Scope and limitations of iodothyronine deiodinases in hypothyroidism

Balázs Gereben,* Elizabeth A. McAninch,* Miriam O. Ribeiro and Antonio C. Bianco

Abstract | Through their coordinated expression and activity, the iodothyronine deiodinases regulate thyroid hormone economy in hypothyroidism. Once heralded as the pathway underpinning adequate thyroid-hormone replacement therapy with levothyroxine, the role of these enzymes has come into question as they have been implicated in both an inability to normalize serum tri-iodothyronine (T_3) levels and the incomplete resolution of hypothyroid symptoms. These observations, some of which were validated in animal models of levothyroxine monotherapy, challenge the paradigm that tissue levels of T_3 and thyroid-hormone signalling can be fully restored by administration of this levothyroxine alone. The low serum T_3 levels observed among patients receiving levothyroxine monotherapy occur as a consequence of relative insensitivity in hypothalamic type 2 iodothyronine deiodinase (DIO2) to ubiquitination. In addition, residual hypothyroid symptoms have been linked to a prevalent polymorphism in the *DIO2* gene that might be a risk factor for neurodegenerative disease. Here we discuss how these novel findings underscore the clinical importance of iodothyronine deiodinases in hypothyroidism and how a better understanding of these enzymes might translate to therapeutic advances in the care of millions of patients with this condition.

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Competing interests
The authors declare no competing interests.

Key points

- Levothyroxine monotherapy at doses that normalize the serum TSH does not universally restore parameters of thyroid hormone economy for patients with hypothyroidism.
- The iodothyronine deiodinases provide a cell-specific, pre-receptor mechanism that controls TH signalling.
- Localized thyroid hormone signalling plays a critical role of in different areas of the brain, as mediated by thyroid hormone transporters and the iodothyronine deiodinases.
- T_4-induced DIO2 ubiquitination normally decreases T_3 production but not in the hypothalamus.
- The levothyroxine dose that normalizes serum TSH in an animal model is lower than the dose that normalizes serum T_3, explaining the increased serum T_4/T_3 ratio observed in levothyroxine-treated patients.
- If patients carrying the Ala92D2 polymorphism derive benefit from combination therapy with levothyroxine and liothyronine, then genotyping for this SNP may become a component of the management of hypothyroidism.

[H1] Introduction

Thyroid hormones are iodinated molecules produced by the thyroid gland that regulate development, growth, energy homeostasis, cardiovascular systems, musculoskeletal systems and cognitive function. Insufficient levels of the thyroid hormones tetraiodothyronine (T_4) and tri-iodothyronine (T_3) result in hypothyroidism, a prevalent condition that affects more than 8 million patients in the USA alone[1] and 1–2% of individuals living within iodine-replete communities.[2]

Over the last 150 years treatment modalities for hypothyroidism have been developed around thyroid hormone (TH) "replacement" through administration of thyroid gland/extracts[3], which remained the mainstay of therapy for nearly a century. However, with the discovery in 1970 that, in humans, iodothyronine deiodinases produce most of the circulating T_3,[4] clinical standards abruptly shifted to align with the assumption that levothyroxine monotherapy would maintain the pool of T_4 and that a group of enzymes known as the iodothyronine deiodinases would provide physiologic regulation of the T_3 availability to tissues.[5]

For the past few decades, clinicians have displayed an almost dogmatic reliance upon the ability of the iodothyronine deiodinases to mediate conversion of T_4 to T_3 and so regulate the availability of serum levels of T_3 among hypothyroid patients treated with levothyroxine.[6] However, it is now understood that ~12% of all patients treated with levothyroxine are biochemically euthyroid (exhibit normal serum levels of thyroid-stimulating hormone [TSH]) but continue to experience residual symptoms of hypothyroidism, including psychological[7-9] and metabolic effects.[10,11] This finding represents a major public-health concern given the high prevalence of hypothyroidism and the fact that not all parameters are restored by levothyroxine monotherapy. With such strong reliance upon the iodothyronine deiodinases and, therefore, the efficacy of levothyroxine monotherapy, clinicians were left with few options to explain or treat these residual symptoms.

As awareness of this subpopulation of patients with hypothyroidism has improved, investigators have made advances into understanding the aetiology of this phenomenon. The serum levels of T_3 might not be fully normalized among such patients owing to insufficient T_4-to-T_3 conversion,[12] which could explain why a minority remain symptomatic despite treatment with levothyroxine. Some patients demonstrate improved well-being and a treatment preference when co-administered levothyroxine and liothyronine;[13,14] however, this issue has remained contentious as the majority of clinical trials failed to demonstrate an objective benefit of combination therapy;[15] as previously reviewed.[16]

Differences within subgroups of patients with hypothyroidism could make them more or less responsive to levothyroxine monotherapy versus combination therapy.[13] Therefore, the iodothyronine deiodinases that were once almost universally accepted as the key clinical strategy to thyroid-hormone regulation in hypothyroidism might actually cause continued symptoms among a substantial proportion of patients.

Here we review the physiologic role of the iodothyronine deiodinases, as well as the changes that occur in hypothyroidism, with particular focus on their role in preserving thyroid hormone economy in the brain. Recent studies indicate that unique biochemical aspects of the iodothyronine deiodinases in the hypothalamus prevent normalization of serum T3 in patients treated with levothyroxine monotherapy. In combination with a prevalent *DIO2* polymorphism, this could also explain the insufficient symptomatic response experienced by an appreciable proportion of the hypothyroid population.

[H1] Animal models of thyroid replacement

Further insight into regulation of iodothyronine deiodinases and tissue-specific thyroid-hormone signalling is required to develop new strategies to treat hypothyroidism. However, studies in humans are limited as they require tissue biopsy. Thus, animal models have been developed that provide the basis for how unique aspects of iodothyronine deiodinase regulation might affect different thyroid-hormone replacement therapies.[17-20]

The applicability of such models reflects the extensive similarities in thyroid physiology between humans and small rodents.[21] Interspecies differences exist but their interference can be minimized by correct experimental planning. For example, the half-lives of T_4 and T_3 are longer in humans (~1 week and 1 day, respectively) than rodents (~8 hours and 2 hours, respectively) and so blood and/or tissue sampling should be planned accordingly.[21] Iodothyronine deiodinases are conserved across species at the amino acid level but they exhibit species-specific differences in tissue distribution. Type 2 iodothyronine deiodinase (DIO2) is expressed in the human thyroid gland and heart but not in these tissues of the rodent.[22,23] Furthermore, DIO2 is the only activating iodothyronine deiodinase in the human brain, whereas both Dio2 and Dio1 are expressed in the rat brain.[24,25]

With these differences in mind, studies in thyroidectomized rats have conclusively shown that only therapy with a combination of levothyroxine and liothyronine could normalize serum and tissue T_3 levels.[18,19] Similar conclusions were obtained for normalization of T_3-dependent biological parameters, such as lipid profile, mitochondrial content and the expression of T_3-target genes, in the brains of thyroidectomized rats.[17] Mouse models were also instrumental in the discovery that preserving the stability of serum T_3 concentration is a biological priority, which is enforced even when Dio1 and/or Dio2 are genetically inactivated in all tissues[26] or in specific tissues.[27,28] Therefore, animal models have provided substantial advances in our understanding of thyroid-hormone replacement therapy.

[H1] Thyroid-hormone signalling

In humans, thyroid hormones are secreted into the circulation predominantly as a prohormone (T_4), with only ~20% secreted as the biologically active form (T_3). As shown in Figure 1, T_4 and T_3 enter virtually all cells through transporters in the plasma membrane and remain in equilibrium between plasma and cells. Once inside a cell, T_3 diffuses into the nucleus and binds to a thyroid-hormone receptor (TRα or TRβ) to modulate gene expression. The T_3–TR complex controls expression of specific sets of T_3-responsive genes, thus promoting T_3-dependent biological effects. Tissues contain different combinations of TRα and/or TRβ as well as other

transcriptional coregulators of the T_3–TR complex. Therefore, the net effect of T_3 depends on these combinations and is highly cell and/or tissue specific.[29,30]

Studies in patients, animal models, and *in vitro* cell models with mutations in the genes encoding TRα or TRβ have highlighted the mechanisms involved in the positive stimulation of gene transcription by T_3.[29] Given the free T_3 concentration in the plasma (about $5x10^{-12}$M) and the affinity of TRs for T_3 (about $1x10^9$L/M), the ratio of occupied to unoccupied TRs is 1:1 in most tissues. Even when unoccupied, TRs are predominantly bound to specific *cis*-acting elements in T_3-responsive genes. Unoccupied TRs exhibit high affinity for negative coregulators of transcription (also known as corepressors), which actively inhibits gene expression. Upon binding to T_3, TRs lose their affinity for corepressors and gain affinity for coactivators, triggering T_3-dependent gene transcriptional activation. Therefore, the clinical syndrome of a hypothyroid individual is largely the result of transcriptional repression of T_3-responsive genes mediated by unoccupied TRs. The goal of thyroid-hormone replacement therapy is to provide sufficient T_3 to relieve TR-mediated gene repression and to promote T_3-dependent transactivation of target genes.[29] The unique role played by the unoccupied TRs is further illustrated by the fact that mice with knockout of both TR genes exhibit only a mild phenotype, mainly because there is no transcriptional repression.[29] Therefore, the intensity of thyroid-hormone signalling depends on the ratio between occupied and unoccupied TRs, which is a function of the plasma T_3 concentration, presence of plasma membrane transporters, iodothyronine deiodinase activity and (ultimately) the nuclear concentration of T_3.

[H2] ***Iodothyronine deiodinases***

Iodothyronine deiodinases are small, highly homologous, integral membrane enzymes that modify thyroid-hormone signalling. They comprise a single N-terminal, trans-membrane segment connected to a larger globular cytosolic domain with a selenocysteine-containing active centre embedded in a thioredoxin-like fold. The molecular structure has been modelled using hydrophobic cluster analysis[31] and confirmed with supportive experimental data in the case of DIO3.[32] The selenium in the active centre provides enhanced substrate affinity and a fast turnover rate for the deiodination reaction.

Both T_4 and T_3 can be deiodinated, a process that results in either activation of T_4 (DIO1 and DIO2) or inactivation of T_4 and T_3 (DIO3). Consequently, the intracellular environment can be enriched with additional T_3 supplied by DIO2 (enhancing thyroid-hormone action) or depleted of thyroid hormones by DIO3 (dampening thyroid-hormone action; Figure 1). A key property of iodothyronine deiodinases in thyroid-hormone signalling is their unique subcellular localization. DIO2 is usually retained in the endoplasmic reticulum;[33,34] however, it is also found to be closely associated with the cell nucleus,[35] but not with the Golgi apparatus.[33] Therefore, the nuclear environment can be greatly affected by DIO2-generated T_3 owing to this physical proximity. By contrast, DIO1 is located in the plasma membrane; it has low affinity for T_4 and so DIO1-generated T_3 rapidly diffuses from the cells and reaches the plasma without appreciably affecting nuclear concentrations of T_3.[34,36]

The inactivating enzyme DIO3 is generally anchored in the plasma membrane, where it is

internalized to become part of vesicles known as early endosomes that can be recycled back to plasma membrane.[34,37] In the rat central nervous system, Dio3 is observed in dense-core vesicles of hypothalamic neurosecretory axon varicosities with the active centre containing the C-terminus of Dio3 at the outer surface of these organelles.[38] However, this peripheral distribution of Dio3 can change depending on oxygen availability. After unilateral induction of ischaemia and hypoxia in the rat brain, Dio3 is found predominantly in the nucleus of the neurons in the pyramidal and granular ipsilateral layers, as well as in the hilus of the dentate gyrus of the hippocampal formation.[39] In isolated mouse hippocampal neurons grown in culture and in a human neuroblastoma cell line, hypoxia redirects active DIO3 to the nucleus via the HSP40 pathway, a shuttle mechanism known to direct proteins to the nucleus. Preventing nuclear DIO3 import by *HSP40* knockdown almost doubles the thyroid-hormone-dependent glycolytic rate and quadruples the transcription of thyroid-hormone target gene *ENPP2*. By contrast, overexpression of HSP40 increases nuclear import of DIO3 and minimizes the effects of thyroid hormones in cell metabolism.[39] Rerouting DIO3 to the nucleus decreases thyroid-hormone signalling and might function to reduce ischaemia-induced hypoxic brain damage.

The corollary is that the activity of DIO2 and DIO3 are viewed as a cell-specific, prereceptor mechanism that controls thyroid-hormone signalling, the intensity of which cannot be predicted on the basis of circulating levels of T_3.[40] For example, cold and/or sympathetic nervous system stimulation of Dio2 expression in rat brown adipose tissue accelerates transcription of T_3-responsive genes, such as *Ucp1* and *Pgc1α*.[41] Furthermore, ectopic expression of DIO3 in the human heart and brain during ischaemia or hypoxia[42] lifts the T_3-dependent transcriptional footprint in these organs, curbing thyroid hormone signalling.[43,44] Although no humans have been identified with loss-of-function mutations in the iodothyronine deiodinases,[45] a few individuals have been identified with defects in selenoprotein synthesis resulting in consequent reduction in DIO2 activity and possible reduction in the activities of DIO1 and DIO3. These patients exhibit increased serum TSH levels, increased serum T_4 levels and low serum T_3 levels, but otherwise display a mild phenotype.[46]

[H2] Effects on brain function

The fundamental impact of thyroid hormones on brain function was established by the striking link between untreated congenital hypothyroidism and developmental retardation of cognitive function.[47-49] Myelination, neuronal and/or glial proliferation, differentiation and neuronal migration represent crucial targets of thyroid-hormone-mediated events in the brain.[48,50,51] Access of thyroid hormones to the brain is selective, with plasma T_4 displaying greater ease of access than T_3 because of the types of transporters expressed in the blood-brain barrier. [52,53]

Uptake of thyroid hormones via the blood–brain barrier and into cells inside the brain parenchyma is mediated by transporters, including MCT8 and OATP1C1.[54-56] These transporters are plasma membrane proteins with multiple membrane spanning domains that have a half-life of several days. Although levels of *Mct8* and *Oatp1c1* mRNA levels are decreased in a rodent model of nonthyroidal illness,[57] there is currently no direct evidence that quick, adaptive changes in the transport of thyroid hormones regulate thyroid hormone availability in the brain. However, the observation that patients with Allan–Herndon–Dudley syndrome express a

mutant MCT8 protein explained the molecular background of this rare X-linked disorder, which is characterized by neurological abnormalities (central hypotonia, hearing problems, rotatory nystagmus and spasticity) and developmental delay.[58,59]

The link between mutations in thyroid hormone transporters and neurological symptoms highlights the critical importance of localized thyroid-hormone signalling in different areas of the brain. In contrast to the majority of tissues, a large portion of T_3 in the rodent brain is produced locally via activation of T_4. A dual-labelling approach was used to assess this process directly[60] and the findings were later confirmed in *Dio2* knockout mice that exhibited ~50% reductions of T_3 levels in the brain.[61] Thus, local metabolism of thyroid hormones (that is, via the DIO2 and DIO3 pathways) is currently viewed as the major factor that regulates the intensity of thyroid-hormone signalling in the brain. Both enzymes exhibit homeostatic regulation in response to changes in serum levels of thyroid hormones. The activity of DIO2 increases but that of DIO3 decreases in the presence of low concentrations of thyroid hormones (for example, in conditions of hypothyroidism or iodine deficiency), a finding that has been interpreted largely as a mechanism that delays adverse effects of hypothyroidism to the brain.[62,63]

Neurons are critically important targets of T_3; however, these cells lack DIO2 and are unable to generate T_3 via this pathway.[64-66] Indeed, enrichment of Dio2 expression is detected in glial cells when examining the transcriptome of astrocytes, neurons and oligodendrocytes.[67] Furthermore, a >95% drop in *Dio2* mRNA and activity occurred in the brain of a mouse model that harboured a transgene conferring glia-specific inactivation of this enzyme.[28] Thus, the current paradigm predicts that T_3 generated by DIO2 located in astrocytes acts in a paracrine fashion to stimulate TRs in neurons.[68-70] This hypothesis has been modelled and validated *in vitro* by co-culturing human DIO2-expressing glioma cells with human neuronal cells that express DIO3.[70] In such a system, glial-cell-generated T_3 (via DIO2 activity) exerts a paracrine effect on the co-cultured neurons and activates T_3-regulated neuronal genes. Such pathways might also affect thyroid-hormone signalling *in vivo*. For example, DIO3 is activated by HIF1-α under conditions of ischaemia and/or hypoxia.[43] Furthermore, DIO2 activity is downregulated and DIO3 expression is activated by the morphogenic hedgehog protein family.[71,72]

Logically, in responding to ischaemia or hypoxia and other cues known to regulate these enzymes, iodothyronine deiodinases enhance or dampen thyroid-hormone signalling in discrete groups of cells or areas of the brain with clear consequences. For example, in rodent models, Dio2 plays a role the generation of TSH-releasing hormone (Trh) and/or Tsh feedback (Figure 2). DIO2 is also implicated in the hypothalamic regulation of nonthyroidal illness, seasonal breeding in birds, and the onset of hearing in rodents.[73-76] Direct evidence suggests that iodothyronine deiodinases play similar roles in humans. For example, in the human foetal hypothalamus, there is a developmental dependency and coordinated expression of DIO2 and DIO3 in combination with the various thyroid-hormone transporters.[77]

In the rodent and avian retina, Dio3 dampens the actions of thyroid hormones, limiting T_3 exposure to the cones and preserving survival and/or patterning of opsins, which is required for

cone function.[78] Some evidence supports a role for iodothyronine deiodinase-mediated thyroid-hormone signalling in the human brain. For example, a correlation exists between the levels of thyroid hormones and enzyme activity in different areas of the developing human brain.[79] During recovery from intraventricular haemorrhage (IVH) among preterm infants, which depends on maturation of oligodendrocytes and myelination, DIO2 expression is decreased and DIO3 expression increased, indicating localized hypothyroidism.[80] Reversing this effect with levothyroxine treatment promotes neurological recovery among infants and in a model of IVH in rabbits and, if confirmed, this approach could improve the neurodevelopmental outcome of preterm infants with IVH.[80]

[H1] What changes in hypothyroidism?

The amount of thyroid hormone entering a cell is equivalent to the amount exiting that cell, characterizing a state of equilibrium. Studies in animal models and human cells grown in culture suggest that there is the net flow of T_4 and T_3 that depends on the type of iodothyronine deiodinase expressed in each given cell type (Figure 1). The expression of DIO2 creates an inward net flow of T_4 and an outward net flow of T_3; expression of DIO3 creates inward net flows of both T_4 and T_3; and the net flow is neutral if no iodothyronine deiodinase is expressed [40].

A corollary is that iodothyronine deiodinase activity also affects circulating levels of T_4 and T_3. In humans, ~70% of the circulating T_3 is produced via the extrathyroidal DIO2 pathway, whereas ~15% originates from the DIO1 pathway. Given that DIO1 is positively regulated by T_3,[81] whereas the opposite is seen for DIO2, the contribution of DIO1 to the circulating T_3 pool is increased among patients with hyperthyroidism. Notably, DIO1 activity is inhibited by propylthiouracil, glucocorticoids and β blockers, which explains at least part of the clinical efficacy of these drugs.[40]

Most circulating T_3 is metabolized via the DIO3 pathway.[82] This pathway acquires especial clinical relevance during pregnancy because DIO3 is highly expressed in the human placenta,[83] and therefore increases the daily requirement for patients on thyroid hormone replacement therapy.[84] Increased expression of DIO3 is also observed in some disease states, including in the liver, lungs, heart and brain of patients experiencing ischaemia or hypoxia.[42] In rare cases, DIO3 is expressed in infantile haemangioma to such an extent that it inactivates T_3 at a faster rate than the hormone can be produced (consumptive hypothyroidism).[85] Similarly, treatment of cancer patients with the tyrosine kinase inhibitors imatinib and sunitinib is associated with the development of hypothyroidism, which seems to be the result of marked overexpression of DIO3 within the tumour cells.[86]

Hypothyroidism is an important example of the mass effect of the iodothyronine deiodinases changing their levels of activity in a coordinated fashion. As a result of low thyroid hormone levels, the activity of DIO2 is accelerated in almost all tissues, increasing whole-body fractional conversion of T_4 to T_3 that helps preserve serum levels of T_3 (the opposite is observed during hyperthyroidism).[87] In addition, DIO3 is a T_3-responsive gene and thus T_3 clearance is reduced in hypothyroidism. These coordinated and reciprocal enzyme responses explain why measuring

serum T_3 concentration is of little diagnostic value among patients with hypothyroidism.

The DIO2-adaptive response to hypothyroidism is possible because of the unique sensitivity of this enzyme to its natural substrate (T_4). DIO2 has a short half-life (about 60 min) that becomes even shorter (20 min) as a result of interacting with T_4 and/or its catalytic activity.[88,89] In other words, by converting T_4 to T_3, DIO2 is inactivated and degraded. This mechanism is explained by an 18-amino acid instability loop unique to the DIO2 protein that is located adjacent to its globular catalytic domain.[71] This loop mediates binding to the hedgehog-inducible protein WSB-1, a ubiquitin ligase adaptor that mediates DIO2 ubiquitination and targeting for proteasomal degradation.[90] Truncation analyses identified a core of six amino acids within the loop as the minimal requirements critical for recognition of DIO2 by WSB-1.[91] The loop explains the short half-life of DIO2, a characteristic that can be transferred between proteins if the loop is fused to an otherwise stable protein.[33]

As with the other iodothyronine deiodinases, DIO2 exists as a dimer maintained by interacting surfaces within its transmembrane and globular cytosolic domains.[92] Upon binding T_4, DIO2 is ubiquitinated with ubiquitin chains formed at the lysine 48 position,[93] which in turn inactivates the enzyme by interfering with globular interacting surfaces critical for dimerization and catalytic activity.[90] This state of inactivity can be transient or permanent, depending on whether ubiquitinated DIO2 is reactivated by DIO2-interacting deubiquitinases (DUBs),[94] or retrotranslocated to the cytoplasm via the p97–ATPase complex and delivered to the proteasomes.[93] The continuous association of DIO2 with this regulatory protein complex supports rapid cycles of deiodination, conjugation to ubiquitin, and enzyme reactivation by deubiquitination, allowing tight control of thyroid-hormone action (Figure 2).

[H1] Treatment of hypothyroidism

[H2] Limitations of levothyroxine monotherapy

Unique aspects of hypothalamic DIO2 define limitations of levothyroxine replacement therapy and the use of TSH as a therapeutic goal.

Circulating T_4 and T_3 exert negative feedback on the secretion of TRH and TSH.[76] In rodents, Tsh-secreting cells co-express Dio2;[95] in the rodent hypothalamus, Dio2 is expressed in specialized glial cells known as tanycytes (Figure 2). These cells are located in the mediobasal hypothalamus, on the floor and infralateral wall of the third ventricle. Tanycytes are distributed from the end of the optic chiasm, along the mammillary recess, and their processes also reach the median eminence, outside the blood-brain barrier.[64,65,76] The presence of DIO2 in both locations is critical for T_4-mediated negative feedback.[28,96] In the pituitary gland, T_4 must be converted to T_3 to suppress secretion of TSH. In the hypothalamus, it is likely that T_3 generation via DIO2 in tanycytes negatively affects TRH expression in the paraventricular nucleus.[97] However, the full extent of the role played by DIO2 at both sites is only just starting to be appreciated.[17] Given that DIO2 activity is increased in most tissues during hypothyroidism and decreased in hyperthyroidism (via ubiquitination), its presence in the median eminence and thyrotroph would seem counterproductive to the feedback mechanism. Thus, for years it remained unclear how fluctuations in plasma T_4 levels could be faithfully transduced in the

thyrotroph and paraventricular nucleus to ultimately regulate serum TSH concentration. [95]

The key new element in this feedback mechanism is the observation that rat hypothalamic Dio2 is stable and largely refractory to the levels of thyroid hormones.[17] In contrast to the rest of the body, DIO2-mediated T4-to-T3 conversion in the hypothalamus is not accelerated during hypothyroidism nor is it diminished by administration of thyroid hormones or hyperthyroidism. Indeed, a sensitivity gradient exists in the DIO2 response to hypothyroidism and thyroid hormones between the hypothalamus and the rest of the brain and body,[17] which is similar to previous observations in the rodent thyrotroph.[95] The corollary of these experiments is that the secretion of Trh and Tsh in rodents is controlled by a steady process of DIO2-mediated T_4-to-T_3 conversion, thus transducing minor changes in serum T_4 levels (Figure 2). By contrast, the rate of Dio2-mediated T_4-to-T_3 conversion in the rest of the body progressively decreases with administration of thyroid hormones because of Dio2 ubiquitination, so that peripheral T_3 production in levothyroxine-treated hypothyroid rats is progressively decreased and self-limiting. As a result, the dose of levothyroxine required to normalize serum TSH concentration is lower than the dose that normalizes serum T_3. Given that the rat model reproduces the findings observed among patients treated with levothyroxine, it is expected that similar pathways in humans provide the mechanistic basis for the observation of increased serum T_4:T_3 ratio in the setting of normalized serum TSH levels that are frequently observed among levothyroxine-treated patients.[12]

Differences in hypothalamic DIO2 susceptibility to ubiquitination explain localized sensitivity to levothyroxine.[17] Both *in vivo* studies in mice harbouring astrocyte-specific inactivation of *Wsb1* and *in vitro* analysis of DIO2 ubiquitination induced by different tissue extracts indicated that DIO2 ubiquitination in the hypothalamus is relatively less or that there is faster de-ubiquitination when compared to other tissues. As a result, in contrast to other DIO2-expressing tissues, the hypothalamus is wired to have increased sensitivity to T_4.

Hypothalamic DIO2 is also sensitive to other stimuli that play a part in the hypothalamic–pituitary–thyroid axis, including nutritional and inflammatory signals.[76] Thus, regulation of DIO2 activity in tanycytes also integrates other signals that override the input provided by circulating thyroid hormone levels. For example, in the animal model of lipopolysaccharide-induced nonthyroidal illness there is upregulation of Dio2 in tanycytes that increases local thyroid-hormone signalling, which in turn suppresses TRH expression despite falling serum thyroid hormone levels.[98,99] Notably, lipopolysaccharide-mediated downregulation of Trh does not occur in mice with global inactivation of *Dio2*, indicating that this pathway is required for this process.[70] Studies in transgenic mice indicate that mice without appreciable Dio2 expression in astrocytes, but with an intact tanycyte Dio2 pathway, can efficiently regulate their hypothalamic–pituitary–thyroid axis.[28]

[H2] Normalization of T_3 levels in hypothyroidism

Many patients receiving levothyroxine fail to achieve serum T_3 levels within the normal range and display elevated serum T_4 levels, resulting in a high serum T4:T3 ratio.[12,100,101] Although this phenomenon has been recognized for decades, clinicians generally adopted the hypothesis that

iodothyronine deiodinases would appropriately regulate the pool of available T_3 at the cellular and tissue level, thus rendering levothyroxine-treated patients euthyroid. However, some investigators have questioned whether a low serum T_3 level and/or a high T_4:T_3 ratio could have clinical implications. Specifically, whether this situation causes the residual symptoms experienced by a minority of patients treated with levothyroxine.[9,102]

The clinical data remain contentious.[15,18,19] However, studies in rats indicate that levothyroxine administration alone does not normalize serum Tsh, serum T_3 and tissue T_3 levels at the same time,[18] and that a serum Tsh level within the normal range coexists with lowered serum and tissue T_3 levels. These issues have were confirmed in an animal study in which thyroidectomized rats receiving levothyroxine monotherapy exhibited low levels of serum T_3 and a high T_4:T_3 ratio.[17] The novelty of this study was not only that these abnormalities in systemic parameters were confirmed but also that tissue-specific markers of hypothyroidism were evaluated. Mitochondrial content and α-glycerophosphate dehydrogenase activity—both known markers of T_3-responsiveness in the liver and skeletal muscle—were normalized in rats receiving levothyroxine plus liothyronine combination therapy but not in those treated with levothyroxine alone. In addition, serum cholesterol levels were normalized in rats receiving combination therapy but not in those receiving levothyroxine monotherapy.

Therefore, in the rodent model, levothyroxine replacement therapy that results in a high T_4:T_3 ratio and a low serum T_3 level seems to exert consequences on markers of hypothyroidism at the tissue level (liver and skeletal muscle). The application of these findings to human studies will be important to determine whether levothyroxine-treated patients experience tissue-specific hypothyroidism and, if so, whether this effect can be reversed with combination therapy.

[H2] Thyroid-hormone homeostasis in the brain

The plasma contributes approximately half of the T_3 present in the rodent brain, whereas the remainder is produced locally via deiodination of T_4.[61] The implication is that a drop in serum T_4 or T_3 levels could negatively affect thyroid-hormone signalling in the brain. However, this effect is largely minimized by the homeostatic actions of the iodothyronine deiodinases.[63] Accordingly, normalization of cerebral cortex, but not cerebellar, levels of T_3 in levothyroxine-treated thyroidectomized rats is achieved when circulating levels of T_4 and T_3 are half that of euthyroid control animals that did not undergo thyroidectomy.[18] In addition, cerebral cortex T_3 levels remain within the normal range over a 20-fold increase in the levothyroxine infusion doses.

These landmark studies provide the rationale for very tight control of thyroid hormone actions in the brain. At face value, they suggest that thyroid-hormone signalling is preserved in the brain of patients with mild hypothyroidism. However, the different behaviour of rat cerebral cortex and cerebellum confirm that thyroid-hormone signalling in the brain is highly compartmentalized (that is, not all brain areas behave in the same way). To address this issue, thyroid hormone action was studied using 16 genetic markers of T_3-responsiveness in the cerebral cortex, cerebellum and hippocampus of thyroidectomized rats treated with either

levothyroxine monotherapy or levothyroxine plus liothyronine combination therapy at doses that normalize serum Tsh levels.[17] All genetic markers were normalized in thyroidectomized rats receiving combination therapy, whereas 10 markers failed to be normalized in those receiving monotherapy, indicative of cerebral hypothyroidism.[17] The animals receiving monotherapy exhibited lowered serum T_3 levels, which might explain some of these observations. An additional non-exclusive explanation is that the reduction in brain Dio2 activity observed among levothyroxine-treated animals (caused by the elevated serum T_4:T_3 ratio) could lead to decreased T3 production via Dio2 and add to the localized hypothyroidism.[17]

These findings challenge the idea that the impact of reduced DIO2 activity on T_3 production following exposure to T_4 is compensated by increased substrate availability, thus preserving or even increasing T_3 production. Although logical, this rationale might not apply to all DIO2-expressing tissue or cell types given that in a cell system in which T_4 to T_3 conversion via DIO2 was monitored while cells were exposed to progressively higher T_4 levels, T_3 production trailed T_4 concentration in the medium only to a limited extent.[95] This was followed by an inflection point at which further elevations in T_4 concentration reduced DIO2 activity, and T_3 production fell abruptly; the level of T_4 at the inflection point depends on the cell type, presumably because of differences in DIO2 ubiquitination.[95] This finding is supported by the observations that Dio2 activity in mice with brain-specific inactivation of *Wsb-1* respond differently to T_4 administration across different brain areas.[17] For example, Wsb-1 mediates Dio2 inactivation in response to levothyroxine administration in brain areas such as the cerebral cortex and hippocampus, whereas in the cerebellum Wsb-1 does not mediate loss of Dio2 activity. Furthermore, levothyroxine-induced Dio2 inactivation is minimal (almost nonexistent), in the hypothalamus.

[H1] Mood disorders and cognition

To what extent do the iodothyronine-deiodinase-mediated mechanisms regulating thyroid-hormone signalling underpin clinical phenotypes observed among patients with hypothyroidism?

These patients exhibit variable degrees of cognitive dysfunction, lethargy, poor motor coordination, memory impairment, depression, and mood disorders. In addition, the efficacy of antidepressant agents among euthyroid individuals is potentiated when associated with the administration of thyroid hormone, either levothyroxine or liothyronine.[103,104] Some clinical studies, but not all,[105] indicate that residual cognitive dysfunction exists among levothyroxine-treated patients with hypothyroidism.[9,53,106,107] Whether these residual symptoms are a consequence of low serum levels of T_3, or a high T4:T3 ratio,[108,109] remains to be determined. In addition, whether combination therapy would restore cognitive function is an interesting hypothesis to be tested.

Some evidence suggests that thyroid hormones act in the brain through serotonergic pathways, a system involved in the pathogenesis of affective disorders and response to psychotropic agents.[110] Arising from discrete brainstem nuclei, serotonin circuits project to a range of cortical and subcortical brain areas, including the prefrontal cortex, hippocampus and amygdala, which

explains their influence on behaviours such as mood, sleep and appetite.[111] MRI spectroscopy and PET studies confirm that thyroid hormones act in the human limbic system, with substantial differences observed in the posterior cingulate cortex and the inferior parietal lobe of hypothyroid versus hyperthyroid individuals.[112,113]

Considering that iodothyronine deiodinases modify thyroid-hormone signalling in the rodent brain,[114] it seems logical to assume that the activity of these enzymes might affect mood and/or play a role in the variability of the response of patients with depression to T_3. In animal models, Dio2 activity in the brain is increased by antidepressants of various classes, including selective serotonin reuptake inhibitors.[115-117] In addition, hippocampal expression of Dio2 and Dio3 is increased in a mouse model that is resistant to stress-induced depression and after antidepressant treatment in mice.[108] However, a mouse with global inactivation of *Dio2* exhibits only a mild motor phenotype and no behavioural phenotype.[61,118] By contrast, a mouse with global inactivation of *Dio3* exhibits brain hyperthyroidism, aggressive behaviour and indifference towards pups.[119] Furthermore, there is a negative correlation between Dio3 expression and T_3 levels in the frontal cortex and hippocampus of a mouse model of depressive behaviour and cognitive deficits, which is partially restored by administration of levothyroxine.[120-122]

The available clinical data are inconsistent for DIO2 and DIO3 but strong for DIO1, which is hardly expressed in the human brain.[24,79] A study of 1447 individuals enrolled from the general population indicated that a polymorphism in DIO1, called C785T, is associated with lifetime depression among white females in high-risk cohorts[123] and with the antidepressant efficacy of T_3 in a trial of the selective serotonin reuptake inhibitor sertraline.[124]

[H1] Genetic risk and brain disease

A prevalent single nucleotide polymorphism in the *DIO2* gene (which results in a single amino acid substitution of Thr for Ala at position 92 in DIO2, thus called Thr92AlaD2) has been identified,[125] with estimates suggesting that ~12–36% of individuals in the general population are homozygous. This variant has been associated with metabolic derangements, including insulin resistance[125,126] and type 2 diabetes mellitus.[127] Furthermore, it has been implicated in mental and psychological disorders,[45] such as mental retardation,[128] bipolar disorder[129] and low IQ.[130] Conversely, Thr92AlaD2 might confer protection from recurrent depression.[131] The clinical relevance of Thr92AlaD2 was strengthened when a large clinical trial reported that it correlated with reduced well-being and a preference for levothyroxine plus liothyronine combination therapy versus levothyroxine monotherapy among carriers with hypothyroidism.[13]

These findings have stimulated much intrigue in the field with most initial hypotheses focusing on a possible defect in thyroid-hormone signalling. However, the single amino acid substitution associated with Thr92AlaD2 does not alter enzyme kinetics when transiently expressed in cells[132,133] and only indirect evidence suggests that the T4-to-T3 conversion via Thr92AlaD2 could be less in patients.[134,135] Perhaps this observation is not unexpected given that the mutation lies not within the catalytic site but rather within the 18-amino acid loop that controls susceptibility to T_4-induced ubiquitination of DIO2.[31] The putative functional abnormality

associated with expression of the variant protein was defined in studies performed in a human embryonic kidney cell line that had been engineered to stably express this mutation. The protein harbouring Ala92D2 has a longer half-life than the native form (Thr92D2) and is ectopically located in the Golgi apparatus.[35] This aberrant subcellular localization was associated with abnormal Golgi structure, such that circular (rather than linear or ribbon-like) stacks of Golgi membrane were observed. These cellular abnormalities resulted in alterations at the transcriptional level that were independent of DIO2-mediated T_3 production given that the cell line studied does not express TRs[136] and that the observed gene expression pattern lacked typical indicators of T_3-responsiveness.[114] Remarkably, when human cerebral cortex samples from patients harbouring the polymorphism were studied, there was overlap in the expression of 81 genes, defining a molecular fingerprint associated with the Ala92D2 variant across these human cell and brain models.[35]

In addition, robust analysis of microarray data derived from the human brain samples demonstrated a gene expression profile reminiscent of that of neurological diseases, with Huntington disease being the top gene expression pathway identified.[35] This finding implies that the polymorphism is a novel risk factor for neurodegenerative disease or impaired cognition, which might explain the neuropsychological impairment displayed by some patients; however, further studies are needed before definite conclusions can be drawn.[35] In particular, it will be important to elucidate whether the high T_4:T_3 ratio observed among levothyroxine-treated individuals with hypothyroidism[12,100] might perturb the cellular abnormalities associated with expression of the variant protein. This relationship could explain the improved well-being and preference for combination therapy recorded in a subgroup of patients because there was no evidence of reduced thyroid-hormone signalling in the samples (T_3-independent).

These findings represent a step towards determination of the relationship between the cellular abnormalities associated with variant protein expression and the associated clinical phenotype. Future studies are needed to fully characterize the molecular basis for the clinical observations. Nevertheless, clinical trials among this subgroup could be warranted to determine whether hypothyroid patients harbouring the polymorphism would benefit from alternate therapeutic strategies. If it is rigorously demonstrated that such patients derive benefit from combination therapy, then genotyping for Thr92AlaD2 could become a routine component of the management of hypothyroidism, thus bringing the concept of personalized medicine to the field.

[H1] Conclusions

Major advances have been made in the treatment of hypothyroidism over the past century; however, refined therapeutic regimens might still be needed to ensure that all patients can be rendered asymptomatic and clinically euthyroid. Once heralded as the ultimate regulators of thyroid-hormone availability and the key to levothyroxine treatment efficacy, iodothyronine deiodinases might actually underpin the inability to normalize serum levels of TSH and T_3 among patients receiving levothyroxine monotherapy and the insufficient symptomatic response experienced by an appreciable proportion of the hypothyroid population. Future studies exploring the role of the iodothyronine deiodinases in hypothyroidism hold real promise

towards determining therapeutic regimens that can normalize systemic and tissue-level parameters for all patients with hypothyroidism, where new approaches might even be genotype-directed.

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Figure 1 | Iodothyronine deiodinases modulate thyroid-hormone signalling in T_3 target cells. T_4 and T_3 enter virtually all cells via membrane transporters. They are then modified in a cell-specific manner by DIOs to either enhance (DIO2) or diminish (DIO3) thyroid-hormone signalling. Consequently, the flow of T_3 diffusing from the cell membrane to the nucleus is increased by $T_3(T_4)$, which represents T_3 generated locally from T_4 via DIO2. By contrast, the DIO3 pathway decreases the flow of T_3 to the nucleus by terminally inactivating T_3 to T_2 and T_4 to rT_3 (rT_3,T_2). DIO2 generates T_3 in a cell compartment adjacent to the nucleus. By contrast, DIO3 resides in the periphery of the cell in the plasma membrane and early endosomes. Once inside cells, T_3 can diffuse to the nucleus to modulate gene expression. Abbreviations: DIO, iodothyronine deiodinase; T_2, di-iodothyronine; T_3, tri-iodothyronine; T_4, tetra-iodothyronine; rT_3, reverse tri-iodothyronine . (Adapted from BiancoLab.org.)

Figure 2 | **T4-induced D2 ubiquitination in thyroid hormone homeostasis**.
In response to thyroid hormone signals from the periphery and DIO2-expressing tanycytes, hypophysiotropic TRH-expressing neurons release TRH into the portal blood. TRH is transported to the anterior pituitary gland where TSH is secreted and stimulates the thyroid gland to produce and secrete T_4 and T_3. Hypothalamic T_3 is generated locally by tanycytes and enters the systemic circulation. T_3 can also be generated in the periphery via DIO1. In most peripheral tissues, exposure to T_4 accelerates inactivation of DIO (UbDIO2) and its targeting to the proteasomal system; however, UbDIO2 can be reactivated and rescued from proteasomal destruction by deubiquitination. Peripheral deiodination is very sensitive to T_4-induced DIO2 ubiquitination: a mild elevation in the serum T_4:T_3 ratio favours DIO2 inactivation and decreases fractional T_4-to-T_3 conversion and peripheral T_3 production. However in the hypothalamus, DIO2 is less susceptible to T_4-induced ubiquitination. Thus, T_4 signaling via DIO2-mediated T_3 production is very effective in the hypothalamus, whereas T_3 production via DIO2 is easily inhibited in the periphery. Abbreviations: DIO, iodothyronine deiodinase; T_2, di-iodothyronine; T_3, tri-iodothyronine; T_4, tetra-iodothyronine; TSH, thyroid-stimulating hormone; TRH, thyroid-stimulating hormone releasing hormone; UbDIO2, ubiquitinated DIO2. (The figure has been modified from[17])

Fig1

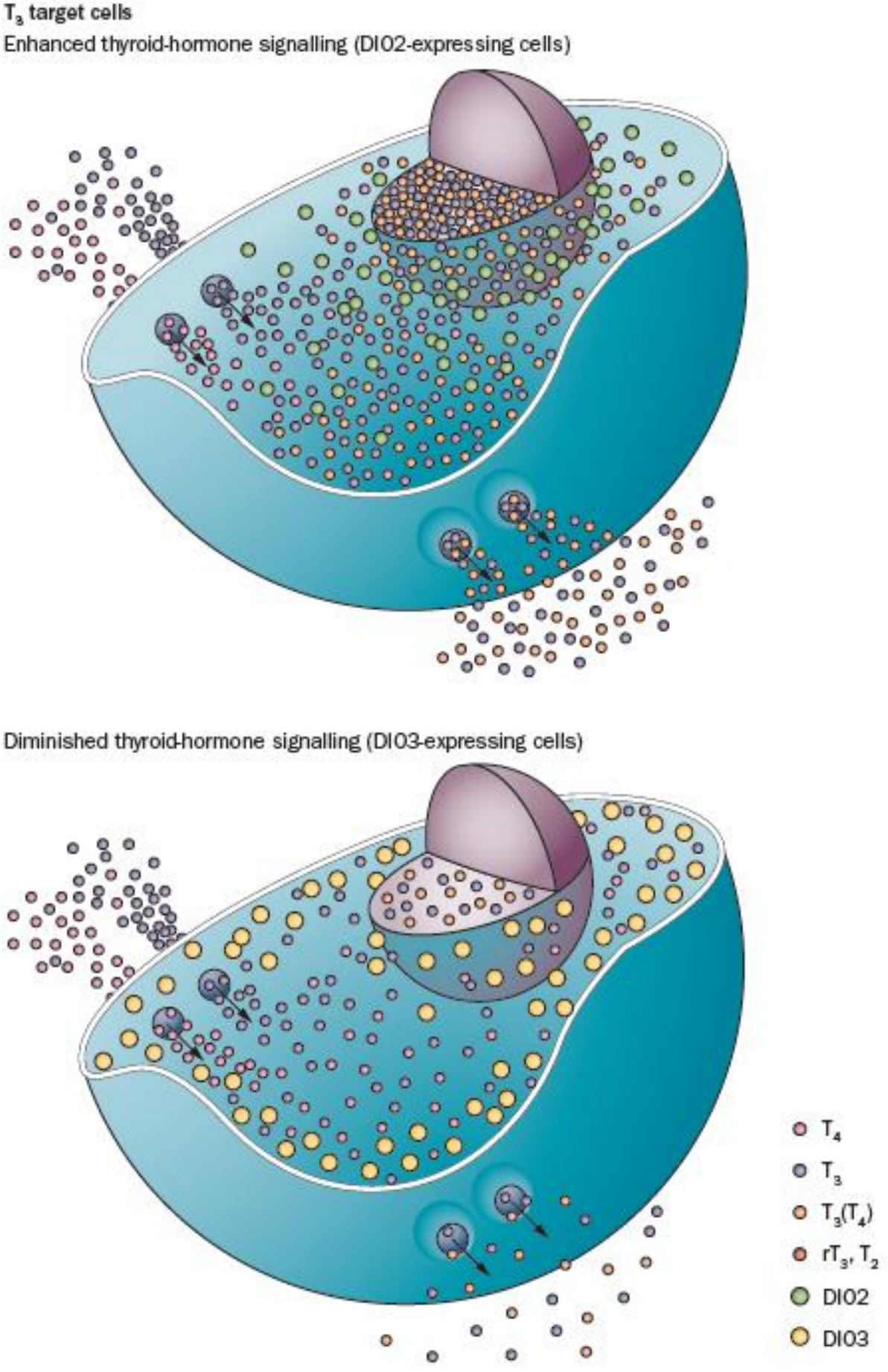
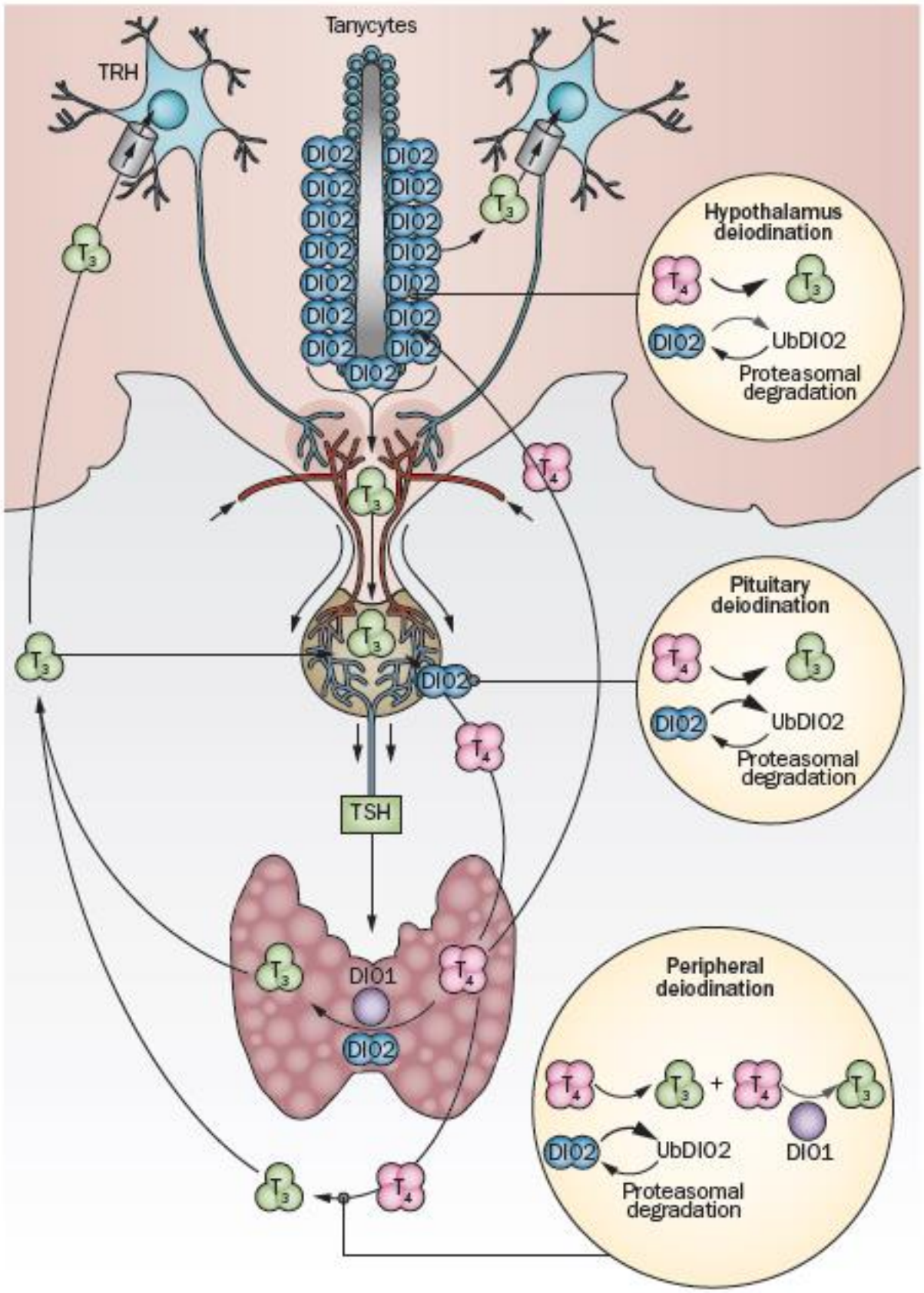

T_3 target cells
Enhanced thyroid-hormone signalling (DIO2-expressing cells)
Diminished thyroid-hormone signalling (DIO3-expressing cells)
T_4
T_3
$T_3(T_4)$
rT_3, T_2
DIO2
DIO3

Fig2


Tanycytes
TRH
DIO2
T3
T4
Hypothalamus deiodination
UbDIO2
Proteasomal degradation
Pituitary deiodination
TSH
DIO1
Peripheral deiodination
+