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The chicken embryo as a model for developmental endocrinology: development of the thyrotropic, corticotropic, and somatotropic axes

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Abstract

The ease of in vivo experimental manipulation is one of the main factors that have made the chicken embryo an important animal model in developmental research, including developmental endocrinology. This review focuses on the development of the thyrotropic, corticotropic and somatotropic axes in the chicken, emphasizing the central role of the pituitary gland in these endocrine systems. Functional maturation of the endocrine axes entails the cellular differentiation and acquisition of cell function and responsiveness of the different glands involved, as well as the establishment of top-down and bottom-up anatomical and functional communication between the control levels. Extensive cross talk between the above-mentioned axes accounts for the marked endocrine changes observed during the last third of embryonic development. In a final paragraph we shortly discuss how genomic resources and new transgenesis techniques can increase the power of the chicken embryo model in developmental endocrinology research.

The chick as a traditional model for developmental endocrinology

For more than 2 millennia, the chicken has been a popular animal model to study embryology and developmental biology (Stern, 2005). This is due to some intrinsic advantages that the chicken embryo offers in comparison with mammalian embryonic models. Fertilized chicken eggs are low in cost and in abundant supply, and the chicken embryo is quite large and easily accessible during its entire amniotic development (Murphy & Clark, 1990). Simply by putting a number of fertilized eggs in the incubator, hundreds to thousands of embryos with a more or less synchronized development can be obtained. Furthermore, as the only endocrine connection with the mother is through hormone deposits in the egg yolk, avian embryonic development is relatively independent of changes in maternal physiology that occur after deposition of the egg, which is particularly interesting in view of endocrinological manipulations. The simplicity of direct manipulation of the embryonic endocrine interior without triggering maternal interference makes the chicken embryo a valuable model for the study of development and function of hormonal systems (Decuyper et al., 1990; Jenkins & Porter, 2004). As such, the chicken embryo is used as a model to study the cellular and subcellular differentiation and maturation of endocrine glands, ontogenic changes in the sensitivity or responsiveness of the glands and their target organs, and the development of top-down and bottom-up regulatory systems (Scanes et al., 1987). In general, the control and function of the endocrine axes are similar in birds and mammals. It should be noted, however, that the development of some endocrine systems, especially the thyroidal axis, differs considerably between precocial and altricial species. On the one hand, this limits the generalizability of the

chicken embryo model to other avian species, including the important order of passerines (see McNabb et al., 1998 and McNabb, 2007 for excellent reviews on the differences in the development of the thyrotropic axis between precocial and altricial birds). On the other hand, in many respects (see for instance Darras et al., 1999), the endocrine changes that occur during late embryogenesis and hatching in the chicken closely resemble those observed in humans, making the chicken a much better model to study these changes than the most common (altricial) mammalian models such as rat and mouse. Since the chicken is a domesticated livestock species of great economical importance, a large part of developmental endocrinological research focuses on the hormonal systems involved in growth, like the thyrotropic, corticotropic and somatotropic axes. Here we present a brief overview of the development of these endocrine axes in the chicken embryo and show how their increased activities during the last week of embryonic development are interrelated. Finally, we will discuss how chicken genomic resources and recent technological advances create new opportunities in developmental endocrinology research.

Ontogeny of the endocrine axes in the chicken embryo

Differentiation of the pituitary gland

Embryonic development takes about 3 weeks in the domestic chicken. By the end of the first week of incubation, both the thyroid and the adrenal glands are supposedly functioning (see reviews by McNabb, 2007, and Jenkins & Porter, 2004). However, developmental changes in circulating hormone levels are not merely the result of the functional differentiation and growth of their respective production sites, but also of

the maturation of regulatory systems, top-down (the instalment of the hypothalamo-hypophyseal axis) as well as bottom-up (the development of feedback interactions). In the embryonic chicken - as in other vertebrates - thyroid hormone (TH) and glucocorticoid secretion are stimulated by the hypophyseal hormones thyrotropin (or thyroid-stimulating hormone, TSH) and corticotropin (or adrenocorticotrophic hormone, ACTH), respectively. As to the first appearance of these hormones in the embryonic pituitary gland, the available data show little coherence for which several factors could be responsible (e.g. the use of various heterologous versus homologous antibodies with different sensitivities, differences in immunostaining protocols, the use of different chicken strains or lines, possible variation in pre-incubation history of the eggs, etc.). TSH β -immunopositive cells have been reported to appear anywhere between day 4.5 and 11 of incubation (Sasaki et al., 2003; Muchow et al., 2005). Using a homologous antibody to chicken TSH β , Nakamura and colleagues (2004) found the first thyrotropic cells on embryonic day 10, but TSH β mRNA – determined by RT-PCR - was expressed somewhat earlier, on day 9. ACTH-immunopositive cells were detected from day 7 onwards (Sasaki et al., 2003), although immunostaining with a homologous antibody to the ACTH precursor peptide pro-opiomelanocortin (POMC) showed no corticotropic cells at this age (Allaerts et al., 1999). Data on the appearance of growth hormone (GH)- and prolactin-immunoreactive cells in the chicken pituitary are again inconsistent, but most studies do show that the somatotropes and lactotropes are the last pituitary cell types to differentiate (Sasaki et al., 2003). A reverse hemolytic plaque assay (RHPA) demonstrated the absence of GH-secreting cells in pituitaries from 10- or 12-day-old embryos (Porter et al., 1995). Nevertheless, it is thought that a significant population of growth hormone-releasing hormone receptor (GHRH-R)-expressing somatotrope precursor cells is present at this

age (Wang et al., 2006). A few GH-secreting cells were found on day 14 and a significant population existed on day 16 (Porter et al., 1995). An immunohistochemical analysis performed by Zheng and colleagues (2006) supports these data, showing the appearance of GH-immunopositive cells on day 14. Using in situ hybridization and RT-PCR, these authors demonstrated the presence of GH mRNA in the pituitary gland from day 12 onwards. A significant increase in pituitary GH mRNA expression was seen on day 16 (Wang et al., 2006). The work of Zheng and colleagues (2006) also confirms an earlier study of Fu and co-workers (2004) demonstrating that in the chicken, unlike in mammals, lactotropes do not differentiate from somatotropes via an intermediate cell type known as somatomammotropes.

The pituitary-specific transcription factor Pit-1, an important regulator in mammalian pituitary development, is expressed very early in chicken embryogenesis, i.e. starting from day 4 or 5 (Van As et al., 2000; Nakamura et al., 2004). This may indicate that this transcription factor plays a similar role in avian pituitary development. Other transcription factors that may be involved in the cellular differentiation of pituitary cell types (e.g. Prop-1, GATA-2, etc.) have received little attention in the chicken so far.

Differentiation of the endocrine hypothalamus

In the chicken, corticotropin-releasing hormone (CRH) is a potent hypothalamic stimulator of both TSH and ACTH secretion, and thyrotropin-releasing hormone (TRH) stimulates the pituitary to release TSH and GH (Decuypere & Scanes, 1983; Kühn et al., 1988; De Groef et al., 2005). Neurons reacting with antibodies generated

against synthetic ovine CRH were first observed in perikarya located in the periventricular part of the hypothalamus and in the median eminence on day 14 of incubation (Jósza et al., 1986). TRH on the other hand, is present in the hypothalamus at a much earlier stage: immunoreactive TRH was demonstrated in the infundibulum of the chick embryo as early as day 4.5 of incubation (Thommes et al., 1985). However, this is probably of little significance for the control of thyroid function, since the vascular connection between the developing hypothalamus and pituitary gland is not yet established at this age (see further). Beginning on day 6.5, immunoreactive TRH neurons of TRH-containing perikarya cross the anterior hypothalamus and terminate upon branches of the primary capillaries in the median eminence. From day 4.5 through 19.5 of embryonic development, there is a gradual increase within the developing hypothalamus in the number of TRH-positive perikarya as well as the amount of immunoreactive TRH per cell (Thommes et al., 1985).

In addition to the stimulatory actions of TRH and CRH on pituitary function, somatostatin (SS) attenuates the induced release of both GH and TSH in the chicken (Geris et al., 2002; 2003; De Groef et al., 2005). To our knowledge, an inhibitory effect of SS on prolactin or ACTH secretion, like it exists in mammals under certain conditions (see for instance Lamberts et al., 1989), has not been thoroughly investigated in the chicken, but the widespread distribution of SS receptor mRNA (at least subtypes SSTR2 and -5) in the cephalic part of the chicken anterior pituitary - where lactotropes and corticotropes are located - certainly leaves that possibility open (De Groef et al., 2007). At day 13 of incubation, immunoreactive SS is detectable in the hypothalamus of the chick embryo (Geris et al., 1998). However, SS-producing

hypothalamic neurons may arise earlier in development, as no earlier stages have been investigated. Ontogenic expression profiles for either hypothalamic growth hormone-releasing hormone (GHRH) protein or mRNA have not been published.

Linking the levels

Although the separate glandular components of the endocrine axes differentiate and synthesize their products early in embryogenesis, the axes only become functional entities when the different levels are “linked”. This linking should be interpreted in the anatomical as well as the functional sense. The latter refers to the target tissue’s acquirement of its responsiveness to the hormones of a higher control centre, *i.e.* the expression of the appropriate hormone receptors in the target cells. The few experiments that have been conducted to address this question suggest that the endocrine tissues become sensitive to their controlling factors even before the hypothalamo-pituitary system is fully functional. (However, this may also reflect a role for early paracrine influences since neuropeptides are often produced locally in the peripheral tissues.) In the chicken, the top-down communication within the thyrotropic axis is established around mid-incubation. Hypothalamic axons terminating on the primary capillary plexus of the median eminence are present on day 6.5 of incubation, but only between days 10 and 12 the definitive hypothalamo-hypophyseal portal vascular plexus is formed and the previously autonomous hypophyseal secretion becomes controlled by the hypothalamus (Daikoku et al., 1973; Thommes, 1987). In this period (between days 11 and 13), thyrotrope numbers in the anterior pituitary increase remarkably and circulating thyroxine (T_4) levels start to augment gradually (Muchow et al., 2005; McNabb, 2007). However, in 6.5-day-old

embryos, TRH or TSH treatment is able to increase circulating T₄ levels, suggesting that even at this very early stage the thyrotropes are readily responsive to TRH (*i.e.* express TRH receptors) and the thyroïdal cells are responsive to TSH (express TSH receptors) (Thommes & Hylka, 1978). The responsiveness of the thyrotropes to CRH stimulation has never been tested in embryos younger than 13 days, at which age CRH is already capable of eliciting a TSH response (Geris et al., 2003) and mRNA encoding the type 2 CRH receptor (CRH-R2), which mediates this interaction, is present in the pituitary gland (De Groef et al., 2006). Binding of immunoreactive TSH to thyroïdal cells was also detected at the earliest stage tested, *i.e.* day 5.5 of incubation (Thommes et al., 1992). The numerical density of TSH-binding cells rises slowly from day 5.5 until day 11.5, followed by a significant increase between day 11.5 and day 12.5 coinciding with the onset of hypothalamo-pituitary control of thyroid function (Thommes et al., 1992).

Likewise, ACTH responsiveness of the adrenal gland is established early in development, as evidenced by the fact that *in vivo* ACTH treatment increases the activity of the enzyme responsible for the conversion of pregnenolone to progesterone in adrenal glands from 4-day-old embryos and stimulates corticosterone (CORT) production on day 5 of incubation (Jenkins & Porter, 2004). At this point, it is not clear which of the five known isoforms of chicken melanocortin receptors are involved in the transduction of ACTH stimuli in the adrenal glands, since all five isoforms are expressed by the adrenals (Takeuchi & Takahashi, 1999) and all show high affinity for ACTH-derived peptides (Ling et al., 2004). The adrenal glands acquire their responsiveness to ACTH well before the different levels of the corticotropic axis connect, which occurs around day 14. The appearance of

hypothalamic CRH at this stage is accompanied by a marked increase in the number of corticotropes and significantly elevated plasma glucocorticoid levels (Wise & Frye, 1973; Jenkins & Porter, 2004). Also, the pituitary gland is known to express type 1 CRH receptor (CRH-R1) mRNA at this stage (De Groef et al., 2006), which is thought to mediate the stimulatory effect of CRH at the level of the corticotropes (De Groef et al., 2003). Pituitary CRH-R1 expression has not been investigated in earlier stages yet.

Embryonic chicken somatotropes can be stimulated to release GH by the hypothalamic hormones TRH and GHRH. GHRH-R mRNA expression is low in pituitaries of 8-day-old embryos, but abundant expression, presumably in a population of somatotrope precursor cells, was noticed on day 12 (Wang et al., 2006). Since TRH receptor expression has only been measured using whole-pituitary RNA (De Groef et al., 2006) and this receptor is also expressed by other cell types such as the thyrotropes, it is difficult to infer the first appearance of TRH receptor mRNA in the somatotropes. On day 16, more embryonic somatotropes respond to a GHRH stimulus than to TRH exposure, but by day 20 the fractions of the somatotrope population responsive to GHRH or TRH are equal (Dean et al., 1997). A stimulating effect of TRH on the *in vivo* circulating concentration of GH was not found in chick embryos at 16 or 18 days of incubation, but was observed during pipping (Decuypere & Scanes, 1983). At a relatively low dose, TRH was shown to be a more effective GH-releasing stimulus than an equimolar dose of GHRH in post-hatch chicks (Darras et al., 1994). Apparently, part of the somatotrope population gradually increases its sensitivity to TRH during maturation. Even though SSTR2 and -5 mRNA expression is very low in pituitaries from 16-day-old embryos compared to later stages (De Groef

et al., 2007), in vitro GH secretion can be attenuated by SS treatment at this stage (Piper & Porter, 1997).

Feedback interactions

An equally important part of the “intra-axial” communication is the bottom-up interaction (feedback signalling) through which peripheral hormones fine-tune their own release by controlling the secretion of the hypothalamic and/or hypophyseal releasing factors.

At the level of the pituitary gland, the thyroid hormone 3,5,3'-triiodothyronine (T_3) significantly decreases pituitary TSH β mRNA levels in 19-day-old embryos in vitro (Gregory et al., 1998). Inhibition of endogenous TH production with methimazole (MMI) increases TSH β mRNA levels in 19-day-old embryos, suggesting that a negative feedback inhibition of TSH production by THs exists at this age (Muchow et al., 2005). THs are also able to suppress in vitro CRH-induced TSH release from embryonic pituitaries (Geris et al., 1999a) and may have an inhibitory effect on thyrotrope function by increasing the expression of the SS receptor types SSTR2 and SSTR5 (De Groef et al., 2007). In contrast with the large number of experiments conducted in mammals, hardly any data are available on the TH feedback effect on the secretion of hypothalamic TSH-regulating hormones in birds. The influence of THs on the synthesis or release of hypothalamic TRH, CRH or SS in the chicken is currently unknown. Lezoualc'h and co-workers (1992) were the first to show that physiological concentrations of T_3 down-regulate transcription driven by the rat TRH promoter in cultured hypothalamic neurons, and that this regulation is TH receptor

(TR) isoform-specific. When chicken TR β 0 and TR α were expressed in cultured embryonic chick hypothalamic cells together with a rat TRH promoter construct, only TR β 0-dependent TRH transcription was modulated by T₃ and resulted in a decrease in TRH transcription. In subsequent experiments, co-expressed chicken TR β 2 was capable of mediating the negative transcriptional effect of T₃ on transcription driven by the rat TRH promoter transfected in the hypothalamus of newborn mice, whereas transcription was T₃-insensitive with chicken TR α 1 (Guissouma et al., 1998). This observation, combined with the fact that TR β 2 mRNA is abundantly expressed in both chicken hypothalamus and the pituitary (thyrotropic cells included) may point to an important role for TR β 2 in mediating TH feedback in the chicken (Grommen et al., 2005; 2008). Interestingly, next to its expected thyroidal location, TSH receptor (TSHR) mRNA is also present in pituitary and hypothalamus (Grommen et al., unpublished observations). The expression of TSHR mRNA in these two tissues could imply a function for TSH – in addition to THs – in regulating its own synthesis and/or secretion. Like in human, chicken hypophyseal TSHR mRNA is not expressed by the thyrotropes, but by the non-endocrine folliculo-stellate cells (Grommen et al., unpublished results). In human, this observation has been interpreted as indicative of a paracrine ultrashort feedback loop: TSH released by the thyrotropic cells will stimulate type 2 iodothyronine deiodinase (D2) activity in the folliculo-stellate cells, and in turn, the resulting local increase in T₃ will inhibit thyrotrope activity (Fliers et al., 2006). However, experimental evidence for the existence of such an ultrashort feedback loop is scarce in chicken as well as in mammals. One should also keep in mind that the presence of both TSHR and TR β 2 in the chicken pituitary and hypothalamus has only been shown at the mRNA level; the expression of the respective receptor proteins remains to be verified. No data are available on the

cellular distribution of D2 (either mRNA or protein) in the chicken pituitary.

Surprisingly little is known about feedback interactions within the adrenal axis in the chicken embryo (reviewed by Jenkins & Porter, 2004). The observed effects of cortisol on immunoreactive ACTH in the pituitary of chicken embryos suggest that negative feedback of adrenal glucocorticoids on pituitary ACTH release is already functional on day 11 (Kalliecharan & Buffett, 1982). A 2-hour *in vitro* CORT treatment of pituitaries taken from 17-day-old embryos caused a marginal decrease in hypophyseal POMC mRNA levels (Vandenborne et al., 2005). The presence of glucocorticoid receptor (GR) and mineralocorticoid receptor (MR) mRNA in the pituitary gland of chick embryos (Porter et al., 2007; Kwok et al., 2007) may also point towards the existence of a negative feedback effect of glucocorticoids at the level of ACTH synthesis or release, though it has not been shown yet that these receptors are expressed by the corticotropes, let alone that they interact with ACTH synthesis, processing or secretion. So far, there have been no reports on inhibitory effects of glucocorticoids at the level of hypothalamic CRH synthesis/secretion in the chicken.

Like for the corticotropic axis, feedback interactions within the somatotropic axis have been investigated mainly in growing post-hatch chicks and adult birds. One study shows that during late embryogenesis, somatotropes are readily sensitive to the inhibitory effect of insulin-like growth factor-I (IGF-I) (Piper & Porter, 1997), but feedback effects at the level of the embryonic hypothalamus (TRH, GHRH) have not been looked at.

In summary, the thyroid and adrenal glands are functional at very early embryonic stages (first week of incubation) and function autonomously (Table 1). During the second week of incubation the cytodifferentiation of the anterior pituitary takes place (except for lactotropes, which differentiate in the third week). When the definite hypothalamo-pituitary portal plexus is formed around mid-incubation, the thyroid gland is controlled by the neuroendocrine axis and there is a general up-regulation of the thyroïdal axis. Somewhat later, with the differentiation of hypothalamic CRH neurons, the adrenals too become controlled by the hypothalamo-pituitary system and the activity of the corticotropic axis is increased. The peripheral glands and the pituitary acquire their sensitivity to hypophyseal and hypothalamic control respectively before the top-down communication is established, but little is known about the precise time at which negative feedback interactions (i.e. bottom-up communication) within the thyrotropic, corticotropic and somatotropic axes become functional.

Endocrine events in the last week of embryonic development

Towards the end of incubation, the thyrotropic, corticotropic and somatotropic axes undergo profound changes, not only stimulating general growth and differentiation of the chick embryo, but also preparing it for its life outside the egg by regulating processes such as yolk sac retraction, the onset of lung respiration, hatching, and the initiation of endothermic responses (Decuyper et al., 1990; McNabb, 2007). The importance of THs in these processes is well established (at least in precocial birds; see McNabb et al., 1998, McNabb, 2007). Plasma T₄ levels increase gradually throughout the last week of embryonic development and reach their maximum around

hatching. Plasma T_3 levels remain very low during most of the embryonic life, but increase abruptly around the transition from chorioallantoic to pulmonary respiration. Factors that inhibit this rise in plasma THs, such as treatment with MMI (e.g. Decuypere et al., 1982) or high concentrations of the dioxin-like PCB 77 (Roelens et al., 2005) are known to delay hatching. Plasma GH, prolactin and CORT levels increase rapidly at the end of incubation (Scanes et al., 1987; Decuypere et al., 1990), resembling – not entirely coincidentally – the ontogenic pattern of T_3 .

Circulating CORT levels start to rise around day 14, most likely because of an increased secretion of ACTH at this time (Jenkins & Porter, 2004). In turn, the differentiating hypothalamic CRH neurons could account for the observed rise in ACTH levels. CORT was identified as a key mediator in the final somatotrope maturation steps in both mammals and chickens. In cultured chicken pituitary cells, CORT induces GH mRNA expression, increases the number of cells containing immunoreactive GH, and augments the population of GH-secreting cells (reviewed by Porter, 2005). Treatment of embryonic chickens with CORT induces premature somatotrope differentiation, and this effect can be mimicked by ACTH injection (Jenkins et al., 2007). Besides CORT, also aldosterone can stimulate GH mRNA expression and increase the number of somatotropes, whereas these effects are abolished by pretreatment with GR and/or MR antagonists (Bossis et al., 2004; Zheng et al., in press). Intriguingly, a recent report demonstrates that the CORT stimulating the initial differentiation of the somatotropes is of hypophyseal origin (Zheng et al., in press). The embryonic pituitary gland was shown to express steroidogenic enzymes, and the spontaneously increasing GH mRNA expression in cultured embryonic pituitary cells was completely blocked by pretreatment with metyrapone, a CORT

conversion enzyme inhibitor. Progesterone was also found to stimulate GH mRNA expression in pituitary cells of 11-day-old embryos, and this effect was significantly inhibited by pretreatment with metyrapone. The authors therefore suggest that progesterone of yolk origin is converted to corticosteroids in the embryonic pituitary gland and that these corticosteroids stimulate somatotrope differentiation in the early stage of chicken development (Zheng et al., in press). Furthermore, GHRH and THs act synergistically with CORT to increase somatotrope abundance in vitro (Porter, 2005). A role for endogenous THs in somatotrope differentiation was demonstrated by treating 9-day-old embryos with MMI, which inhibited somatotrope differentiation on day 14 (Liu & Porter, 2004). CORT does not induce somatotrope differentiation directly: a yet unidentified intermediate protein that is not GHRH-R or Pit-1 seems to be involved (Porter, 2005). Potential candidates are the glucocorticoid-induced leucine zipper (GILZ) and dexamethasone-induced Ras1 (DEXRAS1), showing a similar developmental expression pattern as GH mRNA (Ellestad et al., 2006).

The increasing CORT levels, combined with the rise in plasma GH levels that results from the CORT-induced differentiation of the somatotropes, are thought to be largely responsible for the dramatic increase in circulating T_3 levels before hatching. GH as well as glucocorticoid injection were found to increase circulating T_3 levels in 17-day-old chicken embryos acutely through a reduction of hepatic type 3 iodothyronine deiodinase (D3) (Darras et al., 1992a,b; 1996; Van der Geyten et al., 1999; 2001). Due to its inner ring deiodinating activity, D3 is a TH-inactivating enzyme, and a reduction of this enzyme will cause T_3 to accumulate instead of being broken down to T_2 . Whereas hepatic type 1 deiodinase activity shows a 3-fold increase up to the period of pipping and hatching, hepatic D3 activity decreases more than 10-fold from

day 16 to hatching (Darras et al., 1992a). This observation was later confirmed at the mRNA level (Van der Geyten et al., 2002). The inhibitory effect of GH and glucocorticoids on hepatic D3 activity is induced by a direct down-regulation of hepatic D3 gene transcription (Van der Geyten et al., 1999; 2001).

Presumably, an increased TSH secretion by the pituitary gland causes the gradual rise in plasma T₄ levels that precedes the T₃ peak, but a straightforward assay to measure plasma chicken TSH is still not available. Pituitary TSH β mRNA levels increase towards day 19 of incubation (Gregory et al., 1998; Nakamura et al., 2004; De Groef et al., 2006; Ellestad et al., 2006) and so does the density of TSH β -immunoreactive cells (Nakamura et al., 2004; Muchow et al., 2005). Other possible causes of an increased T₄ release, such as an increased sensitivity of the thyroid gland to TSH stimulation or an augmented sensitivity of the thyrotropes to hypothalamic releasing factors seem unlikely. Although ontogenic receptor mRNA expression profiles are not necessarily representative for changes in receptor protein expression, they show that the expression of thyroïdal TSHR mRNA decreases between days 13 and 14 of incubation and remains low till one day post-hatch (Grommen et al., 2006). Also hypophyseal CRH-R2 and TRH-R1 mRNA expression decreases during the last week of embryonic development (De Groef et al., 2006). The predominant cause of the increased thyroïdal activity during the last week of embryonic development may be an elevated hypothalamic top-down stimulation. Both TRH and CRH levels in the hypothalamic region of the brain increase steadily towards hatching (Geris et al., 1999b; Vandenborne et al., 2005) and the decreasing CRH content of the median eminence towards day 19 is suggestive for an increased CRH secretion (Vandenborne et al., 2005). However, the role of endogenous TRH and CRH in the control of TSH

release towards hatching is still elusive, since all studies investigating the control of TSH secretion in the chicken have made use of exogenous TRH or CRH treatment.

Summarized, the marked changes in the thyrotropic, corticotropic and somatotropic axes that occur during the last week of chicken embryonic development are interrelated. The establishment of a functional corticotropic axis around day 14 is responsible for the final maturation of the somatotropes and – both directly and indirectly through GH – for the rapid accumulation of T_3 in the plasma by down-regulation of D3. Although a number of other factors (e.g. hormone transporters, transcription factors, etc.) are likely to play a role in these developmental changes too, information concerning these molecules is still scarce in the chicken embryo (but see further).

The chicken embryo model in the (post)genomics era

The previous paragraphs show that radioimmunoassays and immunohistochemical studies, in combination with more traditional endocrinological techniques such as gland extirpation and hormone replacement, have provided information about the time of appearance of several hormones in the endocrine axes, the relative amounts of hormones present in each gland at different developmental stages, and a general picture of the pattern of maturation of the endocrine axes (McNabb, 1989). Later technological advances in molecular biology (semi-quantitative and quantitative PCR, RNase protection assay, Northern blot, in situ hybridization,...) have supplemented these data with ontogenic mRNA expression patterns of a variety of hormones, receptors, activating and inactivating enzymes, etc., though protein expression data

concerning receptors are still lagging behind. The recent completion of the sequencing and assembly of the chicken genome represents another major leap forward in avian functional genomics research (Burt & White, 2007; Cogburn et al., 2007) and has reconfirmed the chicken as an exceptional model organism in developmental biology (Stern, 2005; Davey & Tickle, 2007). Its compact genome and its unique evolutionary position with respect to mammals have greatly facilitated the identification of putative regulatory gene regions, which show high sequence conservation to their mammalian counterparts (Stern, 2005). There are long stretches of conserved synteny between chickens and mammals, and the chicken genome exhibits a very low rate of chromosomal translocation. Furthermore, the chicken genome has not undergone recent duplications like that of teleosts and many anurans (Stern, 2005). High-throughput expression proteomics are being used for rapid experimental annotation of the still poorly annotated chicken sequence data. One such recent study (Buza et al., 2007) provides experimental support for the *in vivo* expression of 7,809 computationally predicted proteins, including 30 chicken orthologs of proteins that were only electronically predicted or hypothetical translations in human. Chicken genomic data can also lead to the discovery of new proteins and the reconstruction of peptide evolution, e.g. the recent identification of novel chicken natriuretic peptides providing new insights into the evolution of vertebrate natriuretic peptides (Trajanovska et al., 2007).

The increasingly available genomic resources have also facilitated the cloning and characterization of chicken orthologs of mammalian genes involved in the development of endocrine systems, as exemplified by the very recent cloning of important hormone receptors in the chicken, such as GHRH-R (Porter et al., 2006;

Wang et al., 2006), TSHR (Grommen et al., 2006), and GR and MR (Porter et al., 2007; Kwok et al., 2007). Moreover, the establishment of a large expressed sequence tag (EST) collection and the completion of the chicken genome sequence allow for the chicken embryo model to be used in large-scale screens to assess gene function during embryonic development (Ellestad et al., 2006; Cogburn et al., 2007). The chicken embryo was the first vertebrate model in which high-throughput technology was applied to study gene expression profiles during the final differentiation of the pituitary cell types. Ellestad and colleagues (2006) used microarrays containing 5,128 unique cDNAs expressed in the neuroendocrine system to identify genes involved in the proliferation and differentiation of thyrotropes, somatotropes and lactotropes during chicken embryonic development. A large number of genes, including transcription factors and signalling molecules potentially involved in the initiation of pituitary hormone synthesis were identified, building a foundation for further studies characterizing the precise role of these molecules in pituitary development (Ellestad et al., 2006). Porter and colleagues have also used the microarray technology to identify CORT-responsive genes in chicken embryonic pituitary cell cultures and found 27 genes, previously not demonstrated in the pituitary gland, to be direct CORT targets (cited by Cogburn et al., 2007).

The characteristics of the chicken genome and the accessibility of the chicken embryo allow for easy detection of true homologs of developmentally important genes and manipulation of their expression (Davey & Tickle, 2007). The recent development of new methods for transgenesis has made manipulation of gene expression during chicken development – either transiently or stably - possible. Gene delivery via in ovo electroporation is now a routine procedure to study the role of genes in early chicken

embryogenesis (Davey & Tickle, 2007). Replication-competent and –defective retroviral vectors, and especially lentiviral vectors, have also been used successfully as a method of gene transfer to create transgenic chickens. Recently, considerable progress has been made to generate stable expression of the transgene by using sperm-mediated gene transfer or genetically modified chicken embryonic stem cells (see reviews by Stern, 2005; Cogburn et al., 2007; Davey & Tickle, 2007; Tizard et al., 2007) and the avian research community holds high expectations for the use of genetically modified chicken primordial germ cells and germline stem cells for germline manipulation (Yamamoto et al., 2007; Han, in press). New promoter constructs are being developed continuously to target overexpression to specific tissues. Semple-Rowland and colleagues (2007) showed that it is possible to construct dual promoter lentiviral vectors that reliably express two proteins in a cell-specific manner. These researchers identified two vectors that specifically target expression of both transgenes to chicken retinal cone cells and one vector that specifically targets expression of one transgene to cone cells and the other to rod cells (Semple-Rowland et al., 2007). The stage-specific role of developmental genes can be investigated using the tetracycline-controlled expression method, "tet-on" and "tet-off", which was shown to work efficiently to regulate gene expression in electroporated chicken embryos. Using this technique, the onset or termination of expression of an electroporated DNA can be precisely controlled by timing the administration of tetracycline into an egg (Watanabe et al., 2007).

Besides overexpression or gain-of-function studies, gene silencing or loss-of-function experiments provide critical information to unravel developmental pathways. The development of a simple and efficient system for reverse genetics in the chicken

embryo has been somewhat delayed, but in recent years RNA interference (RNAi) has become a key tool for assessing gene function during avian embryogenesis (Cogburn et al., 2007; Davey & Tickle, 2007; Tizard et al., 2007). Chemically synthesized short interfering RNAs (siRNAs) and long double stranded RNAs have been used to efficiently silence gene expression in the chick embryo, but their effect is transient. Stable long-term expression of siRNAs can be obtained by vector-based RNAi, also allowing the direct linking of a marker gene to the siRNA expression cassette (Das et al., 2006). The most commonly used approach for RNAi entails the transcription of short hairpin RNAs by RNA polymerase III promoters (Tizard et al., 2007). Potent homologous chicken U6 and 7SK can be used for a more efficient transcription (Das et al., 2006, Bannister et al., 2007). Vectors expressing siRNAs in the context of a modified endogenous microRNA (miRNA) have been shown to be the most efficient RNAi method (Das et al., 2006; Chen et al., 2007). Das and co-workers (2006) have generated a chicken RNAi system that utilizes a chicken U6 promoter driving the expression of a modified chicken miRNA operon with embedded siRNA sequences. The efficiency of gene silencing with this system is comparable with the best vectors available for mammalian cells and even allows dual gene silencing from a single plasmid (Das et al., 2006).

To date, gain- and loss-of-function studies are mainly being used to assess the role of developmental genes in *early* chicken embryogenesis, but they hold the exciting prospect of expressing or repressing genes in a tissue- and stage-specific controlled manner to unravel their function in the development of the endocrine systems. Functional knock-outs and knock-ins will contribute significantly to our understanding of pituitary cytodifferentiation and the role of several signalling

molecules in this process. For instance, over- or misexpression of transcription factors (Pit-1, GILZ, DEXRAS1,...) and hormone receptors (GHRH, GR, MR,...) can elucidate their involvement in the differentiation of the somatotropes. Gene silencing of either the TRH or CRH precursor gene or their respective receptors may shed some light on the relative contribution of these hypothalamic hormones in the endocrine changes observed in the period leading to hatching. Pituitary- or hypothalamus-targeted gene silencing of TSHR could provide indications for the existence of controversial TSH-mediated feedback effects. These are just a random few of many possibilities for further research in the field of developmental endocrinology that are made possible by recent technological advances.

General conclusions

The traditional strengths of the chicken embryo for studying development, as well as the high similarity of its endocrine system with its mammalian counterpart, make the chicken a very valuable model organism to study the development of endocrine systems and the endocrinology of developmental processes. As illustrated above with examples concerning the development of the thyrotropic, corticotropic, and somatotropic axes in the chicken embryo, classic endocrinological and molecular-biological technologies have provided us with a wealth of information on the differentiation and maturation of endocrine glands, the anatomical and functional development of top-down and bottom-up communication systems, ontogenic changes in the responsiveness and activity of the glands and their target organs, and the interactions of different regulatory systems to coordinate developmental processes such as hatching/birth. Nevertheless, the available data are still rather fragmentary. In

addition to the classic techniques, high-throughput technologies are now being applied to unify and extend the existing data, to provide insight in the complex endocrine interactions that occur during development, and to identify the variety of factors (hormones, enzymes, receptors, transporters, transcription factors, etc.) involved. The development of tailor-made gain- and loss-of-function methods have made the chicken one of the most versatile experimental models for developmental biology available (Stern, 2005) and we expect that these techniques too will soon be applied in the field of developmental endocrinology.

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Table 1. Development of the thyrotropic, corticotropic and somatotropic axes during the first two weeks of chicken embryogenesis. Day 0 refers to the day on which incubation is started. (HP: hypothalamo-pituitary axis; ir: immunoreactivity)

Day	Peripheral glands	Pituitary gland	Hypothalamus
0			
1			
2			
3			
4			
5	Thyroid and adrenal glands capable of hormone synthesis		
6			
7		ACTH-ir present (corticotropes)	Differentiation of hypothalamic nuclei + TRH-ir neurons present
8			Differentiation of median eminence
9		TSH β mRNA present (thyrotropes)	
10		TSH β - ir present (thyrotropes)	
11			Definitive hypothalamo-pituitary portal plexus formed
12	Thyroid function under HP control	GH mRNA present (somatotropes)	
13			
14	Adrenal function under HP control	GH-ir present + GH secretion detected (somatotropes)	CRH-ir neurons present