What mechanisms control neoteny and regulate induced metamorphosis in urodeles?

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ABSTRACT The Mexican axolotl, like a number of other urodele species, is an obligatory neotene, completing its full life cycle without metamorphosis. Metamorphosis can be induced with thyroid hormone, thyroid stimulating hormone, or stimulation of hypothalamic neurons. Thus, neoteny represents a deviation from the standard course of amphibian ontogeny, affecting the thyroid axis at one or more levels. 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. 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.

KEY WORDS: *axolotl thyroid stimulating hormone, metamorphosis, neoteny, larval reproduction, axolotl thyrotropin releasing hormone*

Introduction

Metamorphosis and neoteny: what are their roles in amphibian life histories?

Metamorphosis is well known as a developmental characteristic of amphibians. It is generally dangerous to speculate on the evolutionary benefit of a developmental or physiological feature, but, metamorphosis is so decisive in the life of amphibian species that a discussion of its presence or absence should include considerations of the evolution and role of metamorphosis in the life histories of the species concerned.

Does metamorphosis necessarily require a shift of habitat?

The basic pattern of amphibian life is an aquatic larval period terminating in metamorphosis, followed by a more or less terrestrial juvenile and adult lifestyle. A sweeping glance over the extant amphibians shows, however, that amphibian species exist in more habitats than the human mind can imagine. For every habitat proposed, one or more amphibian species can be found. According to their name, amphibians should be partly on land, partly in water, but a multitude of species spend their lives in trees, and some can float in the air from one tree to the other. A number of species live in the desert, one or two in sea water. The only habitats that seem closed to amphibians are the deep sea and the high latitudes.

The broad variation in habitats is based on an even greater diversity in life style. Some tree frogs sit on leaves and drop their eggs in the water below. Others lay their eggs in water bodies at leaf bases (Hödl, 1990). These tree frogs usually have a short and specialized larval life, and some have direct development. That is, the larval stage is short and terminates before the young animal leaves the egg. A few species are viviparous: the entire embryonal and larval development is completed before the young leave their mother (or, in some species, father).

Desert species of frogs and toads complete breeding and full larval development and metamorphosis, all within 1-2 weeks after rainfall. Outside these periods they spend yearlong dry seasons underground. Tadpoles of mangrove swamp frogs develop in full strength sea water, apparently able to drink sea water and excrete ions like marine fish.

A number of species spend their whole life in water. Many species metamorphose but abstain from going on land afterwards. The African clawed frog, *Xenopus*, is the internationally best known fully aquatic genus, but examples can be found everywhere of partly or fully aquatic urodeles and anurans.

Abbreviations used in this paper: A, *Ambystoma*; TSH, thyroid stimulating hormone; T4, tetraiodothyronine= thyroxine; TRH, thyrotropin releasing hormone; T3, 3,5,3'-triiodothyronine= triiodothyronine; 5D, 5-I-deiodinase; r-T3, 3,3',5'-triiodothyronine; 5'D, 5'-I-deiodinase; TH, thyroid hormone; mIU, milli-international units; PAGE, polyacrylamide gel electrophoresis; LHRH, luteinizing hormone releasing hormone

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The evolutionary history of amphibians is closely connected to their distribution in ecological niches. The ancient amphibians, with armored surface, were practically extinct by the end of the Triassic period due to competition from the emerging reptiles (see Carroll, 1977). When modern amphibians began to diversify they specialized in border-habitats between water and land, and in environmental extremes, where they to an appreciable extent make use of cutaneous respiration through their moist, well-vascularized skin. *The ability to adapt to specialized habitats justifies the existence of amphibians to such an extent that an appreciable diversification is still going on.*

How ancient is neoteny (everlasting youth)?

Some urodele species go through a complete aquatic life cycle in the larval shape, as neotenic forms. Authors vary, however, with regard to the term used for the concept of neoteny. The above-used term, neoteny, means retention of larval characters beyond larval life. The term *paedomorphosis* is used by some authors and means occurrence of embryonic structures in adult animals. The term *paedogenesis* is seldom used but probably more correct than the others, as it designates reproduction in the larval state. However, the smoother word, neoteny, appears to be widely used by physiologists and will be used in this review.

Neoteny is not an acquired specialization, but appears to have been associated with amphibians at least from the original archaic species (Baird, 1965) and was prevalent among the first modern urodeles (Evans *et al.*, 1988). Thus, neoteny can hardly be considered an adaptation. Considering the many metamorphosing species that are aquatic, both before and after their transformation, the neotenic condition is not prerequisite to a fully aquatic life. However, in their present state, the neotenic forms evidently contribute to amphibian habitation of yet another niche in the supermultitude of niches inhabited by amphibians.

There is no adequate explanation available as to whether larval shape was maintained in the neotenic species or whether there occurred a secondary re-entry into the aquatic habitat in some metamorphosing species. The situation is even more complex because several urodeles display both neotenic and transforming aquatic forms. Those two forms may perform equally well in water. Frolich and Biewener (1992) noted that both the videotaped swimming pattern and the electromyographic record were nearly identical in neotenic and metamorphosed *Ambystoma tigrinum*.

The existence of the neotenic form beside the transforming one is not a simple matter of better fitness, either. Whiteman and Wissinger (1992) compared the growth rates of neotenic and metamorphosing *A. tigrinum nebulosum* over three years. They found that the metamorphosing animals had higher growth rates than the neotenic ones, both in and between the field season. It has been speculated that coexistence of the two forms might increase the chance that the species survive climatic extremes. Many neotenic urodeles inhabit extreme biotopes, e.g., mountainous regions or caves. At high altitudes the climate favors aquatic life, and permanent water forms may be found where competition from fish is absent. The reason for prevalence of neotenic species in caves is less clear. It has been suggested that the streams might hold more food than the terrestrial parts of caves. Another possibility is that in the water prey can be located without vision, i.e., chemically and by the use of lateral line sense organs.

When all the evidence is viewed together, *the existence of the two forms may reflect the fact that neoteny as an option has been present since the evolution of the oldest amphibians, and that the modern amphibians as a whole rely on their ability to colonize specialized habitats.*

What regulatory mechanisms control metamorphosis?

Since neoteny represents an omission of metamorphosis, the mechanism behind it is probably best understood in terms of the regulation of metamorphosis. The breakdown of larval structures and development of new postmetamorphic structures is triggered by thyroid hormone. Larval development is controlled by a classical endocrinological axis (Fig. 1) in which the thyroid gland controls the peripheral tissues, the pituitary frontal lobe controls the thyroid, the brain controls the pituitary and, not least, the tissues to be transformed have a control function of their own.

Thyroid gland

Hormone from the thyroid gland triggers tissue changes. Thyroid hormone, like steroid hormones, traverses the cell membrane and binds to nuclear structures in immediate contact with DNA. It changes transcription from genes which generate larval characters to the transcription of genes which form juvenile characters.

The larval thyroid gland contains hormone as early as the hatching stage (Kaye 1961; Hanaoka *et al.*, 1973). The plasma concentration of thyroid hormone is below detection levels, and the morphological indices only show that hormone is synthesized at a low rate and stored in thyroglobulin.

Minimal amounts of thyroid hormone do however circulate and exert their action, as will be mentioned below.

Pituitary gland

The secretory and synthetic activity of the thyroid is controlled by thyroid stimulating hormone (TSH) from the anterior pituitary. The TSH cells contain stored hormone from early larval stages (Pehlemann, 1974), but, with the methods used up to now, TSH-secretion during larval life has been non-detectable.

Brain

The infinitesimal low activity of the TSH-producing cells of the pituitary gland continues until they in turn receive stimulation. In the typical tetrapod manner the signal is produced by hypothalamic neurons and secreted from the nerve ends in the median eminence, i.e., at a short distance from the anterior pituitary. The neurohormones diffuse into blood vessels and reach the TSH cells via the pituitary portal system.

It is not completely clear which hypothalamic factor is responsible for the stimulation. The tripeptide known as *thyrotropin releasing hormone* in mammals is present in amphibians but may not be the major stimulant. Some of the other well-known hypothalamic hormones appear to have stronger effects, namely gonadotropin-releasing hormone and corticotropin-releasing hormone (Denver, 1988; Jacobs *et al.*, 1988a). The hypothalamic signal may well be mediated by a combination of factors.

Peripheral tissues: hormone activation

In addition to these typical steps in a regulatory pathway, the peripheral target tissues have proven to represent an important part of the regulatory system. Numerous studies during the last 20 years have shown that T_4 (tetraiodothyronine [= thyroxine]) for all practical purposes is a prohormone. Beginning with the studies of

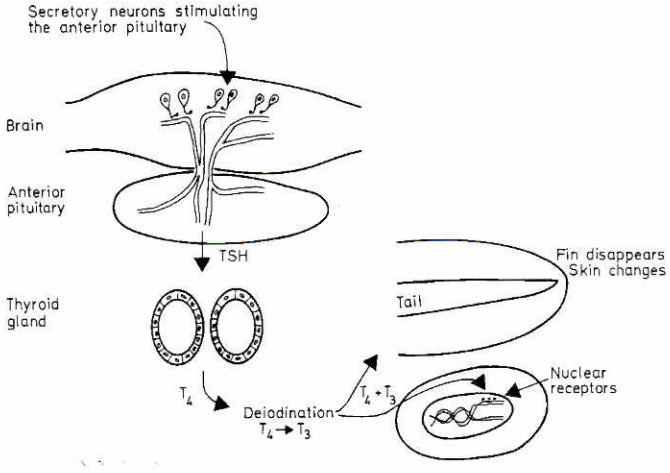

Fig. 1. Diagram of the amphibian thyroid axis.

Kistler *et al.* (1977), it has been shown that thyroid hormone receptors are 10-20 times as sensitive to T_3 (3,5,3'-triiodothyronine) as compared to T_4.

The thyroid gland secretes primarily thyroxin (T_4), but the secreted T_4 is enzymatically deiodinated in the tissues (Fig. 2). One enzyme, called 5D (5-I-deiodinase), removes iodine from the inner ring and thereby changes the molecule to the inactive r-T_3 (3,3',5'-triiodothyronine), while the other enzyme, 5'D (5-I-deiodinase), removes iodine from the outer ring and turns the molecule into the active form, T_3 (3,5,3'-triiodothyronine). Consequently, high thyroid hormone activity relies upon a high 5'D level while 5D will tend to deactivate thyroid hormone.

In early larvae of spontaneously metamorphosing species, the T_4 concentration is below the detection level of existing methods, 5D activity is appreciable, while 5'D activity is low. As metamorphosis approaches and T_4 concentration increases slightly, 5'D increases while 5D decreases (Galton, 1989). Treatment of premetamorphic *Xenopus* tadpoles with as little as 0.5 ng T_4 causes 5'D activity to increase, leading to increased conversion of T_4 to T_3. Thus, at the onset of metamorphosis, the beginning increase of circulating T_4 accelerates $T_4 \rightarrow T_3$-conversion as an integral part of the transformation (Fig. 3).

Peripheral tissues: sensitization of target cells

Metamorphic change in the target tissue, whether it be organ growth, change of synthetic potential, or self-destruction, depends upon the message mediated to the cells by thyroid hormone. Some cells respond to very low levels and initiate their change early in metamorphosis when TH-secretion is only slightly increased (Etkin, 1968). Less sensitive cells respond at correspondingly later stages. That is, reception of the message depends upon the presence and number of receptors.

Estimates of receptor density have revealed a sensitivity build-up comparable to the activation of T_4 to T_3 (Galton, 1989): the increase in circulating TH modulates receptor number in a positive direction, i.e., receptor number increases as hormone level increases. Thus, an increasing thyroxine secretion causes (1) a potentiation of the secreted hormone itself; and (2) an increased hormone sensitivity in the cells, both of which contribute to a progressive hormone activity. *But the origin of this increased activity has not been explained up to now.*

Back to the brain

As described above, the signal for the hormone surge is generated in hypothalamic neurons. Here also, a self activation mechanism appears to be triggered by thyroxine at low, premetamorphic levels. This mechanism is supported by circumstantial, but ample evidence (see further Rosenkilde and Ussing, 1990). The morphological and histochemical evidence points to a hypothalamic maturation comprising neurons of several regulatory centers and culminating at the time of the secretory surge in the thyroid axis. It has been shown that this maturation is prevented by treatment of frog larvae with antithyroid drugs, and restituted with thyroid hormone (Goos *et al.*, 1968a,b).

It therefore appears that the faint premetamorphic thyroxine secretion has the role of preparing cells for a dramatic increase in thyroid hormone activity. These cells, however, are at the same time peripheral target cells for thyroid hormone and the central controllers which set off metamorphic climax. The low premetamorphic level of thyroid hormone may correspond to the conditions met in rats in which the thyrotropic area has been destroyed by hypothalamic lesions (Reichlin *et al.*, 1972). Here, the low level of thyroid hormone exerts a negative feedback inhibition on the TSH cells, keeping the TSH secretion rate minimal. Re-establishing hypothalamic stimulation overcomes this inhibition by lowering the sensitivity of the TSH cells to thyroid hormone.

A general principle

Control of metamorphosis, integrated with the ontogeny of the thyroid axis, is a remarkable feature in amphibian development. It is not however as unique as it appears at first sight. The integration of rising activity in the thyroid axis with maturation of hypothalamic neurons occurs as a perinatal event in higher vertebrates: birds and mammals (see Rosenkilde, 1979). Some teleosts, notably the Japanese flounder, *Paralichthys olivaceus*, go through a metamorphosis controlled by thyroid hormone in striking parallel to amphibians (Inui and Miwa, 1985; Miwa *et al.*, 1988), which includes a corticoid agonist effect (de Jesus *et al.*, 1990) and a prolactin antagonist effect (de Jesus *et al.*, 1994).

Urodele metamorphosis: what is its general pattern, and what special features does it display?

Systematics, evolution, and general experience all provide evidence of appreciable differences between anurans and urodeles. Neoteny, the main theme of this article, is restricted to the urodele subclass. The major body of knowledge on metamorphic processes and their regulation is based on experience from anurans. To what extent can the anuran model of metamorphosis be transferred to urodeles? *It appears that the anuran model, from the somewhat scattered observations on urodele metamorphosis, is very valid for urodeles.*

There may be fewer differences between anurans and urodeles than meet the eye. The external differences are conspicuous: Urodeles do not lose their tail; they are not herbivorous as larvae, but carnivorous in both life periods, and the intestine does not show appreciable change in structure; they have external gills during the whole larval period. There are also a number of internal differences, ranging from dentition to the implementation and performance of the immune system (Ussing and Rosenkilde, 1995).

Still, the main features of metamorphosis are identical. Thyroid hormone triggers a shift in transcription of genes, causing a set of major changes (growth, resorption, redifferentiation) in form and function which take place over a limited time period and initiate a new life period with a renewed body and often a new habitat. Regulation of urodele metamorphosis takes place as in anurans. Hypothalamic factors, apparently the same in the two groups, cause TSH cell activity to increase abruptly from an undetectable level to maximal activity. Thyroid hormone secretion and synthesis increases accordingly, and so does peripheral activation in the form of increased 5'D enzyme activity and receptor mobilization. Reported plasma concentrations in spontaneous urodele metamorphosis vary between extremes within experiments: T_4 varied from non-detectable to 25-30 nM in *Ambystoma tigrinum* (Larras-Regard *et al.*, 1981), and from non detectable to 16 nM in *Eurycea bislineata* (Alberch *et al.*, 1986). T_3 varied much less, between non-detectable and 4 nM in the two studies. The tail can illustrate differences and similarities to anuran metamorphosis (Fig. 1). The elegant fin disappears (although to reappear in the male of some species for ceremonial occasions). There are certain readjustments in the tail musculature, and the skin

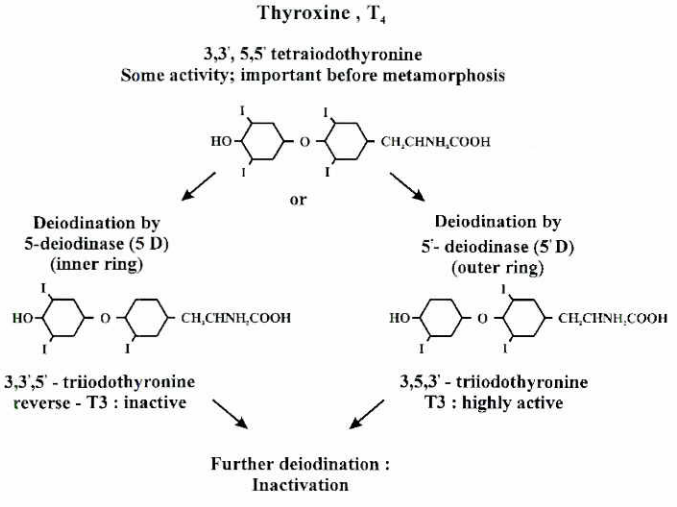

Fig. 2. The thyroid hormones. *Activation and deactivation by enzymatic deiodination.*

changes from smudging fish-like epithelium to the amphibian type, molting in one or a few large pieces with a thickness of one cell layer. The connective tissue shrinks, mainly because the water-binding hyaluronic acid is replaced by chondroitin sulfate and dermatan sulfate (Mathews, 1975). At the cellular level the metamorphic changes are the same as in an anuran.

What features characterize the development of neotenous urodeles (e.g., axolotl)?

Some of the features normally connected with metamorphosis have been studied in larvae of the Mexican axolotl, *Ambystoma mexicanum*, and compared to their occurrence in metamorphosing species. Natural larval to adult hemoglobin change occurred in untreated axolotls at 100-150 days of age, unaccompanied by external metamorphosis. Immersion of the larvae in a T_4 solution of $3x10^{-8}$M, insufficient to induce metamorphosis, led to premature hemoglobin change. Metamorphosis was induced by 5×10^{-8}M (Tompkins and Townsend, 1977).

There are variations in the hemoglobin shift among urodeles, both metamorphosing and neotenic: In *Triturus helveticus*, with frequent neoteny, larval hemoglobin fractions continue to exist when adult-types appear. The hemoglobin shift is thus quantitative rather than absolute, but was retarded when metamorphosis was retarded (Cardellini *et al.*, 1978). The hemoglobin shift may be reflected in Galton's (1992) observation that erythrocytes in axolotl juveniles of $2^{1/2}$-5 months had a comparatively high T_3 receptor number. The receptor number declined as larval cells were replaced by adult-type red blood cells with a low receptor number. Those studies also found some T_3 receptors in the liver of neotenic axolotls, but the number was too low to quantitate. With an appreciably higher receptor number than the other tissues, the erythrocytes may respond to thyroid hormone levels which do not cause other developmental changes. Galton also found 5'D, but again in very low amounts. *It was concluded that T_3 content, low activation of T_4 to T_3, and low receptor number all contributed to neoteny in the axolotl.* The dentition undergoes a partial change from larval to adult types of teeth. Experiments with hypophysectomized larvae show that a short treatment with thyroid hormone can mimic this change, while a complete adult type dentition requires the thyroid hormone concentrations that lead to complete metamorphosis (Clemen and Greven, 1977; Bolte and Clemen, 1991).

In axolotl ontogeny, myosin undergoes a transition from larval to adult isoforms, as it does in a metamorphosing species, *Pleurodeles waltl*. In the axolotl the transition is complete in forelimb muscles, but only partial in dorsal musculature (Saadi *et al.*, 1993). Histoenzymological analyses (Salles-Mourlan *et al.*, 1994) show that the myosin phenotype of the dorsal axis muscle corresponds to a low thyroid activity level. Lung respiration is generally considered a metamorphic feature, but neotenic individuals develop lungs at the same stage as metamorphosing species, and neotenic axolotls can be seen to gulp for air, especially when agitated.

Sexual maturation is completely independent of metamorphosis. This is a prerequisite for the ability to breed in the larval shape, but it also emphasizes that not all tissues need thyroid hormone for maturation. Hypothalamic neurons are thus able to perform the complex activation leading to sexual maturation even though they abstain from the activation leading to metamorphosis. This independence comprises all structures involved in sexual development. A typical example is the ontogeny of the cloacal glands. The cloacal gland is essential in courtship and spermatophore transfer. Sever and coworkers have studied this structure in several transforming and neotenic salamanders. Sever (1992) compared its anatomy in four *Necturus* species and in *Proteus anguinus* and found it fully developed as in metamorphosed species. Likewise, Licht and Sever (1991) found no difference in cloacal anatomy or in the onset of development between metamorphosed and neotenic *Ambystoma gracile*. The above-mentioned observations illustrate a general trend in neotenic urodele ontogeny: *many conspicuous larval characteristics do not transform, a few transform as in metamorphosis in other species (though often comparatively late), while those involved in sexual function mature in an identical way in neotenic and metamorphosing forms.*

What is the connection between the axolotl thyroid axis and induction of metamorphosis?

Spontaneous metamorphosis (in anurans: metamorphic climax) occurs when combined developmental processes cause

hypothalamic neurons to release enough neurohormone to initiate a surge of TSH secretion. *It is tempting to speculate that the main mechanism behind axolotl neoteny is absence of this burst of hypothalamic stimulation.* It is difficult to prove the absence of a short-lasting ontogenic event. Rosenkilde *et al.* (1982) chose the opposite possibility and inquired: Which developmental stage will be the most promising to study in the hope of demonstrating the occurrence of a hormone surge? Limb development in metamorphosing species coincides with the peak of metamorphosis, and, as this feature appears not to be delayed in the neotenic development of axolotl larvae, we decided to try the stage when comparable species metamorphose, specifically the stages when the limbs differentiate. Consequently, axolotl larvae were studied during the period from the emergence of hindlimb stubs to complete toe differentiation. Simultaneous with measurements of thyroxine concentrations, brain tissue was analyzed with a marker for synapse formation. It turned out that in the middle of the period there was a maximum in synapse formation, immediately followed by a period of extremely high thyroxine concentrations in the plasma. This was interpreted as a period of intense hypothalamic stimulation of the pituitary TSH cells, caused by an equally intense neuron maturation. Induction of metamorphosis in older larvae leads to further neuronal changes in the brain, judged by changes, especially in disialogangliosides and gangliosides and their precursors during T_4-induced metamorphosis (Hilbig *et al.*, 1987).

Metamorphosis can be induced at every level of the thyroid axis, and will proceed in the same way, although the rate may, for example, vary with the method. Induced metamorphosis is similar to spontaneous metamorphosis — with a few differences. Some of them are easily explained as methodological differences, while others may contain information on differences between metamorphosing and non-metamorphosing species.

Induction of metamorphosis with thyroid hormone: observations on the sensitivity of the peripheral tissues

As mentioned above, Galton (1992) concluded that low thyroid hormone level, low 5'D activity, and low receptor number all seemed to contribute to neoteny in the axolotl. Successful attempts to induce metamorphosis with thyroid hormone add more angles to this statement. Thyroid hormone is usually given by injection or immersion, although feeding has been used previously. In fact, the first experiment to show the significance of thyroid hormone for metamorphosis was done by Gudernatsch (1912), who fed various (horse) tissues to tadpoles and noticed that those fed thyroid tissue went into premature metamorphosis.

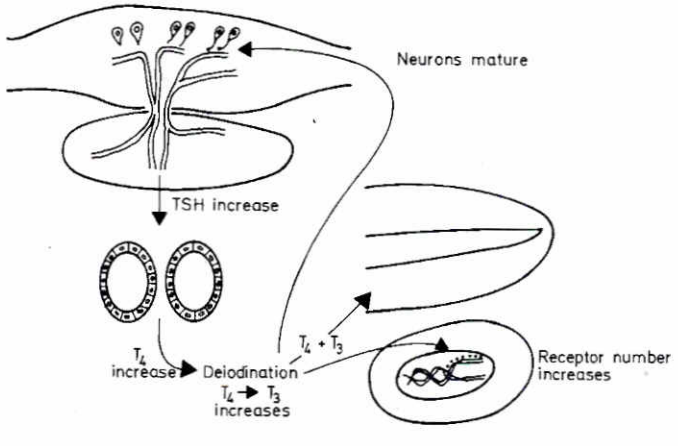

Fig. 3. Focal points of the activity increase in the thyroid axis leading up to metamorphosis.

In our laboratory immersion is the preferred way, because it gives a uniform and reproducible action. Twenty nM T_3 or T_4 generally leads to metamorphosis at a rate which is not increased by higher doses. Toxic effects may be seen from about 80 nM. Induction always starts with a latency period. The first sign that metamorphosis is underway is a distinct weight loss. Axolotls lose 30-40% of their weight during metamorphosis, mostly because water binding in connective tissue decreases. The energy cost of metamorphosis in axolotls is slight, being not more than 10% (Rosenkilde *et al.*, unpubl). Gills and tail fin diminish visibly from 7-9 days. The first molt usually takes place about 11 days from the start of induction. Before metamorphosis, the skin is fish-like, desquamizing in minute scales, while metamorphosed animals shed large pieces of skin in weekly or biweekly molts. At 3 weeks from the start metamorphosis is more or less complete.

Induction of metamorphosis with thyroid stimulating hormone (TSH)

The axolotl thyroid gland contains large amounts of thyroglobulin and thus stored hormone that can be released upon sufficiently strong stimulation (Jacobs *et al.*, 1988b). We induced metamorphosis by injections of bovine TSH, 3 times a week (Andersen and Rosenkilde, unpubl.) Completion of metamorphosis was obtained only after 9 weeks with 75 mIU and after 14 weeks with 7.5 mIU per injection. It is unlikely that the slow response was caused by using heterologous (bovine) TSH, since the thyroid gland was strongly activated, as judged by the uptake of radioactive iodine. Measurement of ^{131}I uptake after 11 weeks of TSH injections showed high uptakes, i.e., strong activation of the thyroid. Thus, the thyroid appears to have a reasonable sensitivity towards TSH. The protracted metamorphosis may be due to decreasing thyroid hormone concentrations, caused by progressive thyroid degeneration. After metamorphosis, the animals developed signs of severe hypothyroidy, and autopsy showed almost complete degeneration of the thyroid. Exhaustion is a possible explanation; autoimmune reaction to the long treatment with bovine TSH may be more plausible. Schultheiss (1980) demonstrated a considerable difference between axolotl and bovine thyrotropin. A pituitary protein isolated by PAGE had an 8 times stronger (based on weight) effect on T_4-release from axolotl thyroid glands than bovine thyrotropin.

Induction of metamorphosis via the brain

Another attempt to get information on neoteny in *Ambystoma* was inspired by Norris and Gern (1976). In a beautiful experiment they induced metamorphosis in *A. tigrinum* from a neotenic population by hypothalamic implantation of thyroid hormone. They showed that metamorphosis could be induced by implantation of 0.2 ng T_4. The amount which led to metamorphosis when implanted in the brain was 10 times smaller than the dose necessary to induce metamorphosis by systemic administration (10x0.2 ng). Norris and Gern measured uptake of radioactive iodine as a parameter of thyroid activity and showed that hypothalamic implantation led to an activation of the thyroid axis not seen after systemic administration of thyroid hormone, and microscopy showed an apparently precocious general activation of the hypothalamus

in immature larvae. This activation had occurred in adult neotenes without hypothalamic stimulation.

Neoteny appears more deeply rooted in *A. mexicanum* than in *A. tigrinum*. Individuals of *A. tigrinum* may transform spontaneously under conditions which can be suspected to cause general disturbance in the central nervous system, e.g., when animals are caught and taken into the laboratory, or during periods of high temperature (Norris *et al.*, 1977). Nevertheless, it seemed worthwhile to test whether metamorphosis could be induced in Mexican axolotls by hypothalamic stimulation. In one experiment, injection of 2 μg T_3 into the hypothalamus (third brain ventricle) led to metamorphosis in all animals, while the same amount of T_4 gave results in all but one larva (Rosenkilde *et al.*, unpubl.) In two further attempts, metamorphic progress was much slower, and transformation was incomplete when the experiments for other reasons had to be terminated.

Jacobs and Kühn (1988) injected synthetic LHRH intravenously in male neotenic axolotls and found increased T_4 with 10 mIU/g and 50 mIU/g LHRH. In another experimental series (Jacobs *et al.*, 1988b) they found increased plasma T_4 after TRH injection in axolotls after metamorphosis in one experiment but not in the other, while neotenic axolotls did not respond in any of the experiments. *The fact that hypothalamic induction is possible shows that the TSH cells of axolotl larvae can respond to some kinds of stimulators, and our experience shows that their own TSH secretion may suffice to induce metamorphosis.*

Possible mechanisms which drive axolotl neoteny

The possible mechanisms of axolotl neoteny can be discussed from the background of the standard pattern of amphibian development.

Why doesn't metamorphosis occur after the endogenous hypothalamic surge?

In rather early larvae, strong hypothalamic stimulation induces a TSH surge which in turn causes thyroid secretion and a high plasma level of T_4. This does not lead to metamorphosis. Is that because T_4 is not deiodinated into the more active T_3: in other words, is 5'D not produced? If so, is the hormone surge too short-lasting to bring about the change?

The surge of thyroxine secretion may be so short that the duration of the stimulation period is determined by the maximal concentration in conjunction with the biological half-life of thyroxine. With a half-life of 14 h (Rosenkilde, 1976), a given amount of thyroxine would be reduced to one fourth after a day. A momentary secretory pulse raising the thyroxine concentration to 40 nM, would be reduced to 10 nM after one day and to 3 nM after two days.

Alternatively, the mobilization of receptors could be too slow. In another context (Rosenkilde, 1985, and unpubl.), we have induced metamorphosis by immersing larvae of stages around the hormone surge in T_3 solutions. Metamorphosis occurred with the same latency as in later larvae. The earliest stages were prior to the surge, and the latest were at a stage corresponding to the middle of the surge at the start of T_3 immersion. Clear indices of metamorphosis were seen after a latency of 11 days (10-12 days) with no difference between larvae of early and later stages. These rather early larvae are thus able to develop sensitivity to thyroid hormone with the same latency as older axolotls. According to the larval stages, none would be past the surge at the end of the experiment. Thus, *low sensitivity to T_3 does not appear to explain axolotl neoteny.* Deiodination of T_4 to T_3 functions or can be induced in neotenic axolotls (Jacobs *et al.*, 1988b). Prahlad and DeLanney (1965) found that metamorphosis was induced equally well with T_4 and T_3, but 3-4 days faster with T_3. However, if the early larvae are not yet able to develop 5'D activity as a response to the T_4 surge, they will not transform T_4 to the much more active T_3. To test this, we immersed larvae of stages around toe development (4-5 weeks old) in 40 nM solutions of either T_3 or T_4 (Rosenkilde, unpubl.). Once again, T_3 caused metamorphosis with short latency. However, T_4 solutions only caused visible metamorphic change after an immersion period of 3 weeks or more, a period appreciably longer than the duration of the T_4 surge. We repeated the experiment with 3-month-old larvae and got the same result. Thus it appears that neoteny in the axolotl is caused by an appreciable delay in the development of 5'D activity.

Hypothalamic inactivity or inhibition?

The attempts to induce metamorphosis by stimulation of brain neurons with thyroid hormone show that the axolotl in stages past the endogenous surge can revive an activation of the thyroid axis from the TSH cells down to the peripheral cells. It is tempting to imagine that the hormone activates hypothalamic neurons to secrete hypophysiotropic hormone, but other interpretations are possible, e.g., that it removes an inhibitor from brain neurons or TSH cells. The existence of such an inhibitor is suggested by an old observation by Rosenkilde (1972) that ectopic grafting of the anterior pituitary in several amphibian species led to increased thyroid activity, judged by the uptake of radioactive iodine as an indicator of increased thyroxine synthesis. In axolotls of adult form (after induced metamorphosis), ectopic grafting of the anterior pituitary was performed in three separate experiments. As often with axolotls, the result shows some variation from one experiment to the other, but in two out of three experiments the group with ectopic pituitary had higher ^{131}I uptake than the controls (Rosenkilde, 1969 and unpubl.; Rosenkilde and Nielsen, unpubl.)

An older study corroborates, in a curious way, the existence of a cerebral inhibition of TSH secretion (Jørgensen and Larsen, 1963). The experiment was part of a study aiming at the regulation of molting in urodeles and used (induced) postmetamorphic Mexican axolotls. In urodeles, molting is dependent upon thyroid hormone. Hypophysectomized specimens do not molt, but treatment with thyroid hormone induces molting. The role of the pituitary in negative feedback regulation was studied in the same experiment. It turned out that injection of a high dose of T_4 would initially cause a series of molts with short intermolt periods, as a result of the high thyroid hormone titer. Then would follow an extremely long period without molts, interpreted as a prolonged negative feedback inhibition. A peculiar trait was that this phenomenon was seen only in intact animals, but not in axolotls with autografted anterior pituitary. Thus, the prolonged negative feedback inhibition seems to depend on normal brain control of the pituitary. Quantitative differences in resistance to metamorphosis could be caused by varying the ratios between agonists and/or antagonists acting at one or more levels of the thyroid axis. Two factors, prolactin and adrenal corticoid hormone, deserve special consideration, because their actions in spontaneous metamorphosis are well established and they are under hypothalamic control.

Prolactin

Prolactin is the classical thyroid antagonist. It retards or prevents metamorphosis of *A. tigrinum*, acting (at least) on peripheral tissues (Platt and Christopher, 1977; Platt *et al.*, 1978). In anuran metamorphosis, prolactin secretion increases to a maximum in late metamorphic climax, and transformation rate is accelerated by injection of prolactin antiserum (Yamamoto and Kikuyama, 1982a,b). That is, prolactin acts in the spontaneously metamorphosing frog and probably also in the urodele. The generally known hypothalamic inhibition of prolactin secretion becomes dominant later, but in metamorphosis prolactin secretion is initiated by strong stimulation (Kawamura *et al.*, 1986). Prolactin could have a role in preventing metamorphosis by a combination of its antimetamorphic action and an initiation of prolactin secretion by hypothalamic stimulation. If the hypothalamic stimulatory waves that activate TSH and prolactin cells in the anterior pituitary are shifted from the pattern that regulates metamorphosis in transforming species, and if the thyroxine surge is of short duration (as suggested above), prolactin could delay the tissue response until the thyroxine concentration is too low to cause metamorphic change.

Corticoids

Demonstrations of agonist effects by corticoid hormone are equally as classical as the prolactin antagonist demonstrations mentioned above. Acceleration of metamorphosis by corticosterone involves an increase in T_3 receptor number (Suzuki and Kikuyama 1983). Carr and Norris (1988) showed a maximum in plasma corticosterone at (spontaneous) midmetamorphosis in *Ambystoma tigrinum*. Schultheiss and Hanke (1972) found that corticoids can provoke some of the changes usually connected with metamorphosis in the axolotl (some water loss and slight cutaneous change), but metamorphosis can not be induced by corticoids alone.

In summary, both prolactin activity and corticoid inactivity could have a role in axolotl neoteny, but it is far from clear whether they are actually involved.

Sensitivity shifts in ontogenesis

At later stages (the stages investigated in most experiments), activity is low in the whole thyroid axis: hypothalamus, TSH cells, and thyroid; and the tissue responsivity is somewhat low compared to amphibians in general. Furthermore, axolotls from some colonies may differ in responsiveness to stimulation of the pituitary TSH cells or the thyroid (see Jacobs *et al.*, 1988b; Galton, 1992; Rosenkilde *et al.*, unpubl.) After induced metamorphosis, the thyroid axis functions at a higher set point than before metamorphosis, at least in axolotls from some colonies (Jørgensen and Larsen, 1963; Jacobs *et al.*, 1988b). The low activity in axolotls from other colonies may be general for the thyroid system, as the TSH cells have low sensitivity to hypothalamic stimulation (Galton, 1992), and the 5'D activity seems also to be lower than in axolotls from more active colonies. This trait is possibly of significance in axolotl neoteny, and it has definitely been of significance in the discussion of the mechanism behind neoteny.

In a facultative neotenic species, *Ambystoma gracile*, larvae exhibited a gradual rise in thyroid activity up to the mean size for metamorphosis. Some metamorphosed spontaneously, others continued neotenic. Both forms exhibited a decline in thyroid activity when they had passed metamorphic size. The higher altitude population had the higher tendency toward neoteny and a lower mean thyroid activity when approaching metamorphic size (Eagleson and McKeown, 1978).

Post metamorphic events

After induced metamorphosis

In our experience there is wide variation with respect to completion of metamorphosis and performance in the transformed state. With identical treatment, some animals will change into a decent-looking salamander with a distinct neck and smooth body shape, regular molting and a slime covered skin. However, some animals show an apparently incomplete transformation, with thick ("larval") neck and conspicuous myomers (as in the larval shape). These animals show a markedly increased mortality at the end of metamorphosis or shortly after. Among the well-proportioned individuals, some have better thyroid function than others. This is easily observed, as the thyroid is indispensable for metamorphosed axolotls (Jørgensen and Larsen, 1963). Thyroid defect can be diagnosed by defective molting, and results in death after some time unless these animals receive supplementary thyroid hormone.

Older axolotls (several years of age) seem to tolerate induced metamorphosis less well than younger specimens. That is, they have higher mortality, and they are more apt to retain larval traits such as a thick neck and metameric pattern.

These observations seem to reveal that some are more neotenic than others. Interestingly, Brandon (1976) found that no individual of *Ambystoma dumerilii* would survive more than 5 months after metamorphosis. This Mexican species is not known to undergo metamorphosis in nature, but some individuals transformed spontaneously in the laboratory, and others did so under T_4 treatment.

Heredity of neoteny as a possibility or an obligation

In an elaborate hybridization experiment involving neotenic *A. mexicanum* and metamorphosing *A. tigrinum,* Humphrey (1967) found that axolotl neoteny predominantly or completely rests with a single recessive gene. In contrast, the *implementation* of neoteny seems to comprise more than one cellular and/or biochemical mechanism. The genetic mechanism segregating neoteny and metamorphosis seems to be extremely labile. Shaffer and Breden (1989) studied allozyme variation in Ozark hellbenders, *Cryptobranchus alleganiensis*. They found a very low genetic variation. Literature studies extended this experience to a great number of species and concluded that most neotenic populations arise as isolated incidents from transforming progenitors.

Neoteny offers some phylogenetic riddles

In view of its long ancestry, it is remarkable that the realization of neoteny seems to rely on different mechanisms in urodele groups. Neoteny probably relies on similar mechanisms in genera such as *Ambystoma*, *Triturus*, and *Pleurodeles*. In particular, the central nervous system and hypothalamus seem to be involved. Thus, spontaneous metamorphosis in hitherto neotenic individuals is often elicited by environmental cues such as temperature increase or disturbance during capture (Norris *et al.*, 1977).

In some species, however, e.g., the southeast European cave salamander, *Proteus*, or the North American mudpuppy, *Necturus*, thyroid hormone induces no significant metamorphic changes. In a number of other species only minor changes have been seen, and attempts to produce further change with extreme doses have

often proved lethal. Most attempts to induce metamorphosis in these species were made long ago, and the treatment has in some instances included factors other than thyroid hormone. A detailed survey is given by Dent (1968).

Galton (1985) studied whether the refractoriness of *Necturus* to thyroid hormone could be explained by inability to convert the weakly active T_4 to the potent T_3, or by absence of T_3-receptors. Her figures show a potency to activate thyroid hormone comparable to that of metamorphosing species. T_3 receptors were below detection level in liver tissue, but were demonstrable in *Necturus* erythrocytes.

Svob *et al.* (1973) cultured tail fin pieces of *Proteus anguineus* and found no differences between controls and pieces cultured with T_4. They also analyzed distribution of iodinated products in thyroid tissue after *in vivo* labeling with ^{125}I. They noted the presence of iodotyrosines, T_3 and T_4. The activity corresponding to T_3 and T_4 is not strong, but histologically the thyroid showed indices of synthetic activity. Svob *et al.* concluded that the thyroid was able to produce hormone but that receptors were lacking. Fox and Durand (1990) observed the cephalic skin of *Proteus anguinus* (age $7^{1/2}$ months-5 years) upon immersion in T_3 solution. They found older animals unresponsive to the treatment, but some of the younger animals showed slight changes, primarily reductions or disappearance of Leydig cells (a strictly larval cell type). The refractoriness to thyroid hormone in these species seems to rely at least partly on absence or paucity of TH receptors, but other changes in the TH effector system may also be involved.

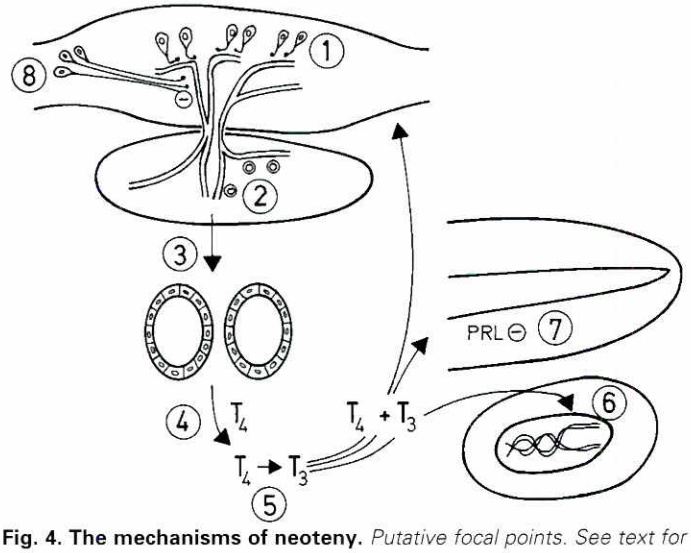

Fig. 4. The mechanisms of neoteny. *Putative focal points. See text for explanation.*

It is striking that neotenic species are systematically and geographically scattered within the urodele subclass. This goes for those in which transforming can be induced as well as for those showing none or only minor response to thyroid hormone. Like the low genetic variation mentioned above, this points to *neoteny as being established on several occasions in urodele evolution.*

Conclusions

The evidence presented in this paper cannot fully explain the mechanism behind neoteny in the axolotl or in other neotenic urodeles. It does, however, point to some processes as probably involved and makes the role of others less probable. Figure 4 repeats the diagram of the thyroid axis with numbers referring to levels of special interest.

The T_4 surge occurring at the stage when toes differentiate shows (1) that TSH stimulating neurons have matured and are able to secrete; (2) that the TSH cells are able to secrete and stimulate the thyroid; (3) that this gland is sensitive to TSH; and (4) able to secrete thyroxine to a high plasma level. The immersion experiments show that both young and older larvae are (5) able to respond to T_3 with metamorphosis, but (6) the ability to activate thyroid hormone by deiodination of T_4 to T_3 is delayed compared to metamorphosing species. Finally, two possible inhibitors are suggested by some experiments. Inhibition by prolactin, most probably (7) at the tissue level, or (8) a cerebral inhibitor, acting at the pituitary stimulating neurons.

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