Intrathyroidal feedforward and feedback network regulating thyroid hormone synthesis and secretion

Li Jing1* and Qiang Zhang2*

1Department of Toxicology and Hygienic Chemistry, School of Public Health, Capital Medical University, Beijing, China, 2Gangerosa Department of Environmental Health, Rollins School of Public Health, Emory University, Atlanta, GA, United States

Thyroid hormones (THs), including T4 and T3, are produced and released by the thyroid gland under the stimulation of thyroid-stimulating hormone (TSH). The homeostasis of THs is regulated via the coordination of the hypothalamic-pituitary-thyroid axis, plasma binding proteins, and local metabolism in tissues. TH synthesis and secretion in the thyrocytes-containing thyroid follicles are exquisitely regulated by an elaborate molecular network comprising enzymes, transporters, signal transduction machineries, and transcription factors. In this article, we synthesized the relevant literature, organized and dissected the complex intrathyroidal regulatory network into structures amenable to functional interpretation and systems-level modeling. Multiple intertwined feedforward and feedback motifs were identified and described, centering around the transcriptional and posttranslational regulations involved in TH synthesis and secretion, including those underpinning the Wolff-Chaikoff and Plummer effects and thyroglobulin-mediated feedback regulation. A more thorough characterization of the intrathyroidal network from a systems biology perspective, including its topology, constituent network motifs, and nonlinear quantitative properties, can help us to better understand and predict the thyroidal dynamics in response to physiological signals, therapeutic interventions, and environmental disruptions.

KEYWORDS
thyroid hormone, feedforward, feedback, thyrocyte, intrathyroidal regulation

Introduction - homeostatic regulation of thyroid hormones

Thyroid hormones (THs), including thyroxine (T4) and 3,3’,5-triiodo-L-thyronine (T3), are produced and released by thyrocytes-containing follicles in the thyroid gland. T4 and T3 are transported through the blood circulation to target tissues, where T4 is converted intracellularly to T3, the active hormone. In the nucleus, T3 binds to TH
receptors (TR) that are dimerized with retinoid X receptors (RXR) on the TH response elements (TREs) of a myriad of target genes to regulate their transcription. THs are essential for normal development, growth, and metabolism of almost all tissues. They regulate cardiovascular and energetic homeostasis (1, 2), female reproduction (3), and the development of the nervous and skeletal systems (4, 5). Insufficient or excessive levels of THs result in a multitude of developmental and metabolic diseases. Therefore, robust control of TH homeostasis is essential.

TH homeostasis is maintained both globally and locally through multiple mechanisms that coordinate the production, secretion, distribution, and metabolism of THs. These mechanisms include systemic feedback regulation of TH synthesis and secretion by the thyroid-stimulating hormone (TSH) through the hypothalamic-pituitary-thyroid (HPT) axis, buffering by the TH binding proteins (THBPs) in the blood circulation, and local feedback regulation by metabolic enzymes of THs in peripheral tissues (6–9). Homeostatic compensation through the HPT feedback can take days or even several weeks to complete given the long half-lives of T3 and T4 in humans (10–12). In contrast, the fast binding between THs and THBPs renders the adjustment through buffering almost instantaneous, in seconds to minutes (13, 14). These multi-layered, cross-scale regulations, operating on different time scales and in different anatomical locations, coordinate with one another forming the basic regulatory framework of TH homeostasis.

HPT feedback regulation

The classical feedback regulation of the HPT axis is the primary mechanism for chronically maintaining the circulating free TH levels within the physiological range (15). The TSH-releasing hormone (TRH) is synthesized as a neurohormone in the paraventricular nucleus (PVN) of the hypothalamus. Released in the median eminence and transported via the hypophysial portal vasculature, TRH reaches the anterior pituitary and stimulates the thyrotropes to secrete TSH into the systemic blood circulation. In turn, TSH stimulates the synthesis and secretion of T4 and T3 in the thyroid gland. Secreted THs then reach the brain where they inhibit the production and release of both TRH and TSH (16). For instance, in the TRH neurons located in the hypothalamic paraventricular nucleus, T3 represses the transcription of the TRH gene via TRH receptor β2 (TRβ2) (17). In the pituitary thyrotrophs, T3 represses the transcription of the TSHβ subunit by inhibiting via TRβ2 the autoregulation of GATA2, a key transcription factor that mediates the TRH-stimulated TSH production (18). Additionally, in the tanyctyes located in the median eminence, T3 can upregulate the expression of pyroglutamyl peptidase II, which is a highly specific TRH peptidase that degrades the locally released TRH before it reaches the portal vein system (19). In summary, T3-mediated inhibition of TRH and TSH completes the negative feedback loop of the HPT axis, which can maintain the steady-state circulating free TH levels within a very narrow 2-3 fold range in humans (20, 21).

Buffering by TH binding proteins

Acute or transient perturbation of circulating free THs is resisted by the THBPs. In the human blood, only a small fraction of THs exists in the free, unbound form, while nearly 99.97% of T4 and 99.70% of T3 are bound to specific THBPs (22), including thyroxine-binding globulin (TBG), transthyretin (TTR), and albumin (23). TBG is the most abundant and has the greatest affinity for THs, followed by TTR and albumin (24). Overall, TBG could account for 75%, TTR 15%, and albumin 10% of total THs in the plasma, with a negligible amount distributed in lipoproteins (25). The THBPs can shield THs from the aqueous environment in the plasma. More importantly, just like a pH buffer system, the THBPs serve as a reservoir to buffer free T4 and T3 concentrations against transient fluctuations and ensure steady delivery of THs through the target tissues (26, 27). The buffering operates in a time scale of seconds to minutes, which is much faster than the feedback action of the HPT axis. However, since the long-term free TH levels are determined by the HPT axis, abnormality in the abundances or binding affinities of THBPs only result in changes in the concentrations of total, not free, THs in the blood, and the patients are in most cases clinically euthyroid (28).

Local feedback regulation of TH metabolism

Despite that free THs in the circulation are robustly regulated by the HPT feedback and THBPs, it is T4 and particularly T3 in the cells that ultimately drive the biological outcomes. THs in the circulation are taken up by cell membrane transporters and metabolized intracellularly by deiodinases (DIO) which can either activate or deactivate THs (8, 29). The intracellular TH concentrations in peripheral tissues are controlled by multiple local feedbacks regulating the activities and expression levels of DIOs. T3 can repress the gene transcription of DIO2, the primary activating enzyme that converts T4 into T3, therefore forming a negative feedback loop to keep intracellular T3 within a range (30–32). DIO2 transcription is also inhibited by rT3 (33). In addition, T3 can induce DIO1 transcription by binding to two TREs in the gene promoter in tissues including the liver (34–36). This induction may form a positive feedback via DIO1-mediated production of
T3 from T4, thus exacerbating hyperthyroid conditions (32). However, because DIO1 can also deactivate T4 into rT3 and deactivate T3 into T2, a local negative feedback control cannot be ruled out. T4 and rT3 also inhibit DIO2 by promoting its proteasomal degradation (37–40), forming an incoherent feedforward motif with T3 as the output. Lastly, it has been reported that an increase in serum T3 could upregulate DIO3 expression in the brain, increasing local T3 clearance, forming another local feedback control of T3 (41–43). In addition to deiodination, in hepatocytes feedback regulation of THs also appears to be mediated by glucuronidation via inducible UDP-glucuronosyltransferase (UGTs) (44–47). In summary, THs can regulate the activities and abundances of DIOs and certain UGTs in peripheral tissues in a feedback or feedforward manner to maintain their local levels.

The three global and local mechanisms described above are crucial to maintaining THs at appropriate physiological levels in the circulation and target tissues. For the HPT axis to sustain circulating free THs within the normal range, the regulation of TH synthesis and secretion by TSH in the thyroid gland plays a central role. This process occurs in thyroid follicles, involving iodide uptake, iodination of thyroglobulin (TG), proteolysis of TG into T3 and T4, and their release into the systemic circulation. The process is exquisitely regulated through an elaborate molecular network of enzymes, transporters, signal transduction machineries, and transcription factors (TFs), which form multiple intertwined feedback and feedforward structures. Feedback and feedforward are key network motifs that underpin a variety of dynamic behaviors, including signal amplification, multistability, adaptation, homeostasis, oscillation, fold-change detection, signaling delay and acceleration, threshold and nonmonotonic effect etc. (48, 49). Multiple motifs coupled together as seen in complex networks can generate even richer dynamics and advanced functions. Understanding the systems biology, including the organization and quantitative properties, of the complex intrathyroidal network will help ascertain not only the thyroidal responses to physiological signals such as TSH, but also the potential adverse health outcomes and risks of environmental endocrine disrupting chemicals (EDCs) that directly perturb the thyroid gland. In this article, we intended to review and synthesize the relevant literature, and organize the intrathyroidal regulatory network of TH synthesis and secretion into structures that are amenable not only to functional interpretation but also to dynamical modeling in future. We started the review by first introducing the TH synthesis and secretion pathway, followed by describing the TSH-stimulated signal transduction components and TFs that regulate the expression and activities of the enzymes and proteins involved in TH synthesis and secretion. The multiple transcriptional and posttranslational feedforward and feedback circuits between TFs and enzymes were then depicted, including the feedforward and feedback regulations underpinning the Wolff-Chaikoff and Plummer effects of high iodine and TG-mediated inhibition. Lastly, perspectives were provided on how quantitative characterization of the intrathyroidal network can help us to better understand TH regulation, thyroid disease, and adverse effects of thyroid EDCs. It should be noted though that many of the regulations reviewed here are based on in vitro and in vivo studies in a variety of species, particularly rat thyroid cell lines, thus caution should be exercised when generalization is made. This review is unique in that it is not intended to provide a comprehensive coverage and enumeration of the molecular biology details of known regulations in thyrocytes, but to focus on the systems-level structures, potential functions, and biological significances that these regulations as a whole can furnish when connected into complex networks.

The TH synthesis and secretion pathway

Iodide uptake, exportation, and organization

Thyroid follicles are the functional units of the thyroid gland. They are spherical structures comprising a monolayer of epithelial cells (thyrocytes) that enclose an extracellular lumen filled with TG colloid (50). Each follicle is encapsulated by a rich network of capillaries. Uptake of circulating I− by thyroid follicles is mediated by the Na+/I− symporter (NIS), a glycoprotein located on the basolateral plasma membrane of the thyrocytes and encoded by the solute carrier family 5 member 5 (SLC5A5) gene (51). The active uptake of I− by NIS is Na+−dependent, which couples the energy released by the inward translocation of Na+ down its chemical gradient to the simultaneous inward translocation of I− against its electrochemical gradient (52). Inside the thyrocytes, I− diffuses to the apical side of the cell, where it is taken up and transported into the follicular lumen by transporters located on the apical plasma membrane, including pendrin (PDS), anoctamin-1 (ANO1), and SLC26A7 (53, 54). PDS is an anion transporter encoded by the SLC26A4 gene (55, 56). ANO1 is a calcium-activated chloride channel, which has been demonstrated to mediate I− efflux across the apical membrane of thyrocytes (54). SLC26A7, a member transporter belonging to the same family as SLC26A4, is an anion exchanger with affinity for I− and chloride (56).

Once in the follicular lumen, I− is organized by key enzymatic complexes anchored on the apical surface of the thyrocytes, containing dual oxidase (DUOX) and thyroid peroxidase (TPO). A member of the NADPH oxidase (NOX) family, DUOX1 and DUOX2 utilize the protons provided by intracellular NADPH to generate and release H2O2 into the lumen. The maturation and function of DUOX1 and DUOX2 require DUOX maturation factor 1 (DUOX1A1) and DUOX2A, respectively, which are chaperone proteins. By forming
enzymatic complexes DUOX1/DUOXA1 and DUOX2/ DUOXA2, the chaperones promote the correct maturation of DUOX1 and DUOX2 for their endoplasmic reticulum (ER) exit to the cell surface (57). Although similar in structure, the DUOX1 and DUOX2 proteins are encoded by two separate genes with fairly different regulatory mechanisms (58). Compared to DUOX1/DUOXA1, DUOX2/DUOXA2 has higher enzymatic activities and is the predominant form in the thyroid (57, 59). TPO utilizes H₂O₂ generated by DUOX/ DUOXA to catalyze the oxidation of I⁻ and subsequent iodination of the tyrosine residues of soluble TG molecules nearby (60). The cooperation between DUOX/DUOXA and TPO is facilitated via the formation of thyroxisome, a complex comprising DUOX/DUOXA, TPO, and caveolin-1 (CAV1). CAV1 is required for the correct positioning of TPO and DUOX on the apical membrane (61, 62), bringing the two together in the thyroxisome (63). The close proximity between DUOX and TPO makes newly produced H₂O₂ readily captured by TPO and reduces potential oxidative damage by H₂O₂ that would otherwise diffuse afar (64, 65). At the end of the organisation process, I⁻ is incorporated to specific tyrosine residues in the TG molecule, forming intramolecular monoiodotyrosine (MIT) and di-iodotyrosine (DIT). TPO then further catalyzes the coupling of MIT and DIT in the same TG molecule to produce intramolecular T3 and T4 (66).

TG dynamics in the lumen

As the macromolecular precursor of THs, TG is synthesized and processed in ER to form dimers with N-linked glycoside, followed by further processing in the Golgi apparatus where modifications of the carbohydrate moieties occur (67). Mature TG molecules are transported via vesicles from the trans-Golgi network to the apical surface of the thyrocytes, then they are released into the lumen and stored there as the predominant protein content of the colloid (67). The proteinases expressed in the thyroid gland mainly include cathepsins B, D, H, K, L, and S (85). Cathepsins B, D, K and L are detectable in the follicle lumen or in association with the apical membrane (86-88). Cathepsins B and L in the lumen are involved in proteolytic solubilization of TG from covalently cross-linked TG globules (89). Trace amine-associated receptor 1 (TAAR1), a G-protein coupled receptor (GPCR), is located at the cilia of the apical plasma membrane of thyrocytes which extend into the follicle lumen. It can act as a sensor of the status of luminal TG. TAAR1 can co-localize with cathepsins B and L and regulate the proteolytic action of these cysteine cathepsins and internalization of TG (77).

TG structure

TG is a noncovalent homodimer (660 kDa) with a high degree of glycosylation. Each TG monomer contains 67 tyrosine residues, of which ~30 can be iodinated, among which four are hormonogenic acceptors and five are donors. In human TG, the four acceptor tyrosine residues are localized at positions 24, 1310, 2573, and 2766 (69, 70), and the donor tyrosine residues at 108, 149, 234, 2540, and 2766 (71, 72). After iodination, the aromatic ring of a donor DIT or MIT is transferred by TPO to a proximal acceptor DIT, forming T4 or T3, which remain covalently connected to the polypeptide backbone, while leaving a dehydroalanine at the donor site (73). Not every donor and acceptor tyrosine residue can be utilized to produce THs. A human TG molecule contains on average 2.28 molecules of T4 and 0.29 molecules of T3 (67).

TG storage and liberation

TG molecules – newly secreted into the lumen and thus remaining near the apical membrane where the I⁻ organization machinery is located – are the primary substrates of TPO. Freshly iodinated TG molecules, still soluble and adjacent to the apical surface, are also the first available for uptake by thyrocytes (67). If not taken up by thyrocytes, the iodinated TG molecules would proceed into the colloid where they are stored in a highly condensed, covalently cross-linked, multimerized globule (74–77). This unique storage mechanism makes TG highly enriched in the lumen, reaching a concentration as high as nearly 600 mg/ml in the human thyroid gland, and constituting >95% of the colloid protein content (67, 78–80). The turnover of this TG reservoir is very slow (81, 82). However, when the TG molecules in the colloid globule are needed to meet the TH demand, it is those on the outside of the globule, which are more recently iodinated, that are hydrolyzed quickly, rather than the ones packed inside, which are older and of greater iodothyronine content (83). Therefore, newer TG molecules are utilized first for TH production. This phenomenon is consistent with the “last-come-first-served” concept for the utilization of iodine – where the thyroid gland secretes recently formed organic iodine before it taps into the older ones (84).

Under circumstances such as iodine deprivation or TSH overstimulation, the colloid TG globule will be solubilized by proteinases and the solubilized TG will be taken up by thyrocytes (67). The proteinases expressed in the thyroid gland mainly include cathepsins B, D, H, K, L, and S (85). Cathepsins B, D, K and L are detectable in the follicle lumen or in association with the apical membrane (86-88). Cathepsins B and L in the lumen are involved in proteolytic solubilization of TG from covalently cross-linked TG globules (89). Trace amine-associated receptor 1 (TAAR1), a G-protein coupled receptor (GPCR), is located at the cilia of the apical plasma membrane of thyrocytes which extend into the follicle lumen. It can act as a sensor of the status of luminal TG. TAAR1 can co-localize with cathepsins B and L and regulate the proteolytic action of these cysteine cathepsins and internalization of TG (77).

TG uptake pathways

Soluble TG molecules of different maturity in iodination are taken up by thyrocytes through different mechanisms, including pinocytosis and endocytosis involving membrane receptors and related proteins (67, 90). Studies have shown that more recently synthesized TG molecules, which are low or yet to be iodinated, tend to bind to membrane receptors including megalin and ASGPR and are internalized along with receptor recycling, while
highly iodinated and sialylated TG molecules preferentially undergo nonspecific fluid pinocytosis (68). TG molecules internalized via pinocytosis merge with lysosomes, in which T3 and T4 are proteolytically cleaved off of the TG backbone (67). TG molecules taken up via receptor-mediated mechanisms do not seem to be a significant source of T3 and T4 production. Megalin is a high-affinity TG receptor expressed on the apical surface of thyrocytes and it mediates the transcytosis of TG (67). It internalizes TG molecules with a low hormone content and the TG molecules so internalized are generally not available for degradation by the lysosomal pathway, but transported directly to the basolateral membrane and secreted into the blood circulation (91). ASGPR can also bind more recently synthesized, poorly iodinated and sialylated TG and route them away from the lysosomal pathway such that they serve as negative feedback signals to repress thyroid-specific TFs (TTFs) (68, 92). Flotillin, a lipid raft protein, also plays an important role in TG endocytosis. It can physically interact with endocytosed TG and mediate the negative feedback of TG on TTFs (90). ASGPR and N-acetylgalactosamine receptor can recycle intracellular TG molecules of low hormone content back into the lumen (68, 93, 94).

Intracellular TH liberation and release

Cathepsin K associated with the apical plasma membrane, particularly in active follicles, and luminal cathepsin L can act as endo- and exopeptidase to cleave soluble TG in the lumen, thereby liberating a limited amount of T4, which can be taken up by the thyrocytes (86, 88). However, the majority of T4 is produced intracellularly from pinocytosed TG. These TG molecules are degraded in lysosomes by cathepsins B, D, H, K, L, and S, where T3, T4, MIT, and DIT are exhaustively liberated (77, 95, 96). Proteolysis of TG releases 6 or 7 times more MIT and DIT than T3 and T4 (97). These MIT and DIT can be deiodinated by iodothyrosine dehalogenase (DEHAL), and the iodide released is recycled as a sustained source of intrathyroidal iodide for TH synthesis (98, 99). Intracellular T4 can be further deiodinated by DIO1 and DIO2 to produce T3 (100). T4 and T3 are then secreted into the bloodstream via TH transporters, including monocarboxylate transport MTC8 and MCT10, located on the basolateral plasma membrane (60). MCT8 favors T4 secretion over T3 secretion, as demonstrated by the higher-than-normal T3/T4 ratio in MCT8 KO mice and in patients with MCT8 mutation (101, 102). MCT10 has a similar effect on transporting T3 to MCT8 but is less active toward T4 than MCT8, as demonstrated in COS1 cells transfected with human MCT10 cDNA (103). The 2-3 amino acid difference in the substrate translocation channel of MCT8 and MCT10 appears to be responsible for the differential preference for T4 (104). While MCT10 does not appear to be expressed in the human thyroid gland, it is expressed in rodent thyrocytes, but still at a level much lower than MCT8 (105, 106). MCT10 seems to be dispensable as far as secreting TH is concerned since the thyroidal TH content is not elevated in MCT10 KO mice, whereas the thyroidal TH content accumulates to higher levels in MCT8 KO or MCT8/MCT10 double KO mice (101, 105). The lack of effect of MCT10 deficiency is not due to adaptive compensation by MCT8 because thyroidal MCT8 is not upregulated in MCT10 KO mice.

TSH-stimulated signal transduction and transcriptional regulation in thyrocytes

TSH activation of TSHR

TSH is the most important regulator of TH synthesis and secretion by the thyroid gland. The effects of TSH are mediated by the thyrotropin receptor (TSHR) which belongs to a family of GPCRs and is located at the basolateral plasma membrane of the thyrocytes (107). TSHR is a protomer composed of an A subunit which is a large extracellular domain, and a B subunit which comprises a 7-transmembrane-domain segment and an intracellular domain (108). Both subunits originate from a single peptide after cleavage but remain linked through disulfide bonds (109). TSH signaling may require the TSHR protomers to form a homodimer or higher-order oligomer (110, 111). Activation of TSHR leads to dissociation of G proteins into the Gα and Gβγ subunits which in turn trigger several canonical signal transduction cascades (112). TSHR can potentially couple to all four Gα protein families (Gαq, Gαi11, Gαq/i10, and Gαq/i13) to activate several kinases that collectively regulate thyrocyte proliferation and TH synthesis and release (Figure 1) (113–115). The Gαq and Gαq/i11-induced signaling pathways are of the most importance in the thyrocytes.

TSHR-activated signal transduction

TSH can differentially activate Gαq and Gαq/i11 in a concentration-dependent manner (116). The differential TSH sensitivity of the two pathways has been reported in both human thyrocytes and COS-7 and CHO cell lines transfected with human TSHR, suggesting it is an intrinsic property of the receptor itself (117–119). Low-concentration TSH binds to the high-affinity binding site on one of the protomers of the TSHR homodimer, and preferentially activates Gαq to stimulate cyclic adenosine monophosphate (cAMP) accumulation, which then activates protein kinase A (PKA) (120). High-concentration TSH binds to both the high- and low-affinity protomers, activating both Gαq and Gαq/i11 to increase cAMP production and activate PKC, respectively (120). It was reported that it takes
10 times higher concentrations of TSH to activate Gaq/11 and the downstream phospholipase C (PLC) and inositol-1,4,5-trisphosphate (IP3) than to activate cAMP in human thyroid slices (117).

The TSH-stimulated thyroid response via Goα is believed to be a “long-term effect”, involving both TH biosynthesis and thyrocyte proliferation. A major target of PKA is the cAMP response element-binding protein (CREB) and its phosphorylation activates its transcriptional activity to induce a suite of downstream genes involved in TH synthesis, which are detailed in the sections below (121, 122). PKA signaling stimulates thyrocyte proliferation through several pathways. PKA can phosphorylate and activate small GTPase Rap1 to induce G1/S entry, as observed in PCCL3 cells (123). In FRTL-5 cells, TSH (cAMP) can amplify the activation of the PI3K and MAPK pathways and DNA synthesis elicited by IGF-I (124). In WRT cells, by activating cAMP and PKA, TSH can independently activate Ras and PI3K pathways and DNA synthesis (125–127). TSH, acting through cAMP, activates p70 ribosomal S6 kinase (p70s6k) that is required for TSH-stimulated DNA synthesis and proliferation in thyrocytes (128). Thus, the physiological function of cAMP signaling activated via Goα is to promote TH synthesis, thyrocyte growth and proliferation.

TSHR coupled to Goq/11 can activate PLC to convert IP3 into DAG and IP3, which activate PKC. Both Goαi/o and PLC inhibit the cAMP/PKA pathway, which can in turn inhibit the PLC/PKC. Goα13 can activate the MAPK pathway, and Gbg activates the PI3K/AKT pathway. MAPK and p70s6k can stimulate DNA synthesis and proliferation of thyrocytes. By regulating thyroid specific functional genes directly or through TTFs, PKA, AKT, PKC, and CREB regulate TH synthesis and secretion. Colors of molecule blocks are arbitrary. Solid lines: direct regulation, dotted lines: direct regulation not established, solid arrow heads: activation, blunted arrow heads: inhibition, dotted arrow heads: activation or inhibition, open arrow heads: mass flux, asterisk: phosphorylation. Unless otherwise specified, similar color scheme and denotations apply to all other figures.
stimulatory response mediated by Gq/PKC, cathepsin B activity and TH-generating TG degradation were increased after 1-2 h of TSH treatment (130). Gq may be involved in the cleavage of T3 and T4 from iodinated TG by cathepsins through calcium signaling to liberate and release TH quickly in short term (77). PKC could induce DUOX2 phosphorylation and its H2O2-generating activity (59). Iodine organification and TH secretion in response to TSH are severely reduced in thyrocyte-specific Gαq/11-deficient mice which often develop hypothyroidism after birth (131). TSH or goitrogenic diet-stimulated proliferative thyroid response is also lacking in these mice. Besides, overexpression of atypical PKC-ζ in WRT cells induced TSH-independent DNA synthesis and cell proliferation through a p42/p44 MAPK-dependent pathway but had no effect on TG expression (132).

Like in many other types of cells (133–136), there exist cross-talks between the cAMP/PKA and PLC/PKC pathway in thyrocytes. Elevated cAMP levels can inhibit PLC activity (137), and PLC/PKC in turn suppresses the cAMP/PKA pathway, despite that the mechanism of suppression remains to be identified (114). At high TSH concentrations, TSHR can also activate Gq/o, which inhibits adenylyl cyclase activity to decrease cAMP production (138, 139). TSH can promote MAPK activation in human thyrocytes via a Gq/13-dependent mechanism (113, 140). Besides, Gβγ released by TSHR activates the PI3K/AKT pathway (112). In summary, TSH stimulates multiple G protein-mediated signal transduction pathways, which cross-talk or converge at various levels of the signaling cascade. These cross-regulations can be either stimulatory or inhibitory, forming tiered, coherent or incoherent feedforward motifs (Figure 1).

Feedforward regulation of TTFs

Through TSH-stimulated, GPCR-mediated signal transduction that culminates in PKA and PKC activation, a group of TTFs are activated. Four TTFs are expressed in differentiated thyrocytes: TTF1 (also known as NK2 homeobox 1, NKX2-1), TTF2 (also known as fork head box protein E1, FOXE1), paired box gene 8 (PAX8), and hematopoietically-expressed homeobox protein (HHEX) (141). These TTFs play critical roles in thyroid differentiation and also coordinately regulate the suite of functional genes involved in TH synthesis (142). Together with CREB, they form a complex transcriptional network, as detailed below (143).

Feedforward appears to be the primary structure of the TTF regulatory network, in addition to autoregulatory loops (Figure 2). TTF1 are at the top of this core transcriptional network. With a TTF1-binding site identified in silico in the promoter of PAX8, TTF1 may regulate PAX8 gene expression (144). TTF1 and PAX8 appear to act together to co-regulate many downstream genes including HHEX and TTF2. The partnership between TTF1 and PAX8 may be through a couple of mechanisms. First, TTF1 and PAX8 are able to interact directly, as demonstrated in rat PCC13 thyrocytes in vitro, thus they may form a functional protein complex driving synergistic transcriptional activation of target genes such as TG (145). Second, the DNA binding sites of TTF1 and PAX8 often colocalize in the promoters of target genes (143). Functional binding sites for both TTF1 and PAX8 have been found in the HHEX promoter, and HHEX expression can be induced (146, 147). The HHEX promoter activity could be increased by 3–4 fold by TTF1 in rat FRTL-5 thyroid cells (146) and by PAX8 in transfected Hela cells (147). In human thyroid tissues, the mRNAs of TTF1 and HHEX were found to be positively correlated (146) and so were the mRNAs of PAX8 and HHEX (147). Another common inducible target of TTF1 and PAX8 is TTF2 (148, 149). PAX8 can bind to one site in the 5′-flanking regions of the TTF2 gene and activate its transcription in FRTL-5 cells (150). In a microarray study using PCC13 cells, TTF2 was downregulated significantly in the presence of a TTF1 inhibitor (151). Taken together, starting from TTF1, mediated through PAX8, and ending on HHEX and TTF2, at least two coherent feedforward motifs are formed among the four TTFs (Figure 2). Lastly, positive autoregulatory loops also exist for TTF1 (152), PAX8 (150) and HHEX (146).

The core network of the four TTFs receives input signals from the basolateral plasma membrane through multiple signal transduction pathways initiated by TSH. In the cAMP/PKA/CREB pathway, CREB acts as a TF to directly regulate the gene expression of PAX8, TTF2, and TTF1. TSH can stimulate PAX8 mRNA and protein expression, likely via the CREB binding sites in the PAX8 gene promoter, as demonstrated in PCC13 and FRTL-5 cells (144, 153). Although no CREs have been determined in the TTF2 gene promoter, in FRTL-5 cells it was found that TSH-stimulated upregulation of TTF2 mRNA levels appears to be mediated by the cAMP/PKA/CREB pathway (154). In contrast to the CREB-mediated upregulation of PAX8 and TTF2 by TSH, TSH downregulates TTF1 mRNA and protein by inhibiting the promoter activity and transcription of TTF1 in FRTL-5 cells (155, 156). The inhibition appears to require the presence of insulin signaling and can be reproduced by forskolin, strongly suggesting the involvement of cAMP/PKA/CREB pathway (155). However, there is no CREB/CREM consensus sequence identified within 2.5 kb of the 5′-upstream regions of TTF1 gene (155). With CREB added to the top of the TTF network, more feedforward motifs are formed, including incoherent ones through the inhibition of TTF1 by CREB (Figure 2).

Other signal transduction pathways activated by TSH also regulate the TTF network. Huang et al. reported that activating the PLC/PKC pathway by using phorbol esters inhibited TTF1 and PAX8 expression in human Nthy-ori-3-1 thyrocytes (157). The Gβγ-activated PI3K/AKT signaling pathway can cause PAX8 exclusion from the nucleus and thus inhibit PAX8-mediated NIS transcription in PCC13 cells (112).
Feedforward and feedback regulation of thyroid functional genes

Binding of TSH to TSHR activates signal transduction pathways that regulate TTF activities, which in turn control the gene expression of many thyroid-specific functional proteins, including NIS, pendrin, TG, TPO, DUOX(A1/2), DIO1 and DIO2, that participate in the synthesis and secretion of THs (158, 159). The TTFs, mainly TTF1, TTF2 and PAX8, converge on the cis regulatory elements of the above target genes and regulate their expression in a feedforward fashion (Figure 3).

NIS

TTF1, TTF2, and PAX8 can bind to the upstream enhancer and promoter regions of NIS and stimulate its transcriptional activity (Figure 3A). TTF1 was demonstrated to bind to a DNA sequence about 200 bp upstream of the transcription start site of NIS in rats and induce promoter activity (160). In a genome-wide screening study in TTF2 knockdown cells, it was discovered that TTF2 can bind to the upstream enhancer region close to an NF1/CTF binding site and these two TFs cooperate to upregulate NIS-promoter activity (161). PAX8 can bind to two enhancers that are upstream NIS promoter to induce NIS transcription, and mediate the Ga/cAMP-stimulated NIS induction in both a PKA-dependent and independent manner (162). In primary human thyrocytes, knockdown of TTF1, TTF2, PAX8 but not HHEX genes markedly inhibited TSH-stimulated NIS mRNA expression (163). For the PKA-dependent response, a CRE-like binding site seems to be required. Conversely, the TSH-stimulated Gβγ/P13K/AKT pathway can somehow induce nuclear exclusion of PAX8 and repress PAX8 binding to the NIS enhancers, thus inhibiting NIS transcription (112). Through activating the P13K/AKT pathway, IGF-1 exerts a synergistic effect on TSH-stimulated NIS expression, which is mediated by
ERK1/2 phosphorylation (164). In addition to transcriptional regulation, NIS also appears to be regulated by TSH posttranslationally. It has been demonstrated that TSH can increase the half-life of NIS protein and plasma membrane translocation, which may be mediated by TSH-stimulated NIS phosphorylation (165).

TG

The transcriptional activity of the TG promoter can be activated by both PAX8 and TTF1 (Figure 3B). This was demonstrated by reporter assays in COS-7 and HeLa cells which were co-transfected with a vector containing a 202 bp fragment from the human TG 5’-flanking region, including the promoter sequence and the transcriptional start site, and vectors containing the cDNAs encoding human TTF1 and PAX8 (166). PAX8 and TTF1 are able to interact directly in vitro and form a functional complex in vivo responsible for the synergistic transcriptional activation of the TG promoter (145). Overexpression of PAX8 in FRTL-5 cells increased TG promoter activity in a reporter assay (153). However, in the same study TTF1 was found to compete with PAX8 for binding to the promoter and plays a minor inhibitory role. TTF2 could interfere with the binding of TTF1 to the TG promoter, inhibiting its transcriptional activation (167). It was suggested that the promoter-specific inhibitory activity of TTF2 is mediated by a C-terminal domain that interacts with a cofactor involved in TTF1 and PAX8-mediated target gene transcription (168). In primary human thyrocytes, knockdown of TTF1, TTF2, PAX8 but not HHEX genes markedly inhibited TSH-stimulated TG mRNA expression (163). HHEX can bind to the TG promoter and function as a repressor to abolish the activating effects of both TTF1 and PAX8 in FRTL-5 cells (159). The HHEX mRNA and protein expression can be downregulated by TSH under certain conditions and this disinhibition may permit TG transcription by TTF1 and PAX8 (159).

TPO

TTF1 and PAX8 can activate the transcription of TPO by binding to specific DNA binding sites that overlap each other in the promoter region (Figure 3C) (169). Under TSH and cAMP activation, TTF2 can directly bind to the TPO promoter in rat FRTL-5 thyroid cells (170), which may interfere with the binding of TTF1 and PAX8 to the promoter, inhibiting the transcriptional activation of TPO (167). In primary human thyrocytes, knockdown of TTF1, TTF2, PAX8 but not HHEX genes markedly inhibited TSH-stimulated TPO mRNA expression (163).

PDS

TTF1 is the main transcriptional activator of the PDS promoter, controlling the expression of PDS in thyroid cells (Figure 3D) (171). Between nucleotides (nt) -1946 and -1938 and between nt -1942 and -1933 on the PDS (SLC26A4) gene promoter exist putative binding sites of TTF1 and TTF2 respectively which are essential for the activity of the PDS
promoter, as demonstrated by mutation analysis in human thyroid follicular carcinoma (LA2) cells (172). TSH-stimulated PKA can phosphorylate PDS to promote its membrane translocation, leading to increased iodide efflux into the follicular lumen (173, 174).

**DUOX(A)1/2**

PAX8 can bind to the 5′-flanking region of the DUOX2 gene to activate its transcription (Figure 3E) (150). TTF2 can also bind to the promoter of DUOX2 but repress its transcription (161). PKC can phosphorylate DUOX2 and induce its H$_2$O$_2$-generating activity, while PKA-mediated phosphorylation of serine 955 of DUOX1 can stimulate its activity (59). When TTF1 was inactivated, the expression of DUOX1 was downregulated, while the expression of DUOX2 remained unchanged, and both DUOX1 and DUOXA1 were upregulated. However, in transient co-transfection experiments, the cloned proximal promoter sequences from the DUOX genes were unresponsive to TTF1 (175).

**DIO1/2**

The promoter of the DIO2 gene contains both CREB-binding site and TTF1 binding site, and TSH and TTF1 were shown to upregulate DIO2 activity (Figure 3F) (176, 177). In primary human thyrocytes, knockdown of TTF1, TTF2, PAX8 but not HHEX genes markedly inhibited TSH-stimulated DIO2 mRNA expression (163). Abolishing PAX8 downregulated DIO1 expression in thyroid cells, suggesting that PAX8 activates DIO1 transcriptionally, which is likely mediated by the selenocystein insertion sequence in the 3′ untranslated region of the DIO1 gene (178). In addition, DIO1 is upregulated by TSH via cAMP, however weather CREB is directly involved is unclear (179). The upregulation of DIO1 and DIO2 by TSH is important in establishing the intrathyroidal TSH-T3 shunt pathway (180) to preferentially stimulate T3 production (181). TSHR-mediated upregulation of DIO2 contributes significantly to the relative increase in intrathyroidal T4-to-T3 conversion and thus T3 production and higher circulating T3/T4 ratio in patients with Graves’ disease and toxic adenomas (182, 183). Plasma free T3 concentration has a clear circadian pattern with a periodicity that lags behind TSH (184, 185), and this periodic rhythm of T3 is partly attributed to the thyroidal T4-to-T3 conversion driven by TSH which itself is under circadian control (180).

**TSHR**

TSHR is under autoregulatory feedforward and feedback control by TSH (Figure 3G). In the “minimal” promoter region of the TSHR gene, there exists a TTF1 binding site, which can mediate its transcriptional activity (186, 187). In FRTL-5 thyroid cells, TSH treatment first upregulated TSHR mRNA moderately in the initial 2 hours and then downregulated it by 75%, and the mRNA recovered in 6 days after TSH withdrawal (188, 189). Treatment with cAMP analogs or activators produce a similar effect, and it seems that low cAMP tends to increase but high cAMP decreases TSHR expression. The TTF1 binding to TSHR promoter shows a similar biphasic trend (190). The initial increase is likely caused by phosphorylation of TTF1 by PKA, which promotes its binding to TSHR promoter and enhances the transcriptional activity (190). However, the subsequent transcriptional repression of TTF1 by CREB is responsible for the downregulation of TSHR. The TTF1 activity on the TSHR gene appears to require the presence of a CRE-like sequence near the TTF1 binding site in the minimal TSHR promoter (186) and the negative effects also require the presence of insulin (156). In primary human thyrocytes, knockdown of TTF1 and TTF2 but not HHEX and PAX8 genes markedly inhibited TSH-stimulated TSHR mRNA expression (163). While likely important for TSHR homeostasis and receptor desensitization, the positive and negative regulations of TSHR by TSH and cAMP form an incoherent feedforward control. This structure explains the transient upregulation of TSHR and may also function to speed up the thyroid response or optimize the response to pulsatile TSH. Changes in TSHR expression in turn affect activation of PKA by TSH forming a positive or negative feedback loop, depending on the direction in which TSHR is regulated.

**ANO1**

The transcription and accumulation of ANO1 at the apical membrane of thyroid follicles were stimulated by TSH in rats (54). The mechanisms underlying ANO1 activation and modulation are just beginning to emerge. It has been shown that calmodulin, protons, cell volume and thermal stimuli can regulate the channel activation (191, 192), and PIP$_2$ regulates the activation and desensitization of ANO1 (193, 194).

**SLC26A7**

The TSH/cAMP signaling cascade suppresses the mRNA and protein expression of SLC26A7 in FRTL-5 thyrocytes, and this suppressive effect may be mediated, at least in part, by downregulation of TTF1 expression under TSH. In addition, TSH induces the translocation of the SLC26A7 protein to the plasma membrane, which may be attributed to the phosphorylation of the STAS domain of SLC26A7 by the TSH signaling cascade (195).

**Regulation between MCT8/10 and TSHR**

The release of THs from TG into the blood circulation can be rapidly regulated, involving direct interaction between MCT8 and TSHR (196). The two can form heterodimeric complex which directly modulates the selectivity of TSHR-mediated signal transduction pathways. MCT8 profoundly reduces the
capacity of TSH to induce $\text{G}_\alpha_{q11}$-mediated signaling at TSHR, without affecting $\text{G}_\alpha_{i1}$-mediated cAMP accumulation (196). Since $\text{G}_\alpha_{q11}$ is involved through calcium signaling in the cleavage of T3 and T4 from TG by cathepsins and their release as an acute effect (77, 130), MCT8, by heterodimerizing with TSHR and interfering with $\text{G}_\alpha_{q11}$ signaling, may inhibit T3/T4 release, while the long-term stimulatory effect of TSH mediated by $\text{G}_\alpha_i$ is spared. Besides, MCT10 plays an important role in maintaining TSHR at the basolateral plasma membrane of the thyrocytes. In murine models lacking MCT10, the localization of TSHR is restricted to vesicles (197).

### Biphasic response to TSH

The specificity of $\text{G}_\alpha$ protein activation appears to depend on the level of TSH, which can biphasically regulate thyrocyte functions (198). In primary human thyrocytes, TSH induced bell-shaped dose-response of HHEX, TG, NIS, TPO, Dio2, and TSHR mRNA expression and secreted TG protein (163, 198). The TSH concentration associated with the peak response is about 10 mU/mL. NIS protein also exhibits a bell-shaped response to TSH (164). The exact mechanism on how these nonmonotonic responses to TSH arise is not entirely clear. It may involve differential regulation of TTFs by TSH. In 2D cultured primary human thyrocytes, TSH concentration-dependently inhibited TTF1, TTF2, and PAX8 mRNA expression modestly (163). In another study also using primary human thyrocytes, TSH at 1 or 5 mU/mL stimulated TTF1, TTF2, and PAX8 mRNA expression while in 3D culture these three genes did not respond to TSH stimulation (199). As described above, the TSH-stimulated signal transduction pathways can form incoherent feedforward motifs to differentially regulate TTFs and thyroid functional genes, and incoherent feedforward is well known for generating nonmonotonic dose responses (200). The biphasic responses may originate from the biphasic cAMP activation by TSH as demonstrated ex vivo in human thyroid tissues (201). In primary human thyrocytes and HEK 293 cells expressing human TSHR, it has been recently demonstrated that while the stimulatory phase of the bell-shaped cAMP response depends on $\text{G}_\alpha_i$, the inhibitory phase requires sufficient TSHR on the cell surface to support homodimer formation and is mediated via $\text{G}_\alpha_{i10}$ (138, 139, 202). Therefore, the incoherent feedforward motif formed by $\text{G}_\alpha_i$ and $\text{G}_\alpha_{i10}$ (Figure 1) is responsible for the biphasic cAMP response and, likely, similar downstream gene expression responses.

The functional significance of this biphasic response of the thyroid to TSH is not clear. Biphasic response has been proposed as part of a mechanism for robust tissue size control against mutations in endocrine systems (203). For example, in the feedback regulation of blood glucose homeostasis, the biphasic proliferative response of pancreatic $\beta$ cells to glucose is proposed to prevent the invasion by mutant $\beta$ cells that have markedly gained sensitivity to glucose. By a similar token, the biphasic response to TSH may play an important role in the homeostatic control of the thyroid gland size against gain-of-function mutations.

### Summary

In summary, starting at TSHR, the TSH signal is transmitted through a complex cascade of signal transduction, transcriptional and posttranslational regulations that diverge and converge at different nodes and layers of the cascade. The divergence and convergence of these regulations primarily form interconnected, coherent and/or incoherent feedforward controls. Feedforward controls are well known for their capability to differentiate transient vs. persistent signals, generate sign-sensitive response delay or acceleration, and produce nonmonotonic dose-response (200). As described above, the TSHR-initiated feedforward signaling cascade has manifested some of these features, including transient transcriptional induction of TSHR and the biphasic response to TSH. However, the complexity does not stop here. As revealed below, a number of negative feedback regulatory pathways also operate on top of the feedforward backbone, providing important functions to thyroid homeostasis.

### The high-iodine effects

While dietary iodine deficiency can result in goiter and hypothyroidism due to the essential role of iodine for TH synthesis, interestingly, excess iodine intake can also lead to inhibition of thyroid functions. Several mechanisms appear to be at play, affecting thyroidal iodide organization, uptake, and recycling, as well as TH liberation, release, and deiodination.

### The Wolff-Chaikoff phenomenon

In the 1940s, Wolff and Chaikoff reported that when large amounts of iodide were administrated intraperitoneally in rats, after an initial increase in the intrathyroidal iodide store, the organization of iodide and thus TH synthesis were inhibited in a couple of hours (204, 205). The inhibitory effect on iodine organic binding, as estimated by the thyroidal $\text{I}^{131}$ accumulation, was also reported in euthyroid and thyrotoxic men, hours after administration of high iodine (206). This phenomenon was named afterward as the Wolff-Chaikoff effect. The inhibition is transient, lasting approximately 26-50 h in rats, followed by adaption and escape of the thyroid from the inhibition despite ongoing iodine excess – the organization of intrathyroidal iodide resumes and TH synthesis returns to near normal levels (207, 208).
The Plummer effect

High iodine also inhibits TH secretion. In humans, serum T4 and T3 quickly decreased after high iodine intake and the decrease could persist for days or even weeks as long as the serum iodide remained high (209–211). The lowered serum THs and secondary increase in TSH can be reversed upon iodine excess removal. The decrease in serum TH levels is attributed to inhibition of TH secretion not synthesis because antithyroid drugs did not produce a similar effect as high iodine intake in comparable period, and the inhibition of iodide organification (and thus TH synthesis) resulting from the acute Wolff-Chaikoff effect is expected to be transient (210). The inhibition of TH secretion by high iodine was named the Plummer effect after the endocrinologist Dr. Henry Plummer, who discovered that high-dose iodine can be used to treat Graves’ disease and reduce postoperative death from thyroid surgery (212, 213). The Plummer effect is more prominent in hyperthyroid patients but also occurs, to a less degree, in euthyroid individuals (214). The Plummer effect seems to be more variable in animal studies. In Wistar rats, high NaI administered daily induced decrease in serum T4 and to a lesser extent in T3 after one day, and by day 6 T4 was normalized with T3 still somewhat lower than normal (102). In another study using Wistar rats, no changes in THs and TSH were observed (215). In Sprague Dawley (SD) rats exposed to NaI in drinking water, T3 and T4 decreased on day 1 but fully recovered in 6 days (216). Therefore, the Plummer effect in rodents may not be as persistent as in humans.

It is worth noting that the literature often confuses the Plummer effect as part of the general Wolff-Chaikoff phenomenon. But as described above, the latter involves the inhibition of iodide organification, whereas the former involves the inhibition of TH secretion (217, 218). Correspondingly, there are mechanistic differences between the two, as further detailed below.

Wolff-Chaikoff inhibition mechanism

The Wolff-Chaikoff effect requires a high intracellular concentration (≥1 mM) of iodide (219). When the intrathyroidal iodide fell well below 1 mM, organic iodination resumes in TSH-stimulated thyroid glands (220). Since iodide is required for TH synthesis and high iodide inhibits TH synthesis through the mechanisms detailed below, it follows that iodide regulates TH synthesis in an incoherent feedforward manner (Figure 4B, top half involving red line).

Iodolipid, such as 2-iodohexadecanal (2-IHDA), is believed to play a major role in mediating the Wolff-Chaikoff inhibitory effect. High iodide inhibits H2O2 production in cultured thyroid cells (221), which is mediated by 2-IHDA (222, 223). The inhibitory action may involve a direct effect of 2-IHDA on the membrane NOX activity (224). More recently, it was demonstrated that the decrease in H2O2 production by high concentrations of 2-IHDA is likely mediated by altered DUOX1/2 expression (225). 2-IHDA strongly inhibits other TSH-stimulated genes including TPO and TG in a concentration-dependent manner, which could be attributed to dysregulation of PAX8, TTF1, and TTF2 expression and promoter binding (225). Thyroidal TPO mRNA expression was observed to decrease after 1 day of acute i.p. administration of 2 mg KI in rats (216). Repeated oral-gavage administration of 1 mg/kg KI every 4 days for 8 days in rats also caused a decrease in TPO mRNA expression (215). The inhibition of expression of these thyroid specific genes could also be mediated by highly iodinated TG as detailed in the section of TG feedback below.

Plummer inhibition mechanism

Excess iodine lowers serum thyroxine quickly, suggesting that it blocks the secretion of preformed hormones stored in the follicular lumen (219). This is believed to result from reduction of the proteolytic cleavage of iodinated TG to liberate T4 and T3 and inhibition of TH secretion from the thyrocytes (226), which form another incoherent feedforward motif (Figure 4B, top half involving blue line). Reduced proteolysis of TG was found in Grave’s patients who received Lugol’s solution and were normalized to euthyroid state, compared with those who were not normalized by the Lugol’s solution treatment (227). Varying susceptibilities of iodinated TG to proteolysis have been reported in animal studies in response to high iodine. It was demonstrated in SD rats that the proteolysis of iodine-rich TG preferentially formed during high iodine diet was reduced (228). Also in SD rats, high iodine inhibited thyroidal cathepsin D activity (229). In another study, no effect on the proteolysis of TG by lysosomes was observed in rats, but guinea-pigs treated with excess iodide exhibited increased TG proteolysis for 3 days after which the effect was reversed to the other direction (230). No inhibition of TG proteolysis was observed in vitro with Wistar rat thyroids.

Feedforward and feedback regulations mediating the high-iodine effects

It is believed that the inhibitory effect of high iodine is an effective homeostatic means by which the thyroid gland prevents the large quantities of iodide that enters thyrocytes from causing excessive TH synthesis and secretion. The inhibition involves blockade of multiple molecular events in the sequential processes of TH synthesis and secretion, including iodide organification, proteolysis of iodinated TG to liberate T4 and T3, and release of T4 and T3 into the blood stream (Figure 4A).
but increased proteolysis was observed in vivo (231). Therefore, there exists species difference in the effect of high iodine on TG proteolysis and further studies are needed.

Lastly, iodide overload decreased MCT8 mRNA and protein expression in rats, thus reducing T4 and T3 secretion into the bloodstream (102, 215). TPO activity appears to be required for the inhibition of MCT8 by high iodide load (102). In the study by de Souza et al, the downregulation of MCT8 was transient, which recovered by day 6. This transient nature of MCT8 downregulation may be responsible for the eventual normalization of serum TH levels in rats despite continuous exposure to high iodine (216).

Wolff-Chaikoff escape mechanism

The thyroid gland is an effective collector of iodine, containing about 10-15 mg of iodine in humans (232). It can handle different iodine loads efficiently to maintain intrathyroidal iodide homeostasis. The escape phenomenon seems to be a result of adaptation in response to high intracellular iodide load, involving negative feedback regulation of multiple processes that participate in the intracellular iodide turnover (Figure 4B, lower half). The main known mechanism mediating the escape is the downregulation of NIS and thus the decrease of iodide uptake. The iodide uptake capacity of thyrocytes is inversely associated with its serum concentration. The reduced iodide uptake through downregulation of NIS by high iodine is exploited to prevent radioactive iodide uptake by the thyroid gland in the event of nuclear accidents or wars (233). NIS is downregulated by high iodide through several mechanisms. First of all, high iodide inhibits the transcription of NIS (216, 234, 235). After exposure to excess iodide, the NIS mRNA level in rat thyroid cells decreased at 6 and 24 h, and the NIS protein level also decreased at 24 h (236, 237). In Wistar rats treated with KI for 8 days, NIS mRNA is downregulated continuously after day 1 (215). In SD rats exposed to high NaI, NIS protein was downregulated for 6 days (216). The repression is mediated by the activation of the PI3K/AKT pathway, which induces nuclear PAX8 exclusion and reduces the binding of PAX8 to the NIS upstream enhancer (237, 238). Second, high iodide resulted in shortening of the NIS mRNA poly (A) tail and caused the transcript to become more susceptible to degradation (239). The destabilization contributed to a shorter half-life of NIS mRNA and thus lower abundance (240). Last, high plasma iodide concentrations led to shortening of the half-life of NIS protein from 4 day to 24 h (216). The iodine organization
activity of TPO appears to be essential for the blockade of iodide uptake elicited by iodine overload. When TPO was inhibited by methimazole, the inhibition of iodide uptake induced by high iodine was also abolished, suggesting that organized iodine is involved in the NIS-mediated escape from high iodine-induced thyroid inhibition (241, 242).

Upregulation of PDS may be involved in the escape process as well. As early as 30 min after acute iodide treatment, the PDS mRNA level was transcriptionally upregulated, which persisted for 48 h in rats (243). PDS mRNA upregulation was also observed in rats between 4-10 days with repeated daily oral gavage of KI (215). Iodide excess also reduced the degradation of PDS protein leading to an increase of the protein half-life (244). After 24 h of iodide treatment, the insertion of PDS into the plasma membrane was increased. The increased PDS density in the plasma membrane is expected to move more iodide out of the thyrocytes to the lumen, reducing its intracellular concentration.

The downregulation of DEHAL and DIO1 may also contribute to the escape from the Wolf-Chaikoff effect. Both acute and chronic iodide administration significantly decreased DEHAL and/or DIO1 mRNA levels and activities in the rat thyroid (245, 246). The downregulation would result in reduced recycling of iodine from MIT, DIT and THs, which may help to alleviate the situation of high intracellular iodide.

Taken together, the three different mechanisms, including the Wolf-Chaikoff inhibition effect, the Plummer effect, and the adaptive escape, work together to fend off the potentially harmful consequence of excess iodine load and ensure thyroid homeostasis.

**TG-mediated feedback regulation**

TG-mediated feedback regulation of thyroid genes and physiological significance

TG is an intrinsic feedback suppressor on the effects of TSH in thyroid follicles (Figure 5). TG accumulated in the follicular lumen regulates TH synthesis by suppressing thyroid-specific gene expression in a concentration-dependent manner (247). Experimental evidence obtained in rat thyroid FRTL-5 cells showed that TG at physiological follicular concentrations suppressed the expression of key genes involved in TH biosynthesis, including TSH thyroid-specific genes and PDS (243, 244, 245). A similar negative feedback regulation by follicular TG was also observed in primary cultures of normal human thyrocytes (251). The TG-mediated gene suppression eventually results in inhibition of TSH-stimulated iodide uptake and H2O2 generation (249, 250, 252).

Besides downregulating gene expression, TG can also upregulate the expression of some genes, including PDS (253). PDS expression was dramatically increased in cells exposed to low-level TG (249, 250). In FRTL-5 cells cultured with TSH and subsequently treated with 1 mg/ml TG, PDS mRNA was induced in 3 h and peaked at 24 h (249); when TG was removed from the culture medium at 24 h, PDS mRNA disappeared 6 h later. In FRTL-5 cells, bovine TG induced upregulation of cathepsin H mRNA, protein, and enzymatic activity (255). In addition, TG

![TG-mediated feedback](image)

**FIGURE 5**

TG-mediated feedback. TG regulates TH synthesis and release by suppressing the expression of NIS, TG, TPO, DUOX(A)2 and upregulating the expression of PDS, MCT8, and CTSi (cathepsin H). I-TG translocates from the follicular lumen into thyrocytes via ASGPR and FLOT. TG inhibits NF1 expression and activity to repress TTF1 gene expression. TG also represses TTF1 and PAX8 expression by augmenting PKC and suppressing PKA activities. Asterisk: phosphorylation.
endocytosis promoted cathepsin H translocation into lysosomes where the proteinase degrades iodinated TG to release T4 and T3. A similar upregulation of MCT8 mRNA and protein was also observed in bovine TG-treated FRTL-5 cells (256). Unlike DUOX2 and its maturation factor DUOXA2, which were repressed by TG feedback, DUOX1 and DUOXA1 expression was largely unaffected by TG (257).

The functional purpose of the TG-mediated feedback is not entirely clear. With the exception of upregulation of PDS, it appears to inhibit processes leading to accumulation of iodinated TG, and promote processes of TG lysosomal degradation and TH release (255). Therefore, the TG-mediated feedback may function to maintain the homeostasis of iodinated TG, rather than THs.

**Effectors of TG-mediated feedback**

The feedback effect of TG appears to depend on its degree of iodination. The expression of NIS, TG, and TPO mRNAs was found to decrease significantly in follicles reconstituted from primary pig thyrocytes and treated with a high dose of iodine for 3 days compared with the control (258). The inhibitory effect of high iodine can be blocked by MMI, an inhibitor of TPO, suggesting that iodinated TG mediates the inhibition of gene expression. Although a direct, TG-independent Wolff-Chaikoff effect cannot be ruled out here, further experiments showed that compared with lowly iodinated TG, highly iodinated TG extracted from the reconstituted follicles inhibited the expression of the above genes in primary pig thyrocytes monolayer culture (258). Highly iodinated TG suppressed TTF1 and PAX8 expression at both mRNA and protein levels, which is likely mediated by augmented PKC and suppressed PKA activities (157).

TG control of thyroid function is an "apical" phenomenon, i.e., it requires receptors in direct contact with high-concentration TG in the follicular lumen. Receptors that were proven to bind TG and transduce the effect of TG on gene expression are ASGPR and flotillin. An antibody against ASGPR can abrogate the inhibitory effect of TG on TTF1 and TPO promoter activity in a luciferase reporter assay in FRTL-5 cells (68). The negative feedback effects of TG on NIS, TG, DUOX2, DUOXA2, TTF1, TTF2, and PAX8 expression were also abolished by siRNAs that specifically knocked down flotillin 1 or flotillin 2 (90). How the TG signal mediated by ASGPR and flotillin is transduced to regulate the downstream genes is not clear. One candidate effector is nuclear factor 1 (NF1). Two NF1 elements were found between -264 and -135 bp in the TTF1 promoter; TG inhibits TTF1 gene expression by decreasing NF1 mRNA and protein expression and the binding activity of NF1 (259). Inhibition of TTFs including TTF1 in turn leads to suppression of TG, TPO, and NIS (260).

**Follicle heterogeneity, cycle, and feedback oscillation**

While the TG-mediated feedback has been considered as an important intrafollicular mechanism for thyroid homeostasis, it is also hypothesized to act as an oscillator that drives cyclic follicular function and renders follicle heterogeneity (247, 248, 257). Follicles in a thyroid gland are highly heterogeneous in morphology and functional states (261, 262). The follicular size, TG content and colloid density of individual follicles vary greatly. ‘Active’ follicles comprise tall columnar epithelia and ‘inactive’ follicles comprise low cuboidal or squamous epithelia (263, 264). The cellular uptake and distribution of iodide also vary widely (265–267).

The heterogeneity may result from genetically different subpopulations of thyroocytes and variations in TSHR expression and downstream pathways (258, 268, 269). The follicle heterogeneity may also result from the operation of the negative feedback of TG autoregulation, which may cause cyclic changes in each follicle's function, termed as thyroid follicular cycle (257, 270). Negative feedback is well-known for its capability of generating sustained oscillation under certain parameter conditions (271). When the colloidal iodinated TG concentration is low, the genes involved in TH synthesis, TG, NIS, TPO, DUOX2, DUOXA2, are not suppressed by TG and thus highly expressed while those genes undertaking TG degradation and TH release such as cathepsin H and MCT8 are slowly expressed. As a result, more iodinated TG is synthesized and stored in the lumen. When the TG concentration reaches a high level, it begins to suppress TH synthesis genes thus downregulating TG synthesis and iodination, and activate genes responsible for TG degradation. As a result, colloidal TG begins to be depleted, and the cyclic pattern repeats. Cycling among follicles can be asynchronous, which leads to follicle heterogeneity. Lastly, given the multiple positive autoregulation loops existing for TTFs (Figure 2), the possibility that the heterogeneity of thyroid follicles arises from stochastic bistable switching of the positive feedback loops between active and inactive states cannot be ruled out (272).

**Perspectives and conclusions**

Homeostatic regulation of THs involves systemic interactions between multiple organs and tissues. As an integral part of this complex process, the thyroid receives physiological, dietary and environmental signals to adjust TH output on demand. These endogenous or exogenous signals impinge on the intrathyroidal molecular network comprising signal transduction pathways, TH-synthesizing and secreting pathways, and circuits regulating gene and protein expression and activities. Dysregulation of this network, either internally or
externally by exogenous disruptors, can lead to pathological thyroid conditions, including goiter, hypothyroidism, hyperthyroidism, and thyroid cancers.

As in many other intracellular systems, the structure of the intrathyroidal network is not linear and unidirectional. It involves multiple feedforward and feedback circuitries that are closely intertwined (Figure 6). The nonlinear properties well-known for feedforward and feedback regulations suggest that the dynamic and stimulus-response behaviors of the intrathyroidal network are not trivial, be the input signal endogenous hormones, nutrients, drugs, or EDCs. For instance, the multiple incoherent feedforward motifs formed by the signal transduction pathways, TTFs and thyroid functional genes may be responsible for the biphasic response of the thyroid to TSH. The incoherent feedforward motif driven by iodide is responsible for the TH synthesis at normal conditions and the Wolff-Chaikoff inhibition under high iodine load. Escaping from the inhibition involves a negative feedback circuit whose function seems to maintain intracellular iodide homeostasis. A potential oscillatory behavior resulting from TG-mediated negative feedback regulation may lead to cyclic follicular function and underpin the observed follicular heterogeneity (257).

More complex functions and behaviors may be expected from this network. Depending on the specific molecular target, i.e., the node in the network, the quantitative consequence of perturbation can be difficult to predict. A better understanding of complex pathways as such requires formulation of mathematical models to simulate the network as nonlinear dynamical systems (49). Such systems biology models can incorporate genetic, epigenetic, environmental and nutritional status to better predict responses of individuals. While there are many models focusing on various aspects of the HPT axis (180, 273–275), models that specifically simulate the molecular regulations in thyroid follicles are still uncommon. Degon et al. constructed a complex model describing the intrafollicular iodine turnover in humans, including uptake, apical transport, organification, endocytosis, recycling, secretion of iodide, upregulation of TPO, TG, NIS and other proteins by TSH, feedback inhibition of H$_2$O$_2$ production and NIS by iodide, and of TG, NIS, and TPO by TG (276). The model was used to understand and predict the condition of dietary iodine deficiency and Wolff-Chaikoff phenomena. More recently, Cohen et al. has used a bioinformatic approach to
map out a regulatory network underpinning the Wolff-Chaikoff phenomena, with the goal of predicting the protective effect of repetitive dosing of iodide as a prophylactic measure (277). The intrathyroidal T4-to-T3 conversion driven by TSH-stimulated DIO2 induction was explored for its role in circadian secretion of T3 (180). Constructing more complex models based on the known intrathyroidal pathways will help identify novel molecular targets for therapeutic compounds that can regulate TH synthesis and secretion with high precision. In addition, such models can be extended to quantitative adverse outcome pathway (qAOP) models to predict dose-response relationship for health risk assessment of environmental EDCs (278–281).

In conclusion, by decomposing the complex molecular network in thyroid follicles into more tractable network motifs, such as feedforward and feedback, and re-assembling them into an organized circuitry structure, we provided a systems-biology perspective on this molecular network that regulates TH synthesis and secretion. This new perspective will help to achieve a better understanding of the network dynamics, and gain novel insights into the regulatory functions, which may provide important clues for developing future thyroid therapeutics and assessing the health impacts of environmental thyroid disruptors.

Author contributions

QZ and LJ conceived and planned the content and structure of the review, LJ wrote the initial draft of the manuscript, and QZ and LJ critically reviewed and revised the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Type 1 iodothyronine deiodinase is a sensitive marker of peripheral thyroid status. This enzyme, located in the outer mitochondrial membrane, mediates the conversion of thyroxine (T4) to triiodothyronine (T3) and reverse triiodothyronine (rT3). 

rT3 attenuates ischemia-reperfusion injury. In a study published in *Biochem Biophys Res Commun* (2018) 506(3):597, researchers investigated the role of rT3 in ischemia-reperfusion injury. They found that rT3 administration significantly reduced brain damage and improved neurological outcomes after ischemia-reperfusion injury.

The study concluded that rT3 may have therapeutic potential for the treatment of ischemia-reperfusion injury, a common complication following cardiac surgery or other conditions that lead to tissue ischemia.

The investigators conducted their research by administering rT3 to ischemic rat brains and comparing the outcomes with those of control groups. They measured various parameters, including brain edema, infarct size, and neurological function, to assess the therapeutic effects of rT3.

Overall, the study highlights the potential of rT3 as a therapeutic agent in ischemic injury, suggesting further investigation into its clinical applications.
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