) for 15 min at 4 C. The supernatant was removed, pooled and vortexed, and then stored at -80C for later use in competitive binding assays.

#### **Competitive [<sup>125</sup>I]-T<sub>3</sub> Binding Assays**

Competitive [<sup>125</sup>I]-T<sub>3</sub> binding assays were conducted as described by Yamauchi and colleagues (27) with modifications. Varying amounts of bacterial lysate containing rbTTR or bullfrog plasma were diluted into 250 ul assay buffer (93 mM Tris-HCl (pH

7.5), 1 mM CaCl<sub>2</sub>). All binding assays were conducted in 12x75 mm borosilicate glass tubes. The [<sup>125</sup>I]-T<sub>3</sub> tracer (50,000 cpm/50 μl assay buffer) was added to the reactions which were then incubated at 0°C for 30 minutes. Competitive binding took place in the presence or absence of unlabeled ligands at final concentrations ranging from 1 μM to 1 nM. The protein-bound [<sup>125</sup>I]-T<sub>3</sub> was separated from free [<sup>125</sup>I]-T<sub>3</sub> by the addition of 100 μl of 10% (m/v) AG-1-X2 anion-exchange resin dissolved in assay buffer, terminating the 0.5-hour incubation period. The tubes containing the incubation mixture and the resin were vortexed briefly before centrifugation (2700g) for 5 min. Two-hundred microliters of the supernatant from each sample were then added to fresh 12x75 mm polypropylene tubes containing Micro Bio-Spin Chromatography Columns (Bio-Rad, Hercules, CA). The columns containing the supernatant were centrifuged (2700 x g) to remove residual resin and further separate protein-bound [<sup>125</sup>I]-T<sub>3</sub> from free [<sup>125</sup>I]-T<sub>3</sub>. Radioactivity was measured in a gamma counter (ICN Biomedicals' Automatic Gamma Counter 4/600 Plus; Aurora, OH). Total binding was determined by counting the product of incubating 50 μl of the [<sup>125</sup>I]-T<sub>3</sub> with 2 μl rbTTR lysate; nonspecific binding was determined by counting the product of incubating 50 μl [<sup>125</sup>I]-T<sub>3</sub> in assay buffer; specific binding (as a percent of total binding) was determined by subtracting the nonspecific binding from each individual sample containing the competitive ligand, and then dividing by total binding.

Titration curves were constructed to determine the appropriate concentration of rbTTR lysate to use in the binding assays. Binding curves were fitted using a hyperbola equation ( $y=(ax)/(b+x)$ ) using Sigmaplot 2001 (SPSS Science, Chicago, IL). This analysis showed that 50% of total binding is achieved with 2.1 ul rbTTR lysate.

Displacement curves were constructed and IC<sub>50</sub> values were calculated for each competitor (see below). Each sample per individual assay and each assay were done in triplicate.

### ***In vivo treatment with PCBs***

#### **Animals**

Bullfrog tadpoles were collected in June of 2001, from Saline Fish Hatchery (Ann Arbor, Michigan) by seining with the permission of the Michigan Department of Natural Resources (collecting permit #C0577 to R.J.D.). They were transferred to tanks in an environmental chamber (25C, 99% relative humidity) and separated into three groups based on Gosner stages of development. Throughout the experiment, tadpoles were fed an excess of tadpole brownies (175g rabbit chow, 75g spinach, 20g agar and 14g Knox gelatin; Nace, 1981, NIH booklet on amphibian care). Animals were cared for following institutional guidelines established by the University Committee on the Care and Use of Animals at the University of Michigan.

#### **Reagents**

PCBs 128 (2,2',3,3',4,4',-Hexachlorobiphenyl; predicted T<sub>3</sub>-like), 153 (2,2',4,4',5,5'-Hexachlorobiphenyl; predicted T<sub>4</sub>-like), and 4008 (4-Hydroxy-2',3,4',6'-Tetrachlorobiphenyl) were purchased from Accu-Standard (purity > 99%; New Haven, CT). Stock solutions of PCBs were dissolved in 100% DMSO at 25-50 mM and stored at +4 C°. Injection stocks were made by adding stock solutions dropwise with stirring to 0.6% saline; the final concentration of DMSO was 1.4%.

## **Injection Paradigm**

We chose to conduct an acute injection experiment because we wished to separate the effects of PCBs on the physiology of the thyroid system from teratogenic effects that might occur if embryos (or mothers) were dosed with the compounds and then followed throughout metamorphic development. Also, while we had previously administered PCBs successfully to leopard frog tadpoles by adding the compounds to their food (29), we did not consider this to be an efficient route for uptake in bullfrog tadpoles in an acute exposure paradigm. Animals were collected from ponds at Saline Fisheries Research Station, transferred to the lab and used in experiments within 3 days of capture.

The mean wet body weight (BW) of bullfrog tadpoles was 14g, and this was used to calculate the injection dose for each PCB. One hundred microliters of vehicle (0.6% saline, 1.4% DMSO) or PCBs (0.30  $\mu\text{g/g}$  BW for PCB4008, 0.36  $\mu\text{g/g}$  BW for PCB128 and PCB153) was injected i.p. L-T<sub>3</sub> immersion treatments received 20nM L-T<sub>3</sub> (sodium salt; Sigma) added directly to the tank water; the L-T<sub>3</sub> was replenished when the tank water was changed. At the end of each experiment (16 hrs. after the last injection), tadpoles were anesthetized in 0.1% benzocaine. Blood was collected into heparinized tubes by cardiac puncture. Plasma was stored at -20C before assay. Subsequently, the animals were sacrificed and brains were removed for extraction and analysis of T<sub>3</sub> content.

The doses of PCBs used (0.30 or 0.36  $\mu\text{g}$  PCB per gram BW) were based on several considerations. These doses would theoretically produce a tissue concentration of 1  $\mu\text{M}$  for each of the three PCBs tested (0.30  $\mu\text{g/g}$  BW for PCB4008, 0.36  $\mu\text{g/g}$  BW for PCB128 and PCB153). We did not attempt to analyze tissue distribution or clearance

rates of the compounds. Doses of 0.30 to 0.36  $\mu\text{g}$  are within the range of reported tissue contents of PCBs for ranid frogs at PCB contaminated sites (e.g., *Rana catesbeiana*: 2.33  $\mu\text{g/g}$  liver; *Rana clamitans*: 3.88  $\mu\text{g/g}$  liver; (30); *Rana clamitans* larvae: 0.2 – 0.8  $\mu\text{g/g}$  BW; (28)). Laboratory injection studies in different animal species were also used as a guideline (e.g., *Xenopus laevis*: 0.05, 5 or 125  $\mu\text{g/g}$  BW; (18); rat: 1  $\mu\text{g/g}$  BW; (31)). Finally, these doses represent 15-30 times the calculated IC50s (see Table 2) for each PCB binding to bullfrog TTR (see Table 2) and thus would be predicted to saturate plasma TTR binding sites.

The following experiments were designed to test for effects of exposure to PCBs on growth and development, and selected measures of thyroid function in bullfrog tadpoles. In Expt. 1 early prometamorphic tadpoles were used to address effects of short term exposure to PCBs on metamorphic development and measures of thyroid function (plasma  $\text{T}_3$  and [ $^{125}\text{I}$ ]- $\text{T}_3$  binding capacity, brain  $\text{T}_3$  content). Late prometamorphic (Expt. 2) and metamorphic climax (Expt. 3) animals were used to test for short term, and possible developmental differences in PCB effects on plasma  $\text{T}_3$  and [ $^{125}\text{I}$ ]- $\text{T}_3$  binding capacity.

**Experiment 1.** Early prometamorphic tadpoles (Gosner stages 39-41) were injected with vehicle or one of three PCBs (128, 153 or 4008). Vehicle-injected tadpoles were further divided into two treatments: plus or minus  $\text{T}_3$  (20 nM) dissolved in the aquarium water. The  $\text{T}_3$  treatment was included as a positive control for metamorphic acceleration (i.e., we could not predict whether PCBs would accelerate, decelerate, or have no effect on development. The  $\text{T}_3$  treatment verified that the animals were responsive, and that development could be accelerated.) Sample sizes were eleven tadpoles per treatment.

Injections were administered i.p. between 4-5:00 p.m. every other day for 10 days (a total of five injections.) Tadpoles were housed in 4L of well water in polypropylene tanks, each equipped with an airstone and lid. A static renewal paradigm was used, with aquarium water changed on days 4 and 7 of the experiment.

**Experiment 2.** Late prometamorphic tadpoles (Gosner stages 39-41) were injected with vehicle or one of three PCBs (128, 153 or 4008). Experiment #2 followed the same injection paradigm as Experiment #1, except that there were 12 tadpoles per treatment group, and the experiment ended after 6 days of treatment. A total of three injections were delivered, and the water was changed on day 3 of the experiment. All animals had reached metamorphic climax (Gosner stage 42-43) by day 6.

**Experiment 3.** In this experiment tadpoles were injected with vehicle, PCB4008 or PCB128 (the L-T<sub>3</sub> immersion treatment was not included in this experiment). There were 7 tadpoles per treatment group, and they were housed in 2 cm water (to allow for lung breathing) in polystyrene tanks with lids. Injections were delivered daily for three days (total of three injections.)

### **[<sup>125</sup>I]-T<sub>3</sub> Binding Assays with Bullfrog Tadpole Plasma**

Bullfrog tadpole plasma was collected in each experiment described above and analyzed in a [<sup>125</sup>I]-T<sub>3</sub> binding assay (modified from Yamauchi, *et al.* (27)). Bullfrog plasma (0.5, 0.25, and 0.125 μl) was diluted into 200 μl assay buffer (93 mM Tris-HCl [pH 7.5], 1 mM CaCl<sub>2</sub>). Fifty microliters of [<sup>125</sup>I]-T<sub>3</sub> in assay buffer (50,000 cpm/50 μl assay buffer) was then added to each tube and incubated for 0.5 hr at 0°C in 12x75 mm borosilicate glass tubes. The protein-bound [<sup>125</sup>I]-T<sub>3</sub> was separated from free [<sup>125</sup>I]-T<sub>3</sub> by the addition of 100 μl of 10% (m/v) AG-1-X2 anion-exchange resin (Bio-Rad, Hercules, CA) dissolved in assay buffer. The tubes were then vortexed briefly before centrifugation at 2700 x g for 5 min. Two hundred microliters of the supernatant from each sample were then added to fresh 12x75 mm polypropylene tubes containing Micro Bio-Spin Chromatography Columns (Bio-Rad, Hercules, CA). The columns containing the supernatant were centrifuged (2700 x g) to remove residual resin and further separate protein-bound [<sup>125</sup>I]-T<sub>3</sub> from free [<sup>125</sup>I]-T<sub>3</sub>. Quantification of radioactivity was measured in a gamma counter (ICN Biomedicals' Automatic Gamma Counter 4/600 Plus; Aurora, OH). Total binding was determined by counting the product of incubating 50 μl of the [<sup>125</sup>I]-T<sub>3</sub> with 0.5, 0.25, and 0.125 μl of untreated, late premetamorphic bullfrog tadpole plasma per 200 μl assay buffer. Nonspecific binding was determined by counting the product of incubating 50 μl [<sup>125</sup>I]-T<sub>3</sub> in assay buffer only, and specific binding (as a percent of total binding) was determined by subtracting the nonspecific binding from each individual sample, and then dividing by total binding. Mean binding for each treatment group was calculated and percent binding was plotted.

### Tissue extraction and radioimmunoassay

Thyroid hormones were extracted from tadpole brain tissue following previously described methods (32,33). Briefly, tissues were homogenized in 3-4 volumes of methanol containing 1mM propylthiouracil. Tissue homogenates were subjected to organic extraction, back extraction into the aqueous phase, and anion exchange chromatography as described (32,33). For estimation of recoveries, 1000 cpm <sup>125</sup>I-labeled T<sub>3</sub> was added to the tadpole homogenates. Recoveries ranged from 30 - 55%, and RIA potency estimates were corrected for recovery.

Radioimmunoassays for T<sub>3</sub> were conducted as described by MacKenzie and colleagues (34) and Denver and Licht (35). Primary antiserum for T<sub>3</sub> was purchased from Endocrine Sciences (Calabasas, CA). The intra- and interassay coefficients of variation were 3.6% and 6.8%, respectively.

### Data analysis

Competitive binding experiments were repeated three to five times for each hormone or PCB. Binding data were analyzed as described by Cortright et al. (36) by fitting the inverse hyperbolic equation using Sigma Plot software. Binding data were fitted to the regression equation below using the computer program SigmaPlot.

$$y = \frac{1 - ax}{b + x};$$

Where  $y$  is the relative intensity of the specific band in the gel as determined by optical density,  $x$  is the peptide concentration,  $a$  is the intensity of the band in the absence of competitor, and  $b$  is the  $K_{i(app)}$ . The  $K_{i(app)}$  values were log-transformed to achieve homogeneity of variance, analyzed by a one way analysis of variance and subjected to

Scheffe's *post hoc* multiple comparison test. Morphological measurements and hormone data were analyzed using one-way and two-way analysis of variance (ANOVA) followed by Scheffe's multiple contrast test ( $p < 0.05$ ). Linear regression was used to analyze plasma T<sub>3</sub> binding capacity.

## Results and Discussion

### Validation of the in vitro rbTTR binding assay.

Titration curves were constructed for [ $^{125}$ I]-T<sub>3</sub> binding to recombinant bullfrog TTR present in bacterial lysate. Binding plateaued at 30% of tracer bound with an ED50 of 2.5  $\mu$ l lysate (15% bound; Fig. 1). Each assay contained three replicates, and the experiment was repeated three times with virtually identical results. Thus, all competitive binding assays were conducted with 2.5  $\mu$ l rbTTR lysate.

To determine if binding of [ $^{125}$ I]-T<sub>3</sub> to rbTTR is comparable to the native TTR, titration curves were constructed for bullfrog tadpole plasma and rbTTR. A plasma pool from Gosner stage 37-38 tadpoles was used, as this stage is known to have elevated plasma levels of TTR protein (compared with later developmental stages; (27)). This experiment demonstrated parallelism between dilutions of bullfrog plasma and rbTTR lysate (Fig. 1). Also, the titers of the rbTTR lysate and bullfrog plasma were comparable.

Competitive binding assays were conducted to compare the affinities of T<sub>3</sub> and T<sub>4</sub> for rbTTR. We calculated an IC50 of 32 nM for T<sub>3</sub>; the IC50 for T<sub>4</sub> was greater than 1000 nM (Fig. 2). These values are comparable to those reported by Yamauchi and colleagues for rbTTR (27).

### PCBs compete for [ $^{125}$ I]-T<sub>3</sub> binding to bullfrog TTR: in vitro binding assays.

We conducted competitive binding assays with different PCBs and found that several compounds exhibited moderate to high affinity binding to rbTTR (Fig. 3; only compounds which exhibited moderate to high affinity binding are shown in the figure;

IC50s for all compounds tested are shown in Table 2). Compounds shown in Fig. 3 are grouped based on their predicted T<sub>3</sub> or T<sub>4</sub>-like structures (2) or hydroxylation. The predicted T<sub>3</sub>-like compounds PCB33, 118 and 128 exhibited high to moderate affinity for rbTTR; whereas, PCB77 exhibited no competitive binding. Only two predicted T<sub>4</sub>-like compounds exhibited moderate to high affinity binding (PCB95, 153) while PCB80, 110 and 127 exhibited no competitive binding. All three hydroxylated PCBs tested (2004, 3003 & 4008) exhibited moderate to high affinity binding to rbTTR.

**Treatment of tadpoles with PCBs alters development, plasma T<sub>3</sub> concentration, brain T<sub>3</sub> content, and plasma [125]-T<sub>3</sub> binding capacity.**

Based on the results from in vitro binding assays (described above) we chose a limited number of PCBs to test for thyroid disruptive activity in bullfrog tadpoles in vivo (Fig. 2). The choice of the compounds tested in these studies was based on their high potency in the bTTR binding assay. Our objective was to test for acute effects of PCBs on tadpole thyroid function, and to determine if potential thyroid disruptive effects may be reflected in effects on development. We administered PCBs to tadpoles by injection beginning at three different development stages (early prometamorphosis - the beginning of hind limb development; late prometamorphosis - hind limb largely developed; metamorphic climax - rapid transformation). We chose these developmental stages because they are associated with an increasing level of thyroid system activity, e.g., low, medium and high plasma thyroid hormone concentrations, respectively.

While treatment with exogenous T<sub>3</sub> beginning at early prometamorphosis accelerated metamorphosis as expected, all PCBs tested significantly slowed

development (Fig. 5, top). There were no significant differences among treatment groups when treatment was begun during late prometamorphosis or metamorphic climax (owing to the already rapid rate of development at these stages; data not shown).

Plasma  $T_3$  concentration was significantly elevated by exogenous  $T_3$  treatment as expected (Fig. 5, middle). Only the hydroxylated compound PCB4008 caused a significant decrease in plasma  $T_3$ , perhaps reflective of decreased plasma  $T_3$  binding capacity (Fig. 4). Plasma  $T_3$  concentration was not altered by PCB treatment in late prometamorphic or climax stage animals (data not shown; although plasma  $T_3$  increased significantly at climax compared to early prometamorphic animals as expected:  $2.3 \pm 0.7$  vs.  $0.3 \pm .17$ ).

Brain  $T_3$  content in animals treated from early prometamorphosis was significantly reduced by  $T_3$ . We hypothesize that this decreased tissue content reflects enhanced type III deiodinase activity, which degrades  $T_3$ . Type III deiodinase is significantly elevated during metamorphosis and is induced by  $T_3$  in brain (37). All PCBs tested caused a significant decrease in brain  $T_3$  content. The mechanism for this decrease is unknown. However, it may be that PCBs mimic  $T_3$  in their effects on brain gene expression, thus enhancing type III deiodinase activity. This could be a plausible mechanism for PCB128 which mimicked  $T_3$  effects on plasma  $T_3$  binding capacity. We have preliminary evidence that PCB128, but not PCB153 or 4008 can compete for binding to  $T_3$  receptors (Schleuter and Denver, unpublished data). Other possible mechanisms include decreased hormone uptake from plasma or decreased synthesis of  $T_3$  from  $T_4$  (i.e., effects on type II deiodinase activity).

Plasma [ $^{125}\text{I}$ ]- $\text{T}_3$  binding capacity was significantly lower at metamorphic climax (Gosner stage 42-43) compared with earlier developmental stages. This reduction in binding capacity is reflected in a significant decrease in plasma TTR concentration at metamorphic climax (see (27)). Treatment with  $\text{T}_3$  beginning in early prometamorphosis (but not in late prometamorphosis) increased [ $^{125}\text{I}$ ]- $\text{T}_3$  binding capacity. These  $\text{T}_3$ -treated animals had reached metamorphic climax by the end of the experiment (when plasma was collected; see Fig. 5) and so this result contrasts with the decrease in plasma [ $^{125}\text{I}$ ]- $\text{T}_3$  binding capacity seen during spontaneous metamorphosis. We do not have an explanation for this result, however it is worth noting that  $\text{T}_3$  treatment generally results in asynchronous development, and thus the lack of a decrease in [ $^{125}\text{I}$ ]- $\text{T}_3$  binding capacity (presumed to reflect plasma TTR concentration) could reflect asynchrony between external morphological changes and liver protein synthesis. The enhanced [ $^{125}\text{I}$ ]- $\text{T}_3$  binding caused by  $\text{T}_3$  could result from enhanced TTR synthesis or decreased clearance. Future studies will address these potential mechanisms.

Treatment with the hydroxylated compound PCB4008 consistently reduced plasma [ $^{125}\text{I}$ ]- $\text{T}_3$  binding capacity at all developmental stages examined (Fig. 4). By contrast, the  $\text{T}_3$ -like compound PCB128 increased plasma  $\text{T}_3$  binding capacity in early prometamorphic tadpoles. Treatment with PCB153 lowered plasma  $\text{T}_3$  binding capacity in late prometamorphic, but not early prometamorphic, tadpoles.

**Significance.** PCBs and other organochlorine compounds (OCs) have been detected in high concentrations in tissues from wildlife from contaminated areas worldwide. The tissue levels of these compounds are often many times greater than those found in water

or sediments and tend to increase with trophic level. For example, tissue PCB contents increased 13-fold from plankton to fish in a Lake Michigan offshore food web (38).

PCBs and other OCs have also been found to accumulate in the tissues of birds from the Great Lakes-St. Lawrence River Basin (38) and eggs of snapping turtles (39). Limited studies show that amphibians, like birds and fishes, bioaccumulate these compounds (30,40-42).

Our results show that PCBs can disrupt thyroid function and metamorphosis in bullfrog tadpoles. Metamorphosis is a thyroid-dependent process, and thus it is likely that the correlation between thyroid disruption and delayed development that we observed in bullfrog tadpoles is a causative relationship. Furthermore, predictions of T<sub>3</sub> and T<sub>4</sub>-like properties of PCBs based on molecular modeling (2) were largely borne out by our competitive binding assays with rbTTR. For example, PCB 33, 118 and 128 exhibited high potency in the rbTTR binding assay but low or no potency in the hTTR binding assay. Similarly, PCB 80, 110 and 127, which have high potency in the hTTR binding assay, had low or no affinity for rbTTR.

In addition to effects on TTR, PCBs could disrupt binding of T<sub>3</sub> or T<sub>4</sub> to other thyroid hormone binding proteins. For example, effects of PCBs on tissue monodeiodinases (enzymes that metabolize thyroid hormone by removing iodine atoms from one or both phenolic rings) have been shown (43,44). Other potential targets include cytosolic thyroid hormone binding proteins (45) and membrane thyroid hormone transport proteins (46). It is also possible that PCBs interact with thyroid hormone receptors, though few studies have addressed this question (25,47). Other sites for

disruption of thyroid status are on hormone clearance through effects on T<sub>4</sub> conjugation via UDP-glucuronoyl transferases (8).

**Conclusions.** The following conclusions can be drawn from our findings: 1) Predictions of T<sub>3</sub>- and T<sub>4</sub>-like properties of the various PCBs based on molecular modeling were largely borne out by competitive binding assays with rbTTR. PCBs that do not exhibit potency in the mammalian TTR binding assay (predicted T<sub>3</sub>-like compounds) are potent competitors for [<sup>125</sup>I]-T<sub>3</sub> binding to rbTTR. None of these T<sub>3</sub>-like PCBs have been tested for their potential to interact with the nuclear TH receptors in any species. We hypothesize that, owing to their T<sub>3</sub>-like properties, these and perhaps other PCBs could act as agonists (or perhaps antagonists) for the TH receptors. 2) There are important species differences between mammals and amphibians in the types of PCBs that can interact with TTR. This suggests that predictions of endocrine disruptive properties of specific PCBs are highly dependent on the species under investigation. 3) PCBs can interact with amphibian TTR and this presents the potential for disruption of TH transport and consequently hormone metabolism and action in tadpoles. Such alterations could negatively impact TH-dependent development in amphibian species, where TH is the primary morphogen controlling metamorphosis.

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**Table 1.** Polychlorinated biphenyls tested and concentration range at contaminated sites in Michigan.

Compound	Reported endocrine disruptive effects in other species#	T <sub>3</sub> or T <sub>4</sub> -like*	Potency in human TTR binding assay**	Concentrations (range) detected at contaminated sites in Michigan*** ng PCB/g sediment = ppb
Aroclor 1254	Decreased plasma T <sub>4</sub> , CORT	NA	NA	NA
PCB 80	ND	T <sub>4</sub>	7.05	ND
PCB 127	ND	T <sub>4</sub>	8.23	ND
PCB 110	ND	T <sub>4</sub>	2.6	40-380
PCB 153	ND	T <sub>4</sub>	0.55	15-181
PCB 95	ND	T <sub>4</sub>	0.51	20-159
PCB 33	ND	T <sub>3</sub>	0.06	11-86
PCB 77	Decreased plasma T <sub>4</sub> , CORT	T <sub>3</sub>	<0.01	2-26
PCB 118	Decreased plasma T <sub>4</sub>	T <sub>3</sub>	<0.04	20-250
PCB 128	ND	T <sub>3</sub>	<0.01	3-41

# Thyroid effects reviewed by Brouwer et al. (8); effects on CORT: Sanders and Kirkpatrick, 1977; Byrne et al., 1988. \* Prediction based on structure-function assay and modeling in Chauhan et al. (26). \*\* Potency relative to T<sub>4</sub> binding which is 1. \*\*\* From Glennemeier and Begnoche (28). Based on samples collected from five contaminated sites in Michigan. NA - not applicable; ND - not determined.

**Table 2.** Competitive binding of iodothyronines and various polychlorinated biphenyls to bullfrog transthyretin (bTTR). Binding assays were conducted with [<sup>125</sup>I]-T<sub>3</sub>. Inhibition constants for human prealbumin (hTTR; competitive binding assays done with [<sup>125</sup>I]-T<sub>4</sub>) are provided for comparison.

Compound	Ki bTTR	Ki hTTR*
3,5,3' triiodothyronine (T3)	32 nM	614 nM
3,5,3',5' tetraiodothyronine (T4)	> 1000 nM	49 nM

**PCB congeners tested**

BZ#	Compound	Ki bTTR	Ki hTTR*	T3 or T4 like**
33	2',3,4-trichlorobiphenyl	30 nM	796 nM	T3
77	3,3',4,4'-tetrachlorobiphenyl	NA	NA	T3
80	3,3',5,5'-tetrachlorobiphenyl	NA	7 nM	T4
95	2,2',3,5',6-pentachlorobiphenyl	155 nM	97 nM	T4
110	2,3,3',4',6-pentachlorobiphenyl	>1000 nM	19 nM	T4
118	2,3',4,4',5-pentachlorobiphenyl	117 nM	> 1000 nM	T3
127	3,3',4,5,5'-pentachlorobiphenyl	NA	6 nM	T4
128	2,2',3,3',4,4'-hexachlorobiphenyl	67 nM	NA	T3
153	2,2',4,4',5,5'-hexachlorobiphenyl	52 nM	90 nM	T4

**Hydroxylated PCBs tested**

ID	Compound	Ki bTTR	Ki hTTR***
2004	4-hydroxy-3,5-dichlorobiphenyl	52 nM	16 nM
3003	3-hydroxy-2',4',6'-trichlorobiphenyl	100 nM	>100 uM
4008	4-hydroxy-2',3,4',6'-tetrachlorobiphenyl	32 nM	33 nM

\* Chauhan et al. (26)

\*\* Based on modeling by McKinney and Waller (2)

\*\*\*Cheek et al. (25)

NA - not active

### Figure Legends

**Fig. 1.** Establishment of the recombinant bullfrog TTR binding assay. **Upper panel.**

Titration curve of bacterial lysate from cells expressing bTTR. Binding assays were conducted as described in Materials and Methods. An effective dose 50 (ED50) of 2.5  $\mu$ l rbTTR lysate was determined from the titration curve and used in all competitive binding assays. **Lower panel.** Parallelism of dilutions of rbTTR lysate and bullfrog tadpole plasma in the [ $^{125}$ I]-T<sub>3</sub> binding assay. See Materials and Methods for methodological details.

**Fig. 2.** Competitive binding of T<sub>3</sub> or T<sub>4</sub> to rbTTR. Competitive binding assays were conducted with [ $^{125}$ I]-T<sub>3</sub> as tracer as described in the Materials and Methods. This analysis showed that T<sub>3</sub> binds with high affinity to rbTTR, whereas T<sub>4</sub> exhibited very low affinity. This result contrasts with binding affinity of human TTR which binds T<sub>4</sub> with high affinity but T<sub>3</sub> with low affinity. Each data point represents the mean of three replicate determinations. Data shown are representative of three separate assays.

**Fig. 3.** Competitive binding of different PCBs to rbTTR. Competitive binding assays were conducted with [ $^{125}$ I]-T<sub>3</sub> as tracer as described in the Materials and Methods. PCBs were added to the reactions to the final concentrations indicated on the x-axis. Each data point represents the mean of three replicate determinations. Data shown are representative of three separate assays. IC50 values were calculated as described in Materials and Methods.

**Fig. 4.** Binding of [ $^{125}$ I]-T<sub>3</sub> to bullfrog plasma collected from tadpoles treated with PCBs. Tadpoles were treated with PCBs (or T<sub>3</sub>) beginning at the developmental stages indicated (top: early prometamorphosis; middle: late prometamorphosis; bottom: metamorphic climax). Plasma was analyzed for [ $^{125}$ I]-T<sub>3</sub> binding as described in Materials and Methods. Each sample was analyzed at three dilutions (0.125, 0.25 and 0.5  $\mu$ l plasma) and each data point represents the mean of three replicates.

**Fig. 5.** Effects of PCBs on metamorphosis, plasma T<sub>3</sub> concentration and brain T<sub>3</sub> content. Treatment with PCBs was begun in early prometamorphosis and continued for 10 days. PCBs were injected, while T<sub>3</sub> was administered in the aquarium water. **Top.** Gosner stages at the end of the experiment. T<sub>3</sub> treatment significantly accelerated metamorphosis, while all PCBs tested slowed development. **Middle.** Plasma T<sub>3</sub> concentrations were determined by RIA. T<sub>3</sub> treatment significantly increased while PCB4008 decreased plasma T<sub>3</sub>. **Bottom.** Brain T<sub>3</sub> content was determined by RIA following extraction as described in Materials and Methods. All treatment significantly decreased brain T<sub>3</sub> content. Bars are the means  $\pm$  SEM for 8 replicates per treatment.

Figure 1

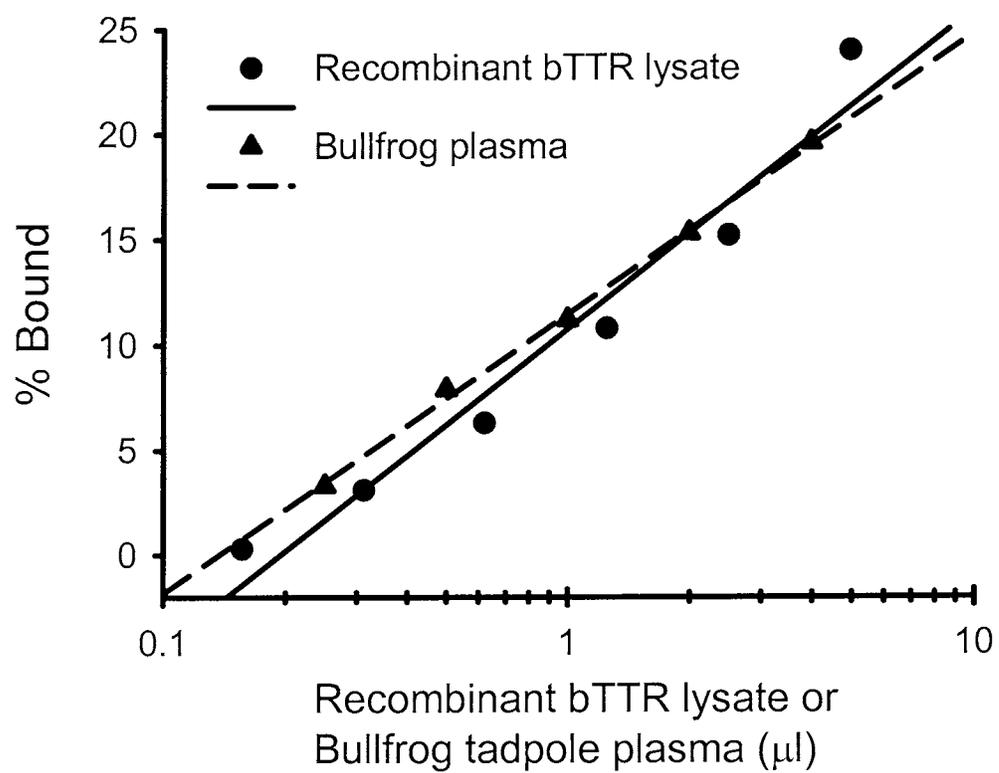
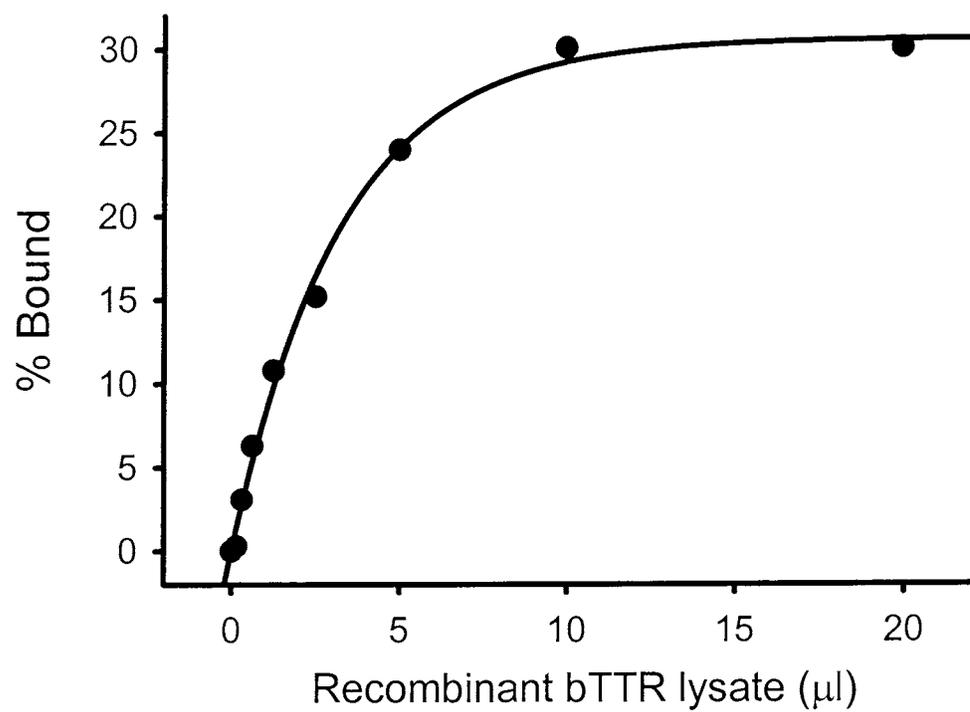


Figure 2

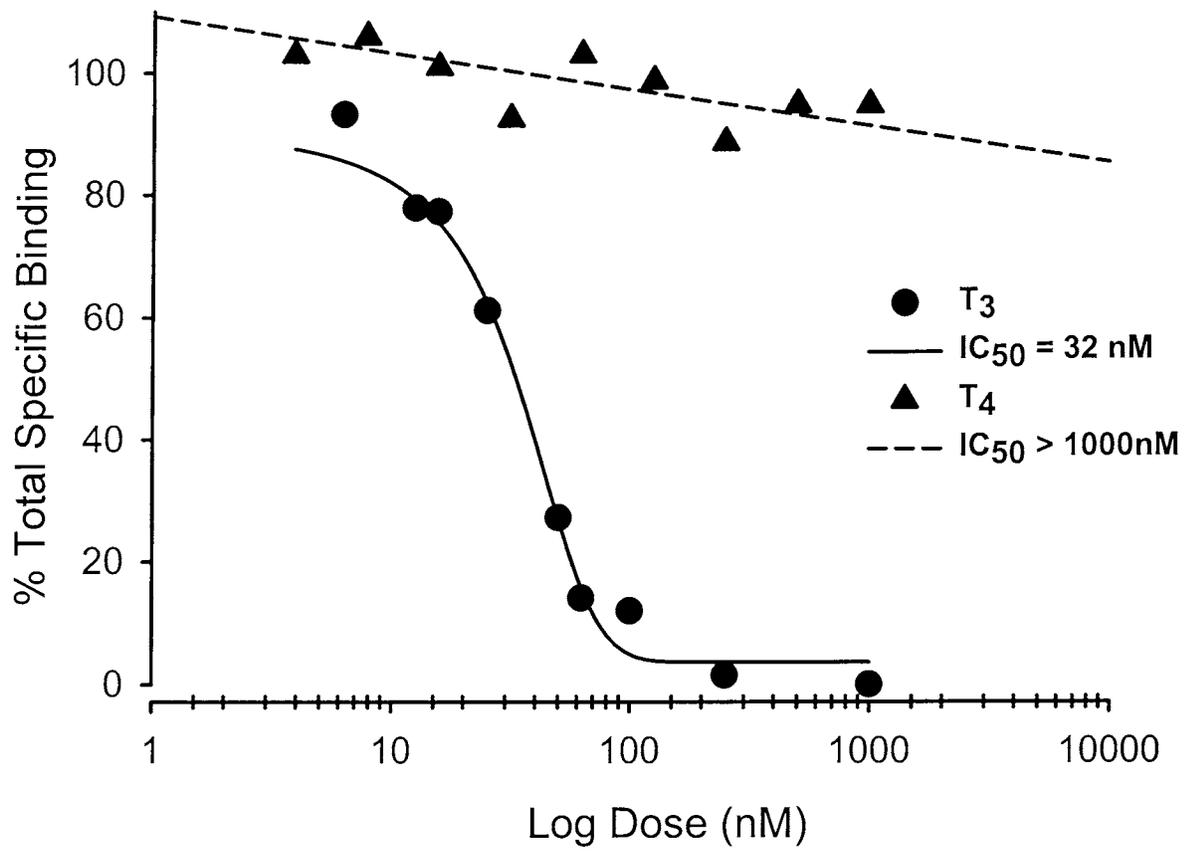


Figure 3

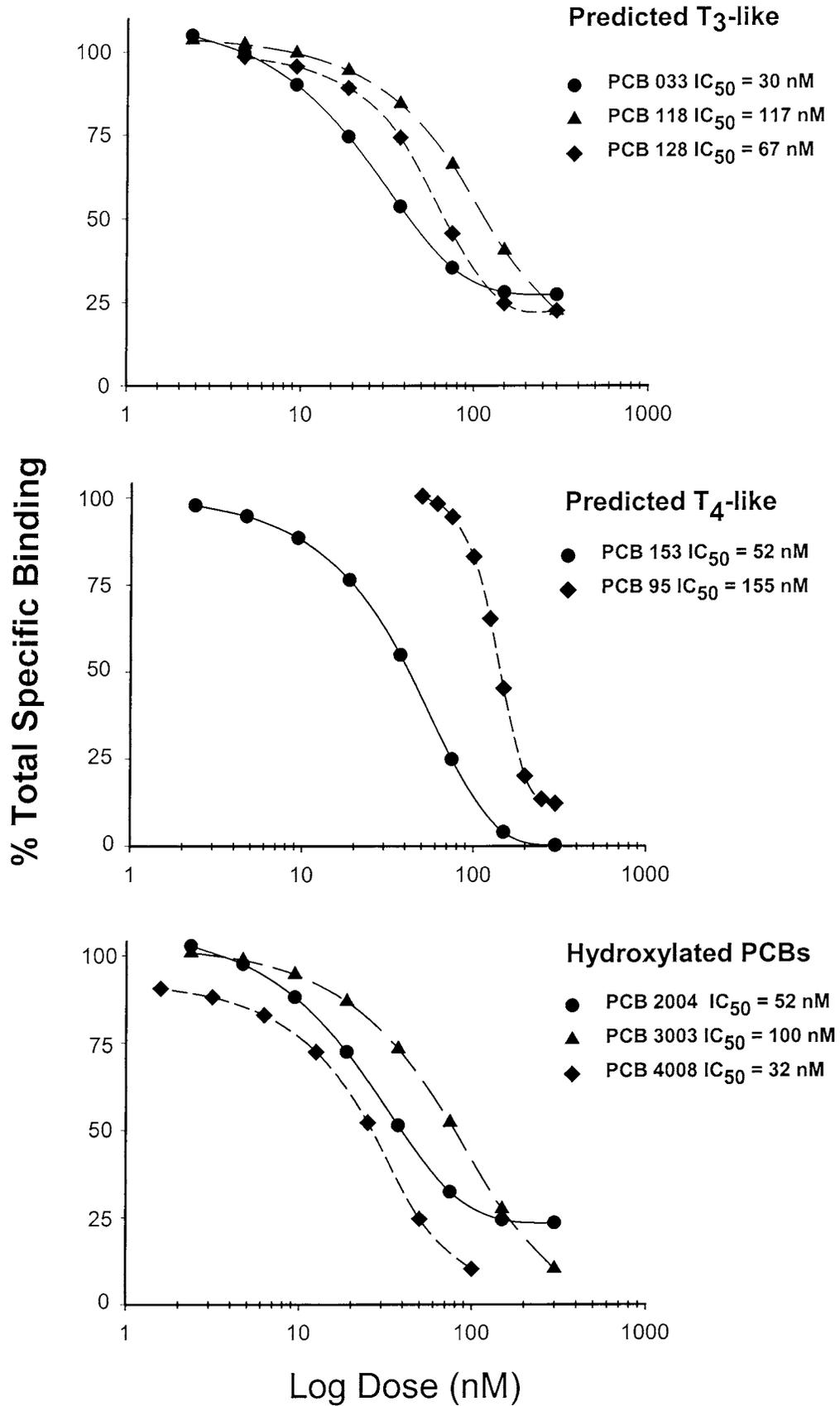


Figure 4

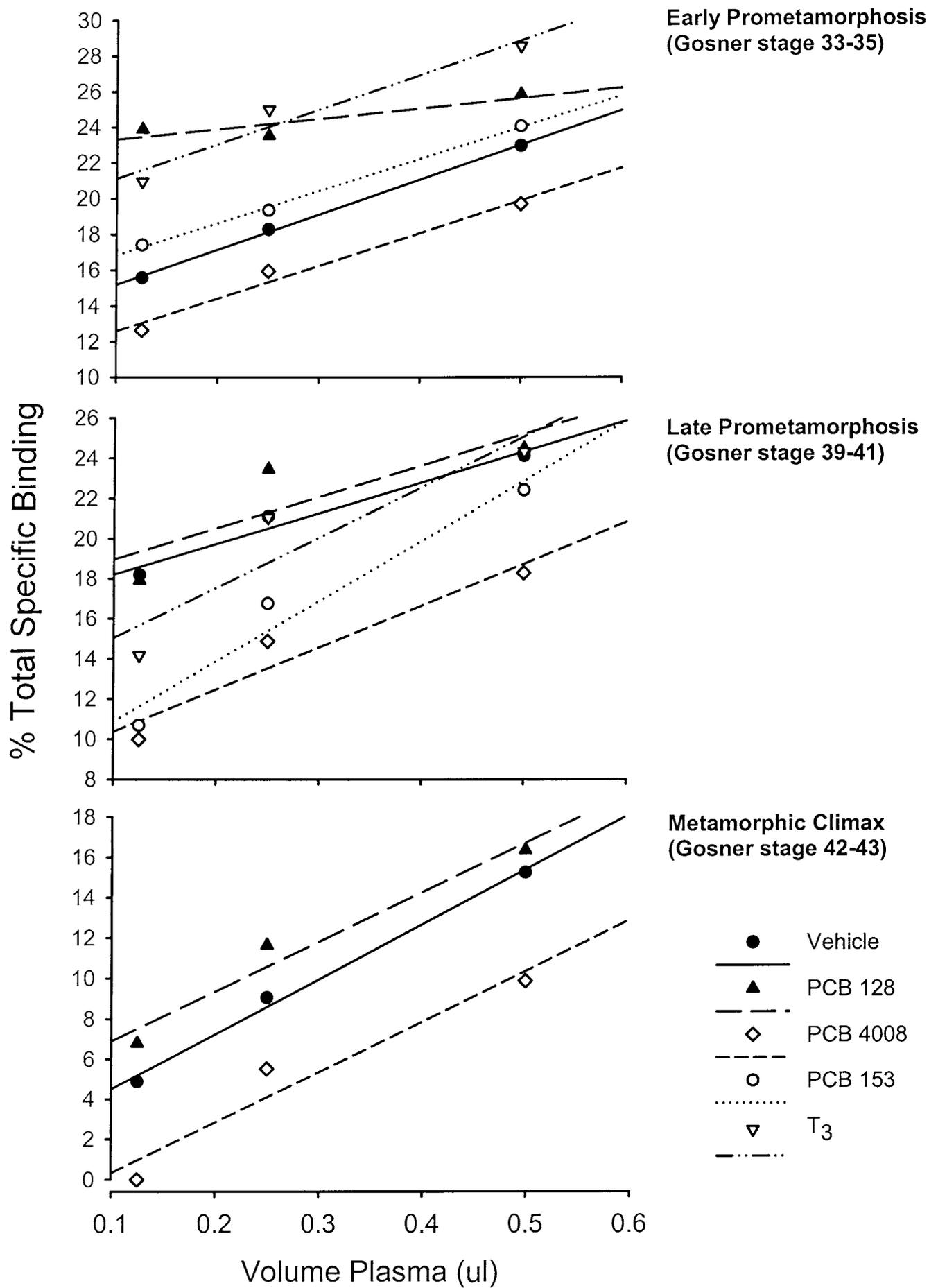
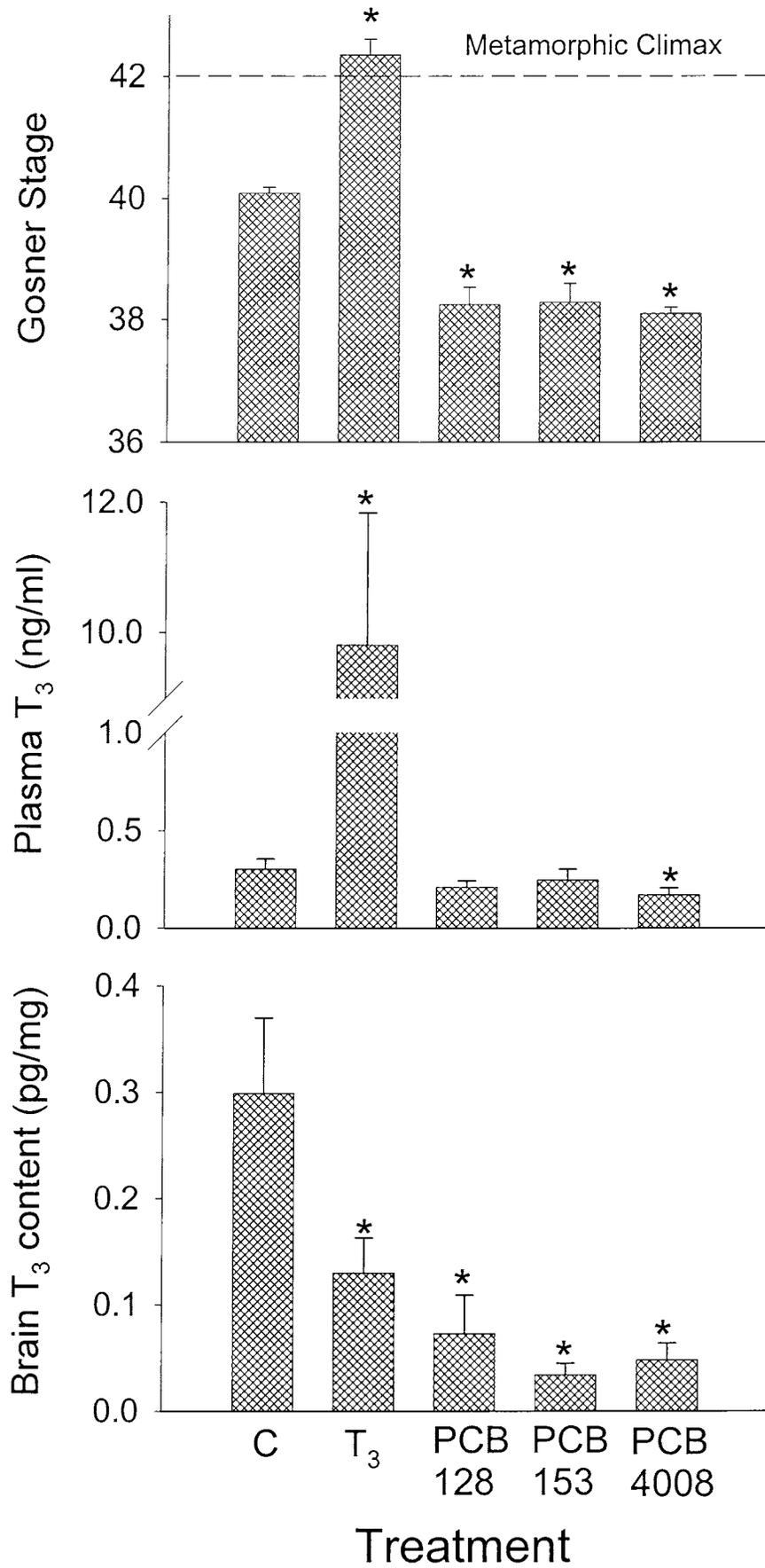


Figure 5



**Part II. Thyroid hormone-dependent cell proliferation in the central nervous system of *Xenopus laevis* during metamorphosis**

**Abstract.** The essential role of thyroid hormone ( $T_3$ ) in amphibian metamorphosis is well known. However, the molecular and cellular mechanisms by which the hormone controls morphogenesis are poorly understood. Towards understanding basic mechanisms of  $T_3$  action in brain development, and to provide baseline data for the analysis of disruption of  $T_3$ -dependent neurological development, we have characterized cell proliferation in the brain of *Xenopus laevis* tadpoles during metamorphosis. We also analyzed the role that  $T_3$  and its receptors play in this process. We quantified cell proliferation by analyzing the incorporation of bromodeoxyuridine (BrdU; a cell birth marker), or the expression of phosphorylated histone H3, a mitosis-associated protein. In premetamorphic tadpoles a low level of cell proliferation was observed in periventricular zones throughout the brain. Addition of 50 nM  $T_3$  to the aquarium water of premetamorphic tadpoles dramatically increased brain cell proliferation by 48 hr. This effect of  $T_3$  on cell proliferation was due to a shortening of the cell cycle. Double immunohistochemistry for BrdU and thyroid hormone receptors (TR) showed that proliferating cells express TR protein. Furthermore, we show by in situ hybridization histochemistry that proliferating cells express mRNA for the TR $\alpha$  subtype. These findings suggest that  $T_3$  could act, in whole or in part, directly on neural stem cells to regulate cell proliferation. By contrast to TR $\alpha$ , several lines of evidence suggest that the TR $\beta$  subtype is expressed outside of the proliferative zone, in cells that are undergoing differentiation and/or migration. During spontaneous metamorphosis the proliferation rate is low in the premetamorphic tadpole brain, reaches a peak during

prometamorphosis and then declines at metamorphic climax. Premetamorphic tadpoles responded to exogenous  $T_3$  by dramatically increasing brain cell proliferation; whereas, beginning in prometamorphosis, when cell proliferation is at a maximum, we observed no effect of treatment with  $T_3$  (50 nM for 48 hr). During prometamorphosis  $T_3$  synthesis and secretion increases dramatically, and we found that at this stage blockade of  $T_3$  synthesis by treatment with the goitrogen methimazole decreased brain cell proliferation, and this effect could be rescued by simultaneous treatment with  $T_3$  (10 nM). Taken together, our data support the conclusion that developmental changes in cell proliferation in the tadpole brain are regulated by  $T_3$ , and that the hormone acts via specific receptors expressed in target cells. Furthermore, the effect of the hormone is to shorten cell cycle time.

## **Introduction**

In vertebrates, thyroid hormone ( $T_3$ ) plays a critical role in postembryonic maturation of the brain. In mammals,  $T_3$  has been shown to influence dendritic branching, axonal density, synapse number, myelination, and cell proliferation (1,2). The lack of TH during fetal and neonatal life is known to result in a condition of severe mental retardation known as cretinism (1,3). Disruption of  $T_3$  signaling in the fetus or neonate could result in impaired neurological development (4). The cellular and molecular mechanisms underlying the action of  $T_3$  on brain cell proliferation are poorly understood. For example, it is unknown whether  $T_3$  acts in a cell-autonomous fashion to increase cell proliferation or by inducing neighboring cells to secrete paracrine-acting growth factors.

In amphibians,  $T_3$  induces metamorphosis, during which time the brain undergoes morphological and biochemical changes that prepare the animal for adult life. Metamorphosis often involves a habitat shift from an aquatic to a terrestrial environment, and the adult frog exhibits dramatically different behaviors and other life history characteristics compared with the tadpole (5). This process requires the creation, modification or elimination of many structures. For example, the Mauthner neurons found on either side of the medulla and the sensory and motoneurons supplying the tail are lost (reviewed by (6)). Conversely, new structures such as the major portion of the retina arises, along with associated ipsilateral visual projections in the diencephalon and mesencephalon (7). Also, the cerebellum undergoes expansion and the neurosecretory neurons in the diencephalon and their projections to the median eminence develop. Thyroid hormone is critical for all of these changes (8-13). Earlier studies using classical histochemical stains showed that thyroid hormone treatment increases apparent mitotic activity of various sites in the nervous system of several amphibian species ((14-18)). However,

there has yet to be a systematic analysis of developmental changes or the effects of T<sub>3</sub> on brain cell proliferation in an amphibian.

Recently, studies on the hormonal regulation of metamorphosis have focused on the molecular basis for T<sub>3</sub> action. A number of genes have been isolated using gene expression screening which exhibit T<sub>3</sub> regulation in the tadpole central nervous system. For example, Denver and colleagues (19) isolated 34 genes from the diencephalon of *Xenopus* tadpoles that are either up- or down-regulated by T<sub>3</sub> and show developmental-dependent changes in expression. The identification and functional characterization of T<sub>3</sub> target genes is critical to understanding T<sub>3</sub> action on the developing CNS, because these T<sub>3</sub>-regulated genes encode downstream effectors of the hormone-receptor complex. Thyroid hormone acts by binding to ligand-activated transcription factors (thyroid hormone receptors; TR) and regulating gene transcription (20,21). In all vertebrates that have been studied, including *Xenopus laevis*, there are two paralogous genes that code for TRs, designated TR $\alpha$  and TR $\beta$  (20-22). In mammals, several functional TR isoforms resulting from differential mRNA processing have been identified (TR $\alpha$ 1, TR $\beta$ 1, TR $\beta$ 2, TR $\beta$ 3). An additional TR $\alpha$  isoform (TR $\alpha$ 2) does not bind ligand and can function as a dominant negative transcriptional repressor. The cell type and developmental stage-specific expression of TR genes suggest that the protein products subserve different functions in brain development (23-25). A number of studies, both *in vivo* and *in vitro*, support distinct functions for TR $\alpha$  and TR $\beta$  proteins (26-32).

In both mammals and amphibians the expression of TR $\beta$  correlates closely with T<sub>3</sub> – dependent morphogenesis (23,33,34). Several lines of evidence support that view that TR $\beta$  plays a role in cell differentiation, and mediates cell cycle arrest when activated by T<sub>3</sub> (35). Over-

expression of TR $\beta$ 1 but not TR $\alpha$ 1 in the mouse-derived neuronal cell line Neuro-2a causes the cells to differentiate in response to T<sub>3</sub> treatment (35).

*Xenopus laevis* possesses four TR genes, two TR $\alpha$  and two TR $\beta$  (designated A or B) owing to its pseudotetraploidy (22). In the tadpole brain, TR $\alpha$  is expressed shortly after hatching whereas the expression of TR $\beta$  rises only during prometamorphosis when plasma TH titers are rising (34). The differential expression pattern of TR genes suggests distinct functions for TR $\alpha$  and TR $\beta$  during tadpole development. A systematic analysis of the developmental expression patterns of TR genes and/or proteins in the tadpole brain will help to elucidate whether the pleiotropic effects of TH are attributable to the differential expression of TR subtypes. In the present study we characterized the profile of brain cell proliferation during metamorphosis of *Xenopus laevis*. We analyzed the thyroid dependence of cell proliferation at different stages of development and the roles that TRs play in this process.

## Materials and Methods

### Animals and animal husbandry:

*Xenopus laevis* tadpoles (obtained from *Xenopus* One, Ann Arbor, MI) were raised in dechlorinated tap water (water temperature, 20-22 °C) and fed pulverized rabbit chow *ad libitum*. Developmental stages were assigned according to the Nieuwkoop and Faber method (36). For the analyses of hormonal regulation of cell proliferation, thyroid hormone and bromodeoxyuridine (BrdU) was administered by direct addition to the aquarium water; water was changed and hormone replenished daily over the treatment period. Tadpoles were treated with 3,5,3'-L-triiodothyronine (T<sub>3</sub>-sodium salt; Sigma-Aldrich, MO) at 50 nM and BrdU at 500 μM for various times (described below). The goitrogen methimazole (Sigma-Alrich, MO) was administered at 1mM for 10 days. At the termination of each experiment tadpoles were euthanized by immersion in 0.1% benzocaine before dissection.

### Experimental design:

The experimental designs are depicted graphically in Figure 1.

**Experiment 1.** We first tested whether T<sub>3</sub> can accelerate cell proliferation in the premetamorphic *Xenopus* tadpole brain. For this experiment we treated NF stage 52-53 tadpoles with or without T<sub>3</sub> (50 nM) plus BrdU for 48 hr. Brains were sagittally sectioned and stained for BrdU.

**Experiment 2.** We next determined the time course for the T<sub>3</sub> effect on brain cell proliferation in order to test whether T<sub>3</sub> causes a shortening of the cell cycle time. Tadpoles at NF stage 54-55 were treated with BrdU for 7 hr. After clearing in BrdU-free water for 24 hr, tadpoles were then treated with or without T<sub>3</sub> for either 24 or 48 hours (n = 6-12/treatment, Fig. 1a).

**Experiment 3.** We examined whether the effect of T<sub>3</sub> on brain cell proliferation is developmental stage-dependent. Tadpoles at different developmental stages (NF 52-53, 54-55, 56-57, 58-59, 60-61, or 62-63) were first treated with or without 50 nM T<sub>3</sub> for 48 hr followed by a brief pulse of 500 μM of BrdU for 3 hr before being sacrificed (n = 6-12 each group, see Fig. 1b). Brains were sampled and processed for fluorescent immunocytochemistry (ICC) for BrdU alone or combined BrdU/TR ICC or *in situ* hybridization (described below).

**Experiment 4.** We tested whether the increase in endogenous thyroid hormone production during prometamorphosis is responsible for the increase in brain cell proliferation (observed in Expt. 2 – see Results). Tadpoles at NF stage 56-57 were treated with or without 1 mM methimazole for 5 days. The methimazole treated group was then divided into two groups, one with and one without 10 nM T<sub>3</sub>. The treatments were then continued for 2 more days (see Fig. 1c). Thus, there were three treatment groups at the end of the experiment: untreated controls, methimazole, and methimazole+T<sub>3</sub>. Brains (n=5 each) were collected and processed for ICC as described below.

#### Thyroid hormone receptor riboprobes and TR antisera:

To generate subtype-specific riboprobes for *Xenopus laevis* TR mRNAs gene specific PCR primers were developed. Fragments of TR $\alpha$  (spanning amino acids 122-384) and TR $\beta$  (spanning amino acids 245-521) were PCR amplified and the PCR products were subcloned into the pGEM-T easy vector (Promega). The orientation and sequence of the cloned products were verified by DNA sequencing. Linearized plasmids were used for riboprobe synthesis (see below).

A rabbit anti-*X. laevis* TR polyclonal serum was a gift of Dr. Yun-Bo Shi. This antiserum was raised against a multiple antigenic peptide corresponding to amino acids 104-123 of *X. laevis* TR $\beta$ .

### Histochemistry

Tadpole heads were fixed in 4% paraformaldehyde in 0.01 M phosphate buffered saline (PBS) overnight at 4<sup>0</sup>C. Brains were removed from the skull and saturated with 30 % sucrose in 0.01M PBS at 4<sup>0</sup>C overnight. Brains were then embedded in OCT compound and frozen at -80<sup>0</sup>C. Brains were then cryosectioned at 20  $\mu$ m and sections were stored at -80<sup>0</sup>C until processing for either ICC or a combination of ICC and *in situ* hybridization histochemistry (ISHH).

### Dual fluorescent immunostaining of BrdU and TR protein:

For double-labeling ICC, tadpole brain sections were first processed for detection of BrdU followed by ICC for TR protein. Briefly, tissue sections were denatured with 2N HCl at 37<sup>0</sup>C for 30 min, rinsed with TBS and blocked with 5% normal goat serum in TBS at room temp. for 15 min. Tissue sections were then incubated with anti-BrdU serum (1:500; generated in rat; Serotec Inc., NC) for 24 hr at rt. Anti-rat-FITC allowed fluorescent detection of primary anti-BrdU immune complexes. For subsequent detection of TR protein, sections were rinsed in TBS and blocked with 5% normal goat serum in TBS for 15 min at rt. Sections were then incubated with rabbit anti-TR serum (1:1000) for 24 hr at 4<sup>0</sup>C. Anti-rabbit-Cy3 allowed fluorescent detection of primary anti-TR immune complexes.

Immunostaining of mitotic cells:

Cells undergoing mitosis were detected using a rabbit antiserum raised against human phosphorylated histone H3 (Upstate Biotechnology, NY). This protein becomes phosphorylated during M phase of the cell cycle and thus is a specific and sensitive marker for mitotic cells (Upstate Biotechnology). Brain sections were first incubated with the primary antibody at 1:500 overnight at 4°C. Primary immune complexes were detected using a secondary antibody conjugated with Cy3 (1:500, Jackson ImmunoResearch Laboratories). Sections were counterstained with a fluorescent green nuclear stain (SYTO 13, Molecular Probes).

In situ hybridization of TRs combined with BrdU ICC:

Linearized plasmid templates were prepared, and sense or antisense digoxigenin (DIG)-labeled TR probes were transcribed *in vitro* using the DIG RNA labeling kit (Boehringer-Mannheim). For ISHH, cryosections were incubated with 1 µg of sense or antisense probe overnight at 55°C. Tissue preparation, hybridization and rinsing followed standard protocols (37). A monoclonal mouse anti-DIG antibody (1:500; Roche) was used to detect RNA hybrids. Primary immune complexes were detected with an anti-mouse secondary antibody conjugated with Cy3. The specificities of the riboprobes were confirmed by the absence of staining by sense probes on consecutive sections and by the absence of staining by antisense probes in premetamorphic animals (for TRβ).

For dual fluorescent immunodetection of TR mRNA (by ISHH) and BrdU-labeled cells (by ICC), sections were immunostained for BrdU following the detection of TR mRNA. Sections were analyzed by confocal microscopy (Zeiss laser scanning confocal microscope). Sections

were scanned at 1  $\mu\text{m}$  thickness using Z-stack to determine if TR mRNAs and BrdU were colocalized.

Quantification and statistical analysis:

Micrographs were scanned and electronic images generated. Images were analyzed in a blinded manner using Scion Image software. The total area of BrdU-immunoreactivity in different regions of the tadpole brain was determined, and these values were compared by one-way ANOVA followed by a post-hoc analysis using Fisher's LSD test. Cells staining positive for H3 were individually counted and differences between treatments compared by one-way ANOVA.

## Results and Discussion

### **Experiment 1. T<sub>3</sub> stimulates cell proliferation in periventricular zones of the premetamorphic tadpole brain.**

Premetamorphic tadpoles at NF stage 53-54 were cultured in water containing 500  $\mu$ M of BrdU with or without 50 nM T<sub>3</sub> for 48 hr. Treatment with T<sub>3</sub> resulted in a dramatic increase in BrdU labeled cells in periventricular zones throughout the tadpole brain (Fig. 2).

### **Experiment 2. T<sub>3</sub> shortens cell cycle time in the premetamorphic tadpole brain.**

Premetamorphic tadpoles at NF stage 53-54 were pulsed with 500  $\mu$ M of BrdU for 7 hours, cleared in BrdU-free water for 24 hours and then treated with or without 50 nM T<sub>3</sub> for 24 or 48 hours. At 24 hr after addition of T<sub>3</sub> there was no difference in BrdU labeling in brains of control or T<sub>3</sub>-treated tadpoles. However, by 48 hr T<sub>3</sub> treatment produced a dramatic increase in the number of BrdU positive cells compared to controls (Fig. 3). The BrdU immunoreactivity seen after 48 hr of T<sub>3</sub> treatment was significantly weaker compared to controls owing to the dilution of BrdU following cell division. This finding supports the view that stem cells in T<sub>3</sub> treated brains have undergone more cell divisions compared with untreated animals (recall that the animals were given a brief exposure to BrdU prior to hormone treatment). The weak and diffuse staining pattern of BrdU-ir in the brain following T<sub>3</sub> treatment was not observed in the attached pituitary, which showed intense BrdU immunoreactivity (data not shown). This result suggests that T<sub>3</sub> stimulated cell proliferation by shortening cell cycling time in the premetamorphic tadpole brain but not in the pituitary.

Because the result with BrdU could might be explained by differences in BrdU uptake caused by T<sub>3</sub> treatment, we used an independent method to detect mitotic cells.

Immunocytochemistry for phospho H3, which identifies cells in M phase, revealed a 4-fold increase over controls in the number of mitotic cells in brains of premetamorphic tadpoles treated with T<sub>3</sub> for 48 hours (Fig. 4), thus confirming our results with BrdU labeling.

### **Thyroid hormone receptor alpha mRNA is expressed in proliferating cells**

Dual labeling for TR $\alpha$  mRNA and BrdU revealed that all BrdU-ir cells express TR $\alpha$  mRNA (Fig. 5). Highest staining for TR $\alpha$  mRNA was seen in BrdU-positive cells, but not all TR $\alpha$  positive cells were BrdU positive. These data support that view that T<sub>3</sub> action on brain cell proliferation is cell autonomous, and may be mediated, at least in part, by the TR $\alpha$ .

### **Experiment 3. Developmental patterns of cell proliferation in *Xenopus* tadpole brain**

Cell proliferation in *Xenopus* tadpole brain during metamorphosis is restricted to the periventricular zones. At premetamorphic stages, the rate of brain cell proliferation is low (Fig. 6). It then peaks at stage 56-57 and remains at a high level during prometamorphic stages. The proliferation rate then declines significantly at late prometamorphic and climax stages (Fig. 6). In addition, the rate of cell proliferation in different regions of the brain changes with development (Table 1). At premetamorphic stages, there are few mitotic cells in the tectum (Table 1, Fig. 7). During prometamorphic stages, more mitotic cells are observed in the tectum. As tadpoles develop, cell proliferation slows in the tectum as evidenced by few mitotic cells (Table 1, Fig. 7).

T<sub>3</sub> treatment of tadpoles during premetamorphosis dramatically increased cell proliferation in periventricular zones throughout the brain, as measured by both phospho H3-ir and BrdU-ir (Fig. 6). However, this effect of T<sub>3</sub> declined with the progression of metamorphosis. By NF stage 56-57 tadpoles became refractory to T<sub>3</sub> treatment, and this persisted through metamorphic climax.

**T<sub>3</sub> increases TR $\beta$  protein expression in premetamorphic tadpole brain and TR $\beta$  is excluded from proliferating cells.**

In premetamorphic tadpoles few BrdU-ir or TR-ir cells are detected (Fig. 8). Treatment of premetamorphic tadpoles with T<sub>3</sub> significantly increased cell proliferation and elevated TR protein expression. However, only a small portion of BrdU-ir cells express TR $\beta$ , as indicated by the yellow cells (Fig. 8). These data suggest that TR $\beta$  does not mediate the effect of T<sub>3</sub> on brain cell proliferation.

**Experiment 4. Endogenous thyroid hormone plays a role in the rise in brain cell proliferation during prometamorphosis**

During prometamorphosis, when the rate of cell proliferation is highest, blockade of endogenous thyroid hormone production by treatment with the goitrogen methimazole reduced brain cell proliferation. Conversely, the addition of 10 nM T<sub>3</sub> reinstated the proliferation rate to the level the controls (Fig. 9). These data support the conclusion that the increase in endogenous thyroid hormone production that occurs during prometamorphosis is responsible for the increase in brain cell proliferation that occurs at this time (Figs. 6&7).

## **Conclusions**

The present study demonstrates that premetamorphic tadpole brains are very sensitive to thyroid hormone in terms of brain cell proliferation. The rise in brain cell proliferation during prometamorphosis is driven by rising titers of thyroid hormone. However, these cells become refractory to the proliferative effects of thyroid hormone by late prometamorphosis. While a proliferative effect of thyroid hormone was observed in the brain, no such effect was observed in the pituitary gland. Our data also support the conclusion that thyroid hormone acts to promote brain cell proliferation in a cell autonomous manner, since thyroid hormone receptors ( $TR\alpha$ ) are highly expressed in regions of active neurogenesis. Furthermore,  $TR\beta$  is not expressed in proliferating cells, supporting the view that this receptor mediates the differentiative actions of thyroid hormone (35).

## References

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Table 1. Relative numbers of BrdU-ir/H3-ir cells in different regions of brains of tadpoles at different stages:

	Telecephalon	diencephalon	tectum/hypothalamus	cerebellum
Stage 52-53	+	+	+	+
Stage 54-55	+	+	+	+
Stage 56-57	+++	+++	++++	+++
Stage 58-59	++	++	+	++
Stage 60-61	++	+	-	+
Stage 62-63	+	+	-	-

## Figure legends

**Fig. 1.** Schematic representations of tadpole treatment paradigms. **Experiment 1.**

Premetamorphic tadpoles (NF stage 53-54) were treated +/-  $T_3$  plus BrdU for 48 hr before sacrifice. **Experiment 2.** Premetamorphic tadpoles (NF stage 53-54) were pulsed with BrdU for 7 hr then treated +/-  $T_3$  for 24 or 48 hr (in the absence of BrdU). **Experiment 3.** Tadpoles at different developmental stages were treated +/-  $T_3$  for 48 hr and pulsed with BrdU for the final 3 hr to label mitotic cells. **Experiment 4.** Early prometamorphic tadpoles (NF stage 55-56) were treated with the goitrogen methimazole (MMI; 1 mM) for seven days before sacrifice. A subset of MMI-treated tadpoles received  $T_3$  for the final 2 days.  $T_3$  was administered by addition to the aquarium water. BrdU was added to the aquarium water to a final concentration of 500  $\mu$ M.

**Fig. 2. Experiment 1.**  $T_3$  induces cell proliferation in the brain of premetamorphic tadpoles (NF stage 53-54). Tadpoles were treated as described in Materials and Methods and Fig. 1. Brains were sectioned sagittally and processed for BrdU ICC.

**Fig. 3. Experiment 2.**  $T_3$  shortens cell cycle time in premetamorphic tadpole brain. Tadpoles were treated as described in Materials and Methods and Fig. 1. Brains were sectioned coronally and processed for BrdU ICC. There were no differences in BrdU-ir between brains from control (a) or  $T_3$  treated (b) animals 24 hr after treatments. After 48 hr there was a significant increase in the number of BrdU-ir cells in brains of  $T_3$  -treated animals (d) compared to controls (c). Also note the faint immunoactivity in 48 hr  $T_3$ -treated animals compared to that of the controls. P, pituitary; IIIV, 3rd ventricle; V, ventricle. Scale bar, 100  $\mu$ m.

**Fig. 4. Experiment 2.** T<sub>3</sub> increases the number of mitotic cells in premetamorphic tadpole brain as determined by ICC for phospho H3. Twenty-four hr after treatments there was no difference in the number of mitotic cells in brains of control or T<sub>3</sub>-treated animals. At 48 hr T<sub>3</sub> caused a 4-fold increase in the number of mitotic cells compared to controls. \* indicates statistically significant difference from the controls (P<0.001; t-test).

**Fig. 5.** Thyroid hormone receptor alpha (TR $\alpha$ ) mRNA colocalizes with BrdU-ir in prometamorphic tadpole brain. Dual labeling histochemistry was conducted for TR $\alpha$  mRNA (red; by ISHH) and BrdU (green; by ICC) on saggital sections of tadpole brain (region shown is the diencephalon, tectum and cerebellum) of an early prometamorphic T<sub>3</sub>-treated tadpole. Note the colocalization of TR $\alpha$  mRNA and BrdU-ir in cords of cells adjacent to the ventricles. Hypo, hypothalamus; Pit, pituitary. Scale bar, 50  $\mu$ m.

**Fig. 6. Experiment 3.** Profiles of brain cell proliferation during normal metamorphosis and following T<sub>3</sub> treatment, measured by the number of cells staining positive for phospho H3. During normal development, cell proliferation is low at premetamorphic stages and peaks during prometamorphosis. Prior to metamorphic climax the rate of proliferation declines to a level that does not differ from premetamorphic stages. T<sub>3</sub> treatment significantly increased cell proliferation at premetamorphic and early prometamorphic stages but failed to alter cell proliferation at later stages.

**Fig. 7. Experiment 3.** Developmental changes in BrdU labeling in the tadpole optic tectum. There are numerous BrdU-ir cells at prometamorphosis (NF stage 56-57; b); whereas, there are

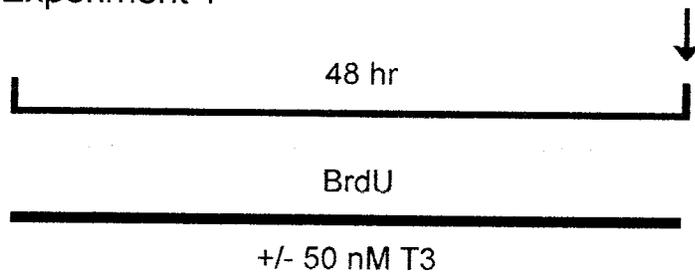
few BrdU-ir cells in premetamorphic (NF stage 52-53; a) or late prometamorphic (NF stage 61-62; c) brains. OT, optic tectum; V, ventricle. Scale bar, 100  $\mu$ m.

**Fig. 8.** Dual fluorescent immunostaining for BrdU (green) and TR $\beta$  (red) in tectum of premetamorphic tadpoles (NF stage 52-53) without (A-C) or with (D-F) T<sub>3</sub> treatment (50 nM for 48 hr). In premetamorphic tadpoles few BrdU-ir or TR-ir cells are detected (A – BrdU, B – TR $\beta$ , C- merge). T<sub>3</sub> treatment of premetamorphic tadpoles significantly increased cell proliferation (D – BrdU) and elevated TR protein expression (E – TR $\beta$ ). Only a small portion of BrdU-ir cells express TR $\beta$ , as indicated by the yellow cells (F – merge).

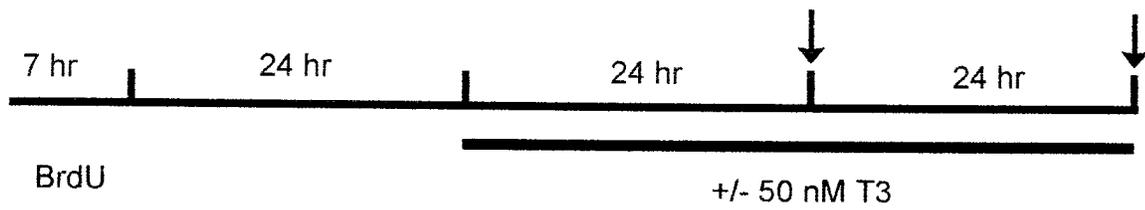
**Fig. 9.** Blockade of endogenous thyroid hormone synthesis by goitrogen treatment reduces brain cell proliferation. Prometamorphic tadpoles (NF stage 56-57) were treated with or without methimazole (MMI; 1 mM) for a week. A subset of animals received T<sub>3</sub> (10 nM) for the final 2 days of the treatment period. MMI treatment reduced brain cell proliferation, as measured by the staining for phospho H3. Addition of exogenous T<sub>3</sub> to the MMI-treated tadpoles restored the rate of proliferation to a level comparable to that of controls.

Figure 1.

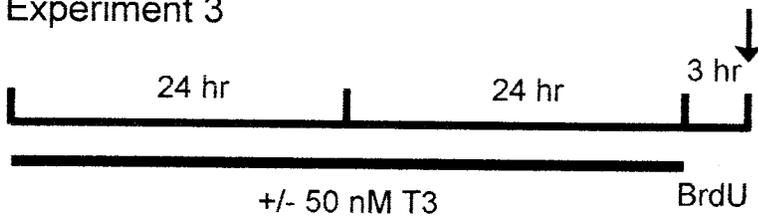
Experiment 1



Experiment 2



Experiment 3



Experiment 4

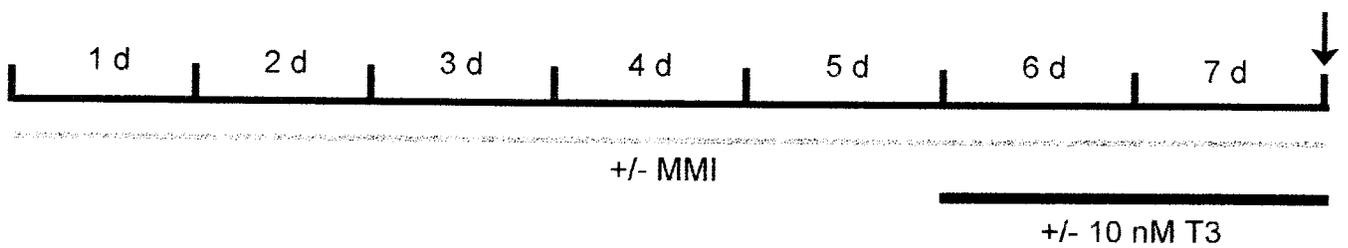
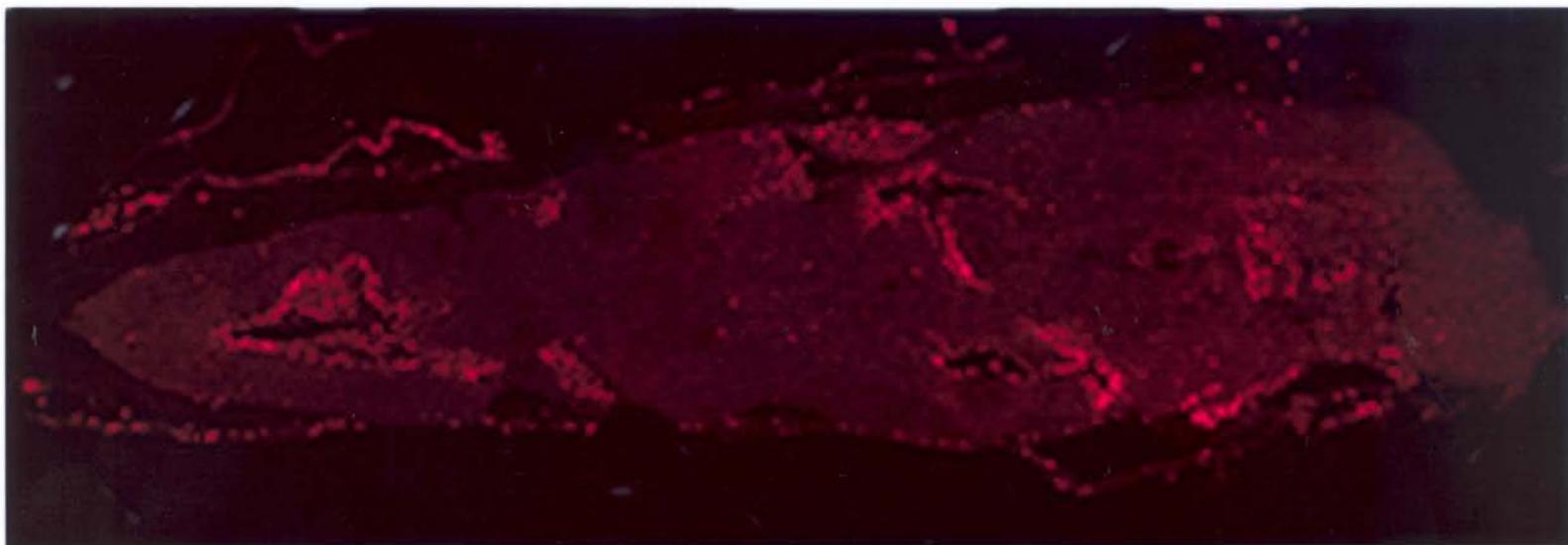


Figure 2

Control



T3-treated

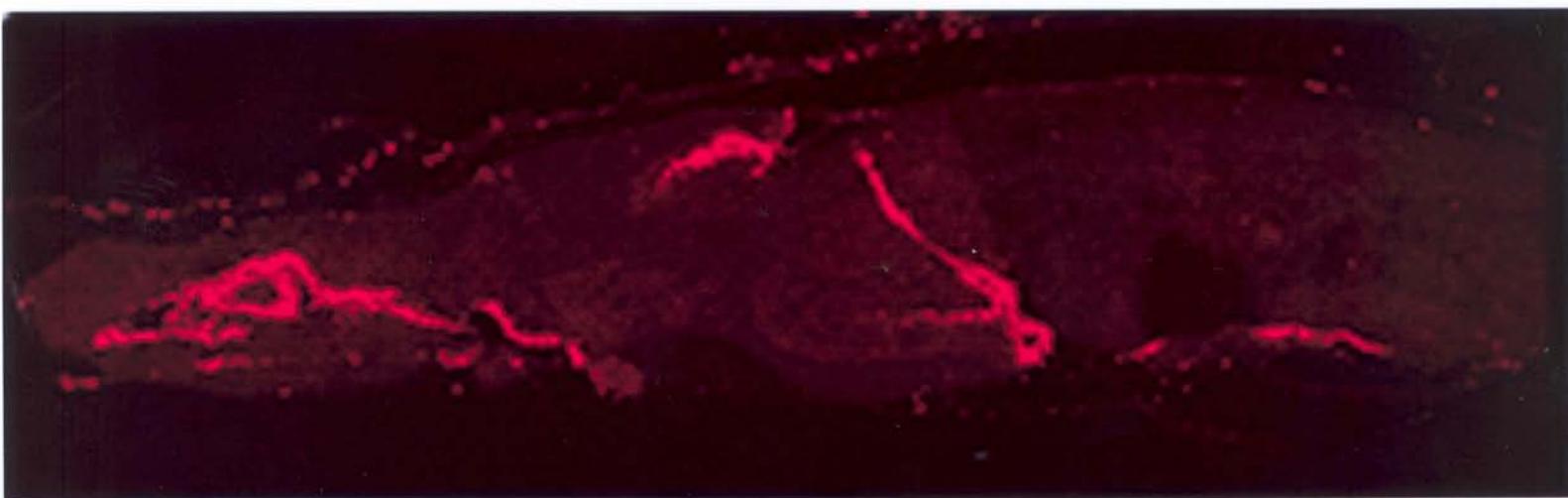


Figure 3

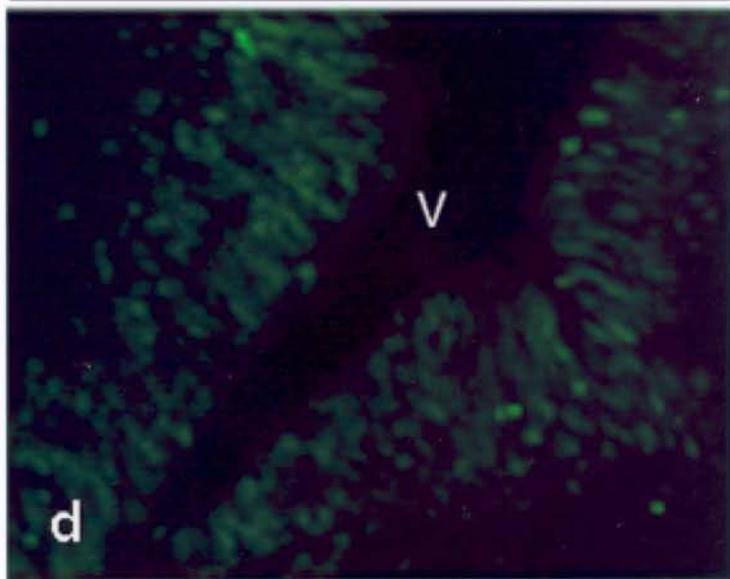
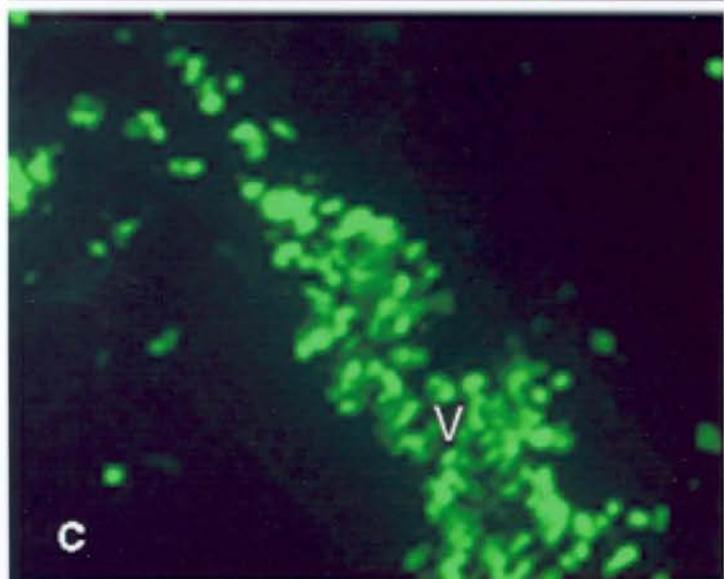
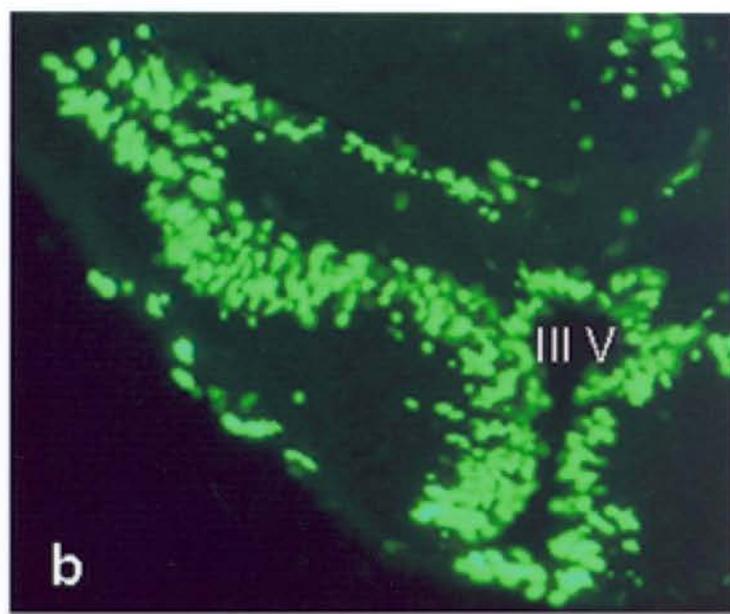
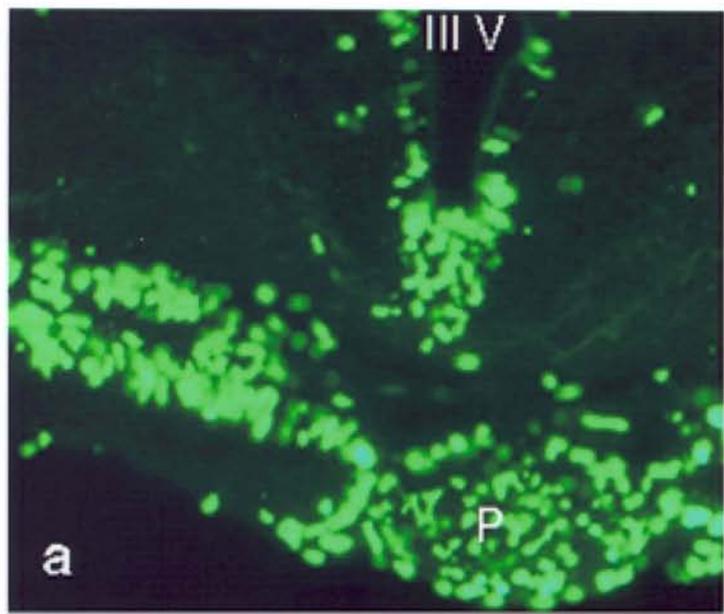


Fig. 4

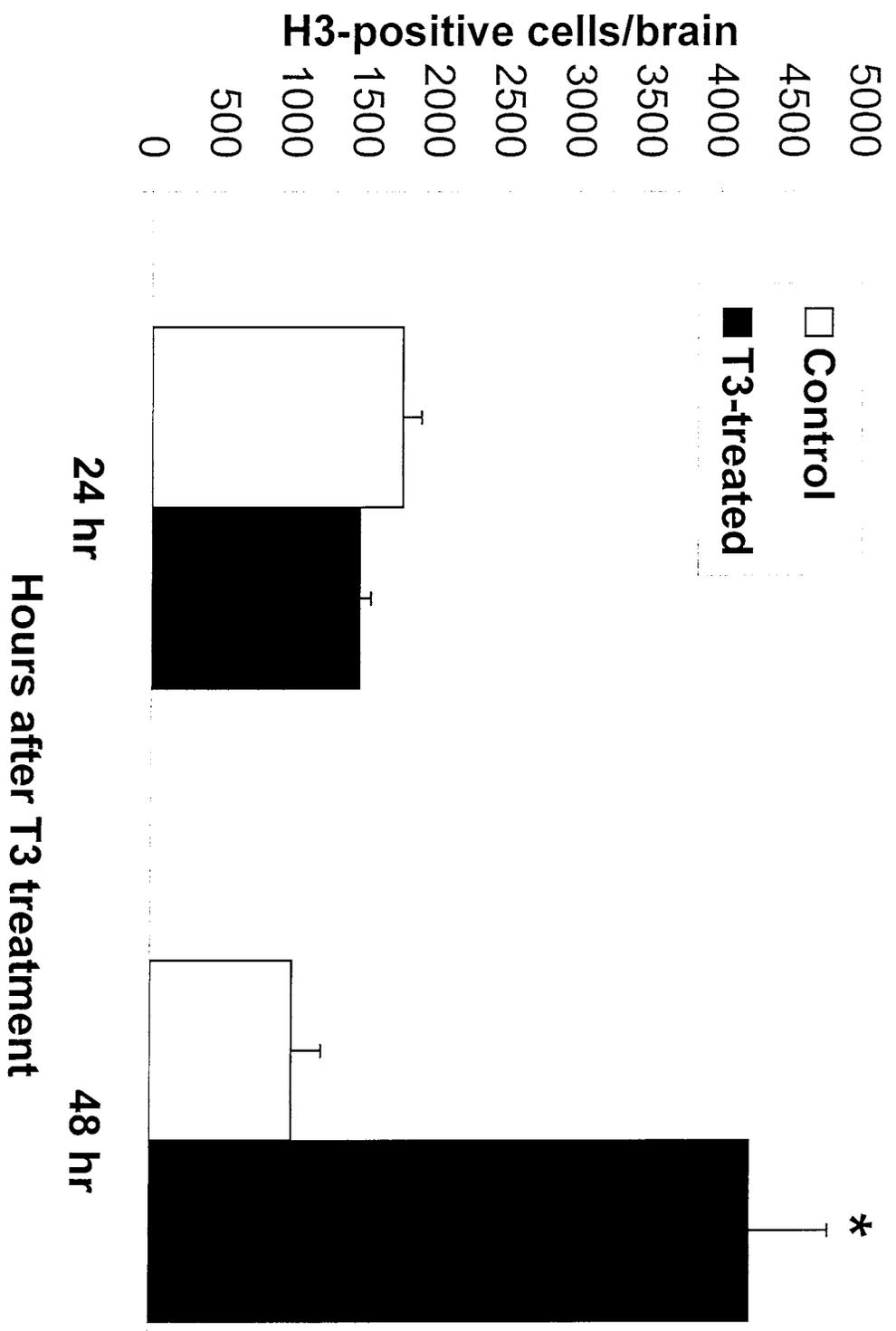


Figure 5

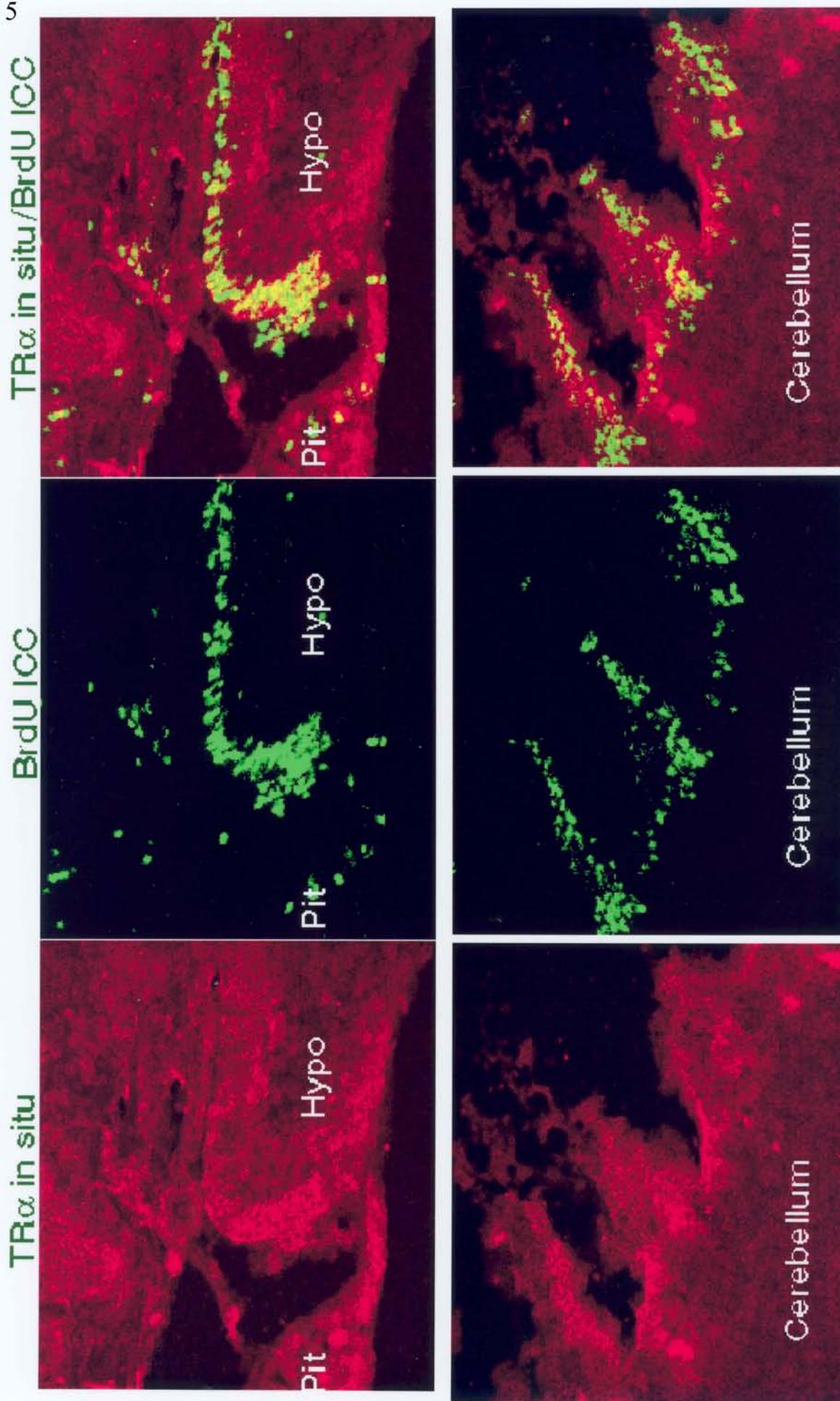


Fig. 6

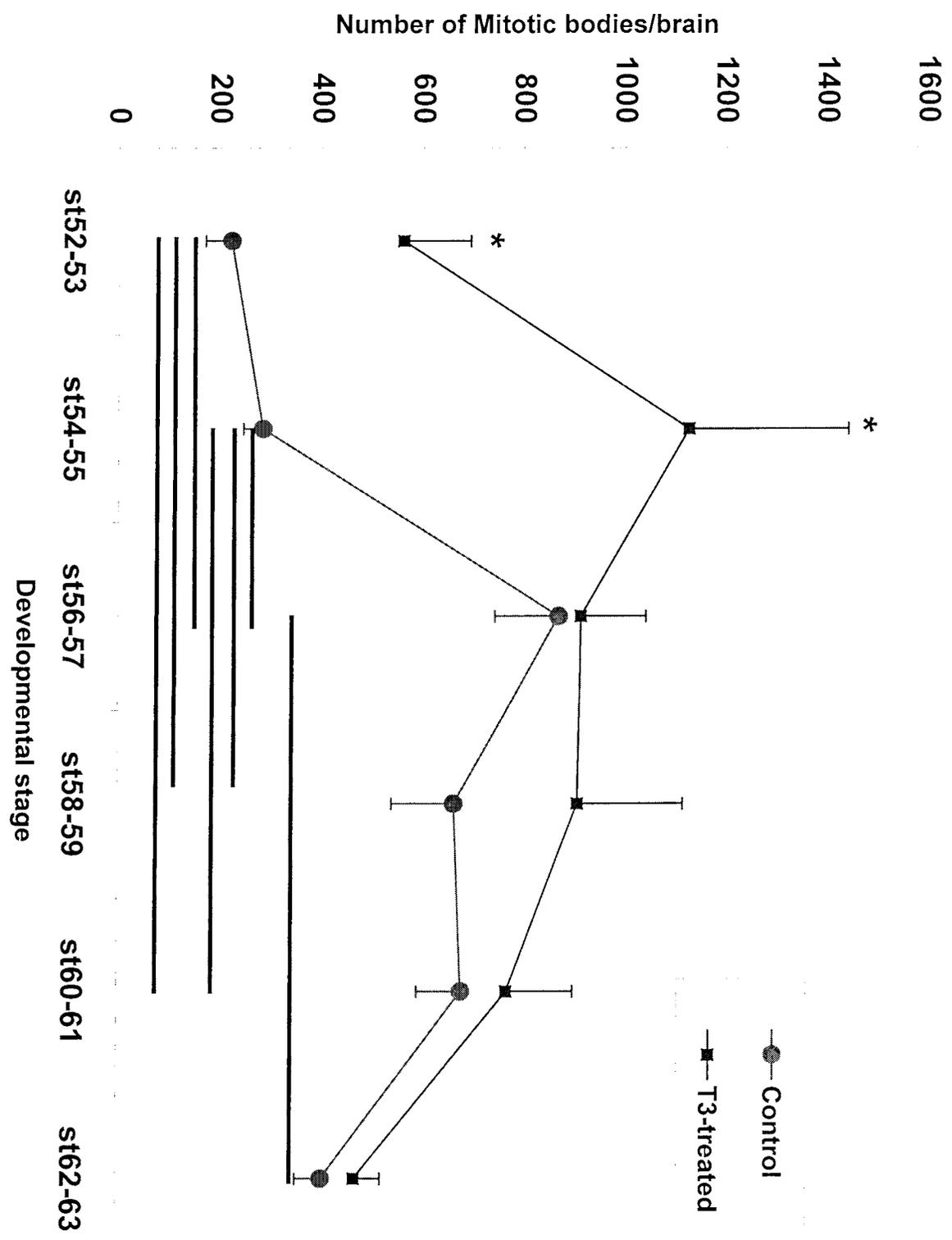


Figure 7

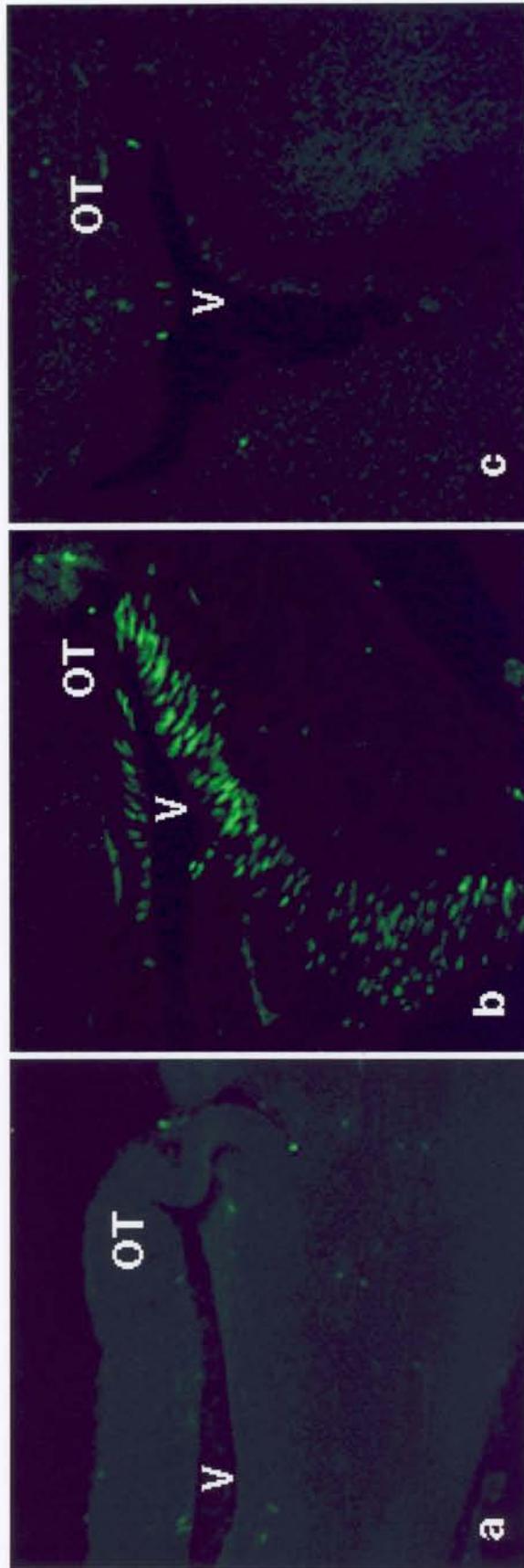


Figure 8

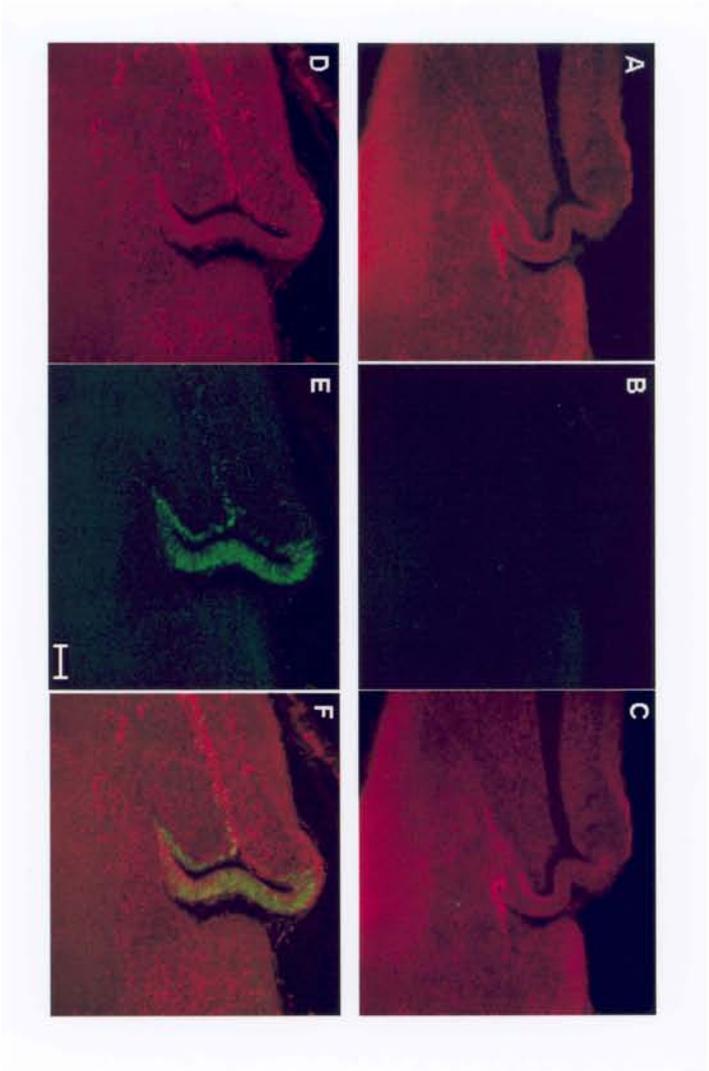


Fig. 9

