

Ontogeny of pituitary thyrotrophs and regulation by endogenous thyroid hormone feedback in the chick embryo

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Abstract

Increased thyroid hormone production is essential for hatching of the chick and for the increased metabolism necessary for posthatch endothermic life. However, little is known about the ontogeny and distribution of pituitary thyrotrophs during this period or whether pituitary thyroid-stimulating hormone (TSH) production is regulated by endogenous thyroid hormones during chick embryonic development. This study assessed the abundance and location of pituitary thyrotrophs and the regulation of TSH β peptide and mRNA levels by endogenous thyroid hormones prior to hatching. TSH β -containing cells were first detected on embryonic day (e) 11, and the thyrotroph population increased to maximum levels on e17 and e19 and then decreased prior to hatching (d1). Thyrotroph distribution within the cephalic lobe of the anterior pituitary was determined on e19 by whole-mount immunocytochemistry for TSH β peptide and by whole-mount *in situ* hybridization for TSH β mRNA. Thyrotroph

distribution within the cephalic lobe was heterogeneous among embryos, but most commonly extended from the ventral medial region to the dorsal lateral regions, along the boundary of the cephalic and caudal lobes. Inhibition of endogenous thyroid hormone production with methimazole (MMI) decreased plasma thyroxine (T₄) levels and increased pituitary TSH β mRNA levels on e19 and d1. However, control pituitaries contained significantly more TSH β peptide than MMI-treated pituitaries on e17 and e19, suggesting higher TSH secretion into the blood in MMI-treated groups. We conclude that thyrotroph abundance and TSH production increase prior to hatching, that thyrotrophs are localized heterogeneously within the cephalic lobe of the anterior pituitary at that time, and that TSH gene expression and secretion are under negative feedback regulation from thyroid hormones during this critical period of development.

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Introduction

Thyroid hormones are involved in the regulation of growth, metabolism, heat production, gonadal development, molting, migration, and hatching in birds (McNabb 2000). Thyroid-stimulating hormone (TSH) plays an important role in the hypothalamic–pituitary–thyroid (HPT) axis in mammals. However, due to a lack of homologous antisera to TSH in birds and the only recent description of an anti-peptide antibody (Iwasawa *et al.* 2002), the physiological role of TSH in the regulation of the HPT axis in avian species has not been intensively researched. Most of what has been learned about the biology of TSH secretion has been through the use of bioassays, heterologous antisera assays, and assays for the α -subunit. Thyrotropin-releasing hormone (TRH) stimulates secretion of TSH-like bioactivity from pituitaries cultured *in vitro* (Scanes 1974), and increases plasma levels of thyroxine (T₄) *in vivo* (Kuhn *et al.* 1988). In addition to TRH, corticotropin-releasing hormone (CRH) has been

shown to induce TSH release in chickens, and somatostatin can diminish the CRH- and TRH-induced TSH response through the type 2 and type 5 somatostatin receptors (Geris *et al.* 1996, 2003a,b, DeGroef *et al.* 2003). Thyroid-stimulating activity has been localized to the cephalic lobe of the anterior pituitary (Brasch & Betz 1971, Radke & Chiasson 1974, 1977), and cells containing immunoreactive TSH have been localized to the cephalic lobe in histological sections stained with antisera against mammalian TSH (Sharp *et al.* 1979, Thommes *et al.* 1983, Mikami 1986, Murphy & Harvey 2001, 2002, Sasaki *et al.* 2003) or with an antiserum raised against a peptide based on the chicken TSH β sequence (Iwasawa *et al.* 2002, Nakamura *et al.* 2004). Similarly, pituitary cells that stained with monoclonal antibodies against the chicken α -subunit and that did not also stain with anti-chicken luteinizing hormone (LH) β and follicle-stimulating hormone (FSH) β monoclonal antibodies were localized to the cephalic lobe (Berghman *et al.* 1993). Thommes *et al.* (1983) reported that the thyrotrophs were first detectable

on embryonic day (e) 6.5 and dramatically increased on e11.5, the time in embryonic development when the HPT axis is thought to become functionally mature in chickens (Thommes *et al.* 1983). However, Murphy & Harvey (2001, 2002) found no immunoreactive TSH in the pituitary on e7, and Nakamura *et al.* (2004) first detected TSH-containing cells on e10. The α -subunit mRNA is expressed as early as e3.5 (Kameda *et al.* 2000), while TSH β mRNA levels are present from e9 and increase at the end of embryonic development (Gregory *et al.* 1998, Nakamura *et al.* 2004). Depression of plasma thyroid hormone levels by goitrogen administration has been shown to increase the size of purported thyrotrophs and the thyroid-stimulating bioactivity of the pituitary, suggesting that thyroid hormones exert negative feedback on TSH production in mature birds (Sharp *et al.* 1979), and treatment of e19 pituitary cells *in vitro* with triiodothyronine (T_3) significantly decreased levels of TSH β mRNA (Gregory *et al.* 1998). However, nothing is known about the regulation of TSH protein production by endogenous thyroid hormones in the developing chick embryo, even though production of thyroid hormones is essential for hatching and progression to endothermic life. Pituitary TSH β mRNA levels increase prior to hatching, coincident with an increase in plasma T_4 and T_3 , raising the possibility that pituitary TSH production is free from the negative effects of thyroid hormones at this critical juncture of development. In the present study we validated an antiserum raised against purified rat TSH β subunit to detect chicken TSH β peptide. This antiserum was used in immunocytochemistry, along with whole-mount *in situ* hybridization, to characterize the distribution of thyrotrophs within the cephalic lobe of the anterior pituitary and to quantify the abundance of thyrotrophs during embryonic development. Finally, TSH β gene and peptide expression were examined following *in vivo* goitrogen treatment of developing embryos, to assess involvement of thyroid hormone negative feedback in controlling TSH production prior to hatching. We report that TSH β gene expression and pituitary content are regulated by negative feedback from endogenous thyroid hormones by the end of chick embryonic development, even though levels of thyroid hormones, pituitary TSH gene expression, and pituitary thyrotrophs all increase prior to hatching. These findings suggest that hypothalamic feed-forward stimulation, rather than a lack of thyroid hormone negative feedback, is the predominant cause of the increased HPT axis activity at the end of embryonic development necessary for hatching and endothermic existence.

Materials and Methods

Animals, incubations and injections

All animals were Avian \times Avian broiler strain chicken embryos obtained from Allen's Hatchery (Seaford, DE,

USA). Eggs were set in a humidified incubator at 37.5 °C, and that day was designated as e0. Ethanol-swabbed eggs were injected with a sterile 1 ml syringe by inserting the needle (25-gauge) into the albumen at the junction of the air cell, which was visualized by candling and marked. Chicken eggs received injections (in 100 μ l sterile water) of 125 μ g of the thyroid hormone synthesis inhibitor methimazole (MMI) on e13, 250 μ g on e15, 500 μ g on e17, and 750 μ g on e19. MMI doses were chosen based on preliminary studies that defined doses effective at suppressing development and hatching but which were non-lethal. Control eggs were injected with sterile water. Water was used as the vehicle because the injections were made into the water-containing albumen. The injection hole was sealed with cellophane tape. Animals were used with approval from our Institutional Animal Care and Use Committee.

Western blotting

Chicken pituitaries were isolated, pooled, and homogenized in either 0.1 M HCl, or a protein extraction buffer (10% glycerol, 15 mM HEPES pH 7.9, 300 mM NaCl, 50 mM KCl, 0.5% deoxycholate, 0.5% CHAPS, 0.5% Triton-X 100, 1 x proteinase inhibitor cocktail (Roche Molecular Biochemicals, Mannheim, Germany) and 2 mM EDTA pH 8.0) in a 1 ml Dounce homogenizer (Wheaton, Millville, NJ, USA). Protein concentrations in pituitary extracts were quantified by the bicinchoninic acid assay (Pierce; Rockford, IL, USA) according to manufacturer's directions and extracts were separated on a 12.5% SDS-polyacrylamide gel using a Tris-Tricine-SDS running buffer under a current of 60 mA. Proteins were transferred overnight to a 0.45 μ m supported-nitrocellulose membrane (Bio-Rad; Hercules, CA, USA) using a 0.2 M phosphate buffer at 22 V. The membrane was washed twice for 5 min in PBS (pH 7.4) and then incubated for 30 min in 0.3% H_2O_2 in PBS. The membrane was washed again for 5 min in PBS and then blocked with 1% normal goat serum (NGS) for 1 h. After blocking, the membrane was incubated with the rabbit anti-rat TSH β antiserum (NIDDK-anti-rBetaTSH-IC-1) diluted 1:10 000 in 0.05% Tween 20-PBS (T-PBS) with 1% NGS for 3 h. The membrane was washed five times for 5 min with T-PBS and then incubated with a goat anti-rabbit biotinylated IgG secondary antibody (Vector Laboratories, Burlingame, CA, USA) in T-PBS with 1% NGS for 2 h. Again the membrane was washed 5 times for 5 min with T-PBS. After washing, the membrane was incubated with Vectastain ABC reagent (Vector Laboratories), an avidin-biotin signal amplification complex, for 30 min. The membrane was washed again with PBS and developed using the Vectastain VIP horseradish peroxidase substrate kit (Vector Laboratories). When statistical tests were applied, bands were scanned and quantified using ImageQuant software (Molecular Dynamics; Sunnyvale,

CA, USA). The NIDDK-anti-rBetaTSH-IC-1 and purified rat TSH were obtained through NHPP, NIDDK and AF Parlow (Harbor UCLA Medical Center, Torrance, CA, USA), L Berghman (Texas A&M University, College Station, TX, USA) and J Proudman (Germplasm and Gamete Physiology Laboratory, USDA, Beltsville, MD, USA) kindly provided the chicken LH and FSH, respectively, used in the validation of the rat TSH β antiserum for chicken TSH β .

Immunocytochemistry (ICC)

Cell culture reagents were from Invitrogen Gibco (Grand Island, NY, USA) unless otherwise noted. Pituitaries were removed from embryos using a dissecting microscope and dissociated by trypsin digestion (1 mg/ml) in SMEM (supplemented with 0.1% BSA, 100 U/ml penicillin and 100 μ g/ml streptomycin) and mechanical agitation at 37.5 °C under 5% CO₂/95% O₂ for 45 min, trituration and gassing every 15 min as previously described (Porter *et al.* 1995). ICC was performed as previously described (Liu *et al.* 2003, Bossis *et al.* 2004), with the following changes. Rat TSH β antiserum (NIDDK-anti-rBetaTSH-IC-1) was used as the primary antiserum at 1:10 000. Cells were washed and then incubated with Vectastain ABC reagent. Finally, the plate was washed again with PBS and developed using the Vectastain VIP horseradish peroxidase substrate kit. Cells were counted using an inverted light microscope and positively stained cells expressed as a percentage of all cells counted. Counting of pituitary cells began in the center of each well, and a minimum of 300 cells was evaluated for each duplicate well for each age in each replicate experiment.

Whole-mount in situ hybridization

The whole-mount *in situ* hybridization procedure, with modifications noted below, was performed as previously described (Bossis & Porter 2000). Briefly, pituitaries were isolated from e19 embryos and fixed. Pituitaries were then dehydrated in an ethanol series, stored overnight, rehydrated the next day, permeabilized with Proteinase K, and then post-fixed. Pituitaries were hybridized with digoxigenin-labelled riboprobes overnight, washed with 2 x and 0.2 x SSC, incubated with alkaline phosphatase-conjugated sheep anti-digoxigenin antibody (1:2000), and developed with 4-nitro blue tetrazolium chloride and 160 μ g/ml 5-bromo-4-chloro-3-indolylphosphate (Roche Applied Science, Indianapolis, IN, USA). The sense and antisense digoxigenin-labelled riboprobes (Boehringer Mannheim, Mannheim, Germany) were generated from a plasmid previously described (Gregory *et al.* 1998).

Whole-mount immunocytochemistry

Pituitaries from e19 embryos were isolated and fixed in 3.7% formaldehyde at 4 °C for 30 min. Pituitaries were

washed in PBS and dehydrated in an increasing methanol series (50%, 75%, 95%, and 100%), quenched with 0.3% H₂O₂ in methanol for 5 min, and stored overnight in methanol at -20 °C. Pituitaries were rehydrated the next day, permeabilized with 0.02% trypsin in 0.1% TritonX-100 in PBS (PBT) for approximately 4 min, and then the digestion was stopped by quenching in 0.1% soybean trypsin inhibitor in PBT with 1 mM CaCl₂ (Sigma, St Louis, MO, USA) for 2 min. Pituitaries were washed in PBT three times for 5 min. The pituitaries were blocked in 2% NGS for 1 h and incubated overnight at 4 °C with the rabbit anti-rat TSH β antiserum (1:5000) in PBS with 1% NGS. Pituitaries were then washed six times for 5 min in PBT and then incubated with biotinylated anti-rabbit IgG (Vector Laboratories) for 2 h. Pituitaries were washed in PBT three times for 5 min, then three times for 10 min, and incubated in Vectastain ABC reagent for 1 h. Pituitaries were washed in PBT three times for 5 min, three times for 20 min, and then developed with Vectastain VIP horseradish peroxidase substrate kit.

Plasma collection and radioimmunoassays (RIAs)

Plasma was collected by removing the shell apical to the air sac junction, visualizing the blood vessels by moistening the inner shell membrane, nicking a vessel with a 25-gauge needle, and drawing the blood into a 1 ml syringe wetted with 0.5 M EDTA. Blood samples were discarded if egg albumen became mixed with blood. The hematocrit was checked by visual inspection. Collected blood samples were transferred to 1.5 ml microcentrifuge tubes on ice containing 2.5 μ l of 0.5 M EDTA. Blood samples were centrifuged, and the plasma was collected and frozen at -20 °C until assayed. RIA was performed using the Coat-A-Count Free T₄ RIA kit from Diagnostic Products Corporation (Los Angeles, CA, USA) according to the manufacturer's protocol. Two different assays were used in attempts to assess plasma levels of TSH, the rat TSH RIA from American Laboratory Products Company (Windham, NH, USA) and the human TSH immunoradiometric assay from Diagnostic Products Corporation. Neither TSH assay proved effective at measuring chicken TSH.

RNA extraction and Northern blotting

Pituitaries were isolated from embryos and snap frozen in liquid nitrogen and stored at -80 °C until RNA extraction. Pituitaries were homogenized in a 1 ml Dounce homogenizer and RNA was isolated using TRIzol according to the manufacturer's protocol (Invitrogen Gibco). RNA was suspended in nuclease-free water, quantified by absorbance at 260 nm, and then stored at -80 °C until use. Approximately 5 μ g of total RNA/sample in RNA Loading Buffer with Ethidium Bromide (Sigma) were separated on 1% agarose-6% formaldehyde gels with

MOPS (Invitrogen Gibco) running buffer at 200 V and transferred overnight to a Zeta-Probe nylon membrane (Bio-Rad, Hercules, CA, USA) by capillary action. The RNA was cross-linked to the membrane in a UV cross-linker and pre-hybridized at 65 °C in PerfectHyb Plus hybridization solution (Sigma) for 3 h in a rotating hybridization oven. ³²P dCTP (Amersham Biosciences, Piscataway, NJ)-labelled TSH β cDNA probes were generated by PCR using *Taq* polymerase (MBI Fermentas; Hanover, MD, USA). The TSH β template: GTGGAG AAGCGGGAGTGTGCCTACTGCCTGGCCACAAC ACCACCATCTGCGCTGGATTTTGCATGACACG GGACAGCAACGGCAAGAAGCTGCTACTCAAA AGTGCTCT and primer: AGAGCACTTTTGAGTAG CAG were synthesized by Sigma-Genosys. PCR products were separated from unincorporated nucleotides by centrifugation through a Spin Column-30 (Sigma) and counted in a beta counter to determine radioactivity. Approximately 1×10^6 counts per minute (c.p.m.) of probe was added per ml of hybridization solution and allowed to hybridize overnight at 65 °C. The membrane was then washed twice at low stringency with 2 x SSC (300 mM NaCl, 30 mM sodium citrate)-0.1% SDS at room temperature. The membrane was washed with 0.2 x SSC-0.1% SDS twice at 65 °C, and then twice with 0.1 x SSC-0.1% SDS at 65 °C for high stringency washes. Then, the membrane was placed between two sheets of transparency acetate inside a film cassette, allowed to expose X-ray film (X-OMAT, Eastman Kodak Co, Rochester, NY, USA) at -80 °C for 2 days with intensification screens and the film was developed. Bands were scanned and quantified using ImageQuant software (Amersham Biosciences).

Statistical analyses

Data are reported as the least squares means \pm S.E.M. Analysis of variance (ANOVA) was used to compare means in the ontogeny experiment and the treatment effects of MMI injections (SAS version 8.02, PROC GLM or PROC MIXED procedures, SAS Institute, Cary, NC, USA). Differences were considered significant at $P < 0.05$.

Results

Validation of TSH β antiserum by Western blotting and preabsorption in ICC

Initial experiments evaluated whether the anti-rat TSH β antiserum could detect chicken TSH β without cross-reacting with the other pituitary glycoprotein hormones, namely LH and FSH, since they all share the same α -subunit. Chicken pituitaries were dissected from three d1, d6, and e18 embryos, and five e20 embryos, pooled and extracted. Protein from heart, liver, spleen, and

muscle tissues of 3 week-old chicks was also extracted. 10 ng of purified rat TSH, 1 μ g each of chicken FSH and chicken LH, and 5 μ g of total protein from pituitary, heart, liver, spleen, and muscle were separated on a polyacrylamide gel and immunoblotted with the anti-rat TSH β antiserum. Two bands of approximately 30 kM_r and 17 kM_r were detected in the chicken pituitary extract (Fig. 1). The rat TSH lane also contained 2 bands of nearly identical molecular weight. The lower band at 17 kM_r is presumably the β -subunit, and the higher band at 30 kM_r likely represents the α/β heterodimer. The antiserum only detected bands in the pituitary extracts and not in other tissues. More importantly, no cross-reaction with either chicken LH or FSH was seen, even though 100-fold more protein was tested for each than for TSH. This indicates that the antiserum recognizes chicken TSH β and does not recognize LH and FSH or the common α -subunit.

Next, the antiserum was further validated for use in ICC. Pituitaries from e17 embryos were dispersed and plated in a 12-well tissue culture plate. Cells were subjected to ICC using anti-TSH β antiserum alone, antiserum preabsorbed for 3 h with rat TSH (0.1 μ g/20 μ l) or chicken FSH (1 μ g/20 μ l). As expected, preincubation with rat TSH completely blocked staining. In contrast, preincubation with chicken FSH had no effect, confirming that the antiserum does not recognize chicken FSH or the common α -subunit. Taken together, these two experiments indicate that the antiserum is specific for chicken TSH β and can be used for both Western blotting and ICC.

Ontogeny of thyrotrophs

Although levels of TSH β mRNA have been characterized during development (Gregory *et al.* 1998, Nakamura *et al.* 2004), changes in the abundance of anterior pituitary thyrotrophs have not been quantified. Pituitaries were isolated from embryos on e11, e13, e15, e17, e19, and d1 and dispersed, plated, and subjected to ICC using the anti-rat TSH β antiserum. Results are shown in Figure 2. TSH β -containing cells comprised $0.7 \pm 0.1\%$, $4.0 \pm 0.4\%$, $5.3 \pm 0.4\%$, $7.0 \pm 0.8\%$, $6.7 \pm 0.7\%$, and $3.9 \pm 0.4\%$ of all pituitary cells on e11, e13, e15, e17, e19, and d1, respectively. Thus, thyrotrophs were rare on e11, and their abundance was maximal on e17 ($P < 0.01$, $n = 4$ separate trials).

Distribution of thyrotrophs within the cephalic lobe

Early reports indicate that thyroid-stimulating activity and immunoreactive TSH are found in the cephalic lobe of the anterior pituitary gland in birds (Brasch & Betz 1971, Radke & Chiasson 1974, 1977, Sharp *et al.* 1979, Thommes *et al.* 1983, Murphy & Harvey 2001). More recently, cells containing the α -subunit but not the β -subunits for LH and FSH were found in the cephalic

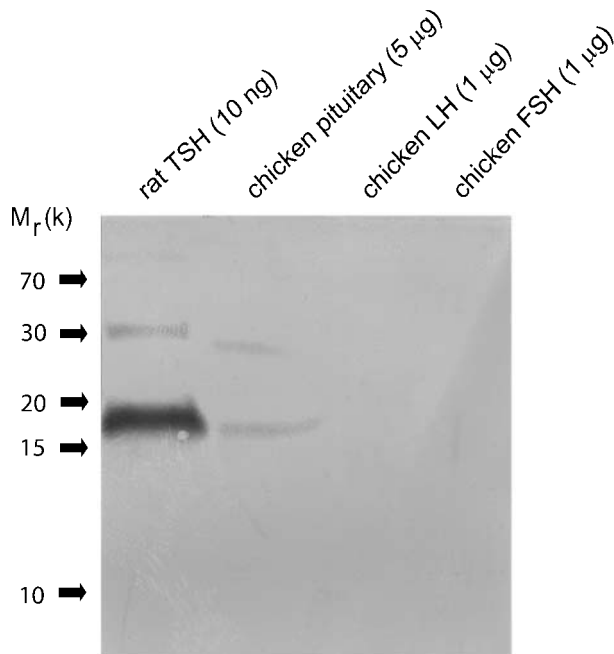


Figure 1 Analysis of antiserum specificity by Western blotting. The antiserum detected two peptide bands of similar size in both the purified rat TSH and the chicken pituitary extract lanes. No bands were observed in the lanes loaded with chicken FSH and LH. The 30 kDa band is presumably the TSH heterodimer and the 17 kDa band is likely the β -subunit.

lobe in sagittal histological sections (Berghman *et al.* 1993). To evaluate three-dimensional distribution of thyrotrophs within the cephalic lobe prior to hatching, whole-mount *in situ* hybridization for TSH β mRNA and whole-mount ICC for TSH β peptide were performed. Pituitaries were isolated on e19, when thyrotrophs were abundant and TSH β mRNA levels were reported to be maximal (Gregory *et al.* 1998). Individual pituitaries were probed with either a TSH β antisense riboprobe or the anti-rat TSH β antiserum. Representative results are presented in Figure 3. The presence of individual thyrotrophs is evident from the specific punctate staining. Thyrotrophs were not distributed uniformly among pituitary glands. TSH β mRNA-containing cells were localized within the cephalic lobe of the e19 pituitary in three general patterns: a focal spot in the ventral medial region of the cephalic lobe (Fig. 3A); a "saddle" pattern extending from the ventral medial to the dorsal lateral regions of the cephalic lobe along the cephalic-caudal boundary (Fig. 3B); and an even distribution within the entire cephalic lobe (Fig. 3C). Hybridization with a sense riboprobe was negative (Fig. 3D). Whole-mount ICC produced similar results. TSH β -containing cells were found in a focal spot in the ventral region of the cephalic lobe (Fig. 3E), in a "saddle" pattern (Fig. 3F), and distributed throughout the cephalic lobe (Fig. 3G). No staining was seen when the anti-rat TSH β

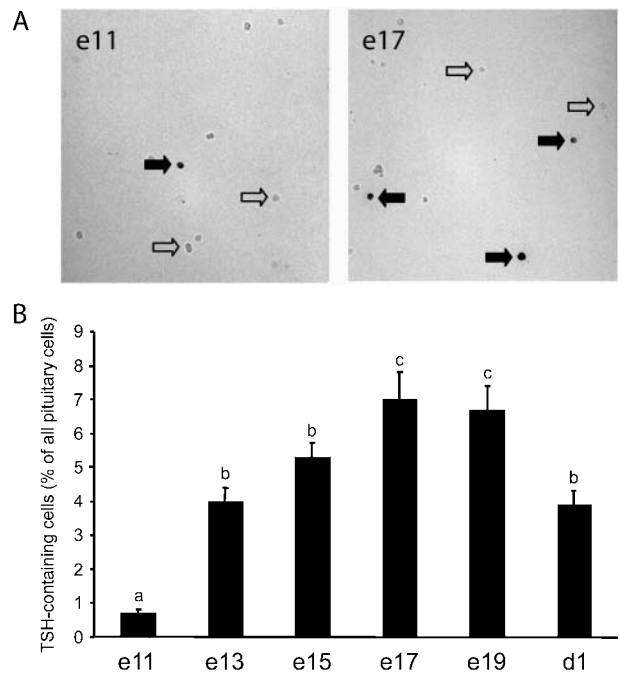


Figure 2 Ontogeny of pituitary thyrotrophs. Anterior pituitary cells were harvested at indicated ages and subjected to ICC for TSH β . Representative results from e11 and e17 are shown (A). TSH β -positive cells are identified by closed arrows and some of the TSH β -negative cells are denoted with open arrows. Quantitative results are reported as the percentage of TSH β positive cells and are the least squares means of four independent replicate trials (B). Data were analyzed by ANOVA, followed by Duncan's multiple range test (SAS). Values denoted by different letters are significantly different from one another ($P < 0.05$). Vertical bars represent the standard errors of the means.

antiserum was omitted (Fig. 3H). Thus, results from both whole-mount *in situ* hybridization and whole-mount ICC indicate heterogeneous distribution of thyrotrophs within the cephalic lobe. To confirm cephalic lobe localization of thyrotrophs, the cephalic and caudal distal ends (approximately one third) of anterior pituitaries were isolated *in situ* within the *sella turcica* on e19, as previously described (Lopez *et al.* 1995). By using only the caudal or cephalic third of each individual pituitary, separation of the lobes was ensured. Pools were then made of caudal and cephalic lobes from at least three embryos for each replicate experiment. Growth hormone (GH)- and prolactin (PRL)-containing cells were identified by ICC, as described previously (Lopez *et al.* 1995, Liu *et al.* 2003, Bossis *et al.* 2004). The lobes were then dispersed, plated, and subjected to ICC for TSH β , GH (a caudal lobe marker), and PRL (a cephalic lobe marker). TSH β - and PRL-containing cells were found among cells isolated from the cephalic lobe, while GH-positive cells were predominantly found in the caudal lobe (Fig. 4). Taken together, these findings from single cell ICC, whole-mount ICC, and whole-mount *in situ* hybridization confirm that

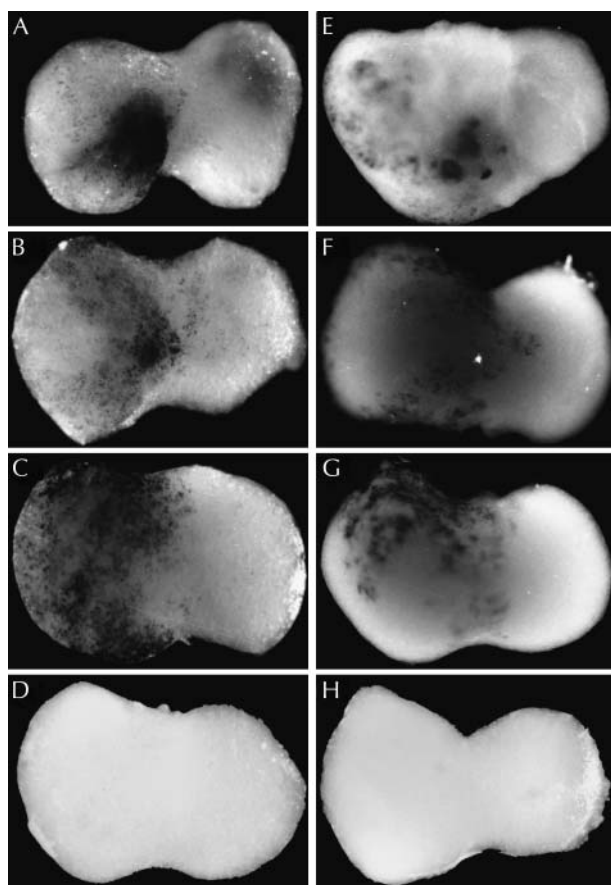


Figure 3 Localization of thyrotrophs within the cephalic lobe of the anterior pituitary. Embryonic day 19 pituitaries were isolated and subjected to either whole-mount *in situ* hybridization (ISH) for TSH β mRNA (A–D) or immunocytochemistry (ICC) for TSH β peptide (E–H). Pituitaries in all fields are oriented with the cephalic lobe to the left. A, B, and C show three patterns of ISH staining for TSH β mRNA within the cephalic lobe. E, F, and G show three similar patterns with ICC for TSH β peptide performed on different pituitaries. D is ISH with a sense strand probe control. H is ICC with no primary antibody.

thyrotrophs are localized to the cephalic lobe of the chicken anterior pituitary gland. Furthermore, results from whole-mount ICC and *in situ* hybridization indicate that thyrotroph distribution is heterogeneous within the cephalic lobe among individual embryos, with a “saddle-shaped” pattern being the most common distribution pattern seen on e19.

Effects of methimazole on thyroid-stimulating hormone in vivo

We reported previously that TSH β mRNA levels and plasma thyroid hormone levels increase prior to hatching (Gregory *et al.* 1998). We also found that T₃ could suppress TSH β mRNA levels in cultures of e19 pituitary cells, indicating the potential for feedback regulation.

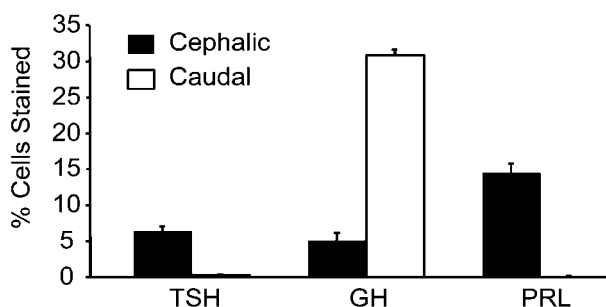


Figure 4 Confirmation of thyrotroph localization to the cephalic lobe. Pools of cephalic and caudal lobes from e19 pituitaries were dispersed and subjected to ICC for TSH β , GH, and prolactin. Cells containing TSH β (thyrotrophs) and cells containing prolactin (lactotrophs) were found in the cephalic lobe, while cells containing growth hormone were found in the caudal lobe. Vertical bars represent the standard errors of means ($n=3$ separate experiments).

However, the contribution of negative feedback from endogenous thyroid hormones on TSH β mRNA levels and pituitary TSH content during this critical period of embryonic development has not been reported. Endogenous thyroid hormone production in the chick embryo was suppressed with MMI injections on e13, e15, e17, and e19. MMI-injected embryos were less developed than the controls and did not hatch. This is consistent with reported effects of MMI on chicken embryos by a continuous flow delivery system (Iqbal *et al.* 1987). To confirm MMI suppression of thyroid hormones, a RIA for free T₄ was performed. In control embryos, T₄ increased more than 6-fold, from 0.025 ± 0.004 ng/dl on e17, to 0.072 ± 0.03 ng/dl on e19, to 0.162 ± 0.044 ng/dl on d1 (Fig. 5). T₄ was undetectable in the plasma of MMI-treated embryos. Next, identical experiments were performed, and pituitaries were collected and pooled from five to seven embryos for each treatment on e17, e19, and d1. Total RNA was isolated from pituitaries of MMI-injected and control embryos on e17, e19, and d1, and analyzed by northern blotting. As seen in Fig. 6, TSH β mRNA levels were increased by MMI treatment ($n=6$ replicate experiments; $P<0.01$; across all ages), with significant differences between MMI and control groups found on e19 and d1 ($P<0.05$). Levels of TSH β mRNA in control groups were: e17, 1.61 ± 0.40 R.D.U. (relative densitometric units; ratio of TSH β / β -actin mRNA in arbitrary densitometric units); e19, 1.21 ± 0.42 R.D.U.; d1, 1.24 ± 0.48 R.D.U. Levels of TSH β mRNA in MMI-treated groups were: e17, 2.00 ± 0.40 R.D.U.; e19, 2.07 ± 0.40 R.D.U.; d1, 2.58 ± 0.42 R.D.U. These results indicate that TSH β gene expression during perinatal development of the chick is under negative feedback regulation from endogenous thyroid hormones. Next, experiments were performed to determine the effect of MMI on TSH β protein levels. Protein was extracted from pituitaries pooled from five to

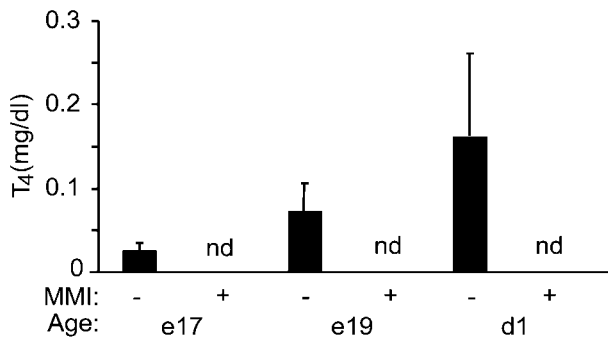


Figure 5 Radioimmunoassay for free T_4 . Blood was collected in tubes containing EDTA. RIA for free T_4 was performed on individual plasma samples ($n=5$ for each group). Means and standard errors for the control groups are presented. T_4 was not detectable in any of the plasma samples from MMI-treated embryos. The detection limit of the RIA was 0.02 ng/dl. nd, not detected.

seven MMI-injected and control embryos on e17, e19, and d1, and analyzed by Western blotting (Fig. 7). Control pituitaries contained more TSH β protein than the MMI treated groups ($n=3$ replicate experiments; $P<0.01$; across all ages), with significant differences between MMI and control groups found on e17 and e19 ($P<0.05$). TSH β protein levels in R.D.U.s (intensity of the 17 kM $_r$ band normalized to the 17 kM $_r$ band of rat TSH β standard in arbitrary densitometric units) for the control groups were: e17, 0.336 ± 0.08 R.D.U.; e19, 0.350 ± 0.08 R.D.U.; d1, 0.246 ± 0.08 R.D.U. Levels of TSH β protein in MMI-treated pituitaries were: e17, 0.108 ± 0.08 R.D.U.; e19, 0.090 ± 0.08 R.D.U.; d1, 0.140 ± 0.08 R.D.U. Thus, inhibition of endogenous thyroid hormone synthesis resulted in decreased pituitary TSH β protein content.

Levels of TSH β protein have not been measured directly in chicken plasma, but in mammals MMI treatment increases levels of TSH in the blood (Rondeel *et al.* 1992). The fact that the MMI-treated pituitaries contained less TSH β peptide than control pituitaries would argue for increased secretion into the blood, consistent with the mammalian model. We attempted to confirm increased levels of plasma TSH by RIA and by western blotting of plasma samples. Unfortunately, our attempts to develop a valid RIA for chicken TSH β using the rat TSH β antiserum and rat TSH as tracer were unsuccessful. Samples of chicken plasma and pituitary extracts failed to displace the rat TSH tracer, presumably due to the much greater affinity of the antiserum for the homologous antigen than for the heterologous chicken TSH β (data not shown). Two commercially available immunoassays for mammalian TSH also failed to detect chicken TSH. Western blotting of plasma samples was hampered by large amounts of albumin present, and prior immunoprecipitation of TSH β resulted in substantial non-specific staining of immunoglobulin fragments in the same molecular weight

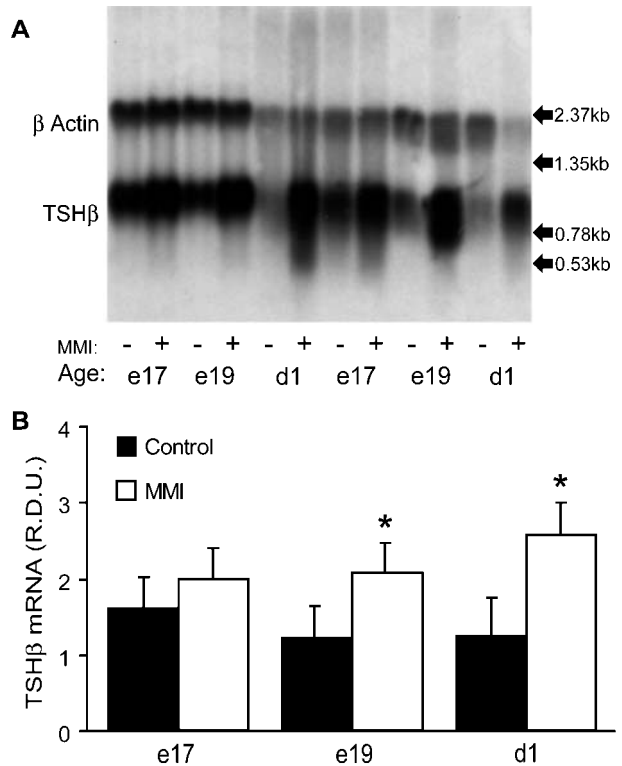


Figure 6 Effect of MMI on TSH β mRNA levels. Pituitaries were collected and pooled from five to seven embryos for each group in each replicate experiment. Total RNA was isolated and analyzed (5 μ g/sample) by northern blotting (A). Arrows indicate locations of standards in an RNA ladder. Autoradiographs were scanned and bands quantified using ImageQuant software (B). Least squares means and standard errors of six independent experiments are shown. Levels of TSH β mRNA (in arbitrary densitometric units) were normalized to β -actin mRNA to yield relative densitometric units (R.D.U.). Results were compared using mixed-model ANOVA (SAS Institute). *, $P<0.05$ compared with control at that age.

range as TSH β (results not shown). Thus, we were unable to directly confirm that inhibition of endogenous thyroid hormone synthesis resulted in increased pituitary TSH secretion into the blood.

Discussion

An increase in thyroid hormone levels is essential for hatching and the transition to posthatch endothermic life during chick development. Despite this critical role for the HPT axis, relatively little is known about the regulation of pituitary thyrotrophs during this period. We reported previously that pituitary TSH β mRNA increases prior to hatching (Gregory *et al.* 1998). However, it was not known whether this increase was due to greater levels of TSH β gene expression in existing thyrotrophs or due to an increase in the number of thyrotrophs.

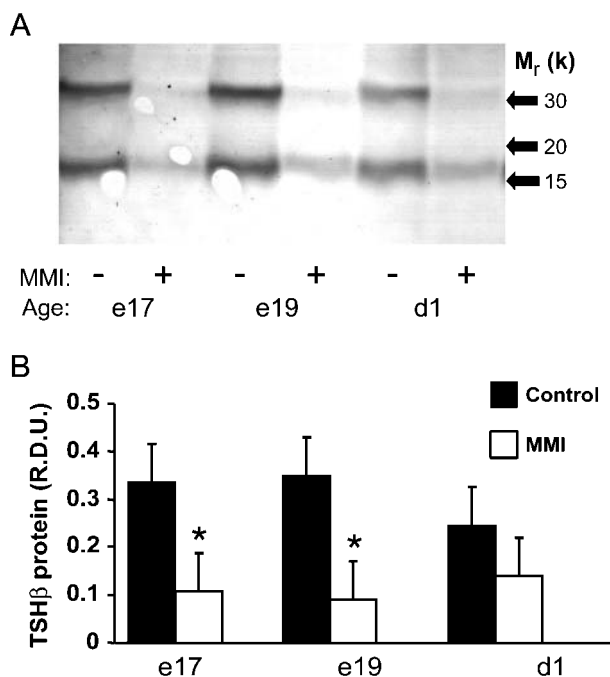


Figure 7 Effect of MMI on pituitary TSH β protein levels. Pituitaries were collected and pooled from five to seven embryos for each group in each replicate experiment. Total protein was isolated and analyzed (20 μ g/sample) by Western blotting (A). Filters were scanned and the 17 kM, bands quantified using ImageQuant software (B). Least squares means and standard errors of three independent experiments are shown. Levels of TSH β peptide were normalized to 5 ng of rat TSH β standard in each western blot and compared using mixed-model ANOVA (SAS Institute). *, $P < 0.05$ compared with control at that age.

Nakamura *et al.* (2004) recently reported that thyrotroph cell density in sagittal sections increased during development. However, absolute numbers of TSH cells could not be determined from this type of analysis. The ontogeny of thyrotrophs during chick embryonic development was quantified in the present study. TSH β -positive cells were less than 1% of the pituitary population on e11, the earliest day pituitaries were examined. The percentage of thyrotrophs increased several fold on e13 and peaked on e17 and e19. The increase in the number of TSH β -containing cells between e11 and e13 follows with the maturation of the HPT axis (Thommes 1987). The percentage of thyrotrophs then decreased by d1, presumably with the appearance of cells from later differentiating cell lineages such as somatotrophs and lactotrophs. We have reported that pituitary GH cells become a significant population on e16 and that pituitary PRL cells first appear on e17 (Porter *et al.* 1995, Woods & Porter 1998). Thus, the peak number of thyrotrophs occurs in the days just prior to hatching. This is the period in development when the animal transitions from ectothermy to endothermy after hatching. During incubation, the temperature of the embryo is maintained by the nesting hen or a commercial

incubator; after hatching, the chicks regulate their own body temperature. Our present findings indicate that the abundance of thyrotrophs increases in preparation for hatching and the transition to an endothermic existence.

Thyrotrophs have been localized in the cephalic lobe of the anterior pituitary by ICC on histology sections and by assessment of biological activity in tissue extracts (Brasch & Betz 1971, Radke & Chiasson 1974, 1977, Sharp *et al.* 1979, Thommes *et al.* 1983, Berghman *et al.* 1993, Murphy & Harvey 2001, 2002, Iwasawa *et al.* 2002, Nakamura *et al.* 2004). However, the three-dimensional distribution of thyrotrophs within the cephalic lobe of the anterior pituitary has not been determined. To accomplish this, *in situ* hybridization and ICC were performed in the present study on whole-mounted pituitary glands. As expected, the cTSH β riboprobe detected TSH β mRNA in the cephalic lobe of the anterior pituitary. Interestingly, different patterns of expression were noted. In some pituitaries, the thyrotrophs were localized to a small region in the medial ventral area of the cephalic lobe; in other pituitaries, cells throughout the entire cephalic lobe were stained for TSH β mRNA. There was also a “saddle-shaped” staining pattern observed, extending from the medial ventral region of the cephalic lobe to the lateral dorsal regions along the cephalic-caudal boundary of the anterior pituitary. Similar patterns were seen when ICC for TSH β protein was performed on whole-mounted pituitaries. The saddle-shaped pattern was most commonly observed. These two sets of experiments, probing for both TSH β mRNA and TSH β peptide, prove conclusively that the thyrotrophs are localized to the cephalic lobe of the chick anterior pituitary. This is consistent with the reports of immunopositive cells in cephalic sections and homogenates using anti-peptide and heterologous antisera (Sharp *et al.* 1979, Thommes *et al.* 1983, Mikami 1986, Murphy & Harvey 2001, 2002, Iwasawa *et al.* 2002, Sasaki *et al.* 2003, Nakamura *et al.* 2004). These findings also indicate that the distribution of thyrotrophs is heterogeneous among individual embryos at this stage of development. Interestingly, in mice a population of thyrotrophs exists transiently during development at the rostral tip of the pituitary in addition to the caudomedial, permanent thyrotrophs (Lin *et al.* 1994). It is unknown whether this is also true in chickens, as only pituitaries on e19 were examined. However, the different patterns of thyrotroph localization noted in the present study using both ICC and *in situ* hybridization make it tempting to speculate that thyrotroph differentiation during chick development initiates in the ventral medial region of the cephalic lobe and proceeds in a dorsal and then rostral fashion so that thyrotrophs are eventually dispersed throughout the cephalic lobe of the anterior pituitary gland.

Classically, thyroid hormones are thought to feed back to suppress the release of TSH from the pituitary. However, this has not been determined during chick embry-

onic development. T_4 and T_3 secretion increases prior to hatching (Gregory *et al.* 1998). Our finding of increased numbers of thyrotrophs and previous findings of elevated TSH β mRNA levels in the pituitary at the end of embryonic development (Gregory *et al.* 1998) raises the possibility that TSH production is not regulated by negative feedback from endogenous thyroid hormones during this period of development. To explore this possibility, endogenous thyroid hormone synthesis was suppressed by injecting eggs with MMI. Effectiveness of MMI injections was confirmed by measuring plasma levels of T_4 . T_4 was below the limit of detection for all three ages of MMI-treated embryos assayed. The control embryos showed increasing levels of T_4 prior to hatching, as expected. MMI treatment increased levels of TSH β mRNA in the pituitary. This increase in TSH β mRNA suggests that TSH β gene expression is normally under negative feedback by thyroid hormones during this period of development. Somewhat surprisingly, control pituitaries contained more TSH β protein than the MMI-treated pituitaries. Thus, MMI-treated pituitaries contain more TSH β mRNA but less TSH β protein. This finding is likely due to increased secretion of TSH protein by the thyrotrophs into the bloodstream in MMI-treated embryos. The pituitary, therefore, is depleted of TSH as compared with control embryos. Alternatively, this may indicate that TSH β protein synthesis was decreased in response to MMI treatment. However, given that TSH β mRNA levels were increased by MMI, this possibility would seem unlikely. Similar findings of decreased staining intensities in purported thyrotrophs were noted when older, post-hatch birds were fed MMI (Sharp *et al.* 1979, Berghman *et al.* 1993). Taken together, the increase in TSH β mRNA levels in combination with diminished pituitary TSH β protein content in MMI-treated embryos clearly indicate that pituitary thyrotrophs are normally under tonic inhibition by endogenous thyroid hormones prior to hatching. Furthermore, these findings indicate that the increase in thyroid hormone levels, in pituitary thyrotrophs, and in pituitary TSH β mRNA and protein levels prior to hatching are not due to a lack of negative feedback inhibition within the HPT axis. Rather, our findings suggest that the increase in thyroid hormone levels critical to hatching and the transition to endothermic life is driven either by an autonomous increase in pituitary thyrotrophs and TSH secretion or by an increase in hypothalamic stimulation of pituitary thyrotrophs. Levels of hypothalamic TRH are known to increase prior to hatching (Geris *et al.* 1998, 1999), supporting the notion that ultimately the hypothalamus controls the metabolic jump to endothermic life.

In conclusion, we report that the number of thyrotrophs increases dramatically between e11 and hatching, that their distribution within the cephalic lobe is heterogeneous on e19 but most commonly in a saddle-shaped pattern, and that TSH β gene expression and protein secretion are

regulated by negative feedback from endogenous thyroid hormones during the perinatal period of chick embryonic development, when the transition to endothermic life occurs. These findings indicate that the increased activity of the HPT axis necessary for hatching and endothermic life is not due to the absence of thyroid hormone negative feedback on pituitary TSH production.

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