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Evolution of ligands, receptors and metabolizing enzymes of thyroid signaling.

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Abstract

Thyroid hormones (THs) play important roles in vertebrates such as the control of the metabolism, development and seasonality. Given the pleiotropic effects of thyroid disorders (developmental delay, mood disorder, tachycardia, *etc*), THs signaling is highly investigated, specially using mammalian models. In addition, the critical role of TH in controlling frog metamorphosis has led to the use of *Xenopus* as another prominent model to study THs action. Nevertheless, animals regarded as non-model species can also improve our understanding of THs signaling. For instance, studies in amphioxus highlighted the role of Triac as a *bona fide* thyroid hormone receptor (TR) ligand. In this review, we discuss our current understanding of the THs signaling in the different taxa forming the metazoans (multicellular animals) group. We mainly focus on three actors of the THs signaling: the ligand, the receptor and the deiodinases, enzymes playing a critical role in THs metabolism. By doing so, we also pinpoint many key questions that remain unanswered. How can THs accelerate metamorphosis in tunicates and echinoderms while their TRs have not been yet demonstrated as functional THs receptors in these species? Do THs have a biological effect in insects and cnidarians even though they do not have any TR? What is the basic function of THs in invertebrate protostomia? These questions can appear disconnected from pharmacological issues and human applications, but the investigation of THs signaling at the metazoans scale can greatly improve our understanding of this major endocrinological pathway.

Introduction

Thyroid hormones (THs) are involved in many biological processes in vertebrates (Holzer and Laudet, 2015), such as energy metabolism (reviewed in Mullur et al., 2014), thermoregulation (Lebon et al., 2001) but also development (Delange, 2001; Flamant et al., 2002; Göthe et al., 1999; Morte et al., 2002). In addition, THs are a critical signal triggering metamorphosis in amphibians as well as in many fishes, and are therefore important hormones that control the life cycle in vertebrates (Laudet, 2011). In humans, defects in THs signaling, either hyperthyroidism (too high THs concentration) or hypothyroidism (too low THs concentration), can lead to severe pathological conditions with a wide variety of symptoms such as tiredness, weight change, mood disorder, developmental delay, tachycardia. In addition, complete lack of THs is lethal. Thyroid cancers are the most common endocrine cancers with increasing incidence (Pacini et al., 2012). The primary treatment for such cancers is the complete or partial removal of the thyroid, leading to a massive disruption of the gland physiology (Pacini et al., 2012). Therefore, TH synthesis, its degradation and its signaling activity are the subject of many investigations.

Two molecules are collectively referred as THs: the native hormone thyroxine (3,3',5,5'-T₄ or T₄), which represents 80% of the hormone synthesized by the thyroid (Sapin and Schlienger, 2003), and the active hormone triiodothyronine (3,3',5-T₃ or T₃) that has a 10-fold higher affinity for the receptor than T₄ (Chopra, 1976). T₄ is used as a precursor by three specific enzymes, the deiodinases that synthesize both active and inactive TH derivatives by catalyzing the removal of iodine atom from T₄. More specifically, two reactions are distinguished. The outer ring deiodination, catalyzed by the deiodinase type II (Dio2, Kuiper et al., 2005), transforms T₄ in target tissues into T₃ (Figure 1). Dio2 is therefore considered as an activating deiodinase. It also turns T₃ into 3,5-T₂, a TH derivative long considered as inactive but for which accumulative evidences suggest a biological role (Lietzow et al., 2016; Mendoza et al., 2013). The inner ring deiodination is catalyzed by the deiodinase type III (Dio3) which is considered as an inactivating deiodinase as it turns T₄ into reverse T₃ (3-3'-5'-T₃ or rT₃) and T₃ into 3-3'-T₂ (Figure 1), compounds that have no biological activity (Chopra et al., 1978; Moreno et al., 2003). This process is also referred as an inner ring deiodination. The deiodinase type I (Dio1) has both outer ring and inner ring deiodination activity but with a lower activity than Dio2 and Dio3. Although it is required to regulate the normal level of TH, its biological role is less clear (Maia et al., 2011).

THs act by binding to specific nuclear receptors: the thyroid hormone receptors (TRs), which belong to the nuclear receptor superfamily. TRs are ligand-dependent transcription factors that bind to DNA. TRs do not bind DNA alone but form a heterodimer with RXR, another nuclear receptor, and it is TR/RXR that forms the real functional unit of gene transcription control (Kojetin et al., 2015). In the classical view, TRs repress their target genes in absence of the ligand and activate them in presence of the ligand (reviewed in Gronemeyer et al., 2004). Some genes undergo a negative regulation by THs and are actively repressed by liganded TRs (Hollenberg et al., 1995; Matsunaga et al., 2015). Additionally, ligand binding influences the receptor binding on DNA (Ramadoss et al., 2014). This suggests that the biological reality of TR transcription regulation is more complex than the classical model (Flamant, 2016). There are two TRs in vertebrates, *TRα* and *TRβ* that emerged from the whole genome duplication events at the basis of this group (Kuraku et al., 2008; Marchand et al., 2001; Smith et al., 2013). Teleost fishes underwent an additional specific whole genome duplication which results in two *TRα* genes, *TRα-A* and *TRα-B* (Escriva et al., 2004; Marchand et al., 2001).

However, TH signaling is not a vertebrate innovation (Paris et al., 2008a) and it is established that TH-signaling was functional at the base of chordates (Paris et al., 2008a) and even possibly at the base of the bilaterians (Bertrand et al., 2004; Wu et al., 2007). Moreover, the investigation in the invertebrate chordate amphioxus (*Branchiostoma sp*) suggests that the 3,5,3'-triiodo-thyroacetic acid (Triac), a deaminated derivative of T3 (Figure 1), was a *bona fide* TRs ligand in the chordate common ancestor (Paris et al., 2008b). Thus, it is useful to describe what the studies of non-classical models bring to our understanding of TH signaling. In this review we will explore our current understanding of TH signaling in several metazoan groups starting from vertebrates toward more distant groups.

Even in vertebrates, the classical ligand can hide an astonishing diversity of active compounds

As introduced above, THs are involved in many biological processes. T3 is well acknowledged and recognized as the canonical ligand of TR in vertebrates. T4 is generally considered only as a precursor of T3 but several evidence, and in particular the recent detailed analysis of mouse strains in which the deiodinase genes have been inactivated, led to the suggestion that it may have a biological function in itself via TRs (Galton, 2017). However we will not discuss much T4 and T3 here as the latest discoveries on their biological role are covered in other chapter of this issue. We will discuss here whether other molecules could be considered as ligand and the role of the deiodinases in the modulation of TH signaling.

As previously mentioned, there are two paralogs of vertebrate TRs, *TRα* and *TRβ*, which originate from the two rounds of whole genome duplication that happened at the base of all vertebrates (Kuraku et al., 2008). This duplication allows the divergence of the two copies in terms of amino acid sequence and regulatory sequence. In the mouse, *TRα* and *TRβ* regulate different subsets of genes, when tested in the same chromatin context, indicating that they have different intrinsic properties (Chatonnet et al., 2013). This divergence between *TRα* and *TRβ* led to a slightly different pharmacology between the two receptors. Indeed, there are some *TRβ* selective compounds such as GC-1 that have been developed for clinical purpose (Bleicher et al., 2008). *TRα* is expressed in the early development whereas *TRβ* is expressed later (Forrest et al., 1991), indicating differences in regulation. In amphibians as well, the timing of TR expression is different between the two paralogs. *TRβ* is late expressed and self-induced by the surge of T3 at metamorphosis because of a thyroid response element in the promoter of the gene (Machuca et al., 1995). This is not the case for *TRα* which is early expressed during *Xenopus* metamorphosis (Eliceiri and Brown, 1994; Kawahara et al., 1991; Yaoita and Brown, 1990). In teleosts, *TRα-A* and *TRα-B* are also differentially expressed as exemplified in the flounder *Paralichthys olivaceus*, where *TRα-A* is expressed earlier at a higher level than *TRα-B* and *TRβ* (Yamano and Miwa, 1998). This illustrates how TR duplication allows the divergence of biological roles between *TRα* and *TRβ*.

The sea lamprey *Petromyzon marinus* has two TRs, TR1 and TR2, whose evolution is not clearly understood yet. Indeed, in a phylogenetic reconstruction they do not group with the *TRα* and *TRβ* but together within an agnathan group at the base of the gnathostomes (Manzon et al., 2014). The most singular feature of lamprey regarding TH pathway is its involvement in metamorphosis. Lamprey is the only vertebrate (to our knowledge) where a decrease of THs, instead of an increase, is observed at the onset of metamorphosis (Youson et al., 1994). Consistently with this peculiarity, THs treatments delay lamprey metamorphosis (Youson et al., 1997) while goitrogen treatments (inhibitors of TH synthesis) induce a precocious metamorphosis (Manzon et al., 2001). This is even more curious since TR1 and TR2 behave as genuine TR and transactivate target genes in presence of

ligand (T4 or T3, Manzon et al., 2014). More curiously, the dynamic expression of lamprey TR1 during metamorphosis matches the remodeling of the tissues, suggesting that TR1 and therefore TH signaling is actively involved in metamorphosis (Manzon et al., 2014). One explanation could be that lamprey TRs behave differently *in vivo* than *in cellulo*, where their activity has been tested, because of a specific co-factor that would repress instead of activate target genes. Alternatively, it is possible that the active compound in this species is not T3 and that the decrease in T3 level corresponds to its transformation into an unknown compound that will be the endogenous TR ligand and surge during metamorphosis.

There is an increasing interest for the T3 derivative 3,5-T2 (Figure 1). Although it is classically considered as an inactive T3 derivative, 3,5-T2 actually exhibits biological activities (for review see: Orozco et al., 2014). It can induce the mRNA synthesis of several genes such as *TSH* (Moreno et al., 1998), *Dio1* (Baur et al., 1997) or *TR β* (Ball et al., 1997). More generally, in mouse, 3,5-T2 has thyromimetic activity on the hypothalamus pituitary axis and lipid metabolism (Jonas et al., 2015) as well as on the liver transcriptome (Lietzow et al., 2016). In the killifish *Fundulus heteroclitus*, 3,5-T2 has been shown to be a ligand for TR β (Mendoza et al., 2013), but this idea is still debated as 3,5-T2 has a very low binding potency on TR in rat and salmon (Darling et al., 1983). Most of the studies on 3,5-T2 are now focused on its non-genomic effect, that is to say the effects that are not mediated by TRs but through cytoplasmic and membrane interactions with proteins (reviewed in Goglia, 2005).

There is another class of TH derivatives of pharmacological interest: the acid acetic analog of TH: Tetrac and Triac (Figure 1). These compounds are synthesized in peripheral tissues such as liver (Rutgers et al., 1989), kidneys, (Flock et al., 1965) and brain (Rall et al., 1957) by deamination of the TH backbone. Although little is known about their metabolism, the TH aminotransferase and the L-amino acid oxydase are likely involved in their synthesis (for reviews see Engler and Burger, 1984; Wu et al., 2005). Because of its short half-life (Goslings et al., 1976; Wu et al., 2005), Triac is considered to have an overall low biological activity (Wu et al., 2005) and is not considered as a true endogenous TR ligand. Nonetheless, Triac has the same affinity for TR α than T3, and a two times higher affinity for TR β than T3 (Messier et al., 2001; Schueler et al., 1990; Takeda et al., 1995), which results in a higher transcription activity (Messier et al., 2001; Schueler et al., 1990; Takeda et al., 1995). As a consequence, clinical use of Triac in hormone replacement therapy is considered to have a better outcome than T4 or T3 treatments (Guran et al., 2009; Kunitake et al., 1989). Another interesting feature of Triac is that it does not require the monocarboxylate transporter 8 (MCT8) for its cellular uptake contrary to T3 (Kersseboom et al., 2014). Therefore, Triac is a very interesting therapeutic molecule for pathologies with mutation of the MCT8 transporter. For instance, this is the case in the Allan-Herndon-Dudley syndrome where T3 uptake to the brain is impaired and T3 treatment is not effective (Kersseboom et al., 2014). This is also true for Tetrac, the acidic derivative of T4 (Figure 1), which has a very low affinity for TR but still has a biological activity in MCT8 deficient mice (Horn et al., 2013). Unfortunately, the question of the cellular uptake of Triac and Tetrac through specific transporters remains poorly investigated. Competition assay with the transporter Oatp14 shows that Triac can inhibit the uptake of T4, suggesting that Oatp14 can also transport Triac (Tohyama et al., 2004). Nevertheless, the question of the transport of Triac was not addressed directly and the affinity of the transporter for T4 is much higher than for Triac (Tohyama et al., 2004). To our knowledge, it is therefore not known how Triac and Tetrac are transported through cell membrane. Thus, although Tetrac and Triac are considered to have a low biological activity and are not viewed as canonical TR ligands, they are endogenous TH derivatives and can exert, in some

extend, a THs signaling (reviewed in Senese et al., 2014). Another compound, the 3-Iodothyronamine (T1AM), can be produced through the decarboxylation of the T4 backbone (Figure 1, for review see Scanlan, 2011). Although its synthesis and biological role remain obscure, some evidence show that they have a biological role (Braulke et al., 2008). This calls for a deeper investigation of T1AM and more generally, the decarboxylated analogs of THs.

Ligands are not the only actors capable of modulating TH signaling. As T4 is produced centrally in the thyroid gland, the deiodinases, expressed in target tissues, are key controllers of THs signaling. The importance of deiodinase is highlighted by the study of amphibian models. One striking feature of amphibian metamorphosis is the late regression of the tadpole tail. How is the tail protected from the remodeling action of thyroid hormones (which at the same time induce limb growth) during a large part of the metamorphosis? The local expression of the deiodinase provides an explanation for this phenomenon since it controls the local amount of available T3 (Becker et al., 1997; Kawahara et al., 1999). First, during early metamorphosis (stage 49, Cai and Brown, 2004) *Dio2* is expressed in the limb buds, which locally turns T4 into T3, enhancing TH signaling and ultimately resulting in limbs growth and development (Brown et al., 2005). At the same time, *Dio3* is highly expressed in the tail resulting in its preservation as *Dio3* transforms T4 and T3 in inactive derivatives (Becker et al., 1997). As a consequence, there is no or little T3 signaling in the tail at this early period, which means limbs and tail can coexist at the same time. Later in metamorphosis (stage 62), *Dio3* expression turns off in the tail, and concomitantly there is a surge of *Dio2* expression. As a consequence, the local level of T3 rises and this induces the regression of the tail cells (Cai and Brown, 2004). The local expression of the deiodinases controls the local availability of THs, which explains how a systemic signaling can be delayed in a specific organ. Interestingly, this regulation at the local level of TH also happens in other species such as teleost fishes, since *Dio2* and *Dio3* are also differentially expressed during their metamorphosis (Campinho et al., 2012; Isorna et al., 2009). Such a local regulation is also critical in the TH effect on seasonality, which will however not be discussed here (for review see Dardente et al., 2014). The deiodinase expression seems overall conserved in vertebrates. For instance, *Dio2* is expressed in the skin of amphibians and fish during their metamorphosis as well as in their brains (Becker et al., 1997; Campinho et al., 2012; Lorgen et al., 2015; Manzon and Denver, 2004). Nevertheless, some species specificities exist, as for instance *Dio1* that has been lost in the amphibian *Rana catesbeiana* (Becker et al., 1997).

In invertebrate chordates, TRIAC is an endogenous active compound....

Because of its prominent role in frog and flatfish metamorphosis and since this process is widespread in metazoans, outside vertebrates, most of the studies on TH are focused on its role in post-embryonic development (Laudet, 2011).

The study of the cephalochordate *Branchiostoma floridae* (Figure 2) shed light on the origin of TH signaling in chordates. Indeed, its metamorphosis (the spectacular transformation of an asymmetric pelagic larva into a symmetrical benthic juvenile) is controlled by TH (Paris et al., 2008a, 2008b). The main difference though, is that the ligand of the amphioxus TR is neither T4 nor T3 which are unable to bind to the receptor, but their derivative Triac (Paris et al., 2010, 2008b). It has been shown that in amphioxus, the TR ligand binding pocket is smaller due to some specific mutations and is unable to accommodate T4 or T3. In contrast, Triac being smaller due to the lack of an amino group can enter into the pocket and regulate the conformational change of the receptor. As a consequence, the Amphioxus TR behaves as a *bona fide* TR: it binds DNA, forms a heterodimer with RXR, binds Triac as a ligand and induces the transactivation of target genes. Triac treatment of amphioxus larvae

effectively leads to a precocious metamorphosis, and treatment with a TR antagonist such as NH3 delays it. TR expression peaks at metamorphosis and there is a thyroid response element in the promoter of the TR, as for TR β in vertebrates (Paris et al., 2008b). Moreover, one of the 5 known deiodinases in *B. floridae* has been biochemically characterized and proven to be specialized in Triac deiodination (Klootwijk et al., 2011), indicating that Triac specialized metabolism effectively exists in this species. Studies of the amphioxus *Branchiostoma blecheri* have shown that Triac signaling regulates C/EBP, a vertebrate TH target gene, suggesting again a conserved TH signaling pathway in cephalochordates (Wang et al., 2009). Together, these data build the hypothesis that the TH pathway and its biological roles appear at the basis of chordates (Paris and Laudet, 2008).

... but not in all known cases since the tunicate TR appears as an orphan receptor.

In tunicates (Figure 2), another invertebrate chordate and sister group of vertebrates, TH also appears to have a role in metamorphosis. However the current situation in this group is unclear. Indeed, the role of TH is quite clear but how this role is mediated remains unknown. Some experiments suggest that TH treatments on larvae accelerate the (already very rapid) metamorphosis and settlement of the larvae, although this is less spectacular than in the case of amphibians or amphioxus (Patricolo et al., 1981, 2001). The presence of T4 in *Ciona intestinalis* has been suggested by immunodetection methods (D'Agati and Cammarata, 2006). This is however not entirely convincing since the specificity of the antibody used is unclear, as it has been assessed only in the context of compounds known in human. Therefore, these data indicate that there probably exists a TH-like compound, but do not formally prove that it is T4. An ortholog of vertebrate deiodinase has been identified in the tunicate *Halocynthia roretzi* with both T4 and rT3 as substrates (D'Agati and Cammarata, 2006; Sheperdley et al., 2004). A clear TR ortholog has been cloned, but this receptor is very divergent, in particular in the ligand binding domain (25% sequence identity with human TRs, whereas the amphioxus LBD display 38% (Carosa et al., 1998). Almost 20 years after its cloning, this receptor remains enigmatic as it behaves as an orphan receptor. None of the known TH derivatives are able to regulate its transcriptional activity. The original authors, as well as ourselves, have tried many different compounds but none of them are able to bind or activate the receptor (Carosa et al., 1998). Therefore, it seems clear that there is a secretion of TH-like compounds in tunicates (Hiruta et al., 2005; Ogasawara et al., 1999), but so far there is no evidence that the effects of TH on tunicates are mediated by the known TR ortholog.

Echinoderms provide another mysterious case...

Echinoderms comprise species such as sea stars, sea urchins, sea cucumbers and sand dollars (Figure 2). Our knowledge of the role of THs in the metamorphosis in echinoderms is reminiscent of the situation observed in tunicates. TH-like compounds are detected in echinoderms tissues and accelerate the complex metamorphosis of the Eiffel-tower-shaped larvae into the benthic juvenile in various sea urchins (Chino et al., 1994; Heyland et al., 2006) and sand dollars species (Heyland et al., 2004; Saito et al., 1998). T4 can also accelerate sea stars development in a dose-dependent manner (Johnson and Cartwright, 1996). Interestingly, nuclear extract of the sea urchin *Hemicentrotus pulcherrimus* can bind T4 (Saito et al., 2012), indicating that a factor within the nucleus, and not the cytoplasm, can bind the hormone. This may therefore be considered as an indication in favor of the presence of a nuclear receptor acting as a TR. And indeed there is a TR ortholog present in echinoderm genomes (Tu et al., 2012). Nevertheless, as in tunicates, the formal demonstration of THs binding to the isolated TR is still lacking in echinoderms. We have tested all the compounds we

could think of in the *Paracentrotus lividus* TR that we have isolated in our lab, without detecting any positive hit (M. Paris, G.H. and V.L. unpublished results). Therefore, as for tunicates, it is impossible to conclude that TH signaling, through TR and transcriptional activation of target genes, is involved in echinoderm metamorphosis as in cephalochordates and vertebrates. In the sea urchin, *Strongylocentrotus purpuratus*, it has been shown that iodine uptake in the larvae is dependent of a peroxidase activity and not the sodium/iodine symporter found in vertebrates (Miller and Heyland, 2013). This may be reminiscent of an active thyroid function and leads to the conclusion that there is a specific iodine metabolism in echinoderms (Miller and Heyland, 2013). In any case, it indicates that the vertebrate model of iodine uptake cannot be generalized in all chordates. But if and how such a metabolism is translated to a hormonal control of life-history transition, is still an open question in these species. One explanation could be an exogenous origin of iodinated compounds, supplied by algae, which are used as hormones (Chino et al., 1994; Heyland and Moroz, 2005).

A loss of TH signaling in ecdysozoa

Genomics data indicate that there is no TR in ecdysozoans, group including insects (Bertrand et al., 2004; Figure 2) or nematodes (Kostrouchova and Kostrouch, 2015). Although some authors suggest a TR ortholog in the crustacean *Daphnia pulex* (Wu et al., 2007), this sequence appears divergent from the other identified TRs and is therefore not a good candidate for a TR ortholog. In addition, no experimental data confirm that the encoded protein behaves as a genuine TR.

This question is of course particularly interesting in the context of the well-known metamorphosis of arthropods and in particular in insects. However, in arthropods, metamorphosis and growth are not controlled by THs but by two other hormones: the juvenile hormone that promotes growth and prevents metamorphosis (Riddford, 1944) and the steroid derivative hormone ecdysone (reviewed in Thummel, 1996). Ecdysone binds the Ecdysone receptor, a nuclear receptor that forms a heterodimer with USP, the ortholog of RXR (Yao et al., 1994). This similarity in the biological function (post-embryonic developmental control) between THs and ecdysone and their mechanisms (nuclear receptor mediated signal) allowed some authors to draw interesting comparisons between the two signaling systems (reviewed in Flatt et al., 2006). As an illustration, TH derivatives T4, T3, rT3 and 3,5-T2 (Figure 1) have been suggested to decrease the oocyte length in the insect *Locusta migratoria* (Davey and Gordon, 1996; Davey, 2000). However, such an observation of the effect of an exogenously provided compound is not a proof that an endogenous pathway is regulating the process. The presence of TH derivative T2 or mono iodinated thyronine in the locust has been suggested but still lacks a clear confirmation (Flatt et al., 2006). More surprisingly, T3 seems to be able to enter insect cells and T3 treatments with a deiodinase inhibitor are less effective to reduce oocyte size than T3 alone, suggesting the existence of a deiodinase-dependent activity (Davey, 2000). Nevertheless, these data are still difficult to interpret. Since there is no TR ortholog in insects, how is this signal integrated? In addition, to our knowledge, no deiodinase has been identified in insect despite the fact that the molecular signature of those enzymes is well known. Moreover, THs bind cell membrane in insects and competes with the juvenile hormone, suggesting that the two compounds may act on the same membrane receptor (Kim et al., 1999). Thus, the TH effect observed in insects might be caused by the displacement of juvenile hormone. Furthermore, as there are no TRs, the TH binding on membrane is reminiscent of the non-genomic pathway mentioned above (reviewed in Goglia, 2005). As a conclusion, TH derivatives have some marginal effects on insects but their mechanism of action remains largely elusive, casting some doubt on the

importance of this pathway in ecdysozoa. Given the absence of TH related genes in insects (*i.e.* TR, deiodinase), the presence of a chordate-like TH signaling remains dubious, and we remain on the opinion that this pathway is not endogenously present in ecdysozoa and has therefore been lost in the common ancestor of this group (see below). Nevertheless, it could be interesting to re-investigate the influence of TH in insects under the light of the non-genomic pathway or the influence of iodinated compounds found in insect food (*i.e.* plant, Eales, 1997; Heyland and Moroz, 2005).

Contrasting evidence about TH signaling in lophotrochozoans

Lophotrochozoans (Figure 2) are the sister taxa of ecdysozoa and cluster animals such as molluscs, annelids and flatworms. The situation regarding TH signaling is very different than for ecdysozoans since their genomes contain clear TR orthologs (Bertrand et al., 2004). Moreover experimental data indicate that TH signaling is possible in this group. The suggestion of a TH metabolism in annelids and molluscs has been proposed in the 50's with experiments showing an incorporation of the iodine isotope I^{131} in their foreguts (Gorbman et al., 1954). For instance, the sea hare *Aplysia californica* harbors a peroxidase gene that is ortholog to the vertebrate thyroid peroxidase (TPO) that is able to incorporate I^{125} into T4, indicating a possible endogenous synthesis of TH (Heyland et al., 2006). Another example comes from the Pacific oyster *Crassostrea gigas*. HPLC experiments have shown that this mollusk contains T4 and T3. In addition its genome contains a TR that can bind DNA on a thyroid response element. However no convincing evidence of activation of this TR by THs has been reported by these authors. Their data indicate a possible weak activation at very high concentration of T4, T3 and Triac (1 μ M, Huang et al., 2015). Therefore, the biological function of this TR ortholog remains unknown although these findings suggest that TH signaling may be functional in lophotrochozoans. At last, this allows to conclude that TR signaling must have been present in the ancestor of all bilaterian animals and that therefore, when it is not present, this means that it has been secondarily lost.

TH at the base of the metazoan tree

Genomic data from the cnidarians *Acropora millepora* (Grasso et al., 2001) and *Nematostella vectensis* (Putnam et al., 2007) or the porifera *Amphimedon queenslandica* (Srivastava et al., 2010) display no clear TR ortholog, suggesting that the diversification of TR from ancestral nuclear receptor did occur only in bilaterians (Bertrand et al., 2004; Tarrant, 2005). Therefore, TR should be considered as a bilaterian-specific gene (Figure 2). However, some studies suggested that T4 and iodinated compounds can induce the strobilation (polyp to medusa transition) of the cnidaria *Aurelia aurita* (Spangenberg, 1971; Spangenberg et al., 1994), despite the absence of identified TR ortholog. This raises the following questions: How is this signaling integrated? Is there a T4-specific metabolism in cnidarians? Is this effect specific or not? The finding that the RXR ortholog of *A. aurita* and its ligand, 9-cis retinoic acid, are clearly involved in strobilation in the very same species is therefore puzzling (Fuchs et al., 2014). If RXR was already involved in metamorphosis control at the base of metazoans, what was the role of TR, once it appears in bilaterians, on the TR/RXR heterodimer? What is the partner, if any, of RXR in cnidarian in controlling the strobilation and other similar metamorphosis-reminiscent processes? Why is RXR always implicated in metamorphosis control in chordates (with TR) in ecdysozoan (with EcR) and in cnidarian (with still an elusive partner, if any, (Holstein and Laudet, 2014)? All these questions remain open and are very interesting paths to explore in the future.

Iodinated molecules in algae: what link with TH signaling?

The synthesis of TH by the thyroid gland, with the contribution of an enormous precursor, thyroglobulin, appears clearly to be vertebrate specific (Holzer et al., 2016). This observation raises the question of how TH derivatives are formed in non vertebrate species such as amphioxus or sea urchin, which are devoid of thyroid gland. In some invertebrate chordates such as amphioxus and tunicates, the existence of an evolutionary precursor of the thyroid gland called the endostyle may provide a possible solution, but in these species there is no gene reminiscent of thyroglobulin (Holzer et al., 2016). In addition, outside chordates there is no sign of endostyle or of thyroglobulin, and the way THs are formed remains a complete mystery. However, the identification of iodine bound to organic molecules in algae, particularly cyclic ones (Barre et al., 2010; Miller and Heyland, 2010) could provide an explanation, and the supply of TH by algae (that is through feeding) have been suggested in sea urchin (Heyland and Moroz, 2005). Under this hypothesis, TR should be considered as a sensor, indicating the food availability and controlling development and life-history transitions depending on food availability. This would make sense as life-history transitional steps are always demanding and energy-consuming processes (Heyland et al., 2004; Wright et al., 2011). Furthermore, the physiological function of TR and TH in most animals in which it has been explored, mammals but also fish, is connected to energy regulation and the control of large physiological equilibria (Habibi et al., 2012; Little et al., 2013).

Conclusion

In this review, we have discussed about the main actors of TH signaling: the ligands, the receptors and the main metabolizing enzymes. We started on vertebrates, for which THs action is best understood, and discussed about related taxa for which evidence is more scarce until we reached the base of the metazoan tree, and even outside in algae (Figure 2). By doing so, we have highlighted the strong bias existing in our knowledge of the pathway (and more generally all hormones): considering the vertebrate situation as the classical situation and using it as the reference to investigate the non-vertebrate situation. This may not be (and even more: this is most probably not) what happened during evolution. Indeed, the roles of TH signaling, and what we consider as a canonical endogenous ligand (*i.e.* T₃) might actually be a vertebrate specificity (Klootwijk et al., 2011; Paris et al., 2008a).

TH signaling is a striking example of what non classical models can bring to our understanding of an endocrinological pathway. Indeed, these models are used to address different kinds of questions that shed a new light on the biological role of THs. In particular these models allow pleading for a complete unbiased chemical and biochemical characterization of the TH-like compounds present. Several key questions on that matter remain unanswered even in mammals, in particular how deaminated derivatives such as Triac and Tetrac are formed. In addition, there are some indications that TH-like compounds may be more diverse outside vertebrates than in vertebrates. For example in amphioxus there are eight iodine symporter related genes that may participate to the transport of TH-like compounds, as well as five genes related to thyroid peroxidase and five deiodinase genes (Paris et al., 2008a), therefore suggesting an exquisite degree of sophistication in the metabolism and transport of these active compounds. Nevertheless, actual biological functions of these genes have not been investigated yet. Another interesting question would be the role of alternative ligands such as 3,5-T₂ and Triac even in mammals. These have been

dismissed as minor players but, as non-genomic action 20 years ago, should perhaps no longer be neglected. Additionally, the non-genomic effects of THs remain to be investigated outside vertebrates, which could shed a new light on the evolution of THs signaling.

Another important avenue is of course the question of the peculiar mode of formation of THs from a huge precursor protein, thyroglobulin, which exhibits a very complex trafficking in a specialized organ, the thyroid gland. This is clearly a specificity of vertebrates, which has hidden how other metazoans produce THs (or extract them from their food). This should be put in a wider perspective, namely the question of iodine supply and storage, and remains to be analyzed in an unbiased manner. We anticipate that this may provide drastic paradigm changes in the way we consider TH formation in vertebrates. The question of whether thyroglobulin is always the only precursor of TH in vertebrate may appear as heterodox but may benefit from a reappraisal.

Lastly, the connection between the three main aspects of TH *in vivo* function, the control of metabolism, the regulation of seasonality and the control of post-embryonic stages transitions (that is metamorphosis) remains a very important question (Holzer and Laudet, 2015). The fact that food availability is central in all these aspects is important to notice, and the role of TH in basic aspects of nutrition biology (from appetite regulation to digestion and growth) may provide a unifying theme for the biological role of these still enigmatic hormones. Diversity is the hallmark of biology: on thyroid hormone studies as in other aspects of our science, we should take advantage of this diversity and use it as an inspiration to explore new avenues.

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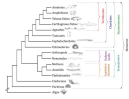
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Legend

Figure 1: The classical TH derivatives. Inner and outer rings are indicated above T4. Red arrows indicate outer ring deiodination performed by Dio2. Blue arrows indicate inner ring deiodination performed by Dio3. Grey arrows indicate deiodination performed by Dio1. Green arrows indicate deaminations. The orange arrow indicates a decarboxylation. The interrogation mark under the T1AM indicates that although possible from TH, the synthesis of this compound remains unknown.

Figure 2: Schematic metazoan phylogeny. The phylogenetic relationships between the main taxonomic groups discussed in this review are represented. Each group is indicated by a bracket. The vertebrate specific TR duplication is indicated.





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Holzer et al., Highlights

The biological role of non-canonical ligands remains to be explored in vertebrates.

Comparative endocrinology shows that T4 and T3 might not be the original TR ligands.

The role of non-canonical ligands remains to be understood outside vertebrates.