Impact of Polychlorinated Biphenyls on Amphibian Development and Endocrine Physiology

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I would also like to thank the organizers. Like the other speakers in this symposium, I also work on hormone action in amphibian development, but today I am going to focus on some recent work that we have done on the potential impacts of polychlorinated biphenyls as endocrine disrupting compounds on amphibian development and endocrine physiology.

It is clear that there are endocrine disruptive compounds that are present in the environment. What is not clear is whether or not these compounds actually effect animal development and physiology, and especially in amphibians there is very little information available on potential effects of these compounds on amphibian development.

Polychlorinated biphenyls or PCBs are compounds that have been shown to disrupt endocrine function in animals and humans. Their use in the USA was phased out in the late 1970s; however, they persist in the environment at high levels because they concentrate in the foodweb and they are resistant to biochemical degradation.

As I mentioned there are a number of studies that have been done in mammals, birds and also fishes that have shown endocrine disruption by PCBs. That is, they can disrupt hormonal signaling perhaps by interacting with proteins that bind hormones. This has been best studied in the case of the reproductive system, and it is well known that PCBs can have estrogenic-like effects.

I think it is worth pointing out that there is no evidence that PCBs or, to my knowledge, any industrially derived compounds can interact with thyroid hormone receptors with any significant affinity. Now that is not to say that there are not compounds out there that can do this, and I will discuss this in just a moment. But currently, the data at least for thyroid disruption, suggests that the disruption occurs at levels other than interactions with the nuclear receptors.

How are amphibians affected by PCBs? It is known that PCBs are teratogenic in amphibians and can also induce mortality, especially at high doses. We also know that PCBs can affect development, growth, and today I am going to show you some data that was published recently from my laboratory, that PCBs can actually affect behavior and may disrupt endocrine function in tadpoles.

My talk is divided into two parts. The first part focuses on a study that we did on a model PCB, that is TCB-77, and we were primarily focusing on disruption of the endocrine stress system in that study. The second part is a study where we have analyzed a number of different PCB congeners for the potential to interact with thyroid hormone binding proteins and thus disrupt thyroid system function in amphibians.

In the first study, we showed that exposure of tadpoles, and in this case grass frog tadpoles, *(Rana pipiens)*, to TCB-77, actually results in a reduction in activity levels. That is, they spend more time resting and less time feeding. There is a potential dose dependent effect here with increasing doses of TCB-77.

I want to mention that we and others have measured sediment levels of various PCB congeners throughout the Great Lakes region throughout Michigan, and there are high levels of these compounds present in sediment in Michigan, and the concentrations that we used in these studies and the studies that I will describe are within the range of concentrations that we found in the environment. We also measured several endocrine parameters, and this one is the concentration or content of corticosterone, and it is a primary stress hormone in amphibians. We found that exposure to PCB results in a decrease in corticosterone content.

Recently we have found that if you expose tadpoles to exogenous corticosterone or if you inhibit

corticosterone synthesis, you can influence feeding behavior. For example, corticosterone treatment increases feeding behavior, and inhibition of corticosterone synthesis reduces feeding behavior. So this is at least one potential mechanism for the behavioral changes that I showed you a moment ago; that is, the reduction in feeding behavior and the more time spent resting.

We also analyzed several other parameters of development and growth, and surprisingly we found that treatment of tadpoles, in this case grass frogs or wood frogs, with 77-TCB actually increased relative growth. This is important because this suggests that the compound is not having a generalized toxic effect, because these animals actually grew better in the presence of TCB-77.

But I want to point out that these are single species environment experiments. That is, they were raised in tanks with only that one species. In just a moment I am going to come back to this, because this is important for understanding how these compounds might affect animals in nature, because these two species occur together in the same ponds and are actually competitors for resources in the natural environment.

To come back to this slide, the reduction in corticosterone seen with higher PCB doses may be a mechanism for the growth enhancement, at least that is one hypothesis that we are testing because we know that if you treat tadpoles with exogenous corticoids you can reduce growth, whereas if you treat them with a corticoid synthesis blocker, you can actually enhance growth rate. So, this is at least one potential mechanism for the differences in growth seen in these single species environment experiments.

Now, this is the reason that I mentioned the single species experiment; we wanted to know whether tadpoles raised together, that is in an environment where they would compete for resources, which is more close to the natural situation, whether exposure to 77-TCB altered their growth rate. And the answer is yes.

This compound reduced their competitive ability, or their competitive response, and they grew worse in the presence of TCB than in the absence. So, if you raise them alone, they grow better, but if you raise them together where they have to compete for resources, they do not do as well. Is this a result of impaired behavior? Possibly. Can this impact competitive interaction in the population structure in nature? That is the question.

So to summarize this part of my talk, we have shown that this compound, 77-TCB, can reduce activity level and feeding rates, reduce whole-body corticosterone content, which may be associated with this reduction in feeding rates because we know that the stress axis plays a very important role in regulating appetite and feeding, not only in amphibians but in humans and other vertebrates as well. We see an enhancement of growth in a single species environment, but impaired growth in a competitive environment, which is more in tune with what is happening in the natural environment.

I did not show you this today, but we have also found that it can impair interrenal responsiveness to ACTH, which is the pituitary hormone that controls the interrenal glands, suggesting that their stress responses may also be impaired.

These are sub-lethal effects of exposure to PCBs that could potentially alter population structure in ways that you would not predict if you were doing single species environment experiments in the laboratory, or simply looking at either cellular or molecular mechanisms. I want to turn to potential roles of PCBs and their disruption of thyroid system function which we already know from the previous talks is very important in amphibian development.

PCBs have structures that are similar to thyroid hormones and have been suggested to be capable of interacting with thyroid hormone binding proteins. There is actually good data to show that they do interact with at least one thyroid hormone binding protein, transthyretin, which is a serum binding protein for thyroid hormone.

We know already that thyroid hormones are very important for development, especially brain

development, which Barbara Demeneix just mentioned, and we also know that they are very important for animal metamorphoses, not only anuran metamorphosis, which we have been focusing on today, but also metamorphosis of flat fishes and metamorphosis of salamanders.

This slide is useful for identifying potential sites for disruption of the thyroid system. Thyroid hormone production is controlled by the neuroendocrine system, that is the hypothalamus and pituitary. Once thyroid hormones are secreted from the thyroid gland they enter the blood where they are bound by the serum binding proteins.

Transthyretin or TTR is found in all vertebrates. Some vertebrates have other thyroid hormone binding proteins which I am not going to talk about today, but the TTR at least serves to transport thyroid hormone in the blood and forms a reservoir for the hormone. The hormone then enters the cells, perhaps through membrane proteins that are poorly understood, that may be amino acid transporters.

Once it enters the cell it can bind to a variety of different cytosolic binding proteins, most of which are enzymes. Some of these proteins are monodeiodinases which can convert thyroid hormone, that is, T4, to the more active form T3, or can degrade T4 and T3 to inactive forms. Then we have heard about this process here already where the thyroid hormone binds to nuclear receptors and can regulate transcription of genes.

So, the question is; where in these pathways do these compounds act, or where are the potential sites for disruption of signaling? Nearly all PCBs or PCB mixtures have been shown to disrupt thyroid function in mammals. A major site for disruption is binding to transthyretin, which results in a reduction in plasma T4 owing to the increased degradation and excretion of T4.

One important point to note is that mammalian TTRs are T4 specific binding proteins. However, amphibian TTR is a T3 specific binding protein as are the majority of vertebrate TTRs. In fact, the specificity for T4 evolved with the eutherian mammals.

This is a graph that shows you a competitive binding assay which we have used to study binding of PCBs to amphibian and mammalian TTRs, and it shows that T3 is much more potent than T4 in displacing radioactive T3. We wanted to know if PCBs could interact with amphibian TTR and also, if they did, then we wanted to compare the binding of the PCBs with predicted T3- or T4-like properties to both amphibian and mammalian TTRs to test the hypothesis that they are actually specific for different classes of PCBs.

Then, we wanted to determine if PCBs could alter development and/or thyroid function in tadpoles, that is; does this have any consequences for animal development and physiology? Finally, we wanted to determine if the PCBs with T3-like properties could bind to thyroid hormone receptors.

It is important to note that as I mentioned in the beginning of my talk, there is no evidence for PCBs having any high affinity interactions with thyroid hormone receptors. However, it is important to point out that the PCBs that have been tested for interactions with thyroid hormone receptors are primarily those have apparent T4-like properties. I will come back to that in just a moment.

These are some competitive binding assays that we have used to study interactions of PCBs with amphibian or bullfrog transthyretin. I just show you this slide to indicate that there are a number of PCBs that have relatively high affinity binding for amphibian or bullfrog transthyretin. If you compare the affinities, 32 nM here for T3, some of these PCBs have equal or even better binding affinity than T3.

This is a complicated table which actually summarizes a lot of data that I had on the previous slide. But I have simplified it by color-coding it, and I am going to tell you the major points. We used competitive biding assays to analyze the interactions of several PCBs that have been predicted to have either T3- or T4-like properties based on modeling by McKinney and Wallace.

In this column here we see the inhibition constants for bullfrog TTR and in this column the inhibition constants for human TTR. What you see in yellow is that those compounds that are predicted to be

T3-like have very high affinity for the bullfrog TTR, but very low affinity for the human TTR. You can see that here also: high affinity and low affinity; high and no activity whatsoever. By contrast, human TTR has very high affinity for those compounds predicted to be T4-like; by contrast, bullfrog TTR has very low affinity or no affinity for these T_4-like compounds.

So, although this is a limited data set, it suggests that these two hormone binding proteins, that is the mammalian versus the non-mammalian, or the bullfrog, have very different specificities for PCB congeners, and this could then result in different classes of PCBs interacting with these thyroid hormone binding proteins and thus causing thyroid disruption.

So, what is predicted from the mammalian studies would not necessarily translate to the amphibian, and vice versa in the case of thyroid disruption. This is a complicated graph which shows the changes in T3 binding capacity. We wanted to know whether or not these PCBs could have any effects *in vivo* in bullfrog tadpoles, and I am going to summarize the data for you.

This shows tadpoles that were treated in early pro-metamorphosis, late pro-metamorphosis and metamorphic climax. We only were able to do two PCBs at this stage. These are the PCBs from the previous table that were shown to have high affinity binding for bullfrog TTR except for this one, which I did not show on the table, but it is a hydroxylated PCB that we found also has high affinity binding.

This is a binding assay with different amounts of plasma. The blue shows the control, and the red here shows the reduction in binding by treatment with PCB 4008. To summarize the results here, PCB 4008 consistently decreased plasma T3 binding, but on the other hand PCB-128, which we showed had a high potency for binding to bullfrog TTR actually consistently increased plasma T3 binding. So it is really impossible to predict from the *in vitro* studies what you would actually get *in vivo*.

We also analyzed the effects on metamorphosis, T3, and brain T3 content. All of the PCBs tested slowed metamorphosis; T3 was included as a positive control. All of the PCBs tended to reduce plasma T3, although it was only significant for PCB 4008, and significantly, all of the PCBs reduced brain T3 content.

But surprisingly, T3 also did this. We know that T3 can induce 5 monodeiodinase expression in the brain which degrades T3. So it may be that the reduction in brain T3 content during metamorphosis is actually a normal thing, caused by the induction of the monodiodinase. Whether the PCBs act the same way by inducing 5 monodeiodinase, we do not know at this point.

Mechanisms of thyroid disruption by PCBs: competition for binding to TTR: yes. Alteration of metabolism, clearance of thyroid hormones: we know from mammalian studies that that is a yes. Do the PCBs compete for binding to plasma membranes, hormone transporters, or cytosolic thyroid hormone binding proteins? We do not know the answer for that, and we also do not know if some PCBs could potentially bind to thyroid hormone receptors.

Now based on the studies that I just showed you a minute ago, we predict that certain PCBs that were not tested previously for binding to thyroid hormone receptors may have T3-like properties. This is a very preliminary study that we did just recently using human thyroid hormone receptor β expressed in a neuroblastoma cell line to test whether or not any of these PCBs could interact with thyroid hormone receptors.

What this graph shows is that PCB-128 which I showed you earlier has high affinity for the bullfrog TTR actually caused a reduction in binding to the thyroid hormone receptor in this assay, but the other compounds which also have some affinity for the bullfrog TTR did not. So again, no one assay is predictive of what effect these compounds may have on physiology or development.

To summarize, PCBs can compete for T3 binding to bullfrog TTR. The T3- and T4-like properties of the PCBs that were predicted from molecular modeling were largely supported by our studies, although we have to do many more congeners to test this prediction further. PCBs slow metamorphosis; they can

alter plasma binding and also decrease T3 concentrations and T3 content in the brain. Finally, some PCBs, or at least one PCB with a T3-like property, may compete for binding for thyroid hormone receptors.

So I started my talk by saying that there was no evidence for any high affinity interactions thyroid hormone receptors. The data that I showed you here do not show that this is a high affinity interaction, because in fact those studies were done with 1.0 micromolar of the compound. But the findings at least suggests that some compounds that may have T3-like properties could potentially interact with thyroid hormone receptors and suggests further studies.

Do the PCBs pose a threat to amphibians and other wildlife populations? They can have lethal effects at high doses, but in nature most of the concentrations are much lower than that. But as I have shown you, they can have sub-lethal effects at low doses which can alter endocrine function, development, growth, and maybe even population structure in natural populations.

I would like to close by mentioning my collaborators, my graduate students Karen Glennemeier and Peter Schleuter who did a lot of these studies, Kiyoshi Yamauchi who supplied us with the recombinant bullfrog transthyretin; and these studies were supported by the Michigan Department of Environmental Quality. Thank you.

Q&A

Yoshizato: We have some time for discussion and questions, please.

Q: There is another plasma thyroid hormone binding protein known as thyroxin binding globulin. Are there data to indicate that thyroxin binding globulin also binds to PCBs, and if it does, how does that change the dynamics of PCB as a thyroid disrupter?

Denver: Yes, thyroxin binding globulin, or TBG, is found only in certain mammals; it is not found in amphibians or non-mammalian vertebrates. To my knowledge there is no evidence that PCBs can interact with any high affinity with TBG.

Q: Another question: you did mentioned that there were cytosolic thyroid hormone binding protein. Did you get a chance to look at that, to see whether that effects…

Denver: No, that is a good question. No, we have not been able to study that. But that is the next obvious thing to study, because these are thyroid hormone binding proteins. There are many of them as you know, and they could potentially be sites for interaction and disruption of thyroid hormone functions.

Q: The affinity of T4 and T3 to TTR is reversed between humans and amphibians. Do you have some molecular explanation for this reversed affinity?

Denver: I personally do not, but there is a group in Australia who has analyzed this problem and studied the structure of the protein and has provided an explanation, but I would not be able to discuss it in great detail. I could give you some references, however.

Q: In your study where you combine the species and have the opposite effect, we know that the corticosteroids are important in metamorphosis or at least they can affect it. Did you measure that outside of that data that you showed?

Denver: Yes, that is a good question. We did not measure corticosteroids in the competition experiment. However, we do know that when you increase tadpole density, that is, the number of animals competing for resources, that it increases corticosterone.

We think that actually is the reason for the growth retardation, because we know that if you do the same experiment with a corticoid synthesis inhibitor, it reverses that effect of the increased density of the tadpoles. So we think that the corticoid axis is very important in tadpole physiology and development.

Q: Is the concentration in the *in vivo* study much to the concentration in the *in vitro* binding study?

Denver: Yes, that is a good question. We tried to match the concentrations that we used *in vivo* to the concentrations that we found were able to displace binding *in vitro*.

I did not mention this, but the way that we administer these compounds is in the food, and we basically treat the food and then give the food to the animals. We did not, however, measure the tissue content or the body burden of PCBs in the animals after the experiment.

Q: Do you know anyone look at the PCB effect on the serum thyroid hormone level in mammals?

Denver: Yes, there has been a lot of work done on that, primarily in rats. As I mentioned briefly, most of the studies have shown that PCBs and mixtures of PCBs disrupt thyroid function, primarily reduce plasma thyroid hormone concentrations.

Q: And they expect basically the same mechanisms?

Denver: Yes, I hypothesize that the same mechanism is occurring, that is, displacement from TTR resulting in increased excretion by the kidney and then perhaps also increased glucurodination and sulfation in the liver, although that has not been tested in amphibians. So I think that the same mechanisms could be occurring, but different classes of PCBs would be causing the same types of effects.

Q: Thank you.

Yoshizato: Due to the time limitation, now I would like to close this session. With the cooperation of the audience here we have had a very exciting, stimulating and enjoyable session. Thank you very much.