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Challenges Confronting Risk Analysis of Potential Thyroid Toxicants

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Screening and testing for potential thyroid toxicants using endpoints of thyroid function, including circulating levels of thyroid hormones and thyrotropin, will not capture toxicants that directly interfere with thyroid hormone action at the receptor. The goals of the present review are to provide a critique of the literature focused on thyroid hormone and brain development as it relates to testing and evaluating thyroid toxicants, and to propose possible solutions to this perceived dilemma.

KEY WORDS: Thyroid hormone; brain development; developmental neurotoxicity; thyroid toxicity; perchlorate; polychlorinated biphenyl

1. INTRODUCTION

There is a long history of clinical observations on children with congenital hypothyroidism (CH) that demonstrate the importance of thyroid hormone in brain development.[1–5] This disorder is caused principally by failure of the thyroid gland to develop properly,[6] which produces severe thyroid hormone insufficiency in these children after birth if it is undiagnosed and uncorrected.[7,8] In addition to post-natal thyroid hormone insufficiency present in CH, recent evidence strongly supports the concept that thyroid hormone is also essential to brain development during fetal life. A growing number of studies indicate that children of women with low serum thyroid hormone during pregnancy have increased incidence of attention deficit, lower global IQ,[9–13] and specific types of visual problems.[14]

The importance of thyroid hormone in brain development, and the irreversibility of the effects of thyroid hormone insufficiency, underscores the importance of identifying environmental agents that may interfere with thyroid hormone action during development. Because developmental studies conducted on humans would clearly be unethical, it is essential to apply valid experimental models to identify such agents. However, there are no experimental endpoints of thyroid hormone action in the developing brain that have been employed to determine the adverse neurodevelopmental consequences of thyroid toxicants. Thus, the ability of potential thyroid toxicants to affect brain development is largely uncharacterized. This is particularly true for agents that are not expected to produce frank hypothyroidism but, instead, produced subtle changes in thyroid hormone action. Therefore, the goals of the present review are to provide a critique of the literature focused on thyroid hormone and brain development as it relates to evaluating potential thyroid toxins and to propose possible solutions to this perceived dilemma.

2. ENDOCRINOLOGY OF THE THYROID SYSTEM

The thyroid system is a classic neuroendocrine axis; the hypothalamus controls the pituitary gland, which in turn controls the thyroid, and feedback

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mechanisms between thyroid secretions and the hypothalamus and pituitary maintain the activity of this axis within narrow limits.[15] 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.[16] 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, liberating T_3 and T_4 into the blood.[17] Thyroxin is the predominant iodothyronine released by the thyroid gland; circulating T_3 is formed largely from peripheral deiodination of T_4.[18] The pituitary glycoprotein hormone, thyrotropin (TSH),[19] regulates the synthesis and secretion of thyroid hormones by activating adeylate cyclase in thyroid follicular cells.[20] However, there are a number of important extrathyroidal processes that combine to maintain circulating thyroid hormones within a relatively narrow concentration range.[18] Although T_4 is the predominant form of thyroid hormone in the serum, T_3 is the active hormone at the receptor. The term "thyroid hormone" will be abbreviated in the remainder of this article to "TH" to include both T_4 and T_3, recognizing the differences between the two.

Normal variation in circulating concentrations of T_4 reflects short-term pulsatile and diurnal variation.[21] Thyroid hormones exert a negative feedback effect on pituitary secretion of TSH,[22,23] and on the hypothalamic secretion of the releasing factor, thyrotropin-releasing hormone (TRH).[24–26] Although it is clear that TRH is a major factor regulating TSH secretion, several hypothalamic factors contribute to TSH regulation, including somatostatin, dopamine, and norepinephrine.[22] Moreover, some investigators suggest that the primary role of TRH in the regulation of TSH secretion is to modulate the set-point around which TH act on the pituitary.[27,28] Thus, circulating levels of TH, 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 Fig. 1.

3. THYROID HORMONE AND BRAIN DEVELOPMENT

3.1. Thyroid Hormone and the Human Neonate

It is well established that TH is essential for brain development during the neonatal period in humans, especially as revealed in the disorder known as congenital hypothyroidism (CH).[8,29–37] Congenital hypothyroidism occurs at a rate of 1 in 3,000 to 1 in 4,000 live births.[30] There are several causes of CH, including thyroid dysgenesis, agenesis and athyreosis, inborn errors of TH synthesis, and, less often, secondary or tertiary hypothyroidism.[30,38] Because CH infants do not present a specific clinical picture early, their diagnosis based solely on clinical symptoms was delayed. For example, before the initiation of routine neonatal screening for TH, only 10% of CH infants were diagnosed within the first month, 35% within three months, 70% within the first year, and 100% only after three years.[39,40] The intellectual deficits as a result of this delayed diagnosis and treatment were profound. One meta-analysis found that the mean full-scale IQ of 651 CH infants was 76.[41] More importantly, the percentage of CH infants with an IQ above 85 was 78% when the diagnosis was made within three months of birth, 19% when it was made between three and six months, and 0% when diagnosed after seven months of age.[41,42]

Because CH is difficult to diagnose on the basis of clinical symptoms alone, and because of the profound consequences both to society and to the individual, universal neonatal screening for circulating T_4 and/or TSH has been implemented by a number of countries.[30,42] This screening program has been enormously successful at identifying CH children rapidly and providing effective therapy, preventing the pervasive and profound neurological deficits previously attributable to CH.[3,30,37,43,44] However, recent studies reveal that specific clinical manifestations of CH persist even if it is diagnosed and treated early.[1,2,5,36,45–52] Although some of the variability in outcome can be attributed to variability in circulating T_4 sustained by treatment, a considerable amount of this variability is attributable to fetal hypothyroxinemia.

3.2. Thyroid Hormone and the Fetus

Thyroid hormones are detected in human coelomic and amniotic fluids as early as at eight weeks of gestation, before the onset of fetal thyroid function

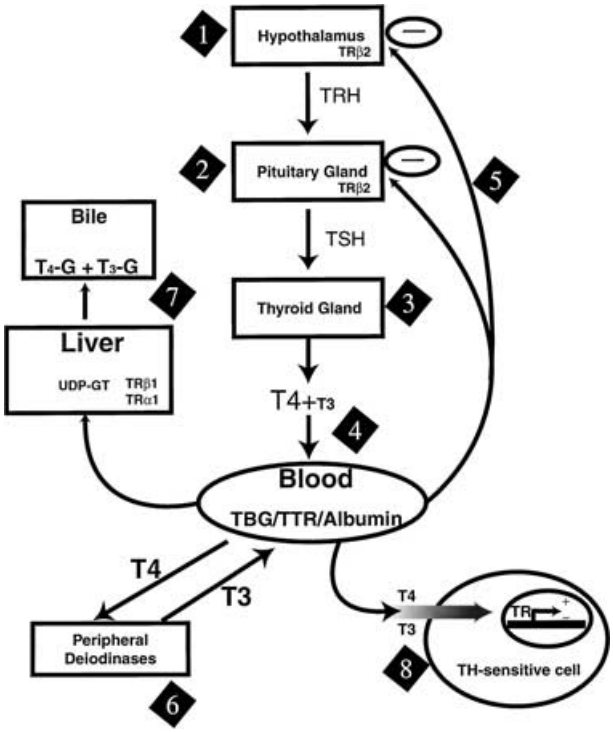

Fig. 1. The hypothalamic-pituitary-thyroid axis.

Numbers in filled diamonds refer to the legend below, which provides 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).[26,111] Although TRH-containing neurons are widely distributed throughout the brain,[241,242] TRH neurons in the PVN project uniformly to the median eminence,[243,244] a neurohemal organ connected to the anterior pituitary gland by the hypothalamic-pituitary-portal vessels,[38] and are the only TRH neurons to regulate the pituitary-thyroid axis.[28,245]

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."[246] TRH selectively stimulates the synthesis of the TSH beta subunit.[246] However, TRH also affects the post-translational glycosylation of TSH, which affects its biological activity.[247–252]

3. Pituitary TSH is one of three glycoprotein hormones of the pituitary gland and is composed of an alpha and a beta subunit.[253] All three pituitary glycoproteins (Luteinizing Hormone, LH; Follicle Stimulating Hormone, FSH; and TSH) share the same alpha subunit.[254] Pituitary TSH binds to receptors on the surface of thyroid follicle cells stimulating adenylate cyclase.[17,253] 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.[17]

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.[255] 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.[256] 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.[255]

5. Thyroid hormones (T_4 and T_3) exert a negative feedback effect on the release of pituitary TSH[21,23,257] and on the activity of hypothalamic TRH neurons.[24,26,258] Although it is clear that thyroid hormone regulates the expression of TSH[259–261] and TRH[24,26,110,111] 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.[262,263] 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 within an individual (pulsatile release), 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.[21,257,264]

6. T_4 and T_3 are actively transported into target tissues.[98,265–272] T_4 can be converted to T_3 by the action of outer-ring deiodinases (ORD, Type I and Type II).[273] Peripheral conversion of T_4 to T_3 by these ORDs accounts for nearly 80% of the T_3 found in the circulation.[257]

7. Thyroid hormones are cleared from the blood in the liver following glucuronidation by UDP-glucuronosyl transferase.[173,174] 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.[98] The receptors for T_3 (TRs) are nuclear proteins that bind to DNA and regulate transcription.[88–90,99,274] 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 three 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.

at 10–12 weeks.[53] In addition, human fetal brain tissues express receptors for TH, and receptor occupancy by TH is in the range known to produce physiological effects as early as nine weeks of gestation.[54,55] Finally, the mRNAs encoding the two known TH receptor types exhibit complex temporal patterns of expression during human gestation.[56] These data indicate that maternal TH is delivered to the fetus before the onset of fetal thyroid function and that the minimum requirements for TH signaling are present at this time. The functional importance of TH in fetal brain development has been recognized more slowly, in part because of the difficulty in correlating what are sometimes subtle differences in maternal TH concentrations with pregnancy outcome.

3.3. Maternal Thyroid Hormones During Pregnancy

The clinical condition of cretinism is the most profound consequence of maternal and neonatal hypothyroidism. There are two forms of cretinism based on clinical presentation.[57,58] Neurological cretinism is characterized by extreme mental retardation, deaf-mutism, impaired voluntary motor activity, and hypertonia.[57] In contrast, myxedematous cretinism is characterized by less severe mental retardation and all the major clinical symptoms of persistent hypothyroidism.[57] 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.[58,59] Several detailed studies of human populations living in geographic regions of severe iodine deficiency have led to the proposal that the various symptoms of the two forms of cretinism arise from TH deficits that occur during different developmental "windows of vulnerability."[57] These studies clearly indicate that TH plays an important role in brain development during fetal development and perhaps before the onset of fetal thyroid function.

Cretinism is an example of severe TH deficits, and the studies and observations mentioned above have contributed greatly to our understanding of the effects of TH on brain development in humans. The effects of subtle, undiagnosed, 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.*[60–65] Early definition of maternal hypothyroxinemia was defined empirically—those pregnant women with the lowest butenol-extractable iodine (BEI) among all pregnant women.[63,66] This work was among the first to report an association between low circulating T_4 in pregnant women and neurological function of the offspring. Pop *et al.*[67] later reported that the presence of antibodies to thyroid peroxidase in pregnant women, independent of TH 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 10th percentile of the normal range had a higher risk of low IQ and attention deficit.[10] These studies also demonstrate that neonatal T_4 levels are not indicative of fetal thyroid status. Excellent recent reviews discuss these studies in detail.[41,66,68,69] Taken together, these data present strong evidence that maternal TH plays a role in fetal brain development prior to the onset of fetal thyroid function. In addition, these data indicate that the consequences to the offspring of even mild and transient maternal TH deficits during pregnancy are neurological and irreversible.[7,8,33,37,70–72] However, despite the increased awareness of the importance of TH during fetal brain development, little is known about the mechanisms by which TH affects the fetus.

4. MECHANISM OF THYROID HORMONE ACTION ON BRAIN DEVELOPMENT

4.1. The Rat as a Model of Thyroid Hormone Action on Brain Development

Many features of the rodent endocrine system make the rat model particularly well suited for experimental studies. Aside from the obvious similarities in the chemistry of TH and dynamic interactions among the levels of the hypothalamic-pituitary-thyroid (HPT) axis,[73] many of the details of the HPT axis in rodents are similar to those in humans. For example, TR expression is measurable in rat brain early in development.[54,74,75] Thyroid hormone from maternal circulation reaches the fetus,[76–80] and recent studies indicate that TH exerts effects on fetal brain development that affect behaviors in the adult.[81] The validity of the rat as a model for the effects of TH on brain development has been recently reviewed.[82–85]

4.2. Thyroid Hormone Receptors are Nuclear Transcription Factors

It is generally believed that the majority of biological actions of TH are mediated by their receptors—nuclear proteins that interact mainly with T_3.[86,87] Thyroid hormone receptors (TRs) are members of

the steroid/thyroid superfamily of ligand-dependent transcription factors,[88–90] indicating that effects on gene expression mediate the majority of biological actions of TH. TRs are encoded by two genes, designated alpha- and beta- c-*erb*A.[91,92] These two genes produce three functional TRs by alternate exon usage: TRα1, TRβ1, and TRβ2.[93–97] Although there are several TR isoforms, the binding affinity for T_3 and for T_4 is not different among the various forms.[98–100] Thus, it is not possible to discriminate between TR isoforms by evaluating 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. In addition, there are ligands that exhibit binding characteristics that differ among the TR isoforms.[101–104]

4.3. Thyroid Hormone Exerts Tissue- and Cell-Type-Specific Effects

Although the responsiveness to TH requires the presence of nuclear TRs, the effects of TH vary from tissue to tissue, even among those tissues that express TRs.[105] Different levels and combinations of TR isoform expression may account in part for this observation,[88,89] but cannot account for all tissue variability in responsiveness to TH. For example, most patients with TH 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 TH actions.[106,107]

Thyroid hormone also exerts variable effects in the brain. For example, TH exerts a negative transcriptional effect on the gene encoding thyrotropin-releasing hormone (TRH).[108] However, this occurs solely in TRH-containing neurons in the hypothalamic paraventricular nucleus [24,109,110] despite the widespread distribution of cells expressing TRH[111] and those expressing TR.[112] This is true also for the gene coding for RC3/Neurogranin, a well-characterized TH-responsive gene in the developing and adult brain.[113,114] RC3/Neurogranin is expressed with TR in many brain areas, but is regulated by TH in a small subset of these areas.[115] Thus, it is unlikely that TH regulation of a specific gene will always be a marker of TH action; rather, studies must focus on the proper gene expressed in specific brain regions at the correct developmental time. An extension of this conclusion is that TH exerts differential effects on developmental processes in different parts of the developing brain and, likely, at different times during development. However, from a toxicological perspective, these selective effects of TH provide a powerful argument for the ability of an exogenous chemical to interfere with, or mimic, TH action.[116]

4.4. TRs Exhibit Specific Temporal and Spatial Patterns of Expression During Brain Development

Young and colleagues[74] demonstrated that the $\alpha-$ and $\beta-$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 the initial stages of fate specification,[117] this suggests that check points of cell division and events contributing to fate specification may be affected by TH and mediated by the TRβ1. In contrast, TRα1 may selectively mediate effects of TH on elements of migration, differentiation, and synaptogenesis because neurons of the cortical plate are undergoing these processes.[118] Thus, TH may influence different developmental processes by different TR isoforms before the fetal thyroid system begins to function on G 17–20.[119]

4.5. TR Function is Modulated by Interactions with Two Types of Regulatory Proteins

Two additional characteristics of the TRs contribute to the mechanisms governing TH action, which contribute to the observed pleiotropic effects. First, TRs can interact with distinct nuclear receptors including those for retinoids (retinoic acid receptors, RARs, and retinoid X receptors, RXRs).[86,88,90] 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.[90] Second, the ability of TRs to affect gene transcription requires them to interact with nuclear co-factors, which are requisite mediators of ligand-dependent transcriptional activation or repression of hormone responsive genes.[120–123] Co-factors are believed to remodel local chromatin structure enabling nuclear receptors to activate or repress gene regulation. Generally, the specific recruitment of a co-factor complex with histone acetyltransferase activity appears to play a regulatory role in activating gene

transcription, whereas the recruitment of a co-factor complex with histone deacetylase activity appears to play a regulatory role in gene repression.[124] Therefore, the sensitivity of a specific gene to regulation by TH may be modulated by the abundance and combination of heterodimer partners and of specific co-factors.

Two kinds of observations support the hypothesis that changes in cellular levels of specific co-factors 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 (i.e., transcriptional squelching[125,126]). This observation indicates that nuclear receptors compete for available co-factors, which, if they are in limited supply, will attenuate the efficacy of hormone-dependent activation of gene expression. Second, overexpression of the co-factor SRC-1 in a human breast cancer cell line (MCF-7 cells) results in an increase in the mitogenic response to estrogen.[127] Thus, the sensitivity of a cell to a specific level of hormone may be determined, at least in part, by the availability of specific co-factors.

There are two categories of nuclear receptor co-factors in general: co-repressors and co-activators.[120,128] In the absence of TH, TRs are able to repress basal transcription via recruitment of the co-repressors SMRT or NCoR.[129,130] In contrast, in the presence of TH, TRs release the bound co-repressor and recruit a co-activator complex that can include SRC-1.[130,131] The ligand-independent repression of basal transcription by TRs appears to account for the observation that TR knock-out mouse models exhibit a relatively mild phenotype compared to animals rendered hypothyroid using goitrogens or surgical thyroidectomy.[132–135] Specifically, because the TR appears to be a constitutive repressor in the absence of TH as ligand, the unliganded TR is predicted to be more damaging to brain development than the loss of the receptor entirely. This hypothesis is strongly supported by Hashimoto *et al.*,[136] who generated a TRβ1 knock-in mutant mouse expressing a TRβ1 unable to bind TH. These homozygous mutant mice exhibited severe neurological deficits that resembled hypothyroidism in wild-type mice.

Taken together, these data indicate that TH action on gene expression—and on specific developmental events—are likely to be highly pleiotropic. The effects of TH on the expression of an individual gene, or on specific developmental processes, will be spatially and temporally tailored. Therefore, studies designed to identify endpoints of thyroid toxicity during development must address this specificity; failure to consider this specificity will likely fail to identify reliable endpoints. However, rather than being a liability, the pleiotropic effects of thyroid hormone can provide significant strength in testing hypothesis about thyroid disruption.[116]

4.6. 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.[31,83–85,137] Much of this work has focused on the post-natal rat. For example, Koibuchi and Chin[83] provide a very clear and compelling argument for studying TH action on cerebellar development, which is almost entirely derived post-natally in the rodent.[138,139] However, it is essential that conclusions about TH action on neurodevelopmental events in the cerebellum not be extrapolated to other brain regions at different developmental times. For example, it is clear that TH affects proliferation of cerebellar granule cells. This was first shown by Nicholson and Altman[140] using ^{3}H-thymidine labeling, and has been shown by labeling with proliferating cell nuclear antigen mRNA (A. Croci, unpublished). However, our lab has not found that TH affects proliferation of cortical neurons on G16, using BrdU labeling or PCNA.[141] This single example clearly indicates that global statements about TH action on brain development should be avoided.

Because there is no *a priori* reason to predict that TH affects specific developmental processes in the early fetal cortex, we recently investigated TH action before the onset of fetal thyroid function using a broad empirical approach.[142] We used the technique of mRNA differential display as a way of identifying TH-responsive genes in the early fetal cortex, which could then guide us in subsequent studies to identify TH-regulated developmental processes. Our underlying rationale was that the lack of information about the molecular mechanism(s) of TH action on fetal brain development has two important consequences. First, we have little appreciation for the molecular events or developmental processes by which TH produces the effects observed in humans and animals discussed above. Second, we have no direct measures of TH action in fetal brain that could be employed in

studies of the neurodevelopmental consequences of thyroid toxicants. Therefore, we cannot directly test the hypothesis that specific chemicals can interfere with TH action. Rather, we are forced to interpret indirect measures of thyroid toxicity, such as circulating levels of TH and thyroid histopathology in terms of neurodevelopment.

We focused on the embryonic day 16 (E16) fetus because fetal thyroid function does not begin until E17;[119] thus, the identified genes would be regulated solely by *maternal* TH. In addition, E16 is the time when most of the neurons of the cerebral cortex are generated and begin to differentiate.[143] We surgically thyroidectomized female rats two weeks before they were mated to allow TH 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 T_4 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 TH and would be physiologically relevant.

We identified a number of genes expressed in the fetal brain that appear to be responsive to maternal TH. Two of these genes, encoding neuroendocrine-specific protein-A (NSP-A)[144,145] and Oct-1,[146–148] exhibited complementary responses to TH. Interestingly, both Oct-1 mRNA and NSP-A mRNA are expressed exclusively in the ventricular zone of the E16 cortex.[142,149] However, Oct-1 mRNA is elevated by T_4 injection, whereas NSP-A mRNA is suppressed by TH. Oct-1 is a member of the POU-domain family of transcription factors[146] 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.[144,145,150] These experiments demonstrated that TH of maternal origin can affect gene expression in the fetus, and they provide "biomarkers" of TH action in the fetal brain.

These observations indicate that although we know that TH is essential for normal brain development, the developmental processes affected by TH, the mechanisms by which TH affects these processes, and the timing of TH action for any one developmental process are poorly understood. Moreover, there are few studies that link TH action in the developing brain with persistent adverse outcomes in the adult. These data gaps produce a situation whereby evaluation of chemical agents for their ability to interfere with the thyroid system during development is compromised. The section below addresses this issue specifically.

5. SPECIFIC CHALLENGES CONFRONTING A RESEARCH PROGRAM EVALUATING THYROID TOXICITY

A number of environmental chemicals have been shown to affect the thyroid system,[151] and the study of environmental goitrogenesis is well developed.[152] However, all known thyroid toxicants have been identified by their ability to influence circulating levels of TH and/or TSH because these are the principal tools used to identify thyroid toxicants. Because there are no validated markers of TH action independent of circulating levels of hormones, chemicals that interfere with TH action without affecting hormone levels would not be detected.

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 TH. This could be envisioned as equivalent to the uterotrophic assay for estrogens[153] or the Hershberger assay for androgens[154] in which specific measurable endpoints (e.g., uterine weight) are highly sensitive to changes in circulating levels of estrogens or androgens. For example, one could imagine a dose response of T_4 replacement in hypothyroid dams or developing rat pups in which a graded effect on specific developmental and neurodevelopmental processes are measured. Ideally, these endpoints would be known to reflect adverse consequences in the adult brain. This kind of information would provide the background to test whether specific chemicals could affect circulating levels of TH and, if so, whether these changes were linked to measurable changes in specific developmental processes. Moreover, this kind of information would provide the basis to design screens and tests for chemicals that interfere with TH action, not simply thyroid function.

Unfortunately, with very few exceptions[155] there are almost no studies in rats in which a dose response of TH is evaluated for its effects on brain development. Rather, nearly all studies make use of potent goitrogens such as propylthiouracil or methimazole that reduce circulating levels of TH to very low or undetectable levels.[31,82,156,157] These studies demonstrate that severe maternal and neonatal hypothyroidism can reduce brain and body weight,[105] and can affect a variety of histological characteristics of the brain.[105,140,158] However, we do not know how

Tissue	Gene	Response to Thyroid Hormone	Reference
Neonatal brain	TRβ1	Increase	100
	RC3/neurogranin	Increase	113, 114, 225, 226
	TRH	Decrease	24, 26, 227
	Myelin basic protein	Increase	228, 229
	Purkinje cell protein-2	Increase	230
	Type II 5′-deiodinase	Decrease	155
Fetal brain	NSP/*s*-rex	Decrease	231, 232
	Oct-1	Increase	231, 232
Pituitary	Growth hormone	Increase	233
	Beta-thrytropin	Decrease	234
Heart	Alpha-myosin heavy chain	Decrease	235
	SERCA2	Increase	236
Skeletal muscle	Alpha-myosin heavy chain	Decrease	235
Liver	Malic enzyme	Increase	237, 238
Testes	Androgen receptor	Increase	239
Ovary	Inhibin	Decrease	240

Table I. A Partial List of Genes Known to be Thyroid Hormone-Responsive in Various Tissues at Different Developmental Stages

sensitive these measures are to subtle changes in circulating levels of TH.

Although individual clinical symptoms lack definitive diagnostic value in humans, experimental systems can be evaluated more thoroughly. Therefore, it seems possible that endpoints of thyroid toxicity exist that could be identified for testing effects of thyroid toxicity. It is known that TH dysfunction produces deleterious effects on many organ systems, including heart, muscle, liver, and brain. A partial list of TH-responsive genes in these various tissues, which could be used in toxicological studies, is shown in Table I. However, few studies to date have begun to expand the endpoints of thyroid toxicity for use in toxicological studies. Moreover, although TH is well known to affect metabolism,[159] body weight,[105] and several aspects of behavior,[160–163] the smallest change in TH required to observe significant effects on these endpoints has not been determined. Thus, the endpoint that provides the lowest effect level for TH itself has not been identified. Likewise in development; TH is known to be essential for brain development, but experimental studies focus on understanding the developmental consequences of TH action[140,164–167] and the mechanisms by which TH acts.[83,85,142,168]

6. 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.[151] Some chemicals selectively interfere with TH synthesis, where others may selectively affect metabolic clearance, serum transport, elimination, or, in principal, 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).[169,170] Because ClO_4 blocks iodide uptake, it reduces TH synthesis and circulating levels of TH. Therefore, perchlorate is expected to produce deleterious effects on an organism solely by reducing TH synthesis and release. In contrast, polychlorinated biphenyls (PCBs) appear to affect the thyroid system at several levels.[171] Specifically, PCBs enhance liver metabolism of TH,[172–175] increasing biliary excretion.[176,177] PCBs interfere with T_4 binding to serum proteins,[178–181] which may also reduce circulating levels of TH. And, PCBs may affect cellular uptake of T_3 and/or T_3 binding to the nuclear receptor.[182–184] Therefore, changes in circulating levels of TH 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 endpoints of thyroid toxicity.

6.1. Perchlorate

Ammonium perchlorate is the principal oxidant for solid propellants in the defense industry.[185,186] Perchlorate contamination of groundwater across the

United States has recently become apparent[187] and, therefore, it is important to determine the level of perchlorate in drinking water that produces an adverse effect in humans. Perchlorate inhibits iodide uptake into the thyroid gland by the sodium/iodide symporter (NIS).[170] This action of perchlorate was the basis of its clinical use in the treatment of hyperthyroidism and its potential toxicity as an environmental contaminant. To establish the dose response in humans for perchlorate inhibition of thyroidal iodide uptake, and short-term effects on circulating TH, Greer *et al.*[188] gave perchlorate in drinking water at 0.007, 0.02, 0.1, or 0.5 mg/kg per day to 37 male and female volunteers for 14 days. In 24 subjects, 8- and 24-hr measurements of thyroidal ^{123}I uptake (RAIU) were performed before exposure, on exposure days 2 and 14 (E2 and E14), and 15 days post-exposure (P15).

In general, these studies allowed the estimation of a true no-effect level of perchlorate of 5.2 or 6.4 μg/kg/day. Considering that a 70 kg adult drinks 2 liters of water per day, this dose would be consumed if the drinking water contained 182–224 ppb. In addition, even the dose of 0.5 mg/kg/day taken for 14 days did not produce changes in circulating levels of T_4 or TSH. Thus, perchlorate levels of 17.5 ppm in drinking water would not be expected to alter circulating levels of T_4 or TSH within a 14-day period even if the adult consumed 2 liters per day. Based on these studies, Greer *et al.* concluded that perchlorate concentrations of 180–220 ppb (and possibly higher) should be of no health concern in iodine-sufficient populations.[188] Although this conclusion is defensible for normal, euthyroid adults, several key aspects of the normal adult thyroid system are significantly different in neonates, which reduces the confidence in this conclusion as it applies to a significant proportion of the normal human population—fetuses, neonates, and infants.

Specifically, Greer *et al.*[188] postulated that 0.5 mg/kg/day of perchlorate failed to influence circulating levels of TH in healthy adults in 14 days of exposure because the normal adult thyroid gland contains a very large storage capacity of unreleased TH. In fact, these authors estimate that there should be sufficient hormone stored in the thyroid gland to last for several months. The case is quite different for a late gestation fetus or neonate. Vulsma *et al.*[189] estimated that the neonatal thyroid gland contains TH equivalent to only a single day's secretion. This estimate was revised by van den Hove *et al.*,[190] who empirically measured intrathyroidal stores of TH in human fetuses and neonates and found that the amount of hormone stored in the colloid is less than that required for a single day. Thus, the concentration of perchlorate sufficient to reduce thyroidal iodine uptake in a fetus or neonate may be sufficient to produce a significant decrement in circulating levels of TH.

Two additional characteristics unique to children should be considered when applying data obtained from normal adults to the potential developmental consequences. First, perchlorate may be concentrated in milk. Perchlorate acts on the NIS,[170] a protein that is induced in lactating breast tissue by prolactin.[191–194] Thus, it is possible that perchlorate is concentrated in milk.[195,196] However, there are no studies to determine the relationship between maternal perchlorate consumption, maternal serum perchlorate concentration, and the concentration of perchlorate in milk. Therefore, the relationship between perchlorate intake in nursing mothers and the dose of perchlorate presented to her infant has not been empirically determined. Minimally, it is clear that perchlorate will reduce iodide uptake into milk, thus reducing the sole source of iodine to the infant. Second, a short period of TH insufficiency may produce permanent neurological deficits in children. Van Vliet reviewed the evidence that a period of TH insufficiency of as little as 14 days may be long enough to produce permanent neurological deficits in neonates.[37]

Thus, there are known differences in the thyroid system between normal euthyroid adults and normal euthyroid newborns and infants that must be considered when interpreting data derived from adult humans. In addition, it is likely that adults can recover from periods of transient hypothyroxinemia without permanent health consequences.[197] In contrast, there is good evidence that the developing brain is quite sensitive to periods of hypothyroxinemia and that the consequences are permanent. For example, long-term studies of children with congenital hypothyroidism that have been treated with T_4 replacement indicate that very subtle differences in circulating levels of T_4 are associated with significant differences in intellectual performance later in life.[1,2] These differences indicate that experimental studies must be performed to address specific neurodevelopmental effects of thyroid toxicants.

Two experimental studies have focused on the developmental effects of perchlorate in rat[198] and in rabbit.[199] In a two-generation rat study,[198] adult Sprague-Dawley rats were provided with drinking water containing target doses of perchlorate of 0, 0.3, 3.0, and 30.0 mg/kg per day. The F1 generation was

maintained on this drinking water after weaning. Reproductive effects were not observed. Microscopic changes in thyroid structure were observed that increased in incidence and severity with increasing dose. Moreover, dose-related changes in TSH and T_4 or T_3 were observed at doses of ammonium perchlorate that were higher than the doses producing significant effects on thyroid histology. Based on these findings, the authors concluded that the no observable adverse effect levels (NOAEL) was 0.3 mg/kg-day.

In the development study performed in rabbits,[199] does were given continual access to ammonium perchlorate in drinking water at target doses of 0, 0.1, 10.0, 30.0, and 100.0 mg/kg per day on gestation days 6 through 28. The does were euthanized on gestation day 29 and fetuses were examined for developmental effects of treatment. Serum TSH, T_4, and T_3 were measured in the does, and the maternal thyroid gland was evaluated microscopically. No effects of treatment were observed on gross inspection of the fetuses (e.g., litter size and weight, etc.). The maternal thyroid gland exhibited an increased incidence of thyroid follicular hypertrophy at doses of 10 mg/kg per day and above. Serum T_4 was significantly decreased in animals treated with 30 mg/kg/day and above. Based on these observations, the authors estimated the NOAEL for developmental effects of ammonium perchlorate at 100.0 mg/kg per day.

In principle, these experimental studies were designed to address an important data gap that exists when human studies are performed. Specifically, human studies cannot address the developmental effects of potential thyroid toxicants because of clear ethical considerations. However, considering the information presented above on the role of TH in brain development, it is not clear how the available published literature on TH action in the developing brain was considered to design studies of the developmental toxicity of perchlorate. Nearly all the literature studying the TH effects on brain development have been performed using rodents; few if any have focused on lagomorphs. Moreover, neither of these studies examined endpoints known to be directly affected by TH, such as the expression of specific genes, cerebellar granule cell proliferation, apoptosis, or others. Although the experimental literature does not provide clear examples of the different sensitivities of various neurodevelopmental endpoints to TH, there are many good examples of neurodevelopmental endpoints that could be employed in studies of the developmental consequences of known thyroid toxicants such as ammonium perchlorate.

In the absence of specific neurodevelopmental endpoints of TH action in a toxicological study, investigators are necessarily in the position of speculating about the potential adverse effects of toxicants using thyroid endpoints in dams and pups. These endpoints, as described above for perchlorate studies, are serum concentrations of hormones (T_4, T_3, and TSH) and thyroid histopathology. The challenge is to make strong inferences about the potential developmental effects on the developing brain based on circulating levels of TH in the dam or in the pup. To categorize observed changes in thyroid endpoints as reflecting compensatory changes or adverse effects, the logic employed is based on the known negative feedback effect of TH on the hypothalamic-pituitary axis. Clearly, the negative feedback system of the HPT axis limits the degree to which circulating levels of TH can change under normal circumstances. However, it is not accurate to assume that the negative feedback system prevents adverse effects of persistent but small changes in circulating levels of TH.

Consider the following. Circulating levels of TSH will increase when the hypothalamic-pituitary system detects a reduction, slight as it may be, in circulating levels of TH. Therefore, if TSH levels are increased, despite measuring "normal" levels of T_4, it demonstrates that T_4 levels are in fact reduced. This may also be true for changes in thyroid histopathology. In the case of the two-generation reproduction study on perchlorate,[198] thyroid histopathology appeared to be a more sensitive indicator of perchlorate action on the thyroid gland than did serum levels of T_4 or TSH. An important question is whether subtle reductions in circulating T_4 that trigger an increase in TSH release in the dam (or pup) is detected as TH insufficiency in other tissues, including the fetal brain.

Recent studies in humans support the interpretation that changes in TSH in the absence of alterations in T_3/T_4 (i.e., subclinical hypothyroidism or hyperthyroidism) are associated with adverse health consequences. Andersen *et al.*[15] demonstrated that individual variation in T_4 levels are much more narrow than the population variance in T_4, which is the basis for the normal reference range. Therefore, an individual can experience a decline or excess in serum T_4 that significantly alters serum TSH, and still possess T_4 levels within the normal range for the population.[15] In addition, long-term follow-up studies of patients given T_4 replacement therapy following thyroid ablation for thyroid cancer or Grave's disease[200] indicate that these patients exhibit a much higher incidence of cardiovascular disease. The interpretation

is that chronic, sustained elevations in circulating levels of T_4 can produce adverse effects on the cardiovascular system. These studies indicate that relatively small changes in circulating levels of TH, identified by changes in serum TSH, do not reflect full compensation for the original decrement in TH. Within a toxicological framework, it is important to distinguish between this kind of compensation and a pathological situation. The difficulty in making this distinction should not be underestimated. We know that the beta-subtype of the TR mediates the negative feedback effect of TH on TRH/TSH.[201] Therefore, serum TSH is a "biomarker" of TH action on the beta TR. In contrast, there are no markers of TH action on the alpha receptor in a study of thyroid toxicity. Therefore, it is an assumption that the hypothalamic-pituitary axis is more sensitive to small changes in TH than are other tissues, including those expressing the alpha receptor predominantly (e.g., heart) or in the developing brain. Attempting to infer adverse effects of even slight reductions in circulating levels of T_4 in dams or in pups based on measures of thyroid function appears to be poorly supported by the kind of studies required to make these inferences.

6.2. PCBs

Polychlorinated biphenyls are a class of industrial compounds consisting of paired phenyl rings with various degrees of chlorination.[202] Before their production was banned in the 1970s, over a billion kilograms of PCBs were produced[203] and they are now ubiquitous, persistent environmental contaminants that are routinely found in samples of human and animal tissues.[202,204] PCB mixtures or individual congeners can reduce circulating levels of TH in animals to varying degrees.[172,205–209] The observation that PCBs are found in human milk is of particular concern. Concentrations of individual congeners reported for milk samples taken from women exposed to background PCB levels and actively breast-feeding their infants range from 38.3 ng/g of lipid[210] to 395 ng/g of lipid.[211] These values correspond to approximately 1.28 μg/ml of milk (3.52 μM) to 13.2 μg/ml of milk (36.3 μM).[212] 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.[213–217] The most commonly noted neurological abnormalities associated with low levels of PCB contamination in humans are hypoactivity and impaired learning.[204] 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.[218,219] For example, Osius *et al.*[220] 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.*[221] 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 TH 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 TH action. The structure of some PCB congeners may resemble that of TH enough to interact with the TH receptor (TR),[222] acting as agonists, antagonists, or mixed agonists.[223]

Because an effect on circulating levels of TH may not accurately reflect an effect on TH action, we recently tested the hypothesis that PCBs interfere with TH action in the developing rodent brain. We initially evaluated the effect of PCB exposure (Aroclor 1254) on circulating levels of TH and on the expression of TH-responsive genes in the developing brain.[116] We found that A1254 reduces circulating levels of T_4 to below the detection limit for the radioimmunoassay, but the TH-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 TH-like effect of A1254. First, RC3/Neurogranin mRNA was elevated only in those regions of the developing brain that others have shown to be TH responsive.[115] In addition, single-cell levels of RC3/Neurogranin mRNA were increased, suggesting a transcriptional mechanism.[116]

We pursued this next in the fetal brain. We found that A1254 had no effect on circulating levels of TH 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 TH-responsive gene expression in the developing brain independent of effects on circulating levels of TH. 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 TH action that can be employed in toxicological studies. For example, TH affects apoptosis of cerebellar granule cells around post-natal day 8 in the rat,[224] perhaps offering a valid toxicological endpoint.

7. CONCLUSIONS

Recent information about the clinical effects of TH insufficiency clearly indicates 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. First, the interpretation of studies in normal adult humans must take into consideration the differences between normal adults and normal children in thyroid economy and in the relative sensitivity and reversibility of these different life stages to TH insufficiency. Second, experimental studies must begin to identify and recruit endpoints of TH action in the developing brain to test whether potential thyroid toxicants may produce adverse neurodevelopmental effects through the TH signaling system. Moreover, chemicals may exist that interfere with TH action in the absence of effects on circulating levels of TH. Risk analysis of potential thyroid toxicants, in the absence of specific neurodevelopmental endpoints, must be viewed with caution as it relates to childhood exposure of these chemicals.

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