Thyroid Hormone, Brain Development, and the Environment

R. Thomas Zoeller

University of Massachusetts

Thank you. First, I would like to thank the organizers for inviting me to speak today. It is a pleasure and an honor for me to participate in this symposium.

First topic of my talk will be on the interface between three pretty large disciplines, or experimental literatures, and that is: thyroid function, brain development, and the environment, and by environment, I really mean environmental toxicology.

The way we think about thyroid hormone and brain development is conditioned by two stark examples: one of metamorphosis in amphibians, the other is cretinism in humans.

Metamorphosis in amphibians represents a very visible and dramatic series of changes that result in a complete transformation in the animal from the aquatic to the terrestrial form. Without thyroid hormone there is no metamorphosis. I would not want to say it is a binary switch, but it is a very dramatic and visible series of events that requires the presence of thyroid hormone. Likewise, cretinism is a very visible and dramatic effect of the lack of thyroid hormone during development. Both of these situations have drawn our attention to the importance of thyroid hormone in brain development. But it has also led us to begin to study the effects of modest changes in maternal thyroid hormone on brain development. Yet these effects are not visible, are not dramatic on the faces of our children, but rather are revealed in population studies.

For example, one study recently published in the "New England Journal of Medicine" by Haddow in 1999, showed that the children offspring of women who exhibited thyroid function in the lower range of the normal range exhibited a 7 to 10 point reduction in full scale IQ and an increased incidence of attention deficit.

These observations, and there are many other studies such as this, suggest that thyroid hormone is important both before and after birth. Yet we know virtually nothing about the role of thyroid hormone in fetal brain development.

So my lab began to study this issue of thyroid hormone in fetal brain development, and our initial focus was to identify thyroid hormone responsive genes in the fetal cortex, and we focused on a time in fetal development when the fetus cannot make its own thyroid hormone, so this represents a story about maternal thyroid hormone and fetal brain development.

We wanted to identify genes that are regulated by thyroid hormone because if we can determine where they are localized, we may understand what brain areas are responsive to thyroid hormone. In addition, if we know the kinds of proteins that are being encoded by these genes, it may give us some insight into the role thyroid hormone plays in specific developmental events.

This strategy was designed based on at least a rudimentary understanding of thyroid hormone action, so what I want to do here is to describe briefly some of the events that regulate thyroid hormone action.

First of all, the thyroid hormone receptor acts in partnership with another receptor. There are two receptors that form a dimer. In the unliganded state, it associates with a protein called a co-repressor. This co-repressor links the thyroid hormone receptor to the transcriptional apparatus and causes repression of target genes.

Thyroid hormone is actively transported into the cell, which is a regulatory step. T_4 is then converted to T_3 by a de-iodinase. That T_3 binds to the thyroid hormone receptor, which causes the release of the co-repressor and recruitment of a co-activator, a protein that links thyroid hormone receptor to the

transcriptional apparatus, which then causes gene expression or regulation of the target gene. Now, this sounds kind of simple, but there are several issues that are important also to consider.

First of all, thyroid hormone action is quite variable. That is, for genes that are regulated by thyroid hormone, the presence of the receptor is required, but it is not sufficient. So we know that the presence of the receptor does not always dictate regulation of thyroid hormone to that target gene.

Here are some of the reasons that we believe there is such heterogeneity. First of all, there are more than one type of thyroid hormone receptor, an α and a β. Also, these receptors can heterodimerize with retinoic acid receptors and other members of the steroid receptor super-family. Also, it is a broad combination of cofactors that might condition the effect of thyroid hormone action mediated by the receptor.

So these observations mean that the effects of thyroid hormone in one cell, or one cell type at a single time during development, cannot be interpreted as a general phenomenon. So, we had to be very empirical in our approach.

This illustrates our initial approach. It is an approach that we used using mRNA differential display that will allow us to identify genes that are either up regulated or down-regulated by thyroid hormone, and this was just our initial approach. The point that I want to make here very quickly is that all the genes that we have identified so far are selectively expressed in this ventricular zone of the cortex on gestational day 16.

This ventricular zone of the cortex is important because there are two kinds of processes occurring. The first is proliferation, second is differentiation. We have mapped the distribution of these genes and I am just giving one example here, but the detail is not really important. In all cases, the expression of the gene is restricted to this ventricular zone and as that zone disappears, so does the gene.

This illustrates this process of proliferation, where cells proliferate in this ventricular zone and once they stop proliferating and begin differentiating they move away from the zone. So, our initial hypothesis was that thyroid hormone regulates proliferation.

However, in a study using bromodeoxyuridine, or BrdU, what we found is no evidence for an effect of thyroid hormone on cell proliferation. We have not only repeated this, but used different measures of cell proliferation and have failed in all cases to see an effect of thyroid hormone.

So if we look at this diagram, we can see that as a neural stem cell begins to differentiate, it has to make a choice about whether to become a neural progenitor or a glial cell progenitor. The switch that seems to be controlling that has to do with a signaling system called “Notch”.

Now in one of our initial screens, one of the genes that we identified as thyroid hormone responsive is a gene called “HES”. HES is a transcription factor that is regulated by Notch Signaling. “Notch” is a receptor that is inserted in the membrane of a cell. It binds to a ligand, and there are several Notch ligands. These ligands are themselves integrated into cell membranes, so this is a signaling system that mediates contact-dependent signaling systems in the cell.

Once the Notch receptor is bound, the Notch intracellular domain is cleaved and becomes free to move into the nucleus, where it combines with another factor or factors to regulate the expression of HES. What studies have shown is that HES expression increases gliagenesis. That is, HES expression marks the production of glia.

What we found is that thyroid hormone increases HES expression. If we treat animals with a drug called propylthiouracil to reduce thyroid hormone, HES expression declines. As we give thyroid hormone back — and this is a transient, acute dose of thyroid hormone — HES expression then increases, and you can see in an *in situ* hybridization study here, the effect of PTU on HES expression.

What is interesting is that there is a typology to the effect of thyroid hormone on HES. What I have represented here is our working hypothesis based on two observations. In the anterior part of the brain, it

is the ventral lateral portion of the cortex that is sensitive to thyroid hormone. In the caudal part of the brain, it is the dorsal lateral. So I have extrapolated here, I doubt that the sensitive area actually looks like this, but that is what we are predicting.

So first of all, there is a typology to thyroid hormone action.

These studies taken together indicate that thyroid hormone of maternal origin exerts specific effects on gene expression in the fetal brain. The ventricular zone of the fetal cortex appears to be a selective target. The thyroid hormone does not appear to influence the number of cells born. Thyroid hormone increases HES expression likely by increasing Notch signaling, and because thyroid hormone does not affect proliferation, we propose that thyroid hormone modifies or adjusts the proportion of neurons and glia that are being produced.

The first implication of this, in terms of endocrine disruption, is that factors that affect maternal thyroid hormone or thyroid function may also affect fetal cortical development. So before the onset of fetal thyroid function, any factor that inhibits maternal thyroid function may affect brain development, and any factor that directly interferes with or modifies thyroid hormone action may affect fetal development.

This concept immediately disturbed me in thinking about the way we test environmental chemicals for their ability to interfere with thyroid function or thyroid hormone action, especially in a brain development paradigm.

As you can see here in this diagram, the hypothalamus controls the pituitary, which then secretes a hormone called TSH, which activates the thyroid gland to produce thyroid hormones T_3 and T_4. Once in the blood, T_3 and T_4 produce a negative feedback effect on hypothalamic and pituitary function.

In toxicological studies, the measures that people take are T_3 and T_4, TSH, and often looking at thyroid histopathology as a measure of some aberration. However, there are two problems with this.

First of all, any chemical that interferes with thyroid hormone action at the tissue may not necessarily affect circulating levels of thyroid hormone or thyroid histopathology. In addition, thyroid histopathology, and the relationship between these hormones and thyroid structure is often confused and difficult for people to interpret in terms of an adverse effect, so the common thought is that it is only an adverse effect if the thyroid exhibits hyperplasia, which would lead to cancer, which really has nothing to do with brain development.

So, we began to test the idea that environmental factors may influence thyroid hormone action directly. We chose to focus on polychlorinated biphenyls, or PCBs. PCBs have a structure that roughly appears like thyroid hormone, but there are several experimental observations that also make this important.

First of all, it is well known that mixtures of PCBs and single congeners can reduce total T_4 as well as T_3 in the blood, and also sometimes affect TSH although that is not as common. Also, in humans, there have been link associations between measures of thyroid function and PCB levels.

So in our first experiment, we focused on a postnatal time point in the cerebellum. This is the reason that we did this. This is an example of a thyroid hormone responsive gene in two different groups; this is a normal group in blue, and the hypothyroid group is in red.

During this limited period of time, there is a reduction of gene expression in the hypothyroid animal. This is transient. If one were to look early in development or late in development, you would not be able to see this effect of thyroid hormone. So we focused on this postnatal day 15 to look at the expression of this gene. The gene itself does not matter that much, just that it is a thyroid hormone responsive gene.

We evaluated 4 doses of PCBs from 0 to 8 µg/kg/day given to the mother from gestational day 6. What we found is a dose dependent decrease in circulating levels of T_4 as well as T_3 in blood of the pup.

You can see here that T_4 levels are really unmeasurable in the highest dose of PCB, yet there is no effect on body weight.

If we had reduced T_4 levels with propylthiouracil, there would have been a reduction in bodyweight, so that was the first dissociation.

When we looked at RC3 mRNA in the postnatal day 15 brain, we found that there was a significant increase in the expression of this gene by PCB. There are two observations that made me suspect that PCBs are acting like thyroid hormone in this system.

First, the topology of thyroid hormone action on RC3 is very specific. There is a specific effect on the dentate gyrus and in the cortex, but not in other parts of the hippocampus or in other parts of the cortex, and that is exactly what we saw with PCBs. So we saw this increase expression in RC3 in dentate gyrus and cortex only.

Second, when we looked at single cell analysis of the cortex, we found that PCB increased the cellular level of RC3/ neurogranin mRNA consistent with a transcriptional effect.

Now, we wanted to look in the fetus. The first thing that we found is that the addition of PCBs did not affect thyroid hormone levels in the mother. So there is no effect of T_4 or T_3 or TSH in the mothers at this time, and this is gestational day 16. However, we saw an increase in expression of HES. I have both HES-1 and HES-5 here, but I am not going to talk about that.

We also saw that PCB exerts the same topological pattern of response on HES expression. We have also looked at several other thyroid hormone responsive genes in the fetus and all have exhibited regulation by PCBs in a manner that is consistent with the hypothesis that PCBs are acting like thyroid hormone.

What this means to me is that PCBs may be altering or adjusting, through not signaling and perhaps via a thyroid hormone dependent mechanism, the proportion of neurons and glia being produced in a specific part of the brain.

In conclusion, the effect of a mixture of PCBs on thyroid hormone-responsive gene expression is not related to their effects on circulating levels of thyroid hormone. I propose that specific congeners or metabolites of PCB congeners within the mixture affect the thyroid system differently. That is, some PCB congeners may be involved in lowering circulating levels of T_4, but that others may interact directly with the receptor.

The possibility that an individual congener may interact directly with the thyroid hormone receptor suggests also the possibility that it could do so in an isoform-specific way, that is act on thyroid hormone receptor α and β differently, or it may exert other pharmacological actions on the thyroid hormonal receptor, none of which could be detected in the traditional screening program. Thank you.

Q&A

Koibuchi: Thank you very much. Now, the paper is open for discussion. Any questions from the floor? Yes, please.

Q: Tom, that was a very nice talk. My question concerns the idea that you have that we may not be able to detect effects of thyroid agonists like the PCBs with traditional test methods, and since we are developing additions to traditional test methods, we want to make sure that we get those right.

It seemed to me in your work that basically, unlike PTU, the PCB congener that you were using seems to act as a direct thyroid agonist. It would appear that it acts that way at the level of the pituitary as well, and that you did get decreases in circulating T_3/ T_4 levels. Would not we be able to detect that just by measuring circulating hormone levels?

Zoeller: I have thought about that a lot. I think the conclusion, at least in my mind, is that if a reduction in thyroid hormone is considered as an adverse effect, then I think it offers some level of protection. But I have never seen a case where people consider a reduction in thyroid hormone level per se as an adverse effect.

The biggest concern that I have is that in the absence of looking at thyroid hormone level itself, and we know, I am sure that in the next year or so, I will find congeners — individual PCB congeners or metabolites — that affect thyroid hormone action in the brain without affecting thyroid hormone levels.

Until we have an appreciation for the structure of chemicals that can bypass the circulating levels of thyroid hormone, we will continue to have this danger. I think at this point thyroid hormone levels will remain one of the test methods of choice, but I think people need to begin to be careful about relying too heavily on thyroid hormone levels, especially if they are not interpreted as adverse.

Koibuchi: Thank you. Next question? Any questions? Yes please.

Q: I have one question concerning the HES mRNA expression controlled by thyroid hormone. You mentioned that the HES mRNA expression is controlled by thyroid hormone, but the HES mRNA expression is also controlled by the Notch signal.

I am wondering whether the Notch messenger expression itself is controlled by the thyroid hormone or not.

Zoeller: That is a very good question and one that is very interesting to us. I do not believe that HES itself is being directly regulated by thyroid hormone. Rather, I think that thyroid hormone is affecting one of the members of this pathway. It could regulate the expression of ligands. There are three different Notch receptors in the ventricular zone at this time, as well as a gamma-secretase activity that does the cleavage.

We are in the process of an iterative experiment to go through each of those sequentially, and right now we do not have an answer for that, but it is a very important issue.

Q: Have you ever checked survey for thyroid hormone responsive element on the promoters of Notch or HES genes?

Zoeller: That is another really good question, and perhaps others here would have a different opinion on this. The TRE, thyroid hormone responsive element on genes is really very variable. There is a canonical sequence that people use in culture, but that canonical sequence is really an artificial sequence and is not very well represented among known thyroid hormone responsive genes, which is actually why we began in this empirical way.

We have actually looked for canonical TREs in a variety of genes, but the failure to find those does not convince me that they are not directly regulated by thyroid hormone.

Q: Thank you very much.

Koibuchi: Next question?

Q: Tom, that was a nice talk. I have a quick question. Did I miss which PCB or which Aroclor you are actually using?

Zoeller: It is Aroclor 1254.

Q: OK. And I wonder whether in fact you have looked at specific metabolites yet, or how you are hoping to go about that, because as you and I have discussed, no one has been able to show that there is any compound yet that we know of that actually binds the thyroid receptor directly. To my knowledge, it is always suggesting that it is changing circulating levels of thyroid. So, I wonder whether you have any evidence yet of which direction we should go with the PCBs.

Zoeller: I do not have a definitive answer. We have a two-pronged approach.

The first is *in vivo* we have cut down this Aroclor mixture to six congeners that represent three different classes that we believe will impact on this system. Those *in vivo* studies are ongoing.

At the same time, we have set up a thyroid hormone receptor-binding assay to begin to look at parents and congeners. So we are taking both of those approaches to determine which, if any, of the PCBs bind to the thyroid hormone receptor.

Q: And have you look at the polybrominated biphenyls?

Zoeller: No, but that is going to be a really important one. I think that polybrominated-diphenylethers really look a lot more attractive to the receptor than PCBs do.

Q: Exactly. Thank you.

Koibuchi: Next please?

Q: Thank you very much for an excellent presentation. I am very interested in the idea that one mechanism by which these environment disrupting chemicals could function is not just directly through the receptor or altering levels of hormone, but in fact because they are able to impact on the activity of different nuclear receptors, that they could do that through altering a common protein in the pathway such as the co-activator or co-repressor. And I would be interested in knowing if your laboratory or others have studied the effect of endocrine disrupters on co-activators or co-regulator proteins.

Zoeller: We actually have done that. We began to look at thyroid hormone regulation of SRC-1/ N-CoR, because those two are well known to interact with thyroid hormone receptor, and in fact in the postnatal day 15 brain, we see robust effects of thyroid hormone on both SRC-1 and N-CoR. PCB failed to affect the expression of those genes.

That was dissociation. But I think your point is well taken, and it may not be just the level of expression, but the way in which the receptor interacts with these co-activators, much like RU486 and the progesterone receptor can alter the way that progesterone receptor interacts with co-repressors and co-activators.

So in our *in vitro* studies, we are making sure to do some functional studies to determine whether it is an agonist, an antagonist, or a mixed agonist.

Q: Thank you.

Koibuchi: Just a quick question regarding the SRC-1 and co-repressor expression regulated by thyroid hormone, which part of the brain did you use? Because in our group we also did RNase protection assay and we did not see any effect of thyroid hormone in the cerebellum.

Zoeller: Right, we had to deal with that in review. There are some differences in the way we did the experiments. That was one thing.

I think also you looked in the cerebellum, and we have really focused on the cortex. We saw limited effects in CA — I know I am going to make a mistake here — in specific sub regions of the hippocampus, and I cannot remember if it was CA1 or CA3. It was a very localized effect, but it was very reproducible.

Koibuchi: Thank you. Any more questions? Thank you very much, Dr. Zoeller.