Pre- and posthatch developmental changes in hypothalamic thyrotropin-releasing hormone and somatostatin concentrations and in circulating growth hormone and thyrotropin levels in the chicken

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Abstract

Thyrotropin-releasing hormone (TRH) and somatostatin (SRIH) concentrations were determined by RIA during both embryonic development and posthatch growth of the chicken. Both TRH and SRIH were already detectable in hypothalami of 14-day-old embryos (E14). Towards the end of incubation, hypothalamic TRH levels increased progressively, followed by a further increase in newly hatched fowl. SRIH concentrations remained stable from E14 to E17 and doubled between E17 and E18 to a concentration which was observed up to hatching. Plasma GH levels remained low during embryonic development, ending in a steep increase at hatching. Plasma TSH levels on the other hand decreased during the last week of the incubation.

During growth, TRH concentrations further increased, whereas SRIH concentrations fell progressively towards those of adult animals. Plasma TSH levels increased threefold up to adulthood; the rise in plasma GH levels during growth was followed by a drop in adults.

In conclusion, the present report shows that important changes occur in the hypothalamic TRH and SRIH concentration during both embryonic development and posthatch growth of the chicken. Since TRH and SRIH control GH and TSH release in the chicken, the hypothalamic data are compared with plasma GH and TSH fluctuations.

Introduction

Both thyrotropin (TSH)-releasing hormone (TRH) and somatostatin (SRIH) are implicated in the control of TSH secretion in the chicken (Lam et al. 1986, Kühn et al. 1987). Besides their effect on the thyroidal axis both hormones also function as potent regulators of growth hormone (GH) secretion in the chicken (Scanes et al. 1981, Harvey et al. 1986, Spencer et al. 1986, Kühn et al. 1988b). These specific actions are already present during embryonic development. The capacity of thyrotropes to respond to TRH administration, measured as an increase in plasma thyroxine (T4) concentrations, is already present as early as day 6-5 of incubation (E6-5) (Thommes & Hylka 1977, Thommes 1987). The GH-releasing activity of TRH is, however, only established around E18 (Kühn et al. 1988a, Darras et al. 1994). The SRIH neuronal system is already fully developed in the hypothalamus before hatching (Blahser & Heinrichs 1982). Both GH and TSH secretion are inhibited by SRIH at the end of the incubation (Lam et al. 1986, Piper & Porter 1997).

Because of their nutritional and hormonal independence from the mother, the chick embryo and the posthatch chick are an excellent model to study the ontogeny of hormonal regulation systems. To date, most studies have focussed on changes at the peripheral level – plasma hormone concentrations or in vitro deiodinase activities (Thommes & Hylka 1977, Hylka et al. 1986, Galton & Hiebert 1987, Darras et al. 1992). Data on the ontogenetic appearance of hypothalamic factors in the chicken brain are, however, restricted to immunocytochemical studies (corticotropin-releasing hormone (CRH): Josza et al. 1986; TRH: Thommes et al. 1985; SRIH: Ambrosi et al. 1992). Accordingly, this paper describes the ontogenetic profile of hypothalamic TRH and SRIH concentrations during chick embryo development and posthatch growth. These profiles are compared with circulating GH and TSH fluctuations. Due to the lack of specific antibodies to chicken TSH (cTSH) a subtractive strategy was used to obtain an index of plasma TSH concentrations at the different developmental stages studied (Berghman et al. 1993).
Material and Methods

Animals

Chickens (Hisex) used in the different studies were purchased as fertilized eggs from a local commercial dealer (Euribrid, Aarschot, Belgium). Eggs were incubated in a forced-draft laboratory incubator at a temperature of 37.8°C with increasing humidity and ventilation from day 14 on, with continuous lighting and a 45° rotation every hour (start of incubation = day 1 (E1)). Posthatch chicks were kept in an acclimated room with a 14 h light:10 h darkness photoperiod. Adult female chickens (Warren), in the middle of their laying period, were housed individually. Water and feed were freely available. The experimental protocols were approved by the ethical committee for animal experiments of the Catholic University, Leuven.

In two independent studies, samples were collected daily during embryonic development starting on day E14. The numbers of animals used in the first study were: E14–E17, n = 30; E18–E19, n = 20; E20, n = 30. Except for E14 and E15 (n = 40), the same numbers of animals were used in the second study. On E20 a distinction was made between animals which were not yet entering the air chamber with their beak (non-pipping (NP)) and those which were (internal pipping (IP)), resulting in 15 animals each. The time interval between NP and IP is approximately 6 h. Further sampling occurred on the 21st day of incubation just after hatching (C0 = chick of zero days old), approximately 6 h. Further sampling occurred on the 21st day of incubation just after hatching (C0 = chick of zero days old), and on 1-day-old chicks (C1). Eight-day-old and 15-day-old chicks were used. Finally adult chickens were added to the study. In both studies the numbers of animals were: C0–C15, n = 10; adult, n = 12. Blood was collected from all animals, by heart puncture in embryonic chicks and by decapitation in posthatch and adult birds. On each occasion the hypothalamic region was separated from the rest of the brain by removing consecutively the telencephalon, the optic lobes, the cerebellum and the brain stem. In the first study TRH concentrations were determined, whereas in the second study SRIH concentrations were analyzed. In the embryonic stages, plasma samples were pooled to ensure completion of each assay. Hypothalamic tissues were pooled similarly to be able to link changes in hypothalamic TRH or SRIH concentrations to plasma hormone fluctuations. This resulted in the first study in 15 samples for the stage E14–E17, in 12 samples for E18–E19 chicks and in 6 samples for E20(NP/IP). The second study contained 11 (E14–E15 and E17–E19), 10 (E16), 8 (E20(NP)) or 12 (E20(IP)) samples per embryonic age studied. Posthatch, individual samples were used in both studies.

Extraction of TRH or SRIH

Hypothalamic regions were weighed and immersed in methanol (1 ml), homogenized and centrifuged at 3000 g for 10 min (4°C). Supernatants were collected and evaporated and vacuum centrifugation (Speed Vac Concentrator, Savant, NY, USA). For the TRH data, the extracts were dissolved in TRH RIA-buffer (0.1 M KCl, 0.1 M KH₂PO₄, 0.01% merthiolate, 0.02% BSA, pH 7.5). Extraction recovery of known amounts of TRH was 104.9 ± 5.3%. For the SRIH data, the extracts were dissolved in SRIH RIA-buffer (0.1 M Na₂HPO₄, 2H₂O, 0.1 M NaH₂PO₄, 2H₂O, 0.05 M EDTA, 1% BSA, 1000 KU Trasylol/ml, pH 7.4). Extraction recovery of known amounts of SRIH was 105.5 ± 13.2%.

Radioiodination of TRH

TRH was iodinated using Chloramine T; 10 µl Chloramine T (1 mg/ml) were added to 10 µl TRH (1 mg/ml in 0.05 M phosphate buffer, pH 7.5), 10 µl 0.5 M phosphate buffer (pH 7.5) and 1 mCi Na¹²⁵I. After 2 min the reaction was stopped by addition of 100 µl metabisulfite (1 mg/ml). The radiolabeled peptide was separated from free iodine by rapid filtration on a Sep-Pak C18 cartridge (Pharmacia, Roosendaal, The Netherlands) eluted with 50% acetonitrile in 0.1% trifluoroacetic acid/water. Further purification of monoiiodinated¹²⁵I-TRH was performed by HPLC on a C4 column (5 µm; 120 × 6 mm, Alltech, Elk, Belgium) using 5% acetonitrile in 0.1% trifluoroacetic acid/water as eluent. The purified labeled hormone was diluted in TRH RIA buffer.

Radioiodination of SRIH

SRIH was iodinated using Chloramine T; 5 µl Chloramine T (1 mg/ml) were added to 10 µl [Tyr¹]-SRIH (0.5 mg/ml in 0.01 M HCl), 50 µl 0.5 M phosphate buffer (pH 7.4) and 1 mCi of Na¹²⁵I. After 30 s the reaction was stopped by addition of 100 µl metabisulfite (1 mg/ml). The radiolabeled peptide was separated from free iodine by rapid filtration on a Sep-Pak C18 cartridge (Pharmacia, Roosendaal, The Netherlands) eluted with 80% acetonitrile in 0.1% trifluoroacetic acid/water. Further purification of monoiodinated¹²⁵I-[Tyr¹]-SRIH was performed by HPLC on a C4 column (5 µm; 120 × 6 mm, Alltech, Elk, Belgium) using a linear gradient (60 min: from 18% acetonitrile in 0.1% trifluoroacetic acid/water to 60% acetonitrile in 0.1% trifluoroacetic acid/water). The purified labeled hormone was diluted in SRIH RIA buffer.

TRH RIA

The TRH RIA was carried out according to van Haasteren et al. (1995). Briefly, radioiodinated TRH (15 000 c.p.m./sample), antibody (1/10 000 (100 µl), kindly donated by Dr T J Visser, Erasmus University, Rotterdam, The Netherlands), assay buffer and 100 µl sample or standard (1.56 to 800 pg) were incubated in a
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final volume of 400 µl in polystyrene tubes for 72 h at 4 °C. Separation of free and bound radioactivity was achieved by immunoprecipitation using Sac–Cel anti-rabbit globulin (Innogenetics, Gent, Belgium). After 1 h incubation at 4 °C and centrifugation, precipitates were counted in a γ-counter (Gammamaster, LKB, Pharmacia). The $E_{D_{80}}$ and the $E_{D_{20}}$ were respectively 10 and 240 pg. The intra- and interassay coefficients of variation were 6.6 ± 0.4% and 15.6 ± 2.1% respectively. Hypothalamic TRH concentrations were expressed as pg/g wet weight.

SRIH RIA

The SRIH RIA was carried out according to Spencer and co-workers (1991). Antiserum to somatostatin (kindly donated by Dr G S G Spencer, Animal and Grassland Research Institute, Reading, UK) was raised in sheep against a somatostatin–human serum α-globulin conjugate (Spencer et al. 1986). On the first day 200 µl antibody (1/40 000) and 100 µl sample or standard (0.0095 to 10 ng) were incubated overnight at 4 °C. After addition of $^{125}\text{T-}[\text{Tyr}^1]\text{-SRIH}$ (25 000 c.p.m.) samples were incubated for another 5 h at room temperature. Separation of free and bound radioactivity was achieved by immunoprecipitation using Sac–Cel anti-sheep globulin (Innogenetics). After 1 h incubation at room temperature and centrifugation, precipitates were counted in a γ-counter (Gammamaster, LKB, Pharmacia). The $E_{D_{80}}$ and the $E_{D_{20}}$ were respectively 0.04 and 1.8 ng. The intra- and interassay coefficients of variation were 10.9 ± 2.3% and 19.0 ± 2.8% respectively. Hypothalamic SRIH concentrations were expressed as ng/g wet weight.

Plasma hormone measurements

Measurement of circulating GH concentrations was carried out as described before (Darras et al. 1992). Due to the lack of a specific antibody to the β-subunit of cTSH a subtractive method was used to obtain an index of plasma TSH levels (Berghman et al. 1999). The RIAs of chicken glycoprotein α-subunit immunoreactivity and chicken lutemizing hormone (cLH) followed the method described by Berghman et al. (1993). The total titer of α-subunit-containing molecules is expressed in relative units (ru). An index of TSH concentrations in the samples is calculated by subtracting cLH values from the concentration of pituitary glycoprotein α-subunit of each individual plasma sample. Since chicken follicle stimulating hormone (cFSH) is approximately four times less effective in inhibiting the (cLH) tracer from binding to the anti-α monoclonal antibody, cFSH levels were not included in the subtraction (Berghman et al. 1993). Finally, TSH is expressed in ru since no homologous standard TSH preparations are currently available. This indirect method, validated by Berghman and colleagues (1993), obviously assumes that free α-subunit is not being secreted under physiological conditions; measurement of the free α-subunit of pituitary glycoproteins is considered a useful indicator of abnormal hormonal production, for example in pituitary adenomas (Presner et al. 1990). For all RIAs, chick plasma dilution and loading tests showed a good parallelism with the respective standard curves.

Statistics

Values represent means ± s.e.m. Statistical analysis for the ontogenetic studies was by the general linear models of SAS (1985), followed by a Scheffé test. Combined data were used to calculate Pearson correlation coefficients between hypothalamic concentrations and plasma hormone levels (SAS 1985).

Results

Figure 1 shows hypothalamic TRH concentrations from the last week of the embryonic development towards newly hatched chicks. TRH was already measurable in hypothalami of E14 chicks. From E17 onwards hypothalamic TRH concentrations increased progressively towards the end of embryonic development. At stage E20 no differences were observed when the chicks switched from allantoic respiration (NP) to lung respiration (IP), whereas at hatching hypothalamic TRH concentrations were increased twofold compared with the IP stage. Together with the TRH data, GH and TSH plasma levels are shown in Fig. 1. GH concentrations remained stable at the different embryonic stages, ending with a steep increase at hatching. Plasma TSH levels on the other hand fell from E14 towards hatching. Plasma GH levels were positively correlated with hypothalamic TRH concentrations ($r=0.705; P<0.001$); plasma TSH indices were, on the other hand, negatively correlated ($r=−0.556; P<0.001$).

Changes in the hypothalamic SRIH concentration during embryonic development are shown in Fig. 2. Two distinct areas can be distinguished. At embryonic stages E14–E17 SRIH concentration was stable. Due to a twofold increase between E17 and E18, a second higher plateau was recorded at the end of the incubation (E18–C0). At day 20 no differences were found between NP and IP animals. One-day-old chicks showed a lower hypothalamic SRIH concentration compared with late-embryonic chicks. Plasma GH and TSH profiles were similar to the data from the TRH study; GH levels increased approximately threefold at hatching whereas TSH concentrations dropped progressively towards the end of the incubation (Fig. 2). No correlation was found between the GH and the SRIH data ($r=0.166; P=0.1$), a negative correlation was observed between plasma TSH and hypothalamic SRIH fluctuations ($r=−0.654; P<0.001$).
As shown in Table 1, hypothalamic TRH concentrations increased gradually posthatch to reach a maximum in adult chickens. Plasma GH levels increased during growth but dropped in adult birds to GH levels recorded in newly hatched chicks (Table 1). Circulating TSH concentrations increased progressively towards adulthood. These changes in plasma GH and TSH were confirmed in the SRIH study (Table 1). In contrast to the TRH data, hypothalamic SRIH concentrations dropped during growth, resulting in mid-embryonic concentrations in adult animals (Table 1). Plasma GH changes did not correlate with the observed changes in hypothalamic TRH ($r = 0.203; P = 0.2$) or SRIH ($r = -0.123; P = 0.4$) concentrations. Plasma TSH indices on the other hand were positively correlated with hypothalamic TRH ($r = 0.487; P < 0.01$) and negatively with SRIH ($r = -0.351; P < 0.05$) concentrations.

Discussion

The present results show the profile of hypothalamic TRH and SRIH concentrations during embryonic development and posthatch growth of the chicken. Both peptides were already detected in E14 chicks, the first developmental stage studied. A tenfold increase in hypothalamic TRH concentrations was observed between E14 and 1 day posthatch, whereas SRIH concentrations were only doubled towards the end of incubation. This progressive rise in hypothalamic TRH is in agreement with the gradual increase in both the number of TRH-positive perikarya and in the amount of immunoreactive TRH per cell towards hatching (Thommes et al. 1985). A gradual but less pronounced increase of hypothalamic TRH concentrations was recorded posthatch. Also in mammals hypothalamic TRH concentrations increase before birth.
and continue to rise during growth (Lamberton et al. 1984, Fuse et al. 1991). As to SRIH, concentrations were low in mid-embryonic stages and peaked around hatching, whereas posthatch a gradual decrease was found. Data obtained in the rat revealed an opposite profile; hypothalamic SRIH concentrations do not change during gestation, whereas in the growth period concentrations decreased. Both observations can, when accompanied by respectively an increased and decreased secretion rate, contribute to the increased circulating GH levels in up to 15-day-old chicks.

Plasma TSH indices dropped towards hatching. This observation is in contrast with the increase in thyrotropes after E10.5 (Thommes et al. 1983) and with the increase in TSHβ mRNA levels around E19 (Gregory et al. 1998). Posttranscriptional regulation may account for the presence of low plasma TSH levels (our data) along with increased TSHβ mRNA levels (Gregory et al. 1998). Since circulating T4 and 3,3′,5-triiodothyronine concentrations increase prior to hatching (Darras et al. 1992), feedback mechanisms through thyroid hormones are likely to take part in this regulation (Gregory et al. 1998). While plasma TSH levels decreased, the availability of hypothalamic TRH increased towards hatching. Due to the huge increase this is probably accompanied by an increased TRH release. However, a negative correlation was observed. This is contradictory to the numerous data on a TSH-releasing activity of TRH during embryonic development (T4 measurement – Thommes & Hylka 1978, Thommes et al. 1984, Kühn et al. 1988b). TRH is, however, not the sole stimulator of TSH release in the chicken. Ovine CRH acts both in the embryonic and the newly hatched chick as a potent TSH regulator (TSH index measurement – Geris et al. 1995, 1996). Further research is needed to clarify if this hormone plays a more important role in the control of plasma TSH levels in the embryonic chick. Other data showed that SRIH lowers the circulating T4 concentration in E18 but not in E16 chicks (Iqbal et al. 1989). At the same period in embryonic development we observed a twofold increase in hypothalamic SRIH concentrations, so a regulatory action of SRIH on plasma TSH levels cannot be excluded. Posthatch, both the increase in TRH and the decrease in SRIH may result in the observed constant elevation of circulating TSH towards adulthood.

Table 1 Hypothalamic TRH and SRIH concentrations and plasma GH and TSH levels from newly hatched chickens (C0) up to adult chickens. The numbers of samples per developmental stage are given in parentheses. Two independent studies were conducted. Data shown are means ± S.E.M.

<table>
<thead>
<tr>
<th>Stage</th>
<th>Study 1</th>
<th>Study 2</th>
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<tbody>
<tr>
<td></td>
<td>TRH (pg/g wet weight)</td>
<td>GH (ng/ml)</td>
</tr>
<tr>
<td>C0 (10)</td>
<td>1453 ± 113c</td>
<td>41.2 ± 7.5b</td>
</tr>
<tr>
<td>C1 (10)</td>
<td>1829 ± 158bc</td>
<td>38.7 ± 3.0b</td>
</tr>
<tr>
<td>C8 (10)</td>
<td>2721 ± 250b</td>
<td>151.2 ± 23.4a</td>
</tr>
<tr>
<td>C15 (10)</td>
<td>3414 ± 278ab</td>
<td>223.2 ± 34.1a</td>
</tr>
<tr>
<td>Adult (12)</td>
<td>4592 ± 737a</td>
<td>66.8 ± 9.8b</td>
</tr>
</tbody>
</table>

Within one hormone measurement, data with a common letter are not significantly different (Scheffé, P<0.05).
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