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An Aryl Hydrocarbon Receptor from the Salamander *Ambystoma mexicanum* Exhibits Low Sensitivity to 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin

Jenny Shoots^{a,†}, Domenico Fraccalvieri^b, Diana G. Franks^c, Michael S. Denison^d, Mark E. Hahn^c, Laura Bonati^b, and Wade H. Powell^{a,*}

^aBiology Department, Kenyon College, Gambier, OH 43022 USA

^bDepartment of Earth and Environmental Sciences, University of Milano-Bicocca, 20126 Milan, Italy

^cBiology Department, Woods Hole Oceanographic Institution, Woods Hole, MA 02543 USA

^dDepartment of Environmental Toxicology, University of California, Davis, CA 95616 USA

Abstract

Structural features of the aryl hydrocarbon receptor (AHR) can underlie species- and population-specific differences in its affinity for 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). These differences often explain variations in TCDD toxicity. Frogs are relatively insensitive to dioxin, and *Xenopus* AHRs bind TCDD with low affinity. Weak TCDD binding results from the combination of three residues in the ligand-binding domain: A354 and A370, and N325. Here we sought to determine whether this mechanism of weak TCDD binding is shared by other amphibian AHRs. We isolated an AHR cDNA from the Mexican axolotl (*Ambystoma mexicanum*). The encoded polypeptide contains identical residues at positions that confer low TCDD affinity to *X. laevis* AHRs (A364, A380, and N335), and homology modeling predicts they protrude into the binding cavity. Axolotl AHR bound one-tenth the TCDD of mouse AHR in velocity sedimentation analysis, and in transactivation assays, the EC₅₀ for TCDD was 23 nM, similar to *X. laevis* AHR1β (27 nM) and greater than AHR containing the mouse ligand-binding domain (0.08 nM). Sequence, modeled structure, and function indicate that axolotl AHR binds TCDD weakly, predicting that *A. mexicanum* lacks sensitivity to TCDD toxicity. We hypothesize that this characteristic of axolotl and *Xenopus* AHRs arose in a common ancestor of the Caudata and Anura.

Graphical Abstract

*Corresponding Author: powellw@kenyon.edu, Phone: +1 740-427-5396, Fax: +1 740-427-5741.

†Present address: Department of Pharmacology, Weill Cornell Medical College, New York, NY 10021 USA

Author Contributions

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Introduction

The aryl hydrocarbon receptor (AHR), a member of the bHLH-PAS protein family¹, plays important roles in a growing list of developmental and physiological processes² in conjunction with structurally diverse ligands, including both synthetic compounds and natural products³. AHR is best known as a nuclear receptor that mediates the toxic effects of environmental contaminants, including dioxin-like chlorinated hydrocarbons and polynuclear aromatic hydrocarbons³. The prototypical xenobiotic agonist is 2,3,7,8 tetrachlorodibenzo-*p*-dioxin (TCDD), which binds to the AHR with high affinity. Following ligand binding, the AHR translocates from the cytoplasm to the nucleus, shedding a complex of cytoplasmic chaperones⁴, and binding the ARNT protein. The AHR:ARNT heterodimer acts as a transcription factor, binding specific regulatory elements associated with the promoters of target genes and interacting with transcriptional cofactors and the RNA polymerase II initiation complex⁵. Prominent targets include phase I and phase II detoxification enzymes comprising the “AHR gene battery”⁶, but many additional transcripts are induced or repressed in direct or indirect fashion^{7, 8}. Interactions with other nuclear proteins and pathways also mediate the biological effects of AHR^{9, 10}. AHR-mediated alterations in gene expression patterns are thought to play a central role in toxicity, especially since organisms in which functional AHR expression has been abrogated or reduced are resistant to TCDD toxicity^{11–13}.

More subtle differences in AHR structure and function can also underlie variations in the sensitivity of different species to TCDD toxicity. Low affinity binding of TCDD by the AHR is associated with reduced toxicity in mouse strains, humans, and several bird species, and recent studies ascribe this low affinity to a small number of amino acids within the ligand binding domain (LBD) that are modeled to protrude into the ligand binding pocket. In mice, for example, a single point mutation within the LBD (A375V; the *AHR^d* allele) is associated with reduced TCDD affinity in the dioxin-insensitive DBA/2J strain^{14–17}. Human

AHR, which has relatively low TCDD affinity, resembles the mouse AHR^d protein with valine at the aligned position^{14–16}. Studies comparing the amino acid sequence and TCDD affinity of bird AHRs identified two critical residues within the ligand binding domain of the chicken and common tern (*Sterna hirundo*, a sea bird): V325 and A381 in the tern AHR are responsible for a 7-fold lower TCDD affinity than the chicken AHR (I325 and S381)¹⁸ and a concomitant reduction in TCDD sensitivity. The combination of residues at homologous positions of AHRs from other birds directly affects binding affinity, representing a robust biomarker of their susceptibility to the toxicity of dioxin-like compounds in dozens of species^{19–21}.

All frogs and toads studied to date are extremely insensitive to toxicity of dioxin^{22–24}. Consistent with the relationship between AHR affinity and TCDD sensitivity observed in mammals and birds, AHRs from the African clawed frog (*Xenopus laevis*) exhibit extremely low TCDD affinity²⁵. Low dioxin affinity results from a unique combination of specific residues within the LBD at the same positions shown to govern variations in TCDD affinity in mammal and bird AHRs²⁶. It is hypothesized that low TCDD affinity is a property shared with other frog and toad AHRs²³, but the AHR binding properties and TCDD sensitivities of other amphibian groups are not well understood.

Because relatively high affinity AHRs are typical of both teleosts, which diverged from the vertebrate lineage prior to amphibians, and of birds and mammals, which evolved later, we hypothesize that the loss of high TCDD affinity emerged uniquely within the amphibian lineage. Less clear is the degree to which this phenotype is shared among the different amphibian groups other than Anurans (frogs and toads). The apparent insensitivity of the tiger salamander (*Ambystoma tigrinum*) to TCDD toxicity²⁷ suggests that it may also be common among members of Caudata, a clade distinct from order Anura^{28, 29}. To help address this question from a mechanistic standpoint, we cloned a cDNA encoding AHR from another salamander the Mexican axolotl (*Ambystoma mexicanum*), evaluating its sequence, its modeled LBD structure, and the binding and transactivation characteristics of the encoded protein to both TCDD and a candidate endogenous ligand, 6-formylindolo[3,2-*b*]carbazole (FICZ).

From both ecological and biomedical perspectives, the Mexican axolotl is uniquely well suited for these studies. Amphibian populations are in global decline³⁰ as a result of various habitat alterations, including chemical contamination, outright destruction, and climate change³¹, and *A. mexicanum* is no exception^{32, 33}. In nature, this critically endangered species³⁴ is restricted to the Xochimilco Wetland near Mexico City^{32, 33}, which is contaminated with numerous endocrine disrupting compounds³⁵ and may be subject to atmospheric dioxin deposition from widespread garbage incineration in the surrounding megalopolis³⁶. However, axolotls are readily maintained in captivity³⁷, and *A. mexicanum* is a widely used laboratory model of vertebrate development and limb regeneration³⁸. Extensive genomic resources, including a deep-sequenced transcriptome and a genetic map and marker collection, combined with the availability of genetically well characterized animals from the NSF-funded Ambystoma Genetic Stock Center^{39, 40}, position *A. mexicanum* as an excellent salamander model for molecular toxicology.

Materials and Methods

AHR Agonists

TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin) was obtained from ULTRA Scientific, and FICZ (6-formylindolo[3,2-*b*]carbazole) from Enzo Life Sciences. [1,6-³H]TCDD (33.1 Ci/mmol, >99% radiopurity) was obtained from Chemsyn Science Labs (Lenexa, KS). AHR agonists were dissolved in dimethylsulfoxide (DMSO; Fisher Scientific) previously extracted with dextran-coated charcoal (Sigma).

cDNA cloning

A. mexicanum larvae at the 2–3 cm life stage were obtained from the *Ambystoma* Genetic Stock Center (Lexington, KY; supported by NSF grant #NSF-DBI-0951484). Total RNA was isolated from a whole animal using RNA STAT-60 (Tel-Test, Inc.). A partial cDNA encoding AHR was amplified by RT-PCR using the GeneAmp Gold RNA PCR Reagent Kit (Applied Biosystems). Reverse transcription of 1 µg total RNA was primed with random hexamers as directed by the manufacturer. The degenerate primers (AHR-A2 and AHR-B1; Table S1) were used successfully in previous studies to amplify partial AHR cDNAs from both fish and amphibians^{25, 41–43}. PCR conditions were: 95°/10 min; 43 cycles of [95°/15 sec, 50°/30 sec, 72°/60 sec]; 72°/7 min. RT-PCR products were cloned into pGEM-T Easy (Promega) and sequenced. The identity of the clones as AHRs was confirmed by BLASTx.

Partial cDNAs were used to design gene-specific primers (Table S2) for the amplification of 5' and 3' ends of each cDNA using the SMARTer RACE cDNA amplification kit and Advantage HF2 DNA polymerase (Clontech) under the following cycling conditions: 94°C/2min.; 30 cycles of [94°/30 sec; annealing/30 sec; 72°/3 min]; 72°C/7'. Annealing temperatures varied with primer sequence. A reaction containing both a 5'-and 3'-RACE primer specific for AHR cDNA was included as a positive control. Sequencing was performed by Retrogen (San Diego, CA). Contiguous full-length sequences were determined with the phred/phrap/cross-match algorithms in MacVector Assembler (MacVector, Cary, NC) using 4–5 clones of each overlapping PCR product.

Sequence alignment and phylogenetic analysis

The translated AHR sequence was aligned with additional AHR amino acid sequences using CLUSTALX⁴⁴. The Neighbor Joining algorithm⁴⁵ with 1000 bootstrap samplings was used to construct a phylogenetic tree. Sequences used in the phylogenetic analysis are indicated in Table S3.

Homology Modeling

The structural model of *A. mexicanum* AHR LBD (residues 283–389) was generated as previously described for *X. laevis* AHRs²⁶, using the NMR structures of the PAS B domains of HIF2α and ARNT (PDB entries 1P97 and 1X0O) as templates in MODELLER version 8v1^{46–48}. The optimal model among the 100 generated was selected on the basis of the best DOPE SCORE⁴⁹. The quality of the model was evaluated using PROCHECK⁵⁰ and the ProSA validation method⁵¹. Secondary structures were attributed by DSSPcont⁵².

The binding cavity within the modeled LBDs was characterized using the CASTp server⁵³. Visualization of the models was accomplished using PYMOL⁵⁴.

AHR Expression Construct

The open reading frame of the *A. mexicanum* AHR was synthesized by Epoch Life Science, Inc. (Missouri City, TX) with XhoI and NotI restriction sites at the 5' and 3' ends, respectively. The sequence was subcloned into the pCMVTNT expression plasmid (Promega). Additional expression vectors used in this study include *X. laevis* AHR1 β ²⁵, and chimeric AHR consisting of *X. laevis* AHR1 β with the ligand binding domain of the mouse AHR²⁶, all in pCMVTNT. *X. laevis* ARNT1 (Open Biosystems, Huntsville, AL) and mouse AHR (provided by Dr. C. A. Bradfield, University of Wisconsin) are in pSPORT.

Velocity sedimentation analysis

TCDD binding was measured by velocity sedimentation on sucrose gradients as described previously²⁶. AHRs were synthesized *in vitro* using the T_NT Quick Coupled Transcription/Translation System (Promega) and diluted 1:1 in MEDMDG buffer (25 nM MOPS, pH 7.5, 1 mM EDTA, 5 mM EGTA, 20 mM Na₂MoO₄, 0.02% NaN₃, 10% glycerol, 1 mM DTT). Lysates were incubated for 18 h at 4° with 2 nM [1,6-³H]TCDD. Proteins were separated on 10–30% sucrose gradients (prepared with MEDMDG) in a Beckman VTi65.2 vertical tube rotor at 65,000 rpm for 90 min. Gradients were fractionated, and the radioactive content of each fraction was determined by liquid scintillation counting in a Beckman LS5000TD instrument. Non-specific binding was measured using T_NT reaction mixtures containing empty pCMVTNT vector [unprogrammed lysate (UPL)]. Specific binding was calculated by subtracting the radioactivity of UPL fractions from that of the corresponding fractions comprising the peak of total binding by proteins of the correct sedimentation coefficients. [¹⁴C]catalase was included on each gradient as an internal sedimentation marker.

Transactivation assay

The transcriptional response of *A. mexicanum* AHR to TCDD and FICZ was measured in a reporter gene assay as described previously^{25, 26}. COS-7 cells were maintained at 37° with 5% CO₂ in Dulbecco's modified Eagle's medium (DMEM; Sigma) and 10% fetal bovine serum (Life Technologies). Cells were cotransfected with expression constructs for each AHR, *X. laevis* ARNT1, the *Renilla* luciferase transfection control pRL-TK (Promega), and the firefly luciferase reporter pGudLuc6.1, derived from the mouse *CYP1A1* enhancer⁵⁵. 30,000 cells were plated in each well of a 48-well plate. After 24 h, 50 ng of an AHR plasmid, 50 ng of the ARNT plasmid, 20 ng of the reporter construct, and 3 ng of pRL-TK were transfected into triplicate wells using Lipofectamine 2000 (Life Technologies). The total amount of transfected DNA was kept constant (300 ng) by addition of pCMVTNT plasmid containing no insert. 5 h after transfection, cells were treated with DMSO vehicle (0.5%) or graded nominal concentrations of agonist up to 500 nM, as previously described for *X. laevis* AHRs^{25, 26, 56}. Cells were lysed after 24 h of TCDD exposure or 3 h of FICZ exposure. FICZ is readily metabolized by the CYP1 enzymes it induces, and the shorter exposure time enables maximum induction of target genes in both cultured cell lines^{57, 58} and transactivation assays²⁶. Luminescence was measured using the Dual Luciferase Assay

kit (Promega) and a TD 20/20 luminometer (Turner Designs). Luminescence values are reported as the ratio of firefly luciferase units to *Renilla* luciferase units [relative luciferase units (RLU)]. The fractional response was then calculated for each AHR at each agonist concentration by subtracting the relative luminescence of vehicle-treated cells from each value and determining its ratio the maximal response level in each concentration-response experiment⁵⁹. The mean and standard error for the fractional response at each concentration were calculated and analyzed by nonlinear regression to determine a single EC₅₀ value for each AHR⁵⁹, constraining the background response to 0 and the maximal response to 1. Nonlinear regression and descriptive statistics were performed using Prism v.6.0b (GraphPad). Calculated r^2 values of the fitted curves resulting from each non-linear regression analysis are provided in the figure legends.

Results

Using RT-PCR with degenerate primers and RACE PCR, we isolated a cDNA for an AHR from the Mexican axolotl *Ambystoma mexicanum*. The corresponding transcript contains 5847 nt plus a polyA tail. An open reading frame of 2564 nt encodes a polypeptide of 853 amino acid residues with a predicted molecular mass of 95.5 kDa. The sequence has been deposited in GenBank with accession number [KM660691](#). The axolotl AHR shares 59% amino acid identity with *X. laevis* AHR1 α and AHR1 β and 50% identity with the mouse AHR. Phylogenetic analysis of the well-conserved N-terminal half (bHLH plus PAS domains; Fig. 1) demonstrates that the sequence is monophyletic with vertebrate AHR1s and is most closely related to the partial sequence derived previously from mudpuppy (*Necturus maculosus*⁴³), another salamander.

We constructed the homology model of the *A. mexicanum* AHR LBD using the PAS B domains of HIF2 α (26% sequence identity in the aligned region) and ARNT (21% identity) as templates, an approach described previously for the *X. laevis* AHRs as well as several fish, mammalian and avian AHRs (Fig. 2)^{26, 60–62}. Both PROCHECK and ProSA analyses indicated that the quality of the model compared very favorably with the previous efforts. 93% of the residues have values of the backbone dihedral angles (ϕ and ψ) within the range of the most favored areas of the Ramachandran plot; the overall G-factors were -0.19 ; and the ProSA z-scores (-4.02) were within the range observed for native protein structures of similar size (Figure S1). Homology models of *A. mexicanum* AHR, *X. laevis* AHR1 β , mouse AHR and chicken AHR share well conserved overall fold and secondary structure elements (Fig. 2B). The DSSPcont attribution of secondary structures (Fig. 2A–C) confirms the common basic composition of a five-strand antiparallel β -sheet (strands G β , H β , I β , A β , and B β), two short alpha helices (C α and D α), one short 3_{10} -helix (E α), and a long α -helical connector (F α), all linked by less ordered loops. CASTp analysis predicts the presence of an internal cavity of about 430 Å³ (Fig. 2C), comparable in both size and shape to the predicted cavities of *X. laevis* AHRs²⁶ as well as mammalian and avian AHRs with high TCDD affinity^{60–62}. Residues N325, A354, and A370 within the LBD of the *X. laevis* AHR1 β are associated with low TCDD affinity, and mutagenesis to more mouse-like (A354S and N325S) or chicken-like (A370S) residues substantially increased TCDD binding²⁶. These residues are conserved at the aligned positions of *A. mexicanum* AHR, N335, A364, and A380 (Fig. 2A), and the homology model also predicts that they protrude into the ligand

binding cavity of the axolotl protein (Fig. 2C). The model identifies two additional residues that differ from high affinity mouse or chicken AHRs and could potentially affect the characteristics of the putative binding pocket, M329 and I339, both in F α (Fig. 2). However, the M329 side chain appears to extend laterally along the cavity core, while I339 seems unlikely to systematically impact ligand binding, since this position contains Met in both low affinity *X. laevis* AHR1 β and high affinity mouse and chicken AHRs. Taken together, the LBD model and sequence suggest the hypothesis that like *X. laevis* AHRs, *A. mexicanum* AHR binds TCDD with low affinity.

We measured TCDD binding by the *A. mexicanum* AHR by velocity sedimentation analysis on sucrose density gradients following incubation with 2 nM [3 H]TCDD (Fig. 3)²⁶. The axolotl AHR exhibited specific [3 H]TCDD binding, with a detectable peak above the unprogrammed T_NT lysate (UPL) surrounding fraction 13. This level of binding was comparable to the low affinity *X. laevis* AHR1 β . Binding peaks of each amphibian protein were approximately one-tenth of either mouse AHR or a previously characterized²⁶ chimeric *X. laevis* AHR1 β containing the LBD from the mouse *AHR^{b-1} allele*¹⁴ (Fig. 3B). Thus, axolotl AHR binds TCDD weakly compared to well-characterized, high-affinity AHRs.

We also compared transactivation properties of axolotl AHR with high- and low-affinity receptors in reporter gene assays. COS-7 cells were co-transfected for heterologous expression of an AHR, *X. laevis* ARNT, and a firefly luciferase reporter gene under control of the mouse CYP1A1 enhancer⁶³. In keeping with the transactivation phenotype predicted by the primary sequence, modeled structure, and TCDD binding properties, *A. mexicanum* AHR exhibited low responsiveness to TCDD, with an EC₅₀ comparable to *X. laevis* AHR1 β and nearly 300-fold greater than that of the mouse:frog chimera (Figure 4, Table 1). This chimeric AHR, which contains the high affinity LBD from mouse *AHR^{b-1} allele*¹⁴, provides a good basis for comparison with amphibian AHRs because it is a high-affinity receptor well characterized in previously published studies²⁶, it is robustly expressed in this heterologous system under control of the same promoter as the *X. laevis* and axolotl receptors, and it substantially preserves the helix-loop-helix and PAS A domains (important regions for ARNT dimerization⁶⁴) from *X. laevis*, the source of ARNT in these experiments.

Although the exact EC₅₀ values for the amphibian AHRs are subject to some uncertainty as a result of TCDD solubility issues at the highest concentrations, the overall trend of low sensitivity of these AHRs is unmistakable. The EC₅₀ for transactivation of axolotl AHR by FICZ, a candidate endogenous agonist^{57, 58}, was also greater than that of the chimeric AHR (Figure 5, Table 1), although as observed previously for the *X. laevis*^{26, 57} and bird⁶⁵ AHRs, the difference is much less pronounced, about 45-fold following 3 h exposure.

Discussion

Frogs and toads (order Anura) are substantially insensitive to the toxic effects of TCDD^{22, 23}. Although rapid elimination contributes to TCDD insensitivity following the onset of feeding in tadpoles^{23, 66}, the low TCDD affinity of *Xenopus laevis* AHRs is well established and likely plays a substantial mechanistic role in all life stages²⁵. Low TCDD

affinity is attributed to three specific amino acid residues bearing side chains that are modeled to protrude into the ligand binding pocket²⁶. Mutation of these residues to resemble high-affinity receptors from mouse or chicken increased TCDD binding and the sensitivity of co-transfected cells to reporter gene induction by TCDD²⁶. In this study, we sought to determine whether an AHR from a member of Caudata, the amphibian order that includes salamanders and newts, shares the fundamental properties of *X. laevis* AHRs that predict low TCDD binding and toxicity. To this end, we isolated a cDNA encoding an AHR from the Mexican axolotl, *Ambystoma mexicanum* and performed structural and functional analyses.

Comparative studies of vertebrate AHRs reveal complexity in the evolutionary history of this gene family, with several instances of gene duplication giving rise to multiple AHR paralogs in various species^{67, 68}. Most prominently, a gene duplication in a common gnathostome ancestor gave rise to AHR1 and AHR2, and possession of one or more copies of each paralog are ordinarily found in many vertebrate classes, including cartilaginous fish, bony fish, reptiles, and birds^{67, 68}. Commonly studied mammals like mice and humans harbor only the AHR1 gene, having apparently lost AHR2, and knockout of the single AHR in mice abrogates toxicity of AHR agonists^{11, 69–71}. However, surveys of recently completed genome projects suggest that AHR2 is retained in diverse mammalian species, including platypus, opossum, and cow⁶⁸. The role of the corresponding gene products in xenobiotic toxicity, development, and physiology is not yet characterized. We found only one AHR in *Ambystoma mexicanum*, a member of the AHR1 clade. Similarly, only AHR1 orthologs have been reported in the mudpuppy *Necturus maculosus*⁴³ and the frogs *Xenopus laevis* and *Xenopus tropicalis*²⁵, and there is no clear evidence for the existence of AHR2 genes in current versions of the two *Xenopus* genomes. We hypothesize that AHR2 was lost in an early amphibian ancestor and the lack of AHR2 maintained in recently derived species, but studies of the *A. mexicanum* and additional amphibian genomes will be required to determine whether the apparent absence of AHR2 can be verified for the axolotl and whether it is a common feature of the entire order or class.

Like *X. laevis*, the axolotl AHR1 possesses low affinity for TCDD, and the structural features of frog AHR that underlie low affinity—N325, A354, and A370²⁶—are also found in the aligned positions of the *A. mexicanum* protein (N335, A364, A380; Fig. 2). The partial AHR cDNA sequence from mudpuppy lacks much of the LBD, but the fragment includes Asn at the position aligned with N335 of axolotl AHR and N325 of *X. laevis* AHR1 β . In contrast, the AHRs of teleost fish bind TCDD with much higher affinity than frog AHRs. AHR2 from zebrafish (*Danio rerio*), for example, exhibits a K_d of 1.1 nM⁷², while *X. laevis* AHR1 α has apparent K_d of 47.2 nM, and AHR1 β is unsaturable²⁵. The AHRs of mammals and many birds also have high affinity for TCDD (e.g. chicken AHR K_d =0.8 nM¹⁸, mouse AHR K_d =2.4 nM^{18, 25}). Taken together, these observations suggest that an ancestral tetrapod AHR exhibited relatively high TCDD affinity, a property that was lost in amphibians after their divergence from the common lineage but maintained in other tetrapods. The conservation of the low affinity residues and phenotype in both salamander and frog AHRs suggests the hypothesis that they arose early in amphibian evolution, prior to the divergence of the orders Caudata (salamander) and Anura (frog and toad), and were

conserved in both lines thereafter. Examination of additional amphibian AHRs from representatives of both orders will be necessary to confirm this hypothesis.

To more precisely establish the evolutionary timing of the emergence of low TCDD affinity AHRs, examination of the third amphibian order, Gymnophiona, would be helpful. Comprised of limbless amphibians known as caecilians, this order exhibited a Gondwanan distribution⁷³, and current molecular phylogenies agree that caecilians were the first group to diverge from the amphibian lineage, prior to the split between Caudatans and Anurans^{28, 29}. If one or more caecilians harbor AHRs that contain the three low-affinity residues and exhibit low TCDD affinity, then the emergence of this genotype and phenotype could potentially be dated to a common ancestor of all extant amphibians. Similarly, a comparative survey of caecilians, frogs, and salamanders could establish the evolutionary timing and degree of conservation of the loss of AHR2 in amphibians.

TCDD binding and transactivation characteristics of the axolotl AHR predict that like frogs, *A. mexicanum* is relatively insensitive to TCDD toxicity. Consistent with this prediction are the relatively high loads of PCBs and TCDD that salamanders have been observed to tolerate. For example, nominal TCDD concentrations up to 3.0 µg/L (9.3 nM) were defined as sub-lethal in larvae of the tiger salamander (*Ambystoma tigrinum*)²⁷. In comparison, the LC₁₀₀ for TCDD in zebrafish, among the least sensitive fish species to dioxin toxicity^{74, 75}, is no more than 1.55 nM⁷⁶. Cave salamanders (*Proteus anguinus*) have also been shown to survive exceptional body and tissue loads of total PCBs of environmental origin⁷⁷.

The loss of high TCDD affinity in amphibian AHRs and associated low sensitivity to TCDD toxicity raises interesting questions about the role of AHR in the development and physiology of these animals. In addition to its toxicological functions, the AHR is known to play numerous important roles in mammals and fish, such as the regulation of cell cycle and circadian rhythms and the development of multiple organ systems, including liver, cardiovascular, reproductive, and immune systems². Although mammalian and axolotl AHRs exhibit dramatic differences in sensitivity to transactivation by TCDD, the EC₅₀ values for the candidate endogenous ligand FICZ are much more similar. This observation mimics those made previously for frog^{26, 57} and bird⁶⁵ AHRs. This differential ligand response is consistent with recent mutagenesis and functional activity studies demonstrating that FICZ can bind within the LBD of the mouse AHR in a fashion different than that of TCDD and related HAHs⁷⁸. Taken together, these studies suggest that distinct, non-toxicological functions of AHR may be more strongly conserved in amphibians than those involving xenobiotics.

This study contributes an important mechanistic perspective to salamander toxicology and amphibian toxicology more generally. Relative to fish, birds, and mammals, reliable molecular biomarkers of contaminant susceptibility, toxicity, and exposure status are lacking in amphibians, especially in species other than *Xenopus laevis*⁷⁹. Similar studies in additional species representing multiple amphibian taxa will be important to determine the degree to which low affinity for TCDD or other xenobiotic AHR agonists is a widespread and predictable phenotype of amphibians that can be broadly generalized for risk assessment purposes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

AHR	aryl hydrocarbon receptor
bHLH-PAS	basic helix-loop-helix Per ARNT Sim
FICZ	6-formylindolo[3,2- <i>b</i>]carbazole
HAH	halogenated aromatic hydrocarbon
LBD	ligand binding domain
PCB	polychlorinated biphenyl
RLU	relative luciferase units
TCDD	2,3,7,8 tetrachlorodibenzo- <i>p</i> -dioxin
UPL	unprogrammed lysate

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Figure 1.

Phylogenetic analysis of *A. mexicanum* AHR. Amino acid sequences of bHLH and PAS domains of each AHR sequence were aligned in Clustal X2. A tree was inferred by the Neighbor-Joining method. Numbers at the branch points represent the bootstrap values based on 1000 samplings. Accession numbers of the sequences are found in Table S3 (online supplemental material).

Sequence and structural model of the *A. mexicanum* AHR LBD. (A) Sequence alignment produced by Clustal W. Only residues that differ from the *A. mexicanum* sequence are shown; dots indicate conserved positions. Variable residues that protrude into the modeled binding cavity are boxed. Color scheme for residues: red, acidic; blue, basic; purple, polar; yellow, Cys; brown, aromatic; green, hydrophobic; orange, Ser, Thr; gray, Pro; white, Gly. Secondary structures attributed by DSSPcont are indicated below: light gray bars for helices and dark gray bars for β -strands. (B) Comparison of cartoon renderings for modeled LBDs of *A. mexicanum* AHR (blue), *X. laevis* AHR1 β (green), mouse AHR (orange), and chicken AHR (magenta). (C) Cartoon representation of the modeled *A. mexicanum* AHR LBD. Residues that both differ from the high-affinity mouse or chicken AHRs and protrude into the modeled binding cavity are labeled blue and shown as sticks. The light green shaded area delineates the molecular surface of the binding cavity identified by CASTp.

Figure 3.

TCDD binding by *A. mexicanum* AHR. (A) Velocity sedimentation analysis. Indicated AHR proteins were synthesized in rabbit reticulocyte lysates, incubated with 2 nM [3 H]TCDD, and fractionated on sucrose density gradients. Radioactivity (dpm) of each fraction was quantified. The experiment was replicated three times. A representative result is displayed. Sedimentation marker [14 C]catalase was present in fractions 14–21 of all replicates. Inset graph depicts the binding peaks of axolotl and frog AHRs on a re-scaled y-axis. (B) Specific binding. TCDD binding was quantified by summing the total radioactivity comprising each peak in panel A. Specific binding was determined by subtracting the radioactivity found in the same fractions in unprogrammed lysate containing no AHR. Bar graph plots mean

specific binding relative to that of frog AHR1 β . Values represent mean $\pm$ standard error; n=3.

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Figure 4.

TCDD-induced transactivation activity of *A. mexicanum* AHR. COS-7 cells were co-transfected with pGudLuc6.1 reporter construct, pRL-TK transfection control construct, and expression plasmids for *X. laevis* ARNT1 and the indicated AHR. Cells were treated with DMSO or TCDD for 24 hours. Each plotted value represents the mean of four replicate assays $\pm$ standard error. (A) Transactivation activity of an AHR is given in relative luciferase units (RLU), the ratio of firefly to *Renilla* luciferase activity at each concentration of TCDD. (B) Fractional induction. For each AHR, relative luciferase expression at each TCDD concentration was normalized to the maximal response, which was assigned a value of 1. Nonlinear regression was used to calculate EC_{50} values for each AHR. r^2 values for the fitted curves are 0.99 for *A. mexicanum* AHR, 0.99 for frog AHR1 β , and 0.87 for the chimeric AHR.

Figure 5.

FICZ-induced transactivation activity of *A. mexicanum* AHR. Fractional induction of reporter gene expression by each AHR following 3-hr FICZ exposure was determined as described in the legend for Figure 1. Values represent the means $\pm$ standard error for three replicates. r^2 values for the fitted curves are 0.99 for *A. mexicanum* AHR, 0.65 for frog AHR1 β , 0.64 for chimeric AHR.

Table 1EC₅₀ for AHR-mediated Reporter Gene Induction¹.

AHR	TCDD (24 h)	FICZ (3 h)
<i>A. mexicanum</i> AHR	22.72 nM	0.45 nM
<i>X. laevis</i> AHR1 β	26.79 nM	0.14 nM
Chimeric AHR	0.08 nM	0.01 nM

¹Values for each AHR were calculated by nonlinear regression of mean fractional induction values for three replicate dose response experiments depicted in Figures 4 and 5.