Journal of Applied Pharmaceutical Science

Available online at www.japsonline.com

Effect of thyroid stimulating hormone on the ultra-structure of the thyroid gland in the Mexican axolotl during metamorphic climax

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ABSTRACT

Anuran metamorphosis has long-served as a model of how thyroid hormones regulate post-embryonic development in vertebrates. However, comparatively little is known about urodele metamorphosis. Twofold approach was utilized in the present study including induction of metamorphosis in the obligatory neotenic Mexican axolotl (*Ambystoma mexicanum*) by applying thyroid stimulating hormone (TSH) treatment and investigating the alternations in the ultrastructure of the thyroid gland that occur during metamorphic climax. The results highlighted the difficulty of inducing metamorphosis in this obligatory neotenic urodele animal and indicated that there are huge variations in the response of experiment animals to the TSH treatment which ranged from complete insensitivity to death. During metamorphic climax induced by TSH treatment, the thyroid follicular cells exhibited evident signs of activation compared with both non-metamorphosed and control animals. These signs include the presence of abundant stacks of rough endoplasmic reticulum on the basement membrane side of the cell, large amount of Golgi complexes, intracellular droplets, dense lysosomal like vacuoles, mitochondria and free ribosomes are observed in the cytoplasm. Colloid vesicles and clear vesicles were also observed in the cytoplasm indicating the production of thyroid hormone.

Key words: Thyroid gland; Ultrastructure; Metamorphic climax; Thyroid stimulating hormone *Ambystoma mexicanum*; Amphibians.

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INTRODUCTION

Amphibian metamorphosis is a well-studied example of a complex developmental process that is regulated by endocrine factors (Rosenkilde and Ussing, 1996, Shi, 2000, Brown and Cai, 2007). However, while the general features of amphibian metamorphosis are broadly conserved (Denver *et al.,*2002), there is considerable variation in the timing, duration, and remodeling patterns that occur across different taxa (Duellman and Trueb, 1994). For example, anurans completely resorb their tails during metamorphosis (Shi, 2000) while urodeles remodel and retain their tails as adults (Duellman and Trueb, 1994). As another example, hind limb formation and growth are intimately linked to metamorphosis in anurans (Shi, 2000), but occur months before metamorphic climax in urodeles (Rosenkilde and Ussing, 1996). Thyroid hormone (TH) control of metamorphosis is broadly conserved across amphibians, and radioimmunoassay data from anurans (frogs; e.g., Leloup and Buscaglia, 1977) and urodeles (salamanders; e.g., Larras-Regard *et al.,* 1981 and Alberch *et al.,* 1986) support the idea that L-thyroxine (T$_4$; relatively inactive form of TH) and 3,5,3′-triiodothyronine (T$_3$; relatively active form of TH) markedly increase at metamorphic climax. TH (T$_3$ or T$_4$) is necessary and sufficient to induce

metamorphosis in anurans (Shi, 2000 and Brown and Cai, 2007) and urodeles (Rosenkilde and Ussing, 1996) and its biological effects are mediated by nuclear receptors (thyroid hormone receptors α and β; TR-α and TR-β).

Some urodeles (but no anurans) are paedomorphic and altogether fail to undergo a conspicuous metamorphosis thus retaining larval morphological traits and completing their life cycles in water as aquatic adults (Petranka, 1998). The Mexican axolotl (*Ambystoma mexicanum*) which has a long history as a developmental model (Smith, 1989) is the prominent example of the paedomorphic urodeles. Despite rarely spontaneously metamorphosing in nature or the lab, the axolotl can, with a large degree of difficulty associated with mass death (Prahlad and Delanney, 1965 and Rosenkilde and Ussing, 1996), be induced to undergo metamorphosis via different kinds of hormonal treatments. It is therefore well established that the axolotl is notoriously difficult to transform especially at young ages as attempts to induce metamorphosis are usually accompanied with high mortality (Rosenkild and Crawford, personal communications and own observation). Although this disadvantage in dealing with the axolotl as an experimental model is yet unexplained, it is likely due to a combination of environmental and /or genetic factors. The common observation is that individuals which exhibit inability to undergo complete metamorphosis usually die within days from the start of hormonal treatment. This is supported by studies conducted on anurans which metamorphose spontaneously where the completion of metamorphosis is a condition for animal survival (Severtsov, 1999). The neoteny appears to be due primarily to low levels of plasma T_4 secondary to a low rate of secretion of thyroid-stimulating hormone. A surge of thyroxine secretion occurs early in larval life but does not lead to metamorphosis, apparently because the enzyme which deiodinates thyroxine to the active form, triiodothyronine, is not yet present. Later in ontogeny, activity in the thyroid axis is low. Hormone treatment can reactivate the thyroid axis at all levels, but some singularities remain. Inhibition at central nervous or peripheral levels may be involved in axolotl neoteny. Therefore, it has been deduced that other factors may also be involved. For example, during anuran metamorphosis plasma concentrations of TH and corticosterone (C) are increased, suggesting a physiological role for both hormones (Kikuyama *et al.,* 1993). It has been known for long that C can enhance TH-induced morphological changes and metamorphosis (Galton, 1990). The T_4-induced morphological changes during tail resorption are markedly retarded when an inhibitor of C synthesis is administered (Kikuyama *et al.,* 1982). Also when C are added to a medium of cultured tail segments of *Bufo japonica* with a subthreshold concentration of T_4, shrinkage of the tail segments occurs in accordance with the amount of C administered. In the absence of T_4 however, C have no effect indicating that they potentiate the action of T_4 (Kikuyama *et al.,* 1993). It is concluded that TH combined with C are essential for metamorphosis in the axolotl and that only high doses of either TH or C can induce morphological changes when injected separately.

Whether or not TH are involved in this C induced transformation or whether corticosteroids are needed in a TH-induced metamorphosis remains to be proven for the Mexican axolotl. According to Kühn *et al.,* (2005) there is entanglement of functions between TH and C in an induced metamorphosis of the neotenic axolotl. On the other hand, the enigma of neoteny in the axolotl cannot be attributed to a deficient interrenal axis since a normal *in vitro* regulation of interrenal corticosterone secretion with corticotropin (ACTH) is present (Gupta and Hanke, 1994). However, a corticotropin-releasing hormone (CRH) challenge is unable to raise plasma levels of thyroxine (T_4), whereas C plasma levels are increased (Jacobs and Kühn, 1989 Kühn *et al.,* 2002 & 2005). This means that the axolotl forms a unique model in the study of metamorphosis and the interaction of the thyroidal and interrenal axis. However, it is generally assumed that the lack of a spontaneously occurring metamorphosis in the Mexican axolotl and the resulting neoteny is due to the low circulating levels of TH but not to the absence of C. These low circulating levels may be due to the relative insensitivity of the thyroid gland to a TSH stimulation compared to the thyroids of metamorphosed axolotl (Darras and Kühn, 1983). In turn this insensitivity may be the result of a deficient hypothalamic stimulation of the thyrotropes by CRH. Indeed, contrary to other amphibians, an intravenous injection of CRH fails to increase Plasma concentrations of T_4 in the axolotl, whereas-probably by a direct action on the corticotropes- circulating levels of corticosterone are elevated (Jacobs and Kühn, 1989; Kühn et al., 2002). The existence of an intact interrenal axis on the other hand was further demonstrated by a normal *in vitro* regulation of interregnal C secretion with ACTH (Gupta and Hanke, 1994).

It has been reported that metamorphic failure in the axolotl is caused by a lack of thyroid stimulating hormone, which is used to induce the thyroid to produce thyroxine in transforming salamanders (Rosenkilde and Ussing, 1996). Several attempts were made to induce metamorphosis in Mexican axolotl by different ways of hormonal treatments and there was always variable degree of mortality associated with variable responses to the treatments among the experimental animals. The most used type of hormonal treatment in inducing metamorphosis is that with T_4 (Rose 1995, Rosenkilde and Ussing, 1996 and Page *et al.*, 2008 & 2009) because it is generally thought to be the primary iodine containing hormone released by the thyroid gland and delivered to other tissues where it is locally converted to T_3 via deiodinase activity (Denver *et al.,* 2002 and Brown, 2005). Other hormonal treatments include TSH or an extract from the axolotl pituitary (Darras and Kühn, 1983), thyrotropin-releasing hormone (Taurog *et al,.* 1974), dexamethasone, a synthetic corticoid (Darras et al., 2002), dexamethasone and thyroxine (Kühn et al., 2004), thyroid hormones and corticosteroids (Kühn *et al.,* 2005). Another method for inducing metamorphosis is to keep the axolotls in shallow water tanks. Some of these axolotls will then, over a period of weeks or monthes, slowly metamorphose into adult salamanders. However, as mentioned before, most attempts at inducing

metamorphosis lead to mass death. This is likely due to the strong genetic basis for neoteny (Shaffer and Voss, 1996, Weisrock *et al.,* 2006). Artificial metamorphosis also dramatically shortens the axolotl's lifespan, if they survive the process. Thus, neoteny represents a deviation from the standard course of amphibian ontogeny, affecting the thyroid axis at more than one level (Denver, 1996). Analysis of the thyroid axis at strategic ontogenic stages and after completed neotenic development suggests that there are a number of deviations, and that the deviations may be temporal and/or quantitative in nature. The present study deals with inducing metamorphosis in the axolotl and at the same time investigating the ultrastructural changes in the thyroid gland during metamorphic climax at which time the thyroid follicle cells exhibit maximal degree of activity (Opitz and Kloas 2010).

MATERIALS AND METHODS

Animals and Husbandry

Sixty Larvae of the axolotl, *Ambystoma mexicanum* aged 46 days with total body length 12±0.7 cm and weighed 50 ± 2 g. were obtained from natural spawning at the Biomedical Services Unite (BMSU), School of Biosciences, Birmingham, UK and used for experimentation. Principles of animal care as regulated by the BMSU were followed. Animals were reared in tanks aerated by running de-chlorinated tap water at a temperature of 19±1℃ under alternate 12 h periods of light and darkness. Four to six days after hatching, the larvae were fed on *Daphnia* and then switched to fresh chopped and intact *Tubifex*. Because the normal table for staging the axolotl ends at hatching (Bordzilovskaya et al., 1989), age, length and weight were considered at the beginning of the treatment. The animals were kept individually in separate tanks. Body weight (g) and tail height (mm) were monitored during a period of 18 days of treatment to determine the time of metamorphic climax. When the survived experimental animals reached the metamorphic climax, all animals were narcotized with a solution of   250 mg/L MS-222 (tricaine methane sulfonate, Sigma, St. Louis, Mo. USA) and killed by decapitation after 18 days from the beginning of the experimentation. The thyroid glands of all survived animals were fixed for investigation. Determining the metamorphic climax stage on morphological basis was conducted according to Cano-Martinez *et al.,* (1994) and Norman (1985).

Hormonal Treatment

50 nM bovine TSH (Sigma, St. Louis, MO) was administered to the animals' rearing water. According to the metamorphic induction studies, the applied TSH treatment is below the threshold of toxicity and induces metamorphosis at a rate that is not accelerated by higher doses and maximizes rate without altering the sequence of morphological and transcriptional events observed at physiologically relevant concentrations.

Transmission electron microscopic (TEM) examination

For TEM, thyroid glands of control larvae, non-metamorphosed experimental and metamorphic climax treated axolotls were separated and 2-mm thick samples of tissue were fixed immediately in 2.5% glutaraldhyde and 2% paraformaldhyde in 0.1M cacodylate buffer (pH 7.4). After rinsing in 0.1 M cacodylate buffer, samples were post fixed in a buffered solution of 1% osmium tetraoxide at 4℃ for 1.5 h. This was followed by dehydration in ascending grades of ethyl alcohol and embedded in epoxy-resin. Ultrathin sections were cut with a glass knife on a LKB Broma ultramicrotome, and mounted on formvar-coated grids, stained with uranyl acetate and lead citrate and then examined using Joel transmission electron microscope.

RESULTS

Effect of TSH treatment on the survival of axolotls

Forty two from the experimental larvae stopped feeding at the end of the latency period and death among them began after ten days from the beginning of the experiment. Data in table (1) show that out of the 50 experimental animals, 8 showed no signs of metamorphosis, 20 died before climax and 22 reached metamorphic climax.

Table 1. Variable responses of the axolotl to TSH treatment in terms of metamorphosis and survival.

Total number of larvae used	60
Control	10
Treated	50
Specimens showed no signs of metamorphosis	8
Specimens died before climax	20
Specimens reached metamorphic climax	22

Change in body weight and tail height

At the beginning of the experiment, weight of the animals were 50±2 g and at the end of the latency period the weight of the animals were in the range of 60±2 g in each of the three groups and the tail height was (15 ± 2 mm). At the end of the experiment i.e. after 18 days, there were evident changes as morphological metamorphosis proceeded. However, these changes were quite variable among experimental animals. Reduction in body weight (Fig.1) reached 50 % and tail height (Fig. 2) by 50% in the metamorphic climax group compared with those recorded at the end of latency period in both controls and non-metamorphosed axolotls.
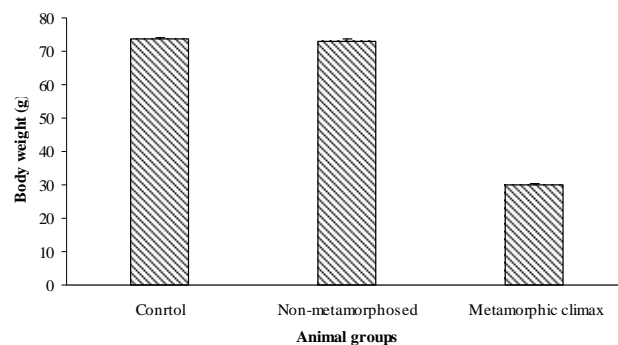

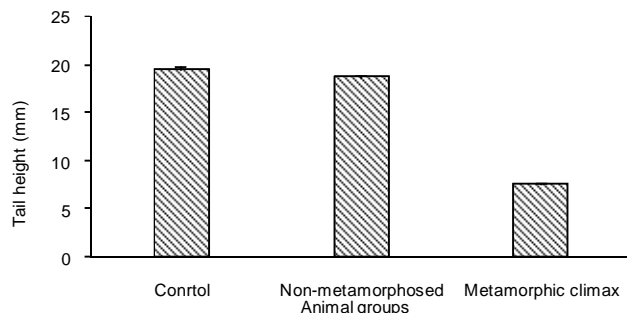**Fig. 1:** Changes in body weight (g) of different animal groups at the end of the experiment i.e. after 18 days.

Fig. 2: Changes in tail height (mm) of different animal groups at the end of the experiment.

Ultrastructure change of the thyroid gland

Thyroid follicle cells of the control group

Examination of semithin sections of thyroid gland of control animals revealed that the thyroid lumen is devoid of colloid. The thyroid follicles are lined by squamous cells (Fig. 3). When examined with TEM, the thyroid follicle cells of larval stage (control group) displayed flat shape with irregularly shaped nuclei, small amount of rough endoplasmic reticulum (RER), free ribosomes, mitochondria and Golgi complexes (Fig. 4).

Thyroid follicle cells of the non-metamorphosed group

The non-metamorphosed experimental larvae (8 larvae) showed the same ultrastructural features of the control group where the follicle cells exhibited the same signs of inactivity. The cells appeared flat with elongated nuclei and irregular nuclear membrane. The lumens of the follicles had no colloid (Fig. 5).

Thyroid follicle cells of the metamorphic climax group

Examination of semithin sections of thyroid gland at this stage showed that the gland is well developed having different features of activity. The lumen of the thyroid follicles are filled with homogeneous colloid with minimal peripheral vacuolation indicating the production of thyroid hormone (Figs. 6 a, b). The TEM examination of the thyroid follicle cells of the metamorphic climax experimental animals revealed that the cells exhibited the spherical shape with prominent nuclei. The nuclei appeared with strongly stained chromatin clumps. Microvilli–like cytoplasmic processes projected into the lumen of the follicle. Endoplasmic reticulum is well developed. Mitochondria and free ribosomes are observed in abundance within the cytoplasm of the follicle cells. Dense osmophilic bodies, colloid vesicles , are present in the cytoplasm. The lumen of the follicles is slightly electron-dense, with large number of microvesicles. Except at tight junction, the follicle cells are widely separated. Abundant stacks of RER are arranged towards the basement membrane side of the follicle cells. Colloid-filled vesicles are present towards the lumen of the follicle. Mitochondria of variable shapes are found in abundance. Clear vesicles, free ribosomes and a well developed Golgi apparatus are present with large quantities in the cytoplasm. The lumens of the follicles are filled with colloid. The basement membrane of the follicle cell is well developed. Large intercellular spaces (except at tight junctions) are seen between the follicle epithelial cells (Figs. 7 & 8).

DISCUSSION

Based on the morphological investigation of this study and others, after thyroid-stimulating hormone (TSH) is administered to an axolotl's rearing water, there is a latency period before metamorphic changes are observed (Prahlad DeLanney 1965, Rosenkilde and Ussing, 1996). During this latency period, growth continues at a normal rate and metamorphic changes, such as weight loss and tissue resorption, are not evident. After this latency period, there is an onset and changes became obvious as morphological metamorphosis proceeds. However, these changes were quite variable among experimental animals, suggesting that even among full siblings raised individually under identical conditions, there is variation in response to TSH treatment.

The variable responses to TSH stimulation can be attributed to the variable sensitivity of the thyroid tissues to the hormone itself (Darras and Kühn, 1983). In turn, this insensitivity may be the result of a deficient hypothalamic stimulation of the thyrotropes by corticotropin-releasing hormone (CRH) (Boorse and Denver 2002, Ito *et al.,* 2004, Okada *et al.,* 2004). Indeed, contrary to other amphibians, an intravenous injection of CRH fails to increase plasma concentrations of T_4 in the axolotl, whereas-probably by a direct action on the corticotropes circulating levels of corticosterone are elevated (Jacobs and Kühn, 1989; Kühn *et al.,* 2002). The existence of an intact interrenal axis on the other hand was further demonstrated by a normal *in vitro* regulation of interregnal corticosterone secretion with corticotropin (ACTH) (Gupta and Hanke, 1994).

The blood concentrations of thyroid hormones are regulated at several different levels. In the axolotl, as in mammals, TSH appears to be the main factor regulating TH secretion from thyroid follicles, and administration of TSH has been shown to cause a rapid elevation of plasma TH levels (Chan and Eales, 1976). Other investigators underscored the dominant role of TSH in gross ultrastructural changes which are paralleled by an increase in circulating TH and reported that other hormones like thyroid releasing hormone can induce significant increase in the number of lysosomes, endoplasmic reticulum (Hoang-Vu *et al.,* 1995). In most *in vivo* studies carried out to date, TSH, even when administered at high doses, does not appear to directly alter T_3 plasma levels, although it does elevate plasma T_4 levels. Thus, there is strong evidence for the independent regulation of T_4 and T_3 plasma levels (Brown *et al.,* 1978; Milne and Leatherland, 1980; Blaschuk *et al.,* 1982; Fok and Eales, 1984; Swanson *et al.,* 1988; Inui *et al,* 1989). Increases in plasma T_4 levels following TSH stimulation are sometimes accompanied by a concomitant increase in plasma reverse T_3 levels (Byamungu *et al.,* 1990). In naturally metamorphosing amphibians, serum T_3 and T_4 gradually rise prior to metamorphic climax (originally proposed by Etkin, 1968 and best documented in anurans (Shi, 2000 and Denver *et al.,* 2002). In contrast, an induced metamorphosis is typically achieved by

Fig. 3: Semithin section of control larva showing thyroid follicles devoid of colloid. x300.
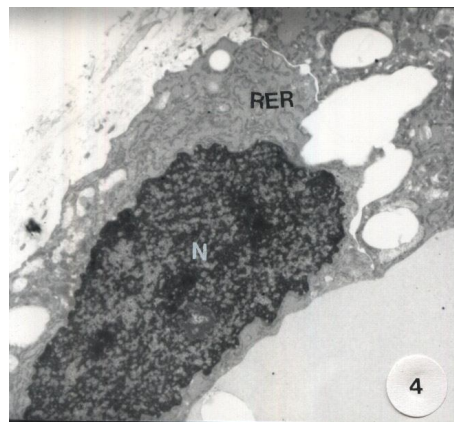

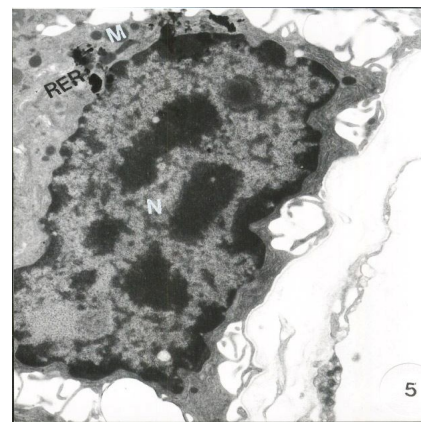**Fig. 4:** TEM of thyroid follicle cell of control larva showing nucleus (N), few RER. x10000.

Fig. 5: TEM of thyroid follicle cell of non-metamorphosed specimen showing nucleus (N), few mitochondria (M) RER. x13000.
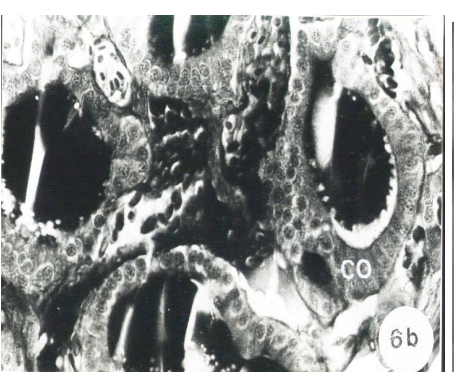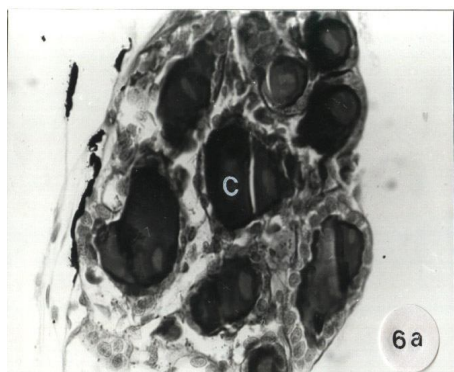
Fig. 6: Semithin sections in thyroid gland of metamorphic climax specimens showing a) Follicles filled with colloid (C) x400. b) Follicles lined with columnar cells (CO) x60
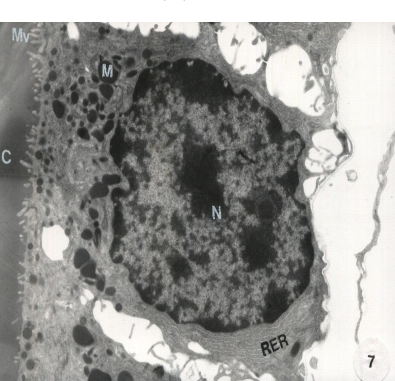

Fig. 7: TEM of thyroid follicle cell of metamorphic climax specimen showing nucleus (N), abundance mitochondria (M), RER, microvilli (Mv) and colloid (C) filled the lumen. x13000

suddenly exposing larvae to a high dose of one form of TH (T_3 or T_4); a practice whose effect on serum T_3 and T_4 levels is not fully understood. Galton (1992) found that juvenile axolotls injected with radiolabeled T_4 had undetectable serum T_3 at 20 h post-injection suggesting that T_4 is not immediately converted to T_3 in large quantities. In agreement with (Galton, 1992) and (Alberch *et al.,* 1985) were unable to detect T_3 in *Eurycea bislineata* larvae immersed in 50 nM T_4 at 24 h post-immersion. Moreover, Alberch *et al.* (1985) observed serum T_4 levels as high as 41 nM during the first 48 h of immersion followed by a leveling off to physiologically relevant T_4 concentrations (Alberch *et al.,* 1986). Thus, in addition to discrepancies that may arise between natural and induced metamorphosis due to gradual versus sudden TH exposure, available studies suggest that the serum TH profiles of induced and naturally climaxing urodeles may be quite different, especially early during their respective TH surges.

Although thyroid-stimulating hormone (TSH) is regarded as the major physiological regulator of tadpoles thyroid gland growth and function (Dumont *et al.* 1992; Vassart and Dumont, 1992), the role of feedback signaling between TH secretion, plasma TH levels and pituitary TSH secretion is still not completely understood (Fagman and Nilsson 2010). What is so far lear is that in response to TSH stimulation, the thyroid tissue of all vertebrates produces and releases the TH which play important

roles in growth and development (Leatherland, 1982; Shi, 1999; Yen, 2001; McNabb, 2007). Upon binding and activation of its membrane receptor, a hallmark of TSH action on thyroid follicular cells is the up-regulation of genes involved in TH synthesis and release (Vassart and Dumont, 1992; De Felice *et al.,* 2004). In addition to the control of TH synthesis and release, TSH also stimulates thyroid growth *via* cAMP-dependent effects on thyroid cell proliferation (Kimura *et al.,* 2001). There is evidence for species-specific differences in the control of thyroid growth by TSH and other growth factors (Kimura *et al.,* 2001). From a comparative perspective, the central role of TSH for regulation of thyroid function and growth appears well conserved across vertebrate classes. Increases in radioiodide uptake and TH release, considered as paradigms of TSH-dependent functional responses, have been demonstrated in experimental animal studies with fish (Leatherland, 1982; Blanton and Specker 2007), amphibian (Shi, 1999), reptilian (Buckingham, 1970), and avian species (McNabb, 2007). Further, a conserved role of TSH for thyroid growth stimulation is suggested by numerous reports of thyroid hyperplasia following treatment of non-mammalian species with anti-thyroidal compounds (Handa and Chiasson, 1980; Bradford *et al.,* 2005; Opitz *et al.,* 2006). However, although thyroidal responses to TSH have been well documented by biochemical and histological techniques, molecular analyses of thyroid tissue in

non-mammalian species are still sparse (Elsalini *et al*., 2003; Grommen *et al*., 2006; Opitz *et al*., 2006).
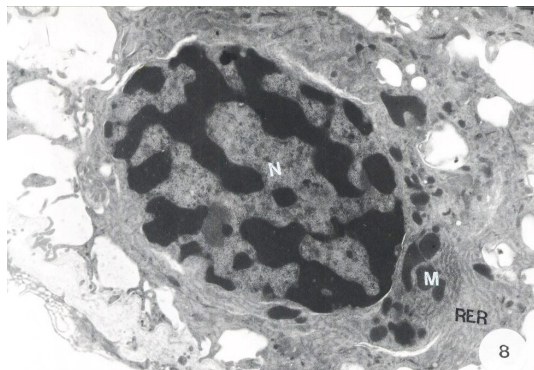

Fig. 8: TEM of thyroid follicle cell of non-metamorphosed specimen showing nucleus (N) with clumping chromatin, abundance   mitochondria (M) RER. microvilli (Mv) and colloid (C) filled the lumen. x13000.

The paucity of references to ultrastructural studies of the amphibian thyroid gland is probably due to the technical difficulties encountered in identifying thyroid tissue in these animals. Even in the later stages of development, the thyroid gland is only just visible macroscopically. The ultrastructural appearance of thyroid follicles reflects their metabolic activity. The follicular epithelial cells become flatter in the resting gland, but columnar epithelium develops under TSH stimulation: the height of the epithelium is inversely related to the diameter of the follicle lumen. TSH-stimulated thyroid follicles exhibited a   peak of activity expressed in the evident increase in surface area of rough endoplasmic reticulum, increase in the lysosomal content, increase in engulfed colloid droplets (Smith and Grau,1986), and increased nuclear size, vacuole size, and nucleoli size (Lai et al., 1980). The effects of TSH on the ultrastructure of the thyroid follicle cell in the axolotl appear to be similar to those found in fishes Smith and Grau, (1986) and mammals (Lai *et al.,* 1980). The abundant number of colloid droplets seen in this study is an evident sign of thyrocyte activation and hence acceleration of TH production induced by the TSH treatment. Other signs of activation induced by TSH treatment included the presence of large amount of Golgi complexes and dense lysosomal like vacuoles.

In both birds and mammals, three phases of thyroid development have been found (McNabb and King, 1993). In phase one, the differentiated thyrocytes appear for the first time in the pharyngeal region and they begin to trap iodide. At this time, the thyroid is prefollicular, and although the thyrocytes are able to synthesize thyroid hormones, the embryo is assumed to have little or no ability to produce endogenous thyroid hormones. In mammals, this early stage of fetal thyroid growth appears to be independent of TSH (Fuse, 1996). In phase two, the thyroid gland has become follicular and has begun to secrete thyroid hormones. Peripheral monodeiodination has begun to occur, and the thyroid gland is under the control of the hypothalamus-pituitary gland thyroid gland axis. In phase three, plasma thyroid hormone levels begin t o increase, eventually reaching the levels seen in adults.

Studying metamorphic remodeling in urodeles at multiple levels of biological organization provides comparative perspective and insights into amphibian metamorphosis (Brown and Cai, 2007). Taken together, the present findings could be interpreted as providing strong supportive evidence for a role of TSH in axolotl metamorphosis. However, there is still no explanation for the evident variable response to the TSH treatment on the axolotl in terms of both metamorphosis and survival.

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