Interpreting Thyroid Toxicity for Risk Assessment

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I. INTRODUCTION

Thyroid hormone is essential for normal brain development. This concept is supported by both clinical and experimental evidence indicating that thyroid hormone excess (1-5) or deficit (6-10) during development is associated with irreversible developmental and neurological abnormalities. Moreover, it is generally believed that the severity of thyroid hormone-induced abnormalities corresponds to the degree to which thyroid hormone levels are abnormal (11-13). However, the key questions within a risk assessment framework are, "how low is too low?" and "how much is too much?" as it relates to circulating concentrations of thyroid hormone. Recent evidence suggests that the answers to these questions depend on the timing of toxicant exposure and the end point(s) evaluated. Moreover, new studies indicate that toxicant exposure can affect thyroid hormone action in the developing brain independent of effects on circulating levels of thyroid hormone in the dam. Therefore, a more formal way to frame the above question is, "what are valid end points of thyroid hormone action that can be used in toxicological studies to accurately assess risk to human health?".

The goal of this review is to provide an analysis of the logic employed by paradigms presently used for the analysis of risk associated with thyroid toxicity, and to place this within the context of emerging insights gained from recent clinical and experimental research. There are two main themes. First, the present method for interpreting thyroid toxicity incorporates only one direct measure of thyroid hormone action, but it is usually ignored; thus, establishing an adverse effect of toxicant is problematic. Second, we have the knowledge base and technology to identify direct measures of thyroid hormone action, and to determine their validity as end points in toxicology studies.

II. ENDOCRINOLOGY OF THE THYROID SYSTEM

The active thyroid hormones, thyroxin (T_4) and triiodothyronine (T_3), are two of the iodothyronines formed in the thyroid gland. These hormones are synthesized in an unusual way in that they are derived from coupling two iodinated tyrosyl residues that make up the larger hormone

"precursor", thyroglobulin (TG). Thyroglobulin is a large glycoprotein containing two identical subunits each of nearly 3,000 amino acids, creating a 660 kDa mature protein (14). Following iodination, the protein is stored in the "colloid", the fluid filling the central core of the thyroid follicle. At the time of hormone release, iodinated TG is taken up into the cell from the colloid, digested by lysosomal enzymes and liberating T_3 and T_4 into the blood (15). Thyroxin is the predominant iodothyronine released by the thyroid gland; circulating triiodothyronines are formed largely from peripheral deiodination of T_4 (16). The pituitary glycoprotein hormone, thyrotropin (TSH) (17), regulates the synthesis and secretion of thyroid hormones by activating adeylate cyclase in thyroid follicular cells (18). However, there are a number of important extrathyroidal processes that combine to maintain circulating thyroid hormones within a relatively narrow concentration range (16).

Normal variation in circulating concentrations of T_4 reflects short-term pulsatile and diurnal variation (19). Thyroxin and/or T_3 exert a negative feedback effect on pituitary secretion of TSH (20, 21), and on the hypothalamic secretion of the releasing factor, thyrotropin-releasing hormone (TRH) (22-24). Although it is clear that TRH is a major factor regulating TSH secretion, several hypothalamic factors contribute to TSH regulation (20). Moreover, some investigators suggest that the primary role of TRH in the regulation of TSH secretion is to modulate the set-point around which thyroid hormones act on the pituitary (25, 26). Thus, circulating levels of thyroid hormones, and the balance between different forms of these hormones, are controlled by a number of processes. Additional details of thyroid endocrinology are diagrammed and described in Figure 1.

III. INTERPRETATION OF THYROID TOXICITY STUDIES

A number of environmental chemicals have been shown to affect the thyroid system (27), and the study of environmental goitrogenesis is well-developed (28). Therefore, considering both the varieties of chemicals affecting the thyroid system and the importance of thyroid hormone in development and in general health, it is important to identify specific chemicals that exert effects on

thyroid function or thyroid hormone action. Several formal reviews on this subject have been published (29, 30).

In principle, the adverse effects of toxicant-induced thyroid dysfunction would most effectively be reflected in the specific effects of low or high circulating concentrations of thyroid hormone. This could be envisioned as equivalent to the uterotrophic assay for estrogens (31), or the Hershberger assay for androgens (32). However, there are few effects of high or low levels of thyroid hormone that are both specific to thyroid hormone and unambiguously "adverse". In adult humans, for example, hypothyroidism is associated with a variety of neurological, behavioral and psychiatric manifestations, but no single feature is diagnostic (33). This is also true for hyperthyroidism (34). Therefore, in adult humans, the diagnosis of hypothyroidism requires "awareness of the clinical features that define a patient's risk for thyroid hormone deficiency and proper use of the two tests usually required to confirm the disorder: serum TSH and free thyroxine (T_4)" (35). This statement implies that the diagnosis of thyroid dysfunction depends principally on biochemical measurements of blood levels of thyroid hormones and TSH in the presence of some of the many symptoms of thyroid hormone excess or deficit.

Although individual clinical symptoms lack definitive diagnostic value in humans, experimental systems can be evaluated more thoroughly. Therefore, it seems possible that end points of thyroid toxicity exist that could be identified for testing effects of thyroid toxicity. It is known that thyroid hormone dysfunction produces deleterious effects on many organ systems including heart, muscle, liver, and brain. A partial list of thyroid hormone-responsive genes in these various tissues is shown in Table 1. However, no studies to date have begun to expand the end points of thyroid toxicity for use in toxicological studies. Moreover, although thyroid hormone is well known to affect metabolism (36), body weight (37), and several aspects of behavior (38-40, 33), the smallest change in thyroid hormone required to observe significant effects on these end points has not been determined. Thus, the end point that provides the lowest effect level for thyroid hormone itself has not been identified. Likewise in development, thyroid hormone is known to be essential for brain development, but these types of studies focus on understanding the

developmental consequences of thyroid hormone action (41-45), or the mechanisms by which thyroid hormone acts (46, 47, 8, 10). As a result, these studies routinely use potent goitrogens such as propylthiouracil or methimazole, or surgical thyroidectomy, to ablate thyroid function, and few report a dose-response designed to identify sensitive end points for thyroid toxicity.

Because sensitive and valid end points of thyroid toxicity have not been identified, the present end points of thyroid toxicity are focused on changes in circulating levels of thyroid hormones and on thyroid morphology. Thyroid morphology has become important to establish a lowest adverse effect level (LOAEL) or no-adverse effect level (NOAEL). In a toxicological paradigm, the issue becomes focused on defining an end point - other than thyroid hormone levels *per se* - that reflect an adverse effect. As is evident in Figure 1, thyroid hormones regulate circulating levels of TSH by negative feedback. Therefore, when thyroid hormones are low, TSH is elevated. Because a sustained increase in TSH can increase thyroid cell size (hypertrophy) and, at higher levels, can increase thyroid cell mitogenesis (hyperplasia) (29, 30, 18, 48), thyroid morphology has become a standard end point for thyroid toxicity studies. However, the general concept is that the risk of thyroid cancer increases under conditions where the rate of cell division is increased (i.e., thyroid hyperplasia). Therefore, thyroid hyperplasia is considered to be reflective of an adverse effect but thyroid hypertrophy is considered to be a physiological adaptation that maintains homeostasis (29, 30).

A weakness in this reasoning is that changes observed in thyroid gland histopathology are not a direct measure of changes in thyroid hormone levels. Rather, it is a measure of changes in thyrotropin levels. Thyrotropin is a direct marker of thyroid hormone action, but it is usually ignored as surrogate measure of thyroid hormone action on all thyroid hormone-responsive genes. This is a weakness for two reasons. First, thyroid histopathology can be evaluated using quite sophisticated computer-assisted morphometric analyses followed by rigorous statistical evaluation, or it can be evaluated in a less formal non-parametric manner. These two approaches are likely to give very different results. Second, it is a weakness because an important marker of thyroid hormone action is being ignored. It is clearly established that thyroid hormone exerts a negative

feedback effect on TSH (49). This effect was shown early to depend on protein synthesis (50-52) and occurs at low doses of T_4 (53-59) and T_3 (53, 60, 58). Finally, periodic (i.e., pulsatile) administration of T_3 is more effective than tonic infusion at producing a negative feedback effect on TSH (61). These early studies are fully consistent with what is known about the molecular mechanisms of thyroid hormone action. It is now clear that the TRβ2 mediates negative feedback on pituitary TSH (62, 63) and on hypothalamic TRH (64). Thus, there is ample evidence that circulating levels of TSH is a valid marker of thyroid hormone action, and that one can expect other thyroid hormone-responsive genes to be affected in similar ways. However, this is usually ignored.

IV. THYROID HORMONE AND BRAIN DEVELOPMENT

Thyroid hormone and the neonate. It is well established that thyroid hormone is essential for brain development during the neonatal period in both humans and animals, especially as revealed in the disorder known as congenital hypothyroidism (CH) (67, 68, 7, 69-71, 1, 72, 73, 13). Congenital hypothyroidism occurs at a rate of 1 in 3000 to 1 in 4000 live births (68). There are several causes of CH, including thyroid dysgenesis, agenesis and athyreosis, inborn errors of thyroid hormone synthesis, and less often, secondary or tertiary hypothyroidism (68, 74). Because CH infants do not present a specific clinical picture early, their diagnosis based solely on clinical symptoms was delayed. In fact, only 10% of CH infants were diagnosed within the first month, 35% within 3 months, 70% within the first year, and 100% only after age 3 (75, 76). As a result of this delayed diagnosis and treatment, the intellectual deficits were profound. One meta analysis found that the mean IQ of 651 CH infants was 76 (77). Moreover, the percentage of infants with an IQ above 85 was 78% when the diagnosis was made within 3 months of birth, 19% when it was made between 3 and 6 months, and 0% when diagnosed after 7 months of age (77, 78).

Because CH is difficult to diagnose on the basis of clinical symptoms alone, and because of the profound consequences, mandatory neonatal screening for circulating thyroid hormones and/or TSH has been implemented by a number of countries (68, 78). Studies now reveal that no clinical manifestations of CH occur if it is diagnosed and treatment initiated within 14 days of birth (13).

This medical profile has become the principal example illustrating the importance of thyroid hormone for normal brain development. However, it has become clear recently that thyroid hormone is also important during fetal development.

Thyroid hormone and the fetus. Thyroid hormones are detected in human coelomic and amniotic fluids as early as 8 weeks of gestation, before the onset of fetal thyroid function at 10-12 weeks (79). In addition, human fetal brain tissues express receptors for thyroid hormone, and receptor occupancy by thyroid hormone is in the range known to produce physiological effects as early as 9 weeks of gestation (80, 81). Finally, the mRNAs encoding the two known thyroid hormone receptor types exhibit complex temporal patterns of expression during human gestation (82). These data indicate that maternal thyroid hormone is delivered to the fetus before the onset of fetal thyroid function, and that the minimum requirements for thyroid hormone signaling are present at this time. The functional importance of thyroid hormone in fetal brain development has been slower to recognize because of the difficulty in correlating what are sometimes subtle differences in maternal thyroid hormone concentrations with pregnancy outcome. However, studies focused on 4 types of maternal abnormalities have led to a better appreciation of the importance of thyroid hormones for the fetus. These include endemic cretinism, autoimmune thyroid disease in pregnant women, subclinical hypothyroidism in pregnant women, and premature birth. These four areas will be reviewed below.

Maternal thyroid hormones during pregnancy. There are two forms of cretinism based on clinical presentation (83, 84). Neurological cretinism is characterized by extreme mental retardation, deaf-mutism, impaired voluntary motor activity, and hypertonia (83). In contrast, myxedematous cretinism is characterized by less severe mental retardation, and all the major clinical symptoms of persistent hypothyroidism (83). Iodide administration to pregnant women in their first trimester eliminates the incidence of neurological cretinism in geographic areas that are severely iodine insufficient. However, by the end of the second trimester, iodine supplementation does not prevent neurological damage (85, 84). Several detailed studies of endemias occurring in different parts of the world, and reviewed by Delange (83), have led to the proposal that the various symptoms of the

two forms of cretinism arise from thyroid hormone deficits occurring during different developmental "windows of vulnerability". This proposal is in contrast to the proposal that neurological and myxedematous cretinism represent two ends of a spectrum of effects. Nevertheless, these studies clearly indicate that thyroid hormone plays an important role in brain development during fetal development and perhaps before the onset of fetal thyroid function.

Endemic cretinism is a case of severe thyroid hormone deficits. The effect of subtle, undiagnosed maternal hypothyroidism, or subclinical hypothyroidism during pregnancy, has been much more difficult to relate to pregnancy outcome. The concept and definition of maternal hypothyroxinemia was developed in a series of papers by Man *et al.* (86-90). Early definition of maternal hypothyroxinemia was defined empirically - those pregnant women with the lowest butenol-extractable iodine (BEI) among all pregnant women (91, 88). This work was among the first to document an association between subclinical hypothyroidism in pregnant women and neurological function of the offspring. Pop et al. (92) found that the presence of antibodies to thyroid peroxidase in pregnant women, independent of thyroid hormone levels *per se,* was associated with significantly lower IQ in their offspring. In addition, subsequent studies have shown that, for pregnant women with undiagnosed hypothyroidism, the children born to women with T_4 levels in the lowest 10^{th} percentile of the normal range had a higher risk of low IQ and attention deficit (93). Excellent recent reviews discuss these studies in detail (91, 94, 95). Taken together, these data present strong evidence that maternal thyroid hormone plays a role in fetal brain development prior to the onset of fetal thyroid function, and that the consequences of even mild thyroid hormone deficits during pregnancy are neurological and irreversible (7, 70, 96, 97, 72, 98, 13). However, despite the increased awareness of the importance of thyroid hormone during fetal brain development, little is known about the mechanisms by which thyroid hormone affects the fetus.

V. MECHANISM OF THYROID HORMONE ACTION ON BRAIN DEVELOPMENT.

Thyroid hormone receptors are nuclear transcription factors. It is generally believed that the majority of biological actions of thyroid hormone are mediated by their receptors - nuclear

proteins that interact mainly with T_3 (99, 100). T_3 receptors (TRs) are members of the steroid/thyroid superfamily of ligand-dependent transcription factors (101-103), indicating that effects on gene expression mediate the majority of biological actions of thyroid hormone. TRs are encoded by two genes, designated α and β c-*erb*A (104, 105). These two genes produce three functional TRs due to alternate splicing: TRα1, TRβ1, and TRβ2 (106-110). Although there are several TR isoforms, the binding affinity for T_3 and for T_4 is not different among the various forms (111-113). Thus, it is not possible to discriminate between TRα1 and TRβ1 by binding to T_3. However, the TRs exhibit a 50-fold greater affinity for T_3 than for T_4, making T_3 the physiologically important regulator of TR action.

Thyroid hormone exerts tissue- and cell type-specific effects. Although the responsiveness to thyroid hormone requires the presence of nuclear TRs, the effects of thyroid hormone vary from tissue to tissue, even among those tissues that express TRs (37). Different levels and combinations of TR isoform expression may account in part for this observation (101, 102), but cannot account for all tissue variability in responsiveness to thyroid hormone. For example, most patients with thyroid hormone resistance syndromes exhibit a mutation in the TRβ gene, but the phenotypes of individuals carrying the same mutation can be different, indicating that other factors contribute to thyroid hormone actions (63, 114).

Thyroid hormone also exerts variable effects in the brain. For example, thyroid hormone exerts a negative transcriptional effect on the gene encoding thyrotropin-releasing hormone (TRH) (115). However, this occurs solely in TRH-containing neurons in the hypothalamic paraventricular nucleus (22, 116, 117) despite the widespread distribution of cells expressing TRH (118) and those expressing TR (119). This is true also for the gene RC3/Neurogranin, a well-characterized thyroid hormone-responsive gene in the developing and adult brain (120, 121). RC3/Neurogranin is expressed with TR in many brain areas, but is regulated by thyroid hormone in a small subset of these areas (122). Thus, it is unlikely that thyroid hormone regulation of a specific gene will always

be a marker of thyroid hormone action; rather, studies must focus on the proper gene expressed in specific brain regions at the correct developmental time.

TRs exhibit specific temporal and spatial patterns of expression during brain development. Young and colleagues (123) demonstrated that the α and β TRs exhibit distinct temporal and spatial patterns of expression in the developing rat CNS. TRβ1 is expressed in the ventricular zone of the cerebral cortex early in development, and TRα1 is expressed in more superficial layers. Because the ventricular zone of the developing cortex contains neural progenitor cells undergoing cell division and fate specification (124), this suggests that check points of cell division and fate specification may be affected by thyroid hormone mediated by the TRβ1. In contrast, TRα1 may selectively mediate effects of thyroid hormone on elements of migration, differentiation, and synaptogenesis. Leonard *et al*. (125) have suggested that TRα2 is expressed exclusively in glial cells, while TRα1 and TRβ1 are expressed predominantly in neurons. Thus, thyroid hormone may influence different developmental processes by different TR isoforms, all well before the fetal hypothalamic-pituitary-thyroid system begins to function on G 17-20 (126).

TR function is modulated by interactions with two types of regulatory proteins. Two additional characteristics of the TRs must be considered to glimpse the range of regulatory mechanisms governing thyroid hormone action. First, TRs can interact with distinct nuclear receptors including those for retinoids (retinoic acid receptors, RARs, and retinoid X receptors, RXRs) (99, 101, 103). Thus, an individual TR protein can dimerize with an individual RAR or RXR forming a heterodimer pair. Interestingly, the type of dimer (TRα1 or TRβ1 homo- or heterodimer, or TRα1/RAR, etc) contributes to the mechanism by which a specific gene is targeted for regulation (103). Second, the ability of TRs to affect gene transcription requires them to interact with nuclear cofactors, which are requisite mediators of ligand-dependent transcriptional activation or repression of hormone responsive genes (127). Cofactors are believed to remodel local chromatin structure enabling nuclear receptors to activate or repress gene regulation. Generally, the specific recruitment of a cofactor complex with histone acetyltransferase activity appears to play a

regulatory role in activating gene transcription, whereas the recruitment of a cofactor complex with histone deacetylase activity appears to play a regulatory role in gene repression (128). Therefore, the sensitivity of a specific gene to regulation by thyroid may be modulated by the abundance of specific cofactors.

Two kinds of observations support the hypothesis that changes in cellular levels of specific cofactors modulate cellular responsiveness to steroid/thyroid hormones. First, ligand-dependent transcriptional activation by one nuclear receptor can be inhibited by ligand activation of another nuclear receptor *in vitro,* even though this second receptor does not directly regulate the affected gene (129, 130). This observation indicates that nuclear receptors compete for available cofactors, which if they are in limited supply, will attenuate the efficacy of hormone-dependent activation of gene expression. Second, overexpression of the cofactor SRC-1 in a human breast cancer cell line (MCF-7 cells) results in an increase in the mitogenic response to estrogen (131). Thus, the sensitivity of a cell to a specific level of hormone may be determined, at least in part, by the availability of specific cofactors.

There are two categories of nuclear receptor cofactors in general: corepressors and coactivators (127, 132). In the absence of thyroid hormone, TRs are able to repress basal transcription via recruitment of the corepressors SMRT or NCoR (133, 134). In contrast, in the presence of thyroid hormone, TRs release their corepressor and recruit a coactivator complex that includes SRC-1 (134, 135). This appears to account for the observation that TR knock-out mouse models have a relatively mild phenotype compared to animals rendered hypothyroid using goitrogens or surgical thyroidectomy (62, 136-138). Specifically, because the TR appears to be a constitutive repressor, associating with a co-repressor in the unliganded state and recruiting a co-activator only after thyroid hormone binding, the unliganded TR would be predicted to be more damaging to brain development than the loss of the receptor entirely. This hypothesis was confirmed by Hashimoto et al. (139) who generated a TRβ1 knock-in mutant mouse containing a

TRβ1 unable to bind thyroid hormone. These homozygous mutant mice exhibited severe neurological deficits that resembled hypothyroidism in wild-type mice.

Taken together, these data indicate that thyroid hormone action on gene expression - and on specific developmental events - are likely to be highly pleiotropic. That is, the effects of thyroid hormone on an individual gene will be spatially and temporally specific, and studies designed to identify end points of thyroid toxicity during development must consider this specificity.

Developmental processes influenced by thyroid hormone. Thyroid hormone is known to affect a number of specific developmental processes, including neuronal proliferation, differentiation, migration and synaptogenesis (6, 8, 140, 9, 10). Much of this work has focused on the postnatal rat. For example, Koibuchi and Chin (8) provide a very clear argument for studying thyroid hormone action on cerebellar development, which is almost entirely postnatally derived in the rodent (141, 142). However, it is essential that generalizations not be made about thyroid hormone action on specific neurodevelopmental events. For example, it is clear that thyroid hormones affect proliferation of cerebellar granule cells. This was first shown by Nicholson and Altman (45) using ^{3}H-thymidine labeling, but has been shown also by labeling with proliferating cell nuclear antigen mRNA (A. Croci, unpublished). In contrast, our lab has not found that thyroid hormone affects proliferation of cortical neurons on G16, using BrdU labeling (E.A. Iannacone, unpublished) or PCNA (A. Croci, unpublished). This single example clearly indicates that global statements about thyroid hormone action on brain development should be avoided.

Because there is no *a priori* reason to predict that specific developmental processes are affected by thyroid hormone in the early fetal cortex, we recently investigated thyroid hormone action before the onset of fetal thyroid function using a broad empirical approach (47). We used a technique known as mRNA differential display as a way of identifying thyroid hormone-responsive genes in the early fetal cortex, which could then guide us in subsequent studies to identify thyroid hormone-regulated developmental processes. Our underlying rationale was that the lack of

information concerning molecular mechanism(s) of thyroid hormone action on fetal brain development has two important consequences. First, we have little appreciation for the molecular events or developmental processes by which thyroid hormone produces the effects observed in humans and animals briefly discussed above. Second, we have no direct measures of thyroid hormone action in fetal brain. Therefore, we cannot directly test the hypothesis that specific chemicals can interfere with thyroid hormone action. Rather, we are forced to attempt to interpret indirect measures of thyroid toxicity, such as circulating levels of thyroid hormones and thyroid histopathology.

We focused on the embryonic day 16 (E16) fetus because fetal thyroid function does not begin until E17 (126); thus, the identified genes would be regulated solely by *maternal* thyroid hormone. In addition, E16 is the time when most of the neurons of the cerebral cortex are generated and begin to differentiate (143). Our paradigm for thyroid hormone manipulation was also novel among studies designed to identify thyroid hormone-responsive genes. Specifically, we surgically thyroidectomized female rats two weeks before they were mated to allow thyroid hormones to decline before pregnancy. Next, on E15, we administered two half-doses of T_4 (12.5 μg/kg each) so that the concentration of thyroid hormone in the dam's blood would not be supraphysiological. We reasoned that this combination of a physiological dose of T_4 and an acute injection paradigm would allow us to identify genes directly responsive to thyroid hormone and would be physiologically relevant.

We identified a number of genes expressed in the fetal brain that appear to be responsive to maternal thyroid hormone. Two of these genes, encoding neuroendocrine-specific protein (NSP) (144, 145) and Oct-1 (146-148), exhibited complementary patterns of responses to thyroid hormone. Interestingly, both Oct-1 mRNA and NSP-A mRNA are expressed exclusively in the ventricular zone of the E16 cortex (149, 47). However, Oct-1 mRNA is elevated by T_4 injection, whereas NSP-A mRNA is suppressed by thyroid hormone. Oct-1 is a member of the POU-domain family of transcription factors (146) that is implicated in the control of neuronal

proliferation. NSP-A is a neural-specific protein associated with endoplasmic reticulum that may be involved in the acquisition of neuronal polarization and differentiation (150, 145). These experiments demonstrated that thyroid hormone of maternal origin can affect gene expression in the fetus, and they provide "biomarkers" of thyroid hormone action in the fetal brain. In addition, because we found that NSP-A and Oct-1 retain their sensitivity to thyroid hormone in adulthood, our results suggest that the concept of "critical windows" of thyroid hormone action apply to specific developmental events, but probably not to thyroid hormone sensitivity *per se*.

From this perspective, it is easy to imagine that thyroid hormone plays an important role in developmental processes such as neuronal proliferation that occur in different brain areas at different times (143). Therefore, the temporal "window" of thyroid hormone sensitivity will depend on the developmental period over which a particular process occurs, and this will differ for different brain areas. For example, in humans, acute disruption of thyroid hormone action selectively during the first trimester might affect assembly of the pool of neurons and glia that will go on to form the adult cortex. However, cerebellar granule cells do not arise until the third trimester, transient exposure to a thyroid toxicant during the first trimester may not affect the cerebellum..

VI. TWO EXAMPLES OF THYROID TOXICITY AND THE INTERPRETATION OF THEIR EFFECTS

A broad range of chemicals is known to affect the thyroid system at different points of regulation (27). For example, some chemicals selectively interfere with thyroid hormone synthesis, where others may selectively interfere with metabolic clearance, serum transport, cellular uptake, hormone action, or combinations of these processes. For example, perchlorate (ClO_4) is an anion that competes for iodide uptake into the thyroid gland via the sodium/iodide symporter (NIS) (151, 152). Because ClO_4 blocks iodide uptake, it reduces thyroid hormone synthesis and circulating levels of thyroid hormones. Therefore, perchlorate is expected to produce deleterious effects on an organism solely by reducing thyroid hormone synthesis and release. In contrast, polychlorinated

biphenyls (PCBs) appear to affect the thyroid system at several levels (153). Specifically, PCBs enhance liver metabolism of thyroid hormone (154-156), increasing biliary excretion (157, 158). They interfere with T_4 binding to serum proteins (159-162), which may also reduce circulating levels of thyroid hormones. And, they may affect cellular uptake and/or receptor binding (163-165). Therefore, changes in circulating levels of thyroid hormone may not be the most sensitive measures of PCB actions on thyroid toxicity. The discussion below is focused on these two examples of thyroid toxicity, perchlorate and PCBs, illustrating the difficulties in interpreting these studies within a risk assessment paradigm, and highlighting the need for the development of valid end points of thyroid toxicity.

Perchlorate. Ammonium perchlorate is the principal oxidant for solid propellants in the defense industry (166, 167). Perchlorate contamination of ground water across the United States has recently become apparent (168), and therefore, it is important to determine the level of perchlorate in drinking water that produces an adverse effect. To this end, Siglin *et al.* (169) performed a 90-day study in rats of ammonium perchlorate on various measures, including body weight, hematology, clinical chemistry, thyroid hormones and thyroid histopathology. Dose groups included a control, and 5 levels of perchlorate: 0.01, 0.05, 0.20, 1.00, and 10.0 mg/kg/day. Body weight, hematology and clinical chemistry all were unaffected by perchlorate. In contrast, total T_4, total T_3, and TSH all were affected in males and females, but the dose at which they first exhibited an effect differed. Specifically, in males treated for 90 days, total T_4 and total T_3 were reduced in all perchlorate-treated groups. However, serum TSH was significantly elevated by only 0.20 mg/kg/day and above. Thirty days after treatment was suspended, circulating TSH and total T_3 was not different from controls, although circulating T_4 remained somewhat reduced. Likewise in female rats treated for 90 days, circulating levels of T_4 and T_3 were significantly reduced by all doses of perchlorate, whereas TSH was elevated only in animals treated with the highest dose of perchlorate (10 mg/kg/day). Thirty days after treatment was suspended, circulating T_4 and T_3 were not different among the treatment groups, while serum TSH was elevated at all doses examined.

Interestingly, the mean absolute thyroid weight was affected only in the high dose groups even when TSH levels were significantly elevated for 90 days.

The authors of this study reasonably consider that the results failed to establish a definitive NOAEL. Body weight was not affected by perchlorate treatment, nor were the clinical measures of hematology or chemistry. Thyroid hormones and TSH were the only measures affected by perchlorate, and they were not uniformly linked to indices of hyperplasia in the thyroid gland. However, the authors also considered setting the NOAEL at the 1.0 mg/kg/day dose because there were no observed effects on the thyroid gland below this dose. They argue that the selection of 1.0 mg/kg/day as the NOAEL requires that the hormonal changes at AP dosage levels of 1.0 mg/kg/day and lower not be considered adverse effects *per se*. Thus, despite the observation that thyroid hyperplasia was not reported at any dosage level for perchlorate, the authors proposed setting the NOAEL on the basis of any observed effect on the thyroid gland.

Considering the information provided in this review, it seems reasonable to propose that, in the absence of any other direct measure of thyroid hormone action, TSH must be used as the single point estimate of thyroid hormone effects. The reasoning for this statement is as follows. First, thyroid hormone is known to regulate circulating TSH directly; thus, changes in circulating levels of TSH can be viewed as a direct action of thyroid hormone. Second, it is clear that, for humans, clinical manifestations of hypothyroidism and hyperthyroidism are not sufficient to diagnose thyroid dysfunction. Rather, it is essential to measure circulating levels of T_3, T_4, and TSH. Moreover, TSH is used as an important biomarker in the therapeutic use of T_4 in hypothyroidism (170). Third, in the case of Siglin *et al.* (169), T_3 and T_4 levels remained significantly reduced despite elevated TSH levels. Thus, in the absence of any other direct measure of thyroid hormone action, one must assume that all thyroid hormone-responsive genes in the body are affected (either up-regulated or down-regulated) in a manner analogous to TSH.

Although it is arguable that recovery of normal hormone levels and thyroid weights from 90 days of perchlorate represents a return to a normal state in an adult animal, this argument cannot be

sustained for a developing animal. Thus, if similar results were obtained in dams, or in pups, one must follow the same line of reasoning except that the thyroid hormone-responsive genes affected by the decline in thyroid hormone are now developmentally regulated. In a developmental context, a period of catch-up or compensation cannot be considered evidence for the return of a normal state. For example, it is clear that experimental animals made hypothyroid during development are hypomyelinated as adults (171-174). However, thyroid hormone effects on the expression of myelin basic protein are transient, reaching a peak on postnatal day 15 in the rat and recovering by postnatal day 30 (37). However, despite the recovery in gene expression, the animals remain hypomyelinated, and exhibit behavioral change characteristic of hypomyelination. Likewise in humans, children with congenital hypothyroidism that exhibit hypomyelination early appear to recover with time, though behavioral characteristics of hypomyelination remain (96). Therefore, within a toxicological framework, fetal end points must be matched to the timing of exposure, and recovery over time after exposure cannot be assumed to be a return to a normal state.

PCBs. Polychlorinated biphenyls are a class of industrial compounds consisting of paired phenyl rings with various degrees of chlorination (175). Before their production was banned in the 1970s, over a billion kilograms of PCBs were produced (176) and they are now ubiquitous, persistent environmental contaminants that are routinely found in samples of human and animal tissues (177, 175). PCB mixtures or individual congeners, to varying degrees, can reduce circulating levels of thyroid hormones in animals (154, 178-182). The observation that PCBs are found in human milk is particularly concerning. Concentrations of individual congeners reported for milk samples taken from women exposed to background PCB levels and actively breast-feeding their infant range from 38.3 ng/g of lipid (183) to 395 ng/g of lipid (184). These values correspond to approximately 1.28 µg/ml of milk (3.52 µM) to 13.2 µg/ml of milk (36.3 µM) (185). Thus, the potential magnitude of PCB exposure to infants through breast milk and other sources justifies concern about potential effects on development.

PCBs are known to be developmental neurotoxicants at environmentally relevant concentrations (186-190). The most commonly noted neurological abnormalities associated with low-levels of PCB contamination in humans are hypoactivity and impaired learning (177). Because the symptoms of PCB exposure can overlap with those of thyroid dysfunction, several investigators have speculated that the neurological consequences of incidental exposure to PCBs are caused by disruption of the thyroid axis (191, 192). For example, Osius *et al.* (193) recently studied 7 to 10 year-old school children in three German municipalities, and found that serum concentrations of individual PCB congeners were associated with circulating TSH. In particular, they found a significant positive correlation between the concentration of the mono-*ortho* congener PCB 118 and TSH. Moreover, they found a significant negative correlation between several PCB congeners and free T_3. There was no correlation between circulating levels of PCBs and T_4. In contrast, Koopman-Esseboom *et al.* (194) measured dioxins and PCBs in human cord blood and breast milk and found that PCB exposure, estimated by toxic equivalents (TEQ), were negatively correlated with circulating T_4 in infants. It is important to recognize that the differences in circulating levels of thyroid hormones associated with PCBs are still within the normal range. Therefore, there is no evidence for overt hypothyroidism resulting from background exposure to PCBs. However, this observation alone does not necessarily mean that there are no adverse consequences of these associations (see below). Specifically, the prediction that PCBs effectively produce neurological deficits by producing hypothyroidism may be wrong, but PCBs may still interfere with thyroid hormone action. The structure of some PCB congeners may resemble that of thyroid hormone enough to interact with the thyroid hormone receptor (TR) (195), acting as agonists, antagonists, or mixed agonists (196).

Because an effect on circulating levels of thyroid hormone may not accurately reflect an effect on thyroid hormone action, we recently tested the hypothesis that PCBs interfere with thyroid hormone action in the developing rodent brain. We initially evaluated the effect of PCB exposure (Aroclor 1254) on circulating levels of thyroid hormone and on the expression of thyroid hormone-responsive genes in the developing brain (197). We found that A1254 reduces circulating levels of

T_4 to below the detection limit for the radioimmunoassay, but the thyroid hormone-responsive genes RC3/Neurogranin and myelin basic protein (MBP) were up-regulated as if T_4 levels were increased. Two elements of our results were consistent with a thyroid hormone-like effect of A1254. First, RC3/Neurogranin mRNA was elevated only in those regions of the developing brain in which others have shown it to be thyroid hormone-responsive (122). In addition, single-cell levels of RC3/Neurogranin mRNA were increased, suggesting a transcriptional mechanism (197). We pursued this in the fetal brain next in the fetal brain. We found that A1254 had no effect on circulating levels of thyroid hormones in the dam, but increased RC3/Neurogranin mRNA in the fetal brain (K. Gauger, C. Herzig, unpublished).

These findings demonstrate that PCBs can exert effects on thyroid hormone-responsive gene expression in the developing brain independent of effects on circulating levels of thyroid hormone. However, these studies do not remedy the overall problem that changes in gene expression are not likely to be considered to be an adverse effect. Therefore, it is essential to identify valid biomarkers of thyroid hormone action that can be employed in toxicological studies. For example, thyroid hormone affects apoptosis of cerebellar granule cells around postnatal day 8 in the rat (198), perhaps offering a valid toxicological end point.

VII. CONCLUSIONS

Information about the clinical effects of altered thyroid hormone levels clearly indicate that very small but persistent changes can produce adverse effects in adults, and can produce permanent changes in brain development. Considering these observations alone, the present logic applied to thyroid toxicity data sets should be reevaluated. Minimally, TSH should be considered a primary target of thyroid hormone action and a surrogate marker of all other thyroid hormone-responsive genes throughout the body. This is especially important in the developing brain. It must be stated, however, that studies focused on understanding the role of thyroid hormone in brain development do not offer a large number of potential end points of thyroid toxicity. It is likely that many, very

specific, developmental processes that all can agree represent an adverse effect can be identified to remedy this problem.

Table 1. A partial list of genes known to be thyroid hormone-responsive in various tissues at different developmental stages.

Tissue	Gene	Response to thyroid hormone	Ref
Neonatal Brain	TRβ1	Increase	(113)
	RC3/Neurogranin	Increase	(120, 121, 199, 200)
	TRH	Decrease	(201, 22, 24)
	Myelin Basic Protein	Increase	(173, 202)
	Purkinje cell Protein-2	Increase	(203)
	Type II 5'-Deiodinase	Decrease	(11)
Fetal Brain	NSP/*s*-Rex	Decrease	(204, 205)
	Oct-1	Increase	(204, 205)
Pituitary	Growth Hormone	Increase	(206)
	Beta-Thrytropin	decrease	(207)
Heart	Alpha-Myosin Heavy Chain	Decrease	(208)
	SERCA2	Increase	(209)
Skeletal Muscle	Alpha-Myosin Heavy Chain	Decrease	(208)
Liver	Malic Enzyme	Increase	(210, 211)
Testes	Androgen Receptor	Increase	(212)
Ovary	Inhibin	Decrease	(213)

FIGURE LEGEND

Figure 1. The Hypothalamic-Pituitary-Thyroid Axis. Numbers in filled diamonds refer to the legend below that provide descriptions of the specific level of the thyroid system.

1. Neurons whose cell bodies reside in the hypothalamic paraventricular nucleus (PVN) synthesize the tripeptide Thyrotropin-Releasing Hormone (TRH) (118, 24). Although TRH-containing neurons are widely distributed throughout the brain (214, 215), TRH neurons in the PVN project uniformly to the median eminence (216, 217), a neurohemal organ connected to the anterior pituitary gland by the hypothalamic-pituitary-portal vessels (74), and are the only TRH neurons to regulate the pituitary-thyroid axis (218, 26).

2. TRH is delivered by the pituitary-portal vasculature to the anterior pituitary gland to stimulate the synthesis and release of Thyroid Stimulating Hormone (TSH) or "Thyrotropin" (219). TRH selectively stimulates the synthesis of the TSH beta subunit (219). However, TRH also affects the post-translational glycosylation of TSH which affects its biological activity (220-225).

3. Pituitary TSH is one of three glycoprotein hormones of the pituitary gland and is composed of an alpha and a beta subunit (226). All three pituitary glycoproteins (Luteinizing Hormone, LH; Follicle Stimulating Hormone, FSH; and TSH) share the same alpha subunit (227). Pituitary TSH binds to receptors on the surface of thyroid follicle cells stimulating adenylate cyclase (15, 17). The effect of increased cAMP is to increase the uptake of iodide into thyroid cells, iodination of tyrosyl residues on TG by thyroperoxidase, synthesis and oxidation of thyroglobulin (TG), TG uptake from thyroid colloid and production of the iodothyronines T_4 and T_3. T_4 is by far the major product released from the thyroid gland (15).

4. Thyroid hormones are carried in the blood by specific proteins. In humans, about 75% of T_4 is bound to thyroxine-binding globulin (TBG), 15% is bound to transthyretin (TTR) and the remainder is bound to albumin (228). TBG, the least abundant but most avid T_4 binder, is a member of a class of proteins that includes Cortisol Binding Protein and is cleaved by serine proteases in serum (229). These enzymes are secreted into blood during inflammatory responses and, in the case of CBP, can induce the release of cortisol at the site of inflammation. The physiological significance of this observation is presently unclear for TBG (228).

5. Thyroid hormones (T_4 and T_3) exert a negative feedback effect on the release of pituitary TSH (230, 21, 19) and on the activity of hypothalamic TRH neurons (22, 231, 24). Although it is clear that thyroid hormone regulates the expression of TSH (232-234) and TRH (22, 118, 24, 117) in a negative feedback manner, it is also clear that the functional characteristics of negative feedback must include more than simply the regulation of the

gene encoding the secreted protein/peptide. In addition, fasting suppresses the activity of TRH neurons by a neural mechanism that may involve leptin (235, 236). This fasting-induced suppression of TRH neurons results in the reduction of circulating levels of thyroid hormone. Because circulating levels of T_4 and of T_3 fluctuate considerably within an individual, and because the radioimmunoassays for T_4 and for T_3 are associated with a fairly high intra-assay coefficient of variation, TSH measurements are considered to be diagnostic of thyroid dysfunction (230, 170, 19).

6. T_4 and T_3 are actively transported into target tissues (237-244, 111). T_4 can be converted to T_3 by the action of outer-ring deiodinases (ORD, Type I and Type II) (245). Peripheral conversion of T_4 to T_3 by these ORDs accounts for nearly 80% of the T_3 found in the circulation (230).

7. Thyroid hormones are cleared from the blood in the liver following glucuronidation by UDP-glucuronosyl transferase (155). These modified thyroid hormones are then eliminated through the bile.

8. T_4 and/or T_3 are actively concentrated in target cells about 10-fold over that of the circulation, although this is tissue-dependent. The receptors for T_3 (TRs) are nuclear proteins that bind to DNA and regulate transcription (101-103, 246, 112). There are two genes that encode the TRs, c-erbA-alpha (TRα) and c-erbA-beta (TRβ). Each of these genes is differentially spliced, forming 3 separate TRs, TRα1, TRβ1, and TRβ2. The effects of thyroid hormone are quite tissue-, cell-, and developmental stage-specific and it is believed that the relative abundance of the different TRs in a specific cell may contribute to this selective action.

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