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Supplement 1:

Some Historical Milestones

Iodine, the essential trace element main component of TH, has been identified as element in 1811 by Courtois and Lusac, and Eugen Baumann identified what he called 'Jodthyrin' after boiling around 1000 porcine thyroids in sulfuric acid, providing first hints on the biochemical nature of TH; for review see [10]. Unacceptable with life-work balance in our societies is the report that Kendall sees the first thyroxine crystals in his microscope on Christmas day 1914 after isolating several grams of pure T4 from approximately three tons of porcine thyroids over months and years [11]. Kendall also proposes a structure for this molecule in 1919, which he called 'oxindole'. Unfortunately, this observation turns out to be quite wrong as indicated by Harington and Barger in 1927 [12], who succeeded in total synthesis and elucidation of the true, correct structure of thyroxine, an iodothyronine. Much later, in 1953, Gross and Pitt-Rivers [7,8] isolated the "real active thyroid hormone" T3. They only needed five-kilogram batches of thyroid gland, and already used in vivo radioactive labelling of thyroid hormones by injecting sodium 131-iodide into rats, which allowed to identify T3 as a separate spot in autoradiograms of tryptic hydrolysates from those treated rats. Claims have been made that such a compound already had been discovered before by a scientist team in Australia [13], one whom (FH) visited Pitt-Rivers and discussed their findings, possibly guiding the London team to success.

Not only adult hypothyroid myxedematous patients but also congenital hypothyroid cretin patients were treated with various thyroid extracts from ovine, bovine, or porcine origin, and during the mid-twenties of the last century, already extracted T4 preparations were given as i.v. solutions and not any more as oral preparations. Synthetic, purified, and clean thyroxine was used as tablet by 1955, and during the recent years, also liquid L-thyroxine preparations in various matrices (aqueous and oily) entered the spectrum of preparations, especially administered to children, hospitalized and intensive care patients, but also individuals with resorption problems. The eminent role of T3 as the active TH was demonstrated after many in vivo experiments using several suitable animal models rapidly indicating TH effects in various forms of "bioassays". The key observation systematically performed by Gudernatsch and reported in 1912 was the acceleration and induction of metamorphosis of amphibian tadpoles after feeding thyroid extracts [15]. Remarkably, this could be facilitated by additional administration of adrenal extracts. Various thyroxine-related compounds were synthesized, tested, and applied with various, sometimes irreproducible success over the next decades. A systematic analysis by Barker and Klitgaard published in 1952 showed a reproducible and quantifiable stimulation of oxygen consumption in various rodent tissues except brain, spleen, and testis [14]. Thus, authors made the premature conclusion that these three tissues are not responding

to and dependent on thyroid hormone, which, obviously, with our knowledge is not true anymore. They just are not responding to TH with this endpoint of action. The remarkable discovery and confirmation of in vivo T3 (and TRIAC) formation by the groups of Braverman, Sterling, and Ingbar [16], demonstrating T3 formation in athyreotic patients after thyroxine administration opens this exciting scenario to better understanding of target cell and tissue-specific effects of TH. Five years later, the evidence for nuclear T3 receptors mediating at least part of TH action in tissue and cell-specific manner was contributed by Oppenheimer's team [17]. A heretic, controversial line of research, demonstrating T3 receptors and action in mitochondria, was not accepted by the mainstream crowd, and some of this data are still cock-eyed observed in the 21st millennium [18,19], especially, after the successful cloning of the genes encoding two different but related T3 receptors (TR) on two different chromosomes in 1986 [20,21]. These bona fide TR represent members of the ligand-modulated family of transcription factors binding to hormone response elements in the promoter, enhancer, or regulatory regions of T3-responsive genes [9]. Not surprisingly, there are many such genes, approximately 8% of the expressed liver transcripts were reported to be T3-responsive, either stimulated or suppressed in their expression.

Supplement 2:

Deiodinases are selenoenzymes enzymes catalyzing reductive THM deiodination

Initially, it was not clear whether reductive TH deiodination by intracellular integral membrane protein deiodinases occurs at random or as mono-deiodination in sequential fashion. The latter reaction has been demonstrated and documented in various model systems and in vitro and in vivo in experimental animals and humans, in part based on monitoring metabolic steps, educts and products using isotope-labeled injected or infused TH, or by analysis employing highly specific immunoassays developed during the seventies and later on in the last century. Such immunoassays were not only developed, validated, and applied for the major TH but also for di- and mono-iodothyronines as well as for their sulfated conjugates [40-43]. Employing these tools, it became obvious that, for example, sulfated TH are generated in the fetus and reach maternal circulation via transplacental passage [43]. Identification, isolation, and characterization of deiodinases generating the active hormone T3 and degrading the prohormone T4, the active hormone T3, and various metabolites turned out to be a challenging issue as enzymes known to cleave aromatic carbon iodine chemical bonds were only described in microorganisms but not in mammals [47-49]. It took the effort of several teams to identify that this peculiar class of enzymes belongs to the family of selenocysteine-containing enzymes, and that indeed three distinct but related enzymes have evolved to catalyze these key reactions of TH action and metabolism in humans [47-51]. In the meanwhile, the structure of one enzyme, DIO3, the key enzyme in degradation of thyroid hormones, has been solved as X-ray structure, at least for the cysteine homologue, and more detailed information on the complex reductive one or two step mechanism of cleavage of the C-I bond liberating iodide has been accumulated [47]. All three deiodinases are of low abundance as hormone metabolizing enzymes. Nevertheless, compared to their turnover rates and capacity to handle TH and their metabolites, at least DIO1 in liver, kidney, thyroid, anterior, pituitary, and several other tissues is much more abundant than theoretically required. Unfortunately, the exact nature of the physiological cofactor(s) required for reductive iodothyronine deiodination, is not finally solved. It is assumed that glutathione and/or thioredoxin-dependent co-factor systems assist during these reductive processes [47]. However, it might be possible that these peculiar enzymes are not regenerated after substrate deiodination and release of product and iodide, thus resembling suicide catalysts of metabolic reactions [48], as observed for some cytochrome P450 enzymes and peroxidases like TPO [52,53]. Characterization of tissue distribution, cellular location, and intracellular compartmentalization of these three deiodinase enzymes is ongoing, and details still need to be worked out. This requires better tools for the unequivocal identification of these very low abundant selenoenzymes in various cells, e.g. highly specific antibodies (which are not yet available for all three enzymes), or

development of highly specific affinity tagging molecules in order to monitor biosynthesis, membrane integration and degradation of deiodinases during development, in different life phases and under specific physiological and pathophysiological conditions. Currently, tracking of their deiodinated iodothyronine products is not yet technically feasible as these small molecules are highly hydrophobic but charged and thus unspecifically interact with various subcellular (phospho-)lipid membranes and/or other hydrophobic cellular structures as well as with surfaces of culture pates and plastic tubes.

Supplement 3:

Is reverse-T3 a T4 metabolite with relevant function?

While biological functions have been assigned to 3,5-T2, the role of rT3, the T4 metabolite generated by reductive deiodination at the tyrosyl ring of T4, either catalyzed by deiodinase 3 or deiodinase 1 (Fig. 1a), is currently unclear [58]. Peculiar to rT3 is its very short half-life probably due to its low binding to serum distributor proteins for thyroid hormones (such as thyroxine-binding globulin, transthyretin and albumin) as well as its high affinity for deiodinase 1 and 2, both removing the 5'-iodine atom and generating 3,3'-T2, which is more stable [48]. Remarkably, the production, degradation, and serum concentration of rT3 are apparently tightly controlled. Increases of rT3 serum concentrations have been found under conditions of non-thyroidal illness [59], carbohydrate depletion, under influence of various drugs inhibiting deiodinase 1 (such as amiodarone, iodinated X-ray contrast agents like iopanoic acid) [60]. During development as well as under conditions of macrophage and leucocyte activation, production of rT3 from T4 is increased while its degradation by deiodinase 1 and 2 are decreased, leading to accumulation, longer half-life, and higher serum, and/or tissue concentrations. So far, only limited functions have been attributed to rT3 such as inhibition of T3 formation by competing at the active site of DIO1 and DIO2. Furthermore, experimental evidence indicates that rT3 might inhibit neuronal migration and neurite outgrowth during early brain development [61]. A further not yet confirmed report indicates rT3 as an avid ligand for the GPR 35 kynurenine and/or chemokine receptor [62]. Generally, rT3 production and accumulation is considered to favour cell and tissue protection from T3 action, T3-dependent cell differentiation, and possibly facilitating proliferation of progenitor and stem cells or cells, which are not terminally differentiated [63, 64]. Thus, rT3 production is high during brain development, in placenta, skin and several other tissues. High rT3 concentrations have been determined in amniotic fluid [65]. All these observations are currently interpreted as rT3 possibly serving as a biomarker that prevents production of active T3 (and 3,5-T2 from the prohormone thyroxine) and counters the 'thyromimetic drive' in those cells, tissues, or exposed organs surfaces. Attempts have been made to monitor rT3 function by various applications in animal experimental models but, due to its short half-life and rapid metabolism, extremely high concentrations had to be used to exert effects, for example, on the HPT axis in rodents [66]. Recently, distinct distribution and localization of the 'inactive' rT3 different from that of T4 and T3 was demonstrated in intestine and muscles of developing tadpoles using matrix-assisted laser desorption/ionization (MALDI)-mass spectrometry (MS) imaging technology [67], supporting the concept of local production of this THM at sites protected from T4 and T3 action (Supplement Figure S3). Potentially, accumulation and increased serum concentration of rT3 might be a self-protective mechanism of tissues and organisms including humans to save energy and resources, to decrease oxygen consumption, thermogenesis, and metabolic turnover, thus allowing

for preparation of tissue regeneration, recovery, or survival until adverse conditions have been managed by the innate or cell-based immune system, adaptation of metabolism and compensatory changes in regulation and metabolism.

Comparably regulated changes between concentrations of active vs. inactive hormone metabolites are well-known in the field of secosteroids (1,25-dihydroxy vitamin D3 vs. 24,25-dihydroxy vitamin D), or active vs. inactive forms of sex steroid, mineralocorticoid and glucocorticoids hormones, retinoid acid metabolites, and various fatty acid derived ligands of de-orphanized nuclear receptors [22, 68-71]. Details of such regulation and adaptation are not fully explained, but an obvious utilization of such local activation and inactivation reactions of hormone precursors, active hormones and their metabolites suggest successful evolutionary adaptation of this principle favouring survival.

A recent publication by Domingues et al. [72] reports on rT3 interaction via the αvβ3 integrin receptor in hypothyroid developing rats. Daily injection of 50 nanogram rT3 per kilogram body weight, between day 12 and 14, alters expression of genes and function of various hippocampal proteins, if these were tested on day 15 in slices originating from these treated brain preparations. The parallel in vitro treatment of slices used 1 nanomolar rT3 concentrations. This complex model is difficult to interpret, and authors propose that rT3 might also inhibit calcium influx in addition to several other actions. Farwell and colleagues [61] reported that rT3 similarly to T4 influences neuronal migration and guidance processes in the developing rat brain by mechanisms involving Dio2 and F-actin polymerization. In contrast, no such effects were observed for T3. Granule cell migration, neurite outgrowth and migration, according to their observations, involved laminin-S guidance structures. These effects were not mediated by classical T3 receptors, but rapid membrane associated processes initiated by T4 and rT3. Possibly, also integrin receptors might be involved. Cettour-Rose et al. [66] tested whether rT3 administration might influence TSH secretion in hypothyroid rats by inhibiting pituitary Dio2. They infused the high amount of 25 nmoles rT3 per 100 g body weight per day for 3 days into hypothyroid rats, which were co-treated with subcutaneous T4 injections. They demonstrated that rT3 infusions indeed inhibited Dio2 activity in pituitary, brain cortex and brown adipose tissue, but TSH concentrations did not increase and both T4 and T3 serum concentrations were not markedly affected. Apparently, systemic administration of rT3 does not influence pituitary Dio2 mediated TSH production and secretion. A summary of putative and reported rT3 effects on various cellular and experimental animal models has recently been reported by Hercbergs et al. [73], but no detailed critical discussions on experimental conditions, reproducibility, and mechanisms underlying those reported effects is provided. Typically, quite high concentrations of rT3 are needed to exert such actions, and in most of these studies, stability of rT3 during incubation and exposure of targets is not reported.

rT3 represents one of the most enigmatic THM. rT3 has been detected early after T3 as constituent in thyroglobulin [74, 75], and after development of chromatographic and immunoassay methods, rT3 concentrations were determined in blood as well as in tissues in concentrations equimolar or sometimes even higher than those of the active hormone T3 (Table 1). rT3 concentrations are tightly regulated under various pathophysiological conditions, typically in inverted manner compared to T3 [40, 41, 59, 76, 85]. This tight regulation and the discovery of the very short biological half-life (only a few hours) [40] raised significant attention for the potential physiological role and putative mechanism of action of rT3. rT3 has been identified as one of the most favorable substrates of deiodinase 1, exhibiting a nanomolar KM value and high Vmax constant [86]. rT3 also is substrate for type 2 deiodinase with almost equal affinity to that of T4, and beyond that, tyrosyl ring deiodination of rT3 to 3'5'-T2 has also been described. However, 3',5'-T2 concentrations are quite low [41,76], and the major metabolic pathway of rT3 leads to the inert 3,3'-T2, which is the most abundant T2 isomer found in blood and most tissues [22]. Based on the high affinity of rT3 for type 1 5'deiodinase, initial hypotheses favoured a role as inhibitor of T3 production from T4 [88]. However, this has not been substantiated and supported by physiologically relevant observations and data [66]. The high abundance of T4 compared to rT3 and the short half-life of rT3, rapidly deiodinated to 3,3'-T2, makes a relevant inhibitory role of rT3 quite improbable. An alternative hypothesis had been put forward, that rT3 might be an inactive metabolite with respect to classical TH action, but a rich source of iodide, which could be delivered to specific compartments such as the fetus (for review see [89]). This hypothesis has not been refuted and receives some support, considering that placental membranes are rich in deiodinase activity and thus could directionally deliver iodide to the fetal compartment and its thyroid during early development, at the same time protecting the fetus from inadequate supply with either the prohormone T4 or active T3 [44]. Whether such a role is also important for brain development and early fetal development of other organs remains to be tested, provided that organs require iodide for specific functions such as phagocytosis-associated iodination of foreign proteins [39, 88, 90]. On the other hand, placental membranes and several other membranes also express sodium iodide symporter and thus are equipped with mechanisms of iodide accumulation independent of deiodinase activity [91]. Chopra developed the first radioimmunoassay for rT3 in 1974, and other labs supported the presence of rT3 in blood and its altered concentrations under various conditions [40]. Typically, starvation, non-thyroidal illness, several drugs like amiodarone, propranolol, and others, lead to increased rT3 serum concentrations, and systematic in vitro and in vivo studies revealed that the majority of rT3 does not originate from thyroid secretion, where rT3 is a minor component of thyroglobulin, but that rT3 is generated from the prohormone T4 by tyrosyl ring deiodination in many tissues such as skin, muscle, intestinal tract, brain, and especially activated leucocytes and macrophages (for recent review see [22]).

During the end of the last century, rT3 determination has frequently been included in thyroid function tests, but recently, rT3 concentrations are only determined under specific conditions [92] and in clinical studies to support specific changes in TH serum profiles in context of impaired THTT due to MCT8 mutations in AHDS patients, or under conditions of consumptive hypothyroidism, or the rare condition of altered TH metabolism due to mutations in selenocysteine binding protein 2 (SBP2). The initial expectation that rT3 to T3 ratios or rT3 to T4 ratios might be indicators of prognosis and outcome in non-thyroidal illness were not supported by several studies. Schmidt et al. [77] recently analyzed clinical practice of rT3 testing and concluded that this test might only be relevant in highly specialized centers but not for clinical routine. Their comprehensive study and analysis of available data revealed a reference range for adult individuals between 90-207 nanograms/liter (tab.1) and a remarkable reduction of numbers of rT3 determinations during the last 25 years [77]. Gomes-Lima and Burman recently commented on the current status of rT3 diagnostics and function [93]. They reminded of elevated rT3 concentrations under conditions of hyperthyroidism, and the typical daily production of 30-40 micrograms of rT3 originating from T4 via deiodinase 3 activity. They discuss various conditions interfering with rT3 concentration and interpretation, especially in clinical context including caloric restriction, major surgery, cardiac failure, or HIV infections.

Supplement 4:

The endogenous acetic acid metabolites of TH – Tetrac & Triac – are biologically active

Soon after the discovery of the classical TH T4 and T3 as iodinated amino acid derivatives, formation of deaminated propionic, acetic acid and formic acid derivatives has been postulated and documented by various chromatographic methods using radioiodine-labelled TH precursors [105-7]. In the context of studying deiodination and activation of the prohormone thyroxine, several other metabolites have been detected, which are distinct from T3 but release (radio-)iodide during incubation with tissue extracts or after administration of radiolabeled T4 or T3 in vivo. One of the first main products identified was Tetrac, and in the same publication, also formation of Triac was documented as product of T3 [107-9]. Follow-up studies suggested mitochondria and/or cytosol as sites of production of these acetic acid derivatives in rat kidney, and evidence has been presented that these products do not form from the postulated intermediates tetraiodothyronamine or triiodothyronamine but possibly the pyruvic acid analogues might be precursors [110]. Enzymes catalyzing the generation of these acetic acid derivatives were found as soluble preparations and FAD cofactors stimulated this activity.

Based on previous observations [7,8], both Tetrac and Triac were found biologically active in the rat goiter prevention assay albeit at lower potency. In contrast to these acetic acid derivatives of TH, the pyruvic acid metabolites were not studied in great detail. Also, formic acid derivatives were only of interest as potential THM analogues. The group of Roche et al., 1955, identified radioactive Triac in kidneys of thyroidectomized rats treated with radiolabeled T3 [111], also supporting in vivo formation of Triac outside of the thyroid gland. While Tetrac and Triac have received attention over the last decades, the propionic and formic acid derivatives were only occasionally analyzed in detail. Ramsden et al., 1974, [36] identified tetraiodoformate by combined gas-liquid chromatography mass spectrometry after incubating rat liver with thyroid hormone. Tetrac represented the main metabolite, but also tetraiodoformate was detected in some of the livers. They speculated that it originated from deaminated tetraiodopropionate via oxidative decarboxylation of the pyruvic acid analogue previously observed by Roche and Michel, 1954 [105, 106] and Myant et al., 1956 [112]. Details of these postulated reactions have not been analyzed.

Tetrac

Tetrac, the physiological T4 metabolite found in human serum in low nanomolar concentrations [82, 113, 114], higher than those of the active hormone T3 (Table 1), represents a neglected THM with dual function, i.e. either as a prohormone for the active T3 ligand Triac (see below) or as a powerful ligand for the cell membrane THM receptor $\alpha v\beta 3$ integrin [115]. Tetrac and the other acetic acid side

chain metabolites are good substrates for deiodinases [116, 117] and thus exhibit short half-lives in serum despite their avid binding to the serum distributor protein TTR [118]. Recently, cellular transport and uptake of Tetrac and Triac received marked attention, because these acetic acid THM are transported by OAPTs and thus bypass MCT8. This is of therapeutic importance in an attempt to rescue T3 (T4) deficiency in the AHDS syndrome caused by MCT8 mutations (see below, [84,119]). High doses of TRIAC (and Tetrac) can bypass (defective) MCT8 in the blood-brain-barrier and reach the neuronal targets during brain development as recently shown in mice [120,121]. Efficiency in restoration of 'normal' brain development, Purkinje cell morphology, parvalbumin-positive neurons and myelination has been demonstrated in mouse, chicken and zebrafish animal models of AHDS [120-123]. Multicentric clinical trials using Triac in AHDS patients are in progress [84,119]. The 'prodrug' Tetrac, after its deiodination to Triac, and even better Triac itself efficiently suppress TSH [124-126] and Triac thus was initially used to ameliorate effects of thyroid hormone resistance caused by mutated TRβ (for recent review see [84].

The second research development around Tetrac centers around its action at the $\alpha v\beta 3$ integrin THM receptor for THM. This receptor exhibits two distinct binding sites for T4 and T3 and is involved in rapid signaling mediated by the MAPK/ERK cytosolic kinase cascades [115]. Davis et al. initially demonstrated its angiogenic action using the chicken chorioallantoic membrane model [126]. Using nanoparticulate formulations of T4, T3 and eventually Tetrac, which cannot pass the cell membrane, rapid and concentration-dependent effects have been documented (for review see [115, 126]. In contrast to the pro-angiogenic effects of both T4 and T3, Tetrac acts as inhibitor to the classical THM T4 and T3 at this receptor, and thus has been developed as promising anti-angiogenic drug in nanoparticular formulations and iodine-free analogs, which cannot be deiodinated and thus have longer half-life [126,127]. Part of this work recently has been confirmed and extended by Schmohl et al. 2015 [128], who characterized the role of the $\alpha v\beta 3$ integrin receptor and its regulation by T4 and T3 on differentiation, migration and tissue invasion of (primary human bone marrow-derived) mesenchymal stem cells in the context of the fibrovascular microenvironment of various solid tumor models. They successfully applied Tetrac as specific inhibitor of these TH-dependent membrane signaling pathways. However, at this point not much information is yet available, whether Tetrac is stable or rapidly metabolized to Triac by deiodinases under these conditions and whether classical TR-mediated Triac effects also contribute to these processes. Considering this promising experimental research developpment the acetic acid side chain metabolites of T4 and T3, endogenously generated or pharmacologically applied Tetrac and Triac, might exert several non-canonical actions which are unexpected from the available classical knowledge of T3-mediated TR action.

On this note also the observation made in amphioxus [129] is of high relevance, that the deiodinase of this early protochordate does not accept T3 as substrate while Triac is a good substrate, suggesting that Triac might be a phylogenetic relict or ancestral ligand in vertebrate evolution. This idea finds strong support by the recent identification of the ancient TSH-primordial glycoproteohormone 'Thyrostimulin', which is functionally active at its recombinantly expressed TSH receptor stimulating T4 synthesis [130,131].

Triac

Biological effects of Triac were studied in several models. Of interest for human application were basic rodent experiments comparing Triac and T4 action on pituitary TSH secretion, and hepatic induction of TH response genes such as *Dio1*. One of the most extensive studies was performed by the group of Burger et al. [124], who administered T4 and Triac to hypothyroid rats over six days by i.p. injection. Triac rapidly decreased TSH concentrations already after six hours, and this suppression persisted beyond the time of Triac application. Similarly, hepatic Dio1 activity was stimulated, but the time courses of effects exerted by 10 nmoles Triac vs. 2 nmoles T4/100 g body weight/day were remarkable. Authors concluded that endpoints for thyromimetic activity respond differently and in a tissue-specific context, as illustrated by the response of Dio1 and the expression of transcripts of *spot 14* in the same organ, i.e. liver. Differences between T4 and Triac effects were discussed in context of distinct intracellular kinetics of uptake and metabolism, different binding to cytosolic proteins, and also different half-lives of transcripts and proteins responding to thyromimetic activity. Obviously, Triac has a much shorter half-life (ca. 6 h) than T4 [125,126,132], however, this cannot explain those differences in action. Meanwhile it is known that cellular uptake of iodothyronines is distinct from those of acetic acid derivatives, and these features may be clinically useful in treatment of AHDS, where MCT8 expression is missing or impaired. Clinical utility and option of these approaches have been recently reviewed in detail by Groeneweg et al. [84,119]. Differences in thyromimetic activities of Triac compared to T3 have also been interpreted as distinct interaction with and activation of TRα vs. TRβ [133]. But at this point, no clear evidence is presented whether TR beta1 and TR beta2 forms show distinct Triac activation, which might be involved in altered pituitary and brain sensitivity compared to T3. Structure analysis (X-ray crystallography) supports higher Triac affinity for TRβ compared to the ligand T3, an observation relevant for several TRβ mutants [133]. Cellular uptake of Triac is independent of MCT8, and Triac administration in MCT8/OATP1C1 double-knockout mice rescued several deficits in brain development of these mice [121]. Similar positive effects were also demonstrated in chicken model [123] and zebrafish model [134], where MCT8 was genetically inactivated. The disadvantage of Triac use in clinic practice might be due to its short half-life, which requires multiple dosing, and the necessity to administer Triac already in utero if MCT8 deficiency is

suspected. Whether Triac might reach all relevant brain structures and neurons deficient in MCT8 function requires further detailed studies. Timing and dosage of such treatment is a matter of intensive research unless other THM will be identified for intervention. Kinetic properties of T3 vs. Triac in humans and rats have been compared by Groeneweg et al. [84] (Table 1). Similarly, data on deiodination, sulfate and glucuronide conjugation of Triac have been recently summarized and discussed [84].

Higher affinity of Triac for TRβ isoforms has been explained by the amino acid difference between TRα1 (SER277) and TRβ1 (ASN331) in the TR ligand binding site, the prominent difference in the ligand binding domain of these two receptor isoforms [133]. Whether higher affinity for TRβ isoforms also manifests as higher transcriptional activation is still questionable. Many of these studies addressing these issues did not consider different half-lives and metabolic fates of T3 vs. Triac in those in vitro or in vivo assays using distinct TH response elements such as DR4. Studies on physiology and pathophysiology as well as endogenous activity of Triac in humans are not conclusive yet. Increases of Triac (and Tetrac) were reported during fasting and non-thyroidal illness [114], data that need to be interpreted with caution, as most of the analytics used radioimmunoassays with cross-reactivities with conjugates of Triac and Tetrac or T4 and T3. Probably, mass spectrometry analysis distinguishing between TH precursor, acetic acids and their conjugates needs to be employed to better understand alterations in serum and tissue concentrations of acetic acid derivatives and various clinical conditions [22].

Of interest might be a recent observation that, in contrast to pituitary TSH, hypothalamic TRH expression and secretion might not be affected by administration of Tetrac or Triac [120]. While studies on clinical use of Triac in AHDS are ongoing, Triac has been initially used in patients with TH resistance [135,136]. Typical daily Triac treatment suppresses TSH, T4, T3 and rT3 concentrations. Major side effects of these treatments in terms of excessive thyromimetic activity were not observed, as summarized by Groeneweg et al., 2017 [84]. Cardiac function was not much affected while some bone endpoints and liver parameters responded in a thyromimetic manner. Triac reaches cardiomyocytes and leads to expected thyromimetic effects albeit at lower potency than typically expected for T3, if properly dosed. Possibly, the weaker interaction and activation of TRα1, the dominant T3 receptor in heart, might protect this organ from excessive thyromimetic activity of Triac. Several other target tissues of Triac action such as adipose tissue, bone, skin, muscle, kidney, and brain have been reviewed and comprehensively discussed by Groeneweg et al. 2017 [84].

Changes in sex hormone binding globulin and ferritin have been observed by some but not all authors (for review see [84]). Interpretation of published data needs to carefully consider the quite distinct responses to Triac, exerted in hypothyroid animal models vs. euthyroid conditions, and also

in thyroidectomized or athyreotic patients compared to patient with intact thyroid function. Here, additive and/or competitive effects might be observed if endogenous T3 and TH are present, while under conditions of thyroidectomy and hypothyroidism, classical thyromimetic effects might prevail. Similar considerations need to be made for applications of other thyromimetic compounds such as 3,5-T2 or synthetic T3 analogues.

Clinicians need to take into account and be reminded that Triac (Tiratricol) is widely used as weight-reducing, slimming drug without medical prescription and OTC or internet distribution [137]. In such cases, over-dosing and chronic abuse might lead to severe thyromimetic side effects beyond wanted control of body weight and composition. Typically, Triac highly interacts in most currently used T3 immunoassays, therefore, obscure laboratory findings in clinical medicine of thyroid patients and other individuals need to be questioned and monitored appropriately. Mass spectrometry clearly distinguishes between T3 and Triac in serum. Whether Triac administration or abuse impacts on traditional hepatic readouts of TH action is controversial.

Supplement 5:

Potential functions of THM Sulfates

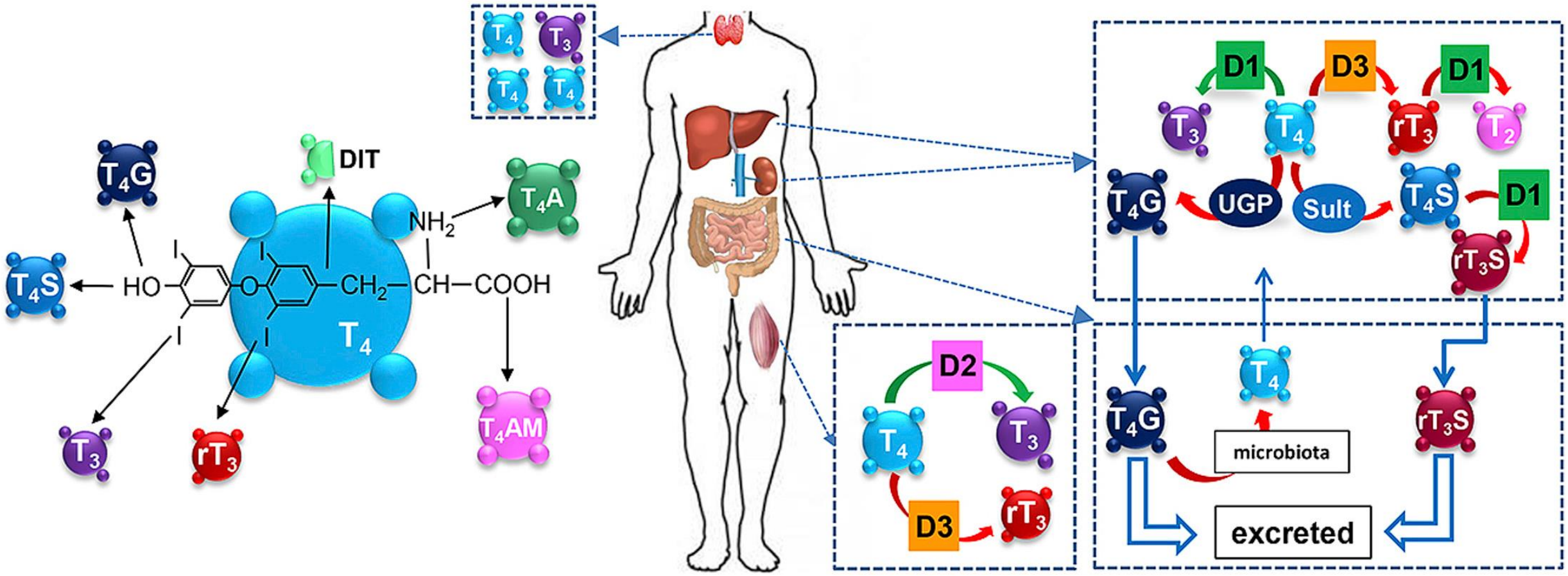
Among the variety of THM 4'O-sulfated metabolites received attention in various contexts from time to time. An important observation was that 4'O-sulfation of various TH precedes and markedly facilitates their deiodination [208, 209]. More than that, sulfation of T4 and T3 significantly increased their metabolism by tyrosyl-ring deiodination while 4'-O-sulfated T4 does not undergo anymore phenolic ring deiodination. This means, that the prohormone T4 after sulfation preferentially is deiodinated by DIO1 to yield rT3-sulfate which has very short half-life and is immediately further deiodinated to yield 3,3-T2 sulfate, that either is eliminated directly or after cleavage of the sulfate bond by members of the (ubiquitous) sulfatase family. T4 sulfation in the consequence implies an irreversible modification targeting this metabolite for elimination. No evidence has been presented for cleavage of T4 sulfate to 'regenerate' the prohormone. On the other hand, T3 sulfation similarly facilitates its tyrosyl-ring deiodination (also by the less specific DIO1) to generate 3,3-T2-sulfate with the same implication as described above, i.e. elimination. However, compared to T4-S there is good evidence that T3-S is a good substrate for the sulfatase family [210] which then regenerates T3. Thus T3-sulfation generates a 'reservoir' for active T3 under various (patho-) physiological conditions (see below). Available information thus implies a distinct fate for the sulfated prohormone T4-S (i.e. degradation and elimination) and for T3-S (i.e. reservoir) regeneration of the active T3 with the possibility of its degradation).

The relevant role of THM-sulfation is evident from different observations. Inhibition of hepatic DIO1 in vivo leads to a marked increase in sulfation of TH and concentration of various sulfated THM increases in serum [211]. Imparing expression and function of (hepatic) DIO1 e.g. by Se-deficiency, starvation, CHO-withdrawal or various drugs and DIO1 inhibitors results in increased formation and serum concentration of sulfated THM, increased half-life and enterohepatic recirculation of THM-S [212-214]. Whether these adaptations reflect attempts to retain iodinated THM in the organism or to generate a 'reservoir' of another prohormone form of T3, ie. T3-S, requires further studies, which need availability of improved analytical tools such as LC-MS/MS based profiling of circulating and tissue THM and their sulfates, the thyronome.

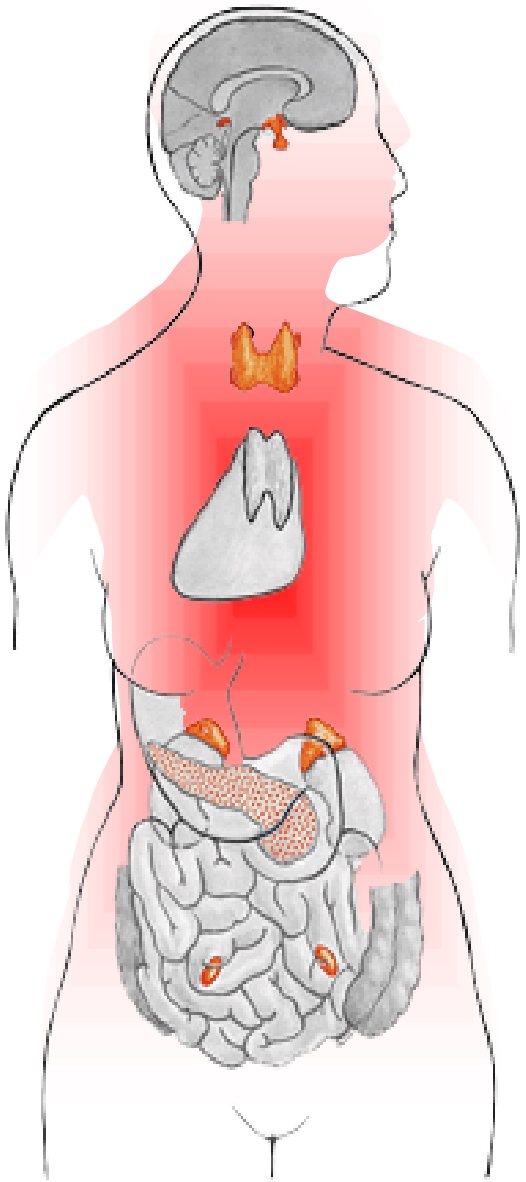
Another condition where sulfated THM receive attention is pregnancy, placental function and maternal-fetal communication. Several decades ago, Jimmy Wu and colleagues described the appearance of a sulfated diiodothyronine THM ('compound W') appearing in sera of pregnant women and presented evidence for its fetal/placental origin, representing a THM or elimination /degradation product of the fetal compartment disposing excess unwanted TH to maternal

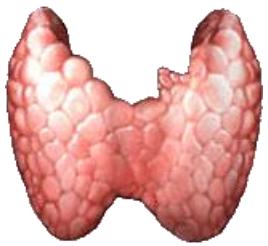
circulation and elimination [214]. The exact nature of compound W remained elusive except for its relation to T2 and its sulfation state [43, 214]. Recently, a more precise assay was developped and concentrations of compound W were described in more detail [215]. Still further work is needed to better understand the biochemical nature and processes leading to its formation and placental export into maternal serum and its fetal/placental function. Several years ago, R. Ekins postulated the hypothesis [216], that a major part of maternal/fetal import of TH and THM to the fetal compartment during pregnancy represents an efficient way to deliver the essential trace element iodine to the fetus (apart from its direct import by NIS) [94] and that especially the high expression of DIO3 in placental membranes during pregnancy is a clever way to prevent unwanted fetal exposure to excess maternal TH and at the same time to use iodide liberated from T4 during deiodination to rT3 for fetal supply. Whether stoichiometry and physiology fit with this hypothesis has so far not been tested.

Supplement Figure 1: TH Synthesis & Metabolism

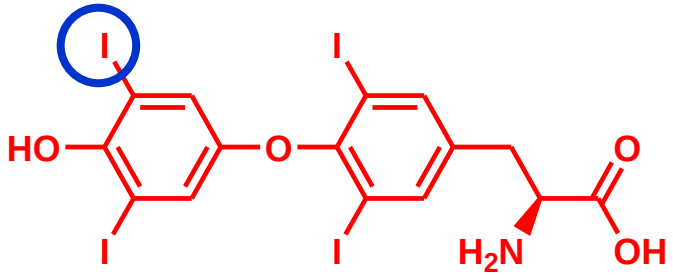


Supplement Figure 2A: The "hot" Thyroid hormones T4, T3, & 3,5-T2





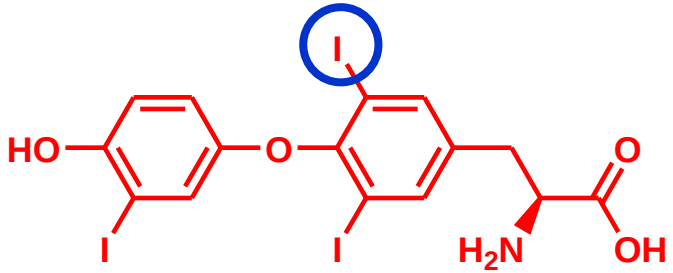
T4 (Thyroxine, 3,3′,5,5′-Tetraiodothyronine)



5′ Deiodinases (DIO1, DIO2)

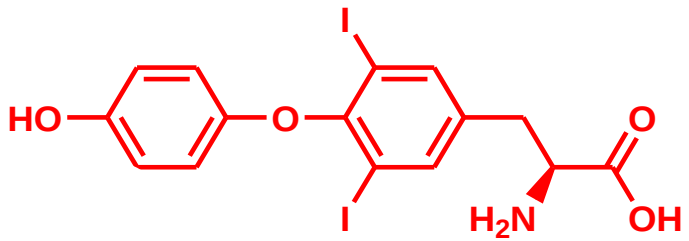
T3 (3,3′,5-Triiodothyronine)

active hormone

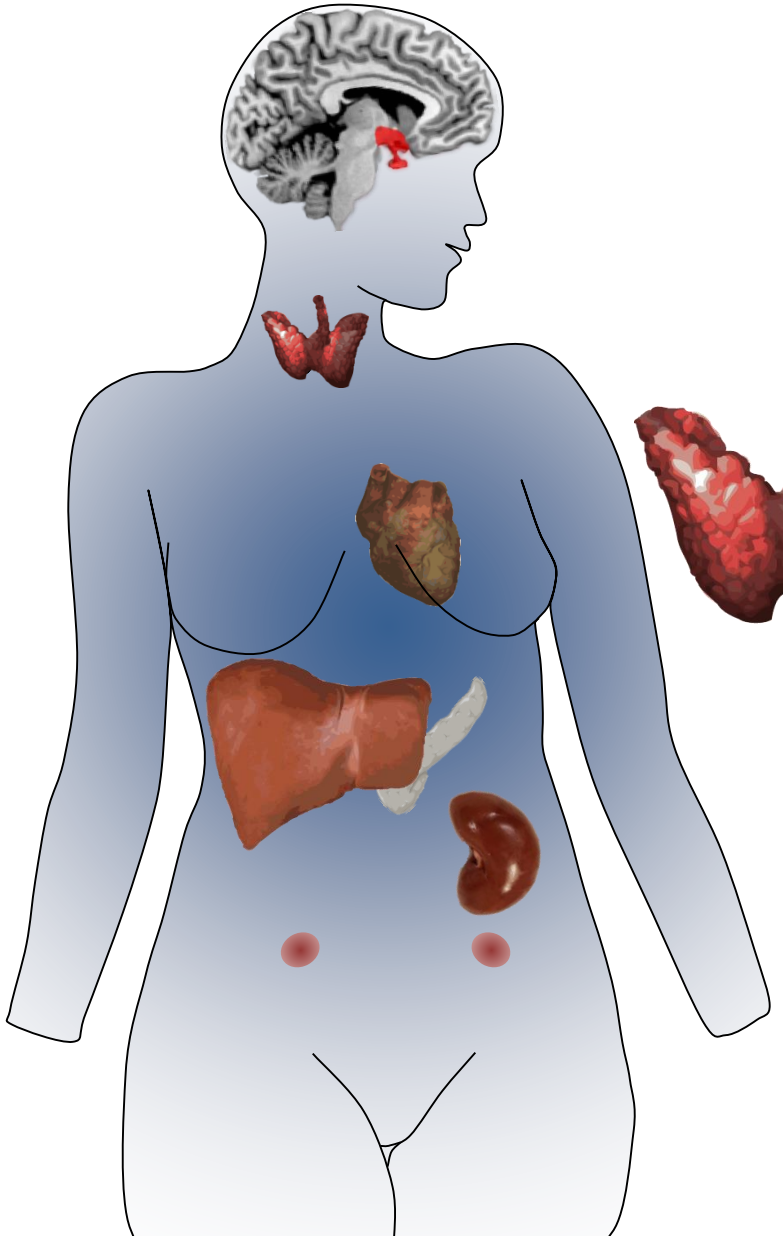


5′ Deiodinases (DIO1, DIO2)

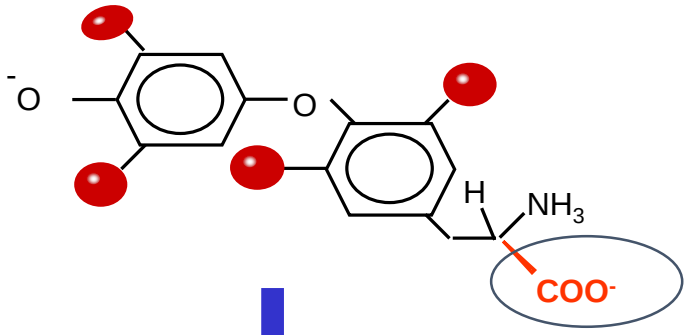
3,5-T2 (3,5-Diiodothyronine)



Supplement Figure 2B: Thyroid hormones & Thyronamines

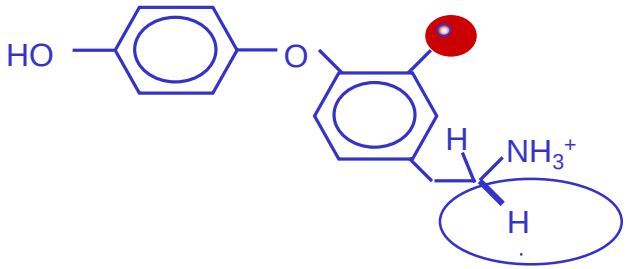


T_4 (Thyroxine,
3,3',5,5'-Tetraiodothyronine)



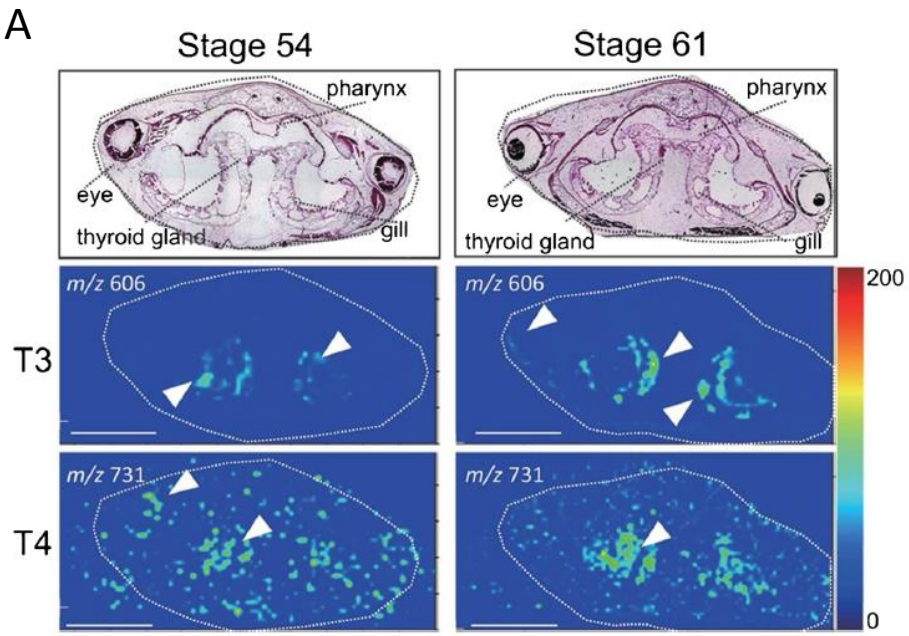
Deiodinases &
Decarboxilase

3-T_1AM (3-Iodo-Thyronamine)

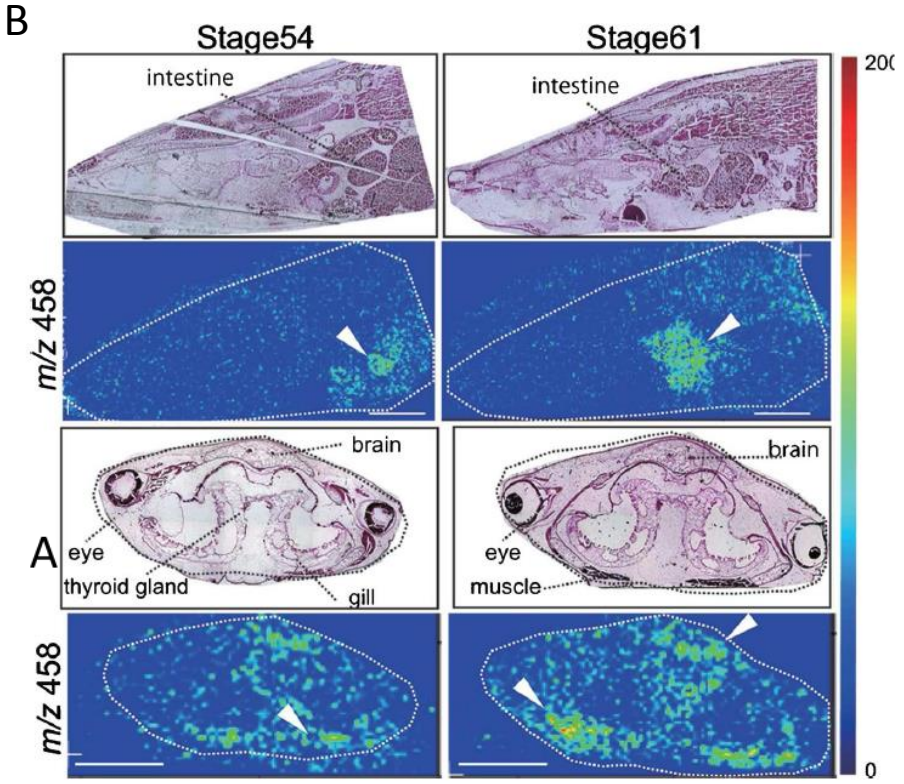


the "cool" thyroid hormone

Supplement Figure 3: Localization of T4, T3 and rT3 in tadpole tissues during metamorphosis



Visualization of T4 and T3 during metamorphosis.
The molecular ions of T3 (m/z 605) and T4 (m/z 731) were demonstrated and the relative amount with the same threshold set at a maximum intensity count of 200 were compared. White arrows show the specific distribution of T3 and T4. T3 were distributed in gill and eyes, and T4 were detected in eyes and inside of the gills.
Scale bar: 2.5 mm



Visualization of rT3 in tadpoles.
We were able to observe rT3-specific ions at m/z 458. By monitoring the value, we visualized rT3 localization in both stages 54 and 61. White arrows show the specific distribution of rT3; which were distributed in intestine, brain, and muscle.
Scale bar: 2.5 mm

Legends for Supplementary Figures

Supplement Figure 1: Thyroid Hormone Metabolic Pathways. The amino acid derived thyroid (pro-) hormone T4 may undergo several metabolic transformations to active and inactive THM. Abbreviations: T4A: Tetrac (Tetraiodothyroacetic acid); T4AM: Tetraiodothyronamine (not yet identified ex vivo); rT3: 3,3‘,5‘-triiodothyronine (reverseT3); T4S: T4-O-sulfate; T4G: T4-glucuronide; DIT: Diiodotyrosine; D2: Deiodinase Type 2; D3: Deiodinase Type 3 (Inactivating). From van der Spek AH, Fliers E, Boelen AMol Cell Endocrinol. 2017 Jan 18. pii: S0303-7207(17)30029-1. doi: 10.1016/j.mce.2017.01.025. (reproduced with permission).

Supplement Figure 2A. The “hot” Thyroid hormones T4, T3, & 3,5-T2. The human thyroid produces all endogenous T4 and to some extent T3. The majority of T3, the bioactive hormone, is generated in responsive tissues by the two 5‘-Deiodinases DIO1 and DIO2, the thyromimetic ‚hot‘ THM, 3,5-T2 has been found in blood and tissues, but its formation from the postulated precursor, T3, has not yet been formally proven in vitro. Red shading illustrates thermogenic (‘hot’) action of high concentrations of exogenous 3,5-T2 administered to experimental animals.

Both figures 2A and 2B were designed by Peter J. Hofmann, IEÉ.

Supplement Figure 2B. Thyroid hormones & Thyronamines. 3-T1AM, the ‘cool’ THM found in blood and tissues, is generated from T4 and THM via DIO and Ornithine Decarboxylase (ODC) activities. Details of its endogenous formation and mode(s) of action require further studies. Blue shading illustrates anapyrexic action (‘cool’) of high concentrations of exogenous 3-T1AM, administered to experimental animals.

Both figures 2A and 2B were designed by Peter J. Hofmann, IEÉ.

Supplement Figure 3: Localization of T4, T3 and rT3 in tadpole tissues during metamorphosis. Visualization of T4 and T3 during metamorphosis by mass spectrometry imaging [67]. A) The molecular ions of T3 (m/z 605) and T4 (m/z 731) were demonstrated and the relative amount with the same threshold set at a maximum intensity count of 200 were compared. White arrows show the specific distribution of T3 and T4. T3 was distributed in gill and eyes, and T4 was detected in eyes and inside of the gills. B) Visualization of rT3 in tadpoles. rT3-specific ions were observed at m/z 458. By monitoring the value, rT3 localization was visualized in both metamorphic stages 54 and 61. White arrows show the specific distribution of rT3, which were distributed in intestine, brain, and muscle. Scale bars: 2.5 mm. (Reproduced with permission [67]).