

Topic 1.14

Nonmammalian nuclear receptors: Evolution and endocrine disruption*

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Abstract. Most research to identify endocrine-disrupting chemicals and their impacts has relied on mammalian models or in vitro systems derived from them. But nuclear receptors (NRs), the proteins that transduce hydrophobic hormonal signals and are major mediators of endocrine disruption, emerged early in animal evolution and now play biologically essential roles throughout the Metazoa. Nonmammalian vertebrates and invertebrates, many of which are of considerable ecological, economic, and cultural importance, are therefore potentially subject to endocrine disruption by synthetic environmental pollutants.

Are methods that rely solely on mammalian models adequate to predict or detect all chemicals that may disrupt NR signaling? Regulation of NRs by small hydrophobic molecules is ancient and evolutionarily labile. Within and across genomes, the NR superfamily is very diverse, due to many lineage-specific gene and genome duplications followed by independent divergence. Receptors in nonmammalian species have in many cases evolved unique molecular and organismal functions that cannot be predicted from those of their mammalian orthologs. Endocrine disruption is therefore likely to occur throughout the metazoan kingdom, and a significant number of the thousands of synthetic chemicals now in production may disrupt NR signaling in one or more nonmammalian taxa. Many of these endocrine disruptors will not be detected by current regulatory/scientific protocols, which should be reformulated to take account of the diversity and complexity of the NR gene family.

ENDOCRINE DISRUPTION AND NUCLEAR RECEPTOR EVOLUTION

Nuclear receptors (NRs) are evolutionarily related multidomain proteins that bind to specific DNA motifs and up- or down-regulate transcription of specific genes. Most NRs bind tightly to hydrophobic hormones and other ligands, which effect a conformational change in the receptor, causing it to switch into transcriptionally active or repressive modes [2]. Many plant and synthetic chemicals, including pesticides, plastics components, and industrial by-products, have been found to bind to and thereby mimic, block, or otherwise disrupt the natural activity of vertebrate NRs [2,3].

The premise of this paper is that current biological systems carry the traces of the evolutionary history by which they were created. A historical perspective on the nuclear receptor superfamily allows us to organize and interpret the diversity of NRs and their properties in a biologically meaningful way, to discover patterns in the functional and evolutionary dynamics of NRs, and to predict the distribution of receptors in taxa that have not been well studied. Evolutionary analysis can, therefore, yield useful

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knowledge for understanding and predicting the effects of environmental endocrine disruptors (EDs). In this paper, I review knowledge of nuclear receptor evolution and survey the NR complement of major nonmammalian taxa, with a focus on implications for efforts to identify and manage endocrine-disrupting synthetic chemicals.

ANCIENT AND CONTINUING RECEPTOR DIVERSIFICATION

The NR superfamily, like other gene families, is the product of an ongoing process of genomic diversification within and among species. There are over 1000 nuclear receptor sequences in the SMART database of protein family domains [4]. The genomes of humans, *Drosophila melanogaster* and *Caenorhabditis elegans* contain 49, 21, and ~270 NRs, respectively [5]. NRs have a conserved domain structure and identifiable sequence homology to each other in their DNA-binding domains (DBDs) and ligand-binding domains (LBDs). BLAST searches indicate no statistically significant similarity to any other gene family. This pattern indicates that all NRs evolved by descent from a single ancestral NR—the origin of which remains unknown—through a series of gene duplications.

Because gene sequences diverge independently after duplication, the evolutionary relationships among NRs form a tree-like nested hierarchy, and the techniques of phylogenetic systematics can be used to reconstruct the history of NR diversification. As shown in Fig. 1, analysis of a large set of nuclear receptor sequences indicates that there are five evolutionary classes of nuclear receptors: (1) the vitamin D receptor (VDR), ecdysone receptor (EcR), retinoic acid receptors (RARs), thyroid hormone receptors (ThRs), and a number of receptors involved in cholesterol and xenobiotic metabolism; (2) the peroxisome proliferators activated receptors (PPARs) and several related orphans, which are involved in cell differentiation, development, and metabolism; (3) NGFI-B and related “orphan” receptors (receptors with no known ligand), which function in development and immunity; (4) the reproductive receptors, including steroidogenic factor 1 (SF-1), the gonadal and adrenal steroid and related receptors of vertebrates, and germ-cell nuclear factor (GCNF); and (5) the retinoid x receptors (RXRs) and related orphans, most of which are involved in development. Most of the groups are well supported, although certain relationships (such as placement of the RARs and ThRs) are sensitive to alignment and analysis parameters. This classification, like an earlier parsimony analysis [6], is different from the results of an earlier NR phylogeny/classification, which used a smaller number of sequences and the less reliable neighbor-joining method of phylogenetic inference [7]. The most important differences are the existence of distinct classes 1 and 2 and the close relationship of the gonadal/adrenal steroid receptors to SF-1 and GCNF.

NR diversity is ancient and therefore widespread. Five groups of receptors, all in the RXR and SF-1 classes, have been identified in both diploblastic Cnidaria (jellyfish, corals, and anemones) and triploblastic animals (Fig. 1). This finding indicates that the NR superfamily had already achieved considerable diversity before the advent of the bilaterally symmetric metazoa. By the time the lineages of protostomes (arthropods, nematodes, mollusks, annelids, etc.), and deuterostomes (chordates and echinoderms) split from each other at least 670 million years ago [8], the fundamental diversity of the nuclear receptor superfamily had been established: 15 groups of receptors, representing all five classes, are found in the genomes of both *D. melanogaster* and *Homo sapiens*. Only DAX-1, SHP, ThRs, and RARs of vertebrates have no known protostome orthologs; based on current evidence, these are “recent” chordate-specific novelties. These findings indicate that most NRs are ancient and distributed throughout the Eumetazoa, making the vast majority of animal species potential targets for endocrine disruption.

There have also been many lineage-specific gene duplication events that created new receptors in certain taxa. For example, there was considerable additional diversification in the vertebrate lineage, indicated by the presence of two to four retained duplicates in vertebrates for each arthropod receptor (Fig. 1). This pattern is consistent with the serial genome duplication hypothesis, which states that the vertebrate genome is the result of two serial rounds of genome duplication [9]. Duplications that gen-

erated taxon-specific receptors also occurred in other lineages, including teleosts, nematodes, and arthropods, as detailed later in this paper.

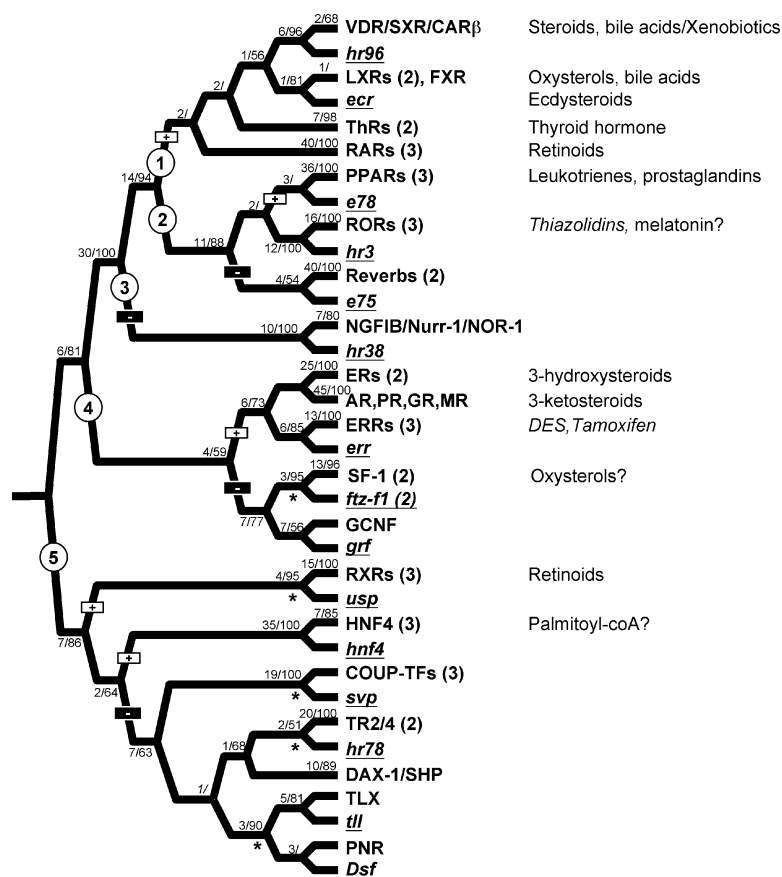


Fig. 1 Phylogeny of the nuclear receptors. A reduced consensus is shown of the 12 most parsimonious trees from analysis of 163 NR sequences of *H. sapiens*, *D. melanogaster*, *Bombyx morii*, *Gallus gallus*, *Brachydanio rerio*, *Xenopus laevis*, and *Acropora millepora*. Roman type indicates vertebrate sequences; insect NRs in underlined italic. Asterisks indicate the branch to which Cnidarian sequences connect. Numbers in parenthesis show the number of human paralogs in each group. Ligands, when known, are to the right of the tree; synthetic ligands are in italics, and question marks indicate unconfirmed results. On this phylogeny, an ancestral ligand-activated receptor is the most parsimonious reconstruction, as shown by four dark boxes (–) that indicate loss of ligand binding if the ancestral receptor was ligand-dependent, and five light boxes (+) that indicate gain of ligand binding if the ancestral receptor was ligand-independent. Node labels show unweighted branch support values [53]/bootstrap values >50 %. Sequences were downloaded from the EBI SMART database [4]; the DBDs and LBDs were extracted and then aligned using ClustalX 1.7 [54]. Parsimony analysis was conducted using PAUP* [55] with a stepmatrix that downweights conservative substitutions and a heuristic search strategy of 100 random taxon additions followed by TBR branch swapping. Reconstruction of change in ligand-binding status assumes parallelism is more likely than reversal; different assumptions would change the placement but not the number of changes.

THE ANCESTRAL NUCLEAR RECEPTOR: ORIGIN AND FUNCTION

When did the first nuclear receptor appear, and what was its function? No nuclear receptors have been found in any non-metazoan species, including the completely sequenced genomes of eubacteria, ar-

chaea, yeast, and plants. Because NRs have been identified in the three major divisions of eumetazoans—cnidarians, protostomes, and deuterostomes—we can conclude that the first NRs emerged some time after the divergence of metazoans from fungi but before the Cnidarian-Bilateria divergence (Fig. 2).

It has been argued that the ancestral NR was a ligand-independent receptor and that ligand binding was acquired later during NR evolution [10]. This scenario is possible, but there is no evidence to favor it over the alternative explanation of an ancestral ligand-activated receptor. Both ligand-binding and ligand-independent receptors have been identified in protostomes, deuterostomes, and cnidarians [11]; the inferred age of ligand-binding is therefore the same as the inferred age of ligand-independent receptors. Mechanistically, either scenario is plausible, although neither a complete loss of ligand activation nor a gain of ligand binding from a more ancient orphan has been unambiguously established in the evolution of NRs.

The most reliable means for inferring the characteristics of ancient proteins is by reconstructing ancestral functional states using phylogenetic methods. Based on the parsimony criterion, the best-supported hypothesis of ancestral function is the one that requires the fewest parallel gains or reversals on a gene family tree. As Fig. 1 shows, the NR phylogeny weakly supports an ancestral ligand-binding receptor if the tree is rooted between groups 4 and 5; the same would be true if it were rooted with an ancient ligand-binding NR like RXR in a basal position. If the tree were rooted on an ancient orphan receptor like COUP, an ancestral orphan receptor would be weakly favored. There is presently no way to determine which of these receptors should be used as the root, so current evidence does not support either scenario over the other.

Whatever the ligand-binding characteristics of the first NR were, there is no doubt that ligand binding is very ancient. Ligand binding must have evolved very early in the radiation of the animal phyla from an ancient eumetazoan ancestor, as shown by the presence of conserved ligand binding in Cnidaria and Bilateria. It also must have very early in the diversification of the NR superfamily into its five classes, as Fig. 1 shows. Virtually all receptors inherit the capacity to be ligand-activated and there-

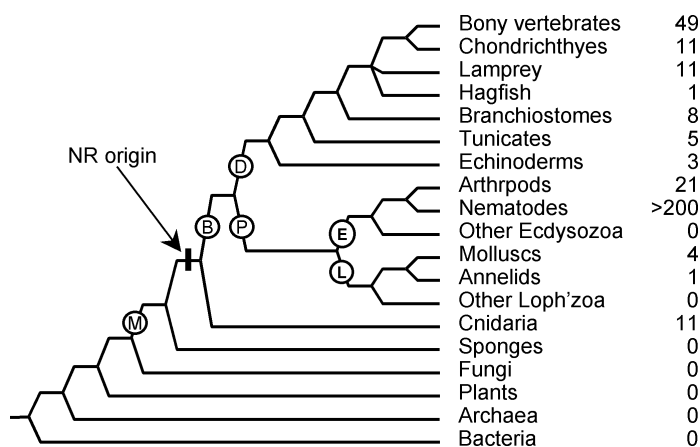


Fig. 2 Taxonomic distribution of nuclear receptors. A reduced phylogeny of living kingdoms and selected metazoan taxa is shown, with the number of NR paralogs that have been sequenced from each group. Number of NRs in jawed vertebrates, arthropods, and nematodes are derived from the complete genomes of *H. sapiens*, *D. melanogaster*, and *C. elegans*, respectively. Absence of NRs in plants, fungi, and prokaryotes is also supported by whole genome sequences. Other NR numbers in metazoa are inferred from screening efforts and are likely to underestimate the total NR complement of each group. M, Metazoa; B, Bilateria; P, Protostomia; D, Deuterostomia; E, Ecdysozoa; L, Lophotrochozoa, within the last century. These substances serve as NR ligands because their stereochemistry allows them to fit by chance into NR binding pockets, which have not been selected over the long term to exclude binding of these substances.

fore are potential targets for endocrine disruption. Further, the ancient nature of ligand binding makes it likely that endogenous ligands and endocrine disruptors will be discovered for many orphan receptors, as has already occurred for many [12,13].

PLASTICITY IN THE EVOLUTION OF LIGAND BINDING

Since the first ligand-binding receptor, there has been remarkable diversification of receptors' ligand-specificity. All NR ligands are hydrophobic and of relatively small size, but beyond these commonalities they are very diverse. High-affinity endogenous ligands for NRs include a variety of cholesterol-derivatives (steroid hormones, vitamin D, bile acids and other cholesterol metabolites), retinoids, modified amino acids (thyroid hormone), prostaglandins, leukotrienes, and several kinds of fatty acids and benzoates.

Many of the gene duplications by which NRs diversified have been followed by the evolution of novel ligand affinity. This is true even for the relatively recent chordate-specific duplications. For example, the vertebrate gonadal/adrenal steroid receptors and the members of the VDR and LXR/FXR groups are each activated by a unique hormone or group of hormones that do not bind to the others with high affinity. (The estrogen receptors α and β are an exception, although they do vary in their affinity for endocrine disruptors [14]).

Receptors have also evolved affinity for new ligands after speciation events. The ecdysone receptor of arthropods is orthologous to the FXR, LXR α , and LXR β of vertebrates, which bind oxysterols and bile acids [15], but vertebrates do not respond to ecdysteroids. A similar pattern is found with more recent divergences: the principal ligand for the teleost androgen receptor, for example, is not testosterone (T) or 5 α -dihydro-T as it is in tetrapods, but 11-ketoT [16]. It is thus impossible to predict the ligands or EDs that will bind to a receptor by extrapolation from other—even closely related—receptors or model species from other classes or phyla.

PARALLEL EVOLUTION OF LIGAND SPECIFICITY AND PROMISCUITY

There is a surprising lack of consistency in the evolution of certain types of ligands on the NR phylogeny. Steroids are bound by the adrenal/gonadal steroid receptors as well as by NRs in the distant VDR group, but the receptors that intervene in the tree between these groups are orphans or bind nonsteroidal ligands. Similarly, RXRs and the distantly related RARs are both activated by retinoic acids. The ability to bind steroids or retinoids must, therefore, be either an ancestral character lost and then regained in the more recent receptor group or a parallel acquisition in the NR classes in which it is found.

The use of steroids and similar molecules as high-affinity endocrine ligands has evolved in parallel in numerous other gene families. In plants, brassinosteroids bind to membrane receptor kinases and function as critical regulators of plant development [17]. Steroids regulate reproduction in the fungus *Achlya ambisexualis* [18], but fungi lack NRs. And in nitrogen-fixing symbiotic bacteria, the unrelated protein NodD is an intracellular transcriptional regulator that is activated by flavonoids excreted by the partner plant. NodD has a mode of action very similar to that of the NRs; NodD is antagonized by several pesticides and industrial chemicals that also disrupt vertebrate steroid receptors [19]. All these receptors are potential—and ecologically important—targets for environmental endocrine disruptors (EDs).

That several unrelated families of proteins independently evolved the ability to act as high-affinity receptors for steroids and chemically similar compounds suggests that these substances have intrinsic properties that pre-adapt them to long-distance signaling functions. Steroids are small and lipid soluble, which allows them to cross cell membranes. Their chair-like polycyclic structure makes them rigid enough to establish stereospecific binding relationships with receptors, and their structural complexity allows for the synthesis of hundreds of unique compounds by substitution of small moieties at various points around the steroid nucleus. Finally, steroids are metabolically cheap to make, since cholesterol

and other steroid-precursors are essential to membrane biochemistry and other fundamental processes in eukaryotes.

NRs, in turn, have evolved their affinity for one or a few specific ligands in the context of the endogenous and exogenous chemical background of the organisms of which they are a part. Virtually all NRs are potentially promiscuous, as shown by the phenomenon of endocrine disruption and the existence of synthetic pharmaceuticals that bind receptors with very high affinity. This pattern of endogenous specificity and xenobiotic promiscuity is likely to have an evolutionary explanation. The function of most receptors is to transduce an endocrine signal, which indicates the internal state or environmental context of the organism, into a specific cellular response. (There are several broad-specificity receptors, but these regulate metabolism of the ligands that bind to them rather than more fundamental developmental or physiological responses). Receptors that are activated or antagonized by other compounds present in the organisms' endogenous chemistry or diet would add noise into the information processing capacity of the cell and organism, impairing its ability to regulate its development or respond to environmental conditions. Natural selection is, therefore, expected to drive receptors towards greater specificity for their primary ligands. In contrast, xenobiotic drugs and industrial chemicals have been created within the last century. These substances serve as NR ligands because their stereochemistry allows them to fit by chance into NR binding pockets, which have not been selected over the long term to exclude binding of these substances.

This promiscuity for evolutionarily novel ligands predicts that a nontrivial portion of recently developed hydrophobic synthetic chemicals of small to moderate size will bind to and disrupt nuclear receptors. There are an estimated 87 000 synthetic substances in commerce, plus thousands more formed and released as by-products of chemical production, use, and combustion [20]. Many of these—including polymers and hydrophilic molecules—are unlikely to be NR ligands. But a substantial number have shapes that may attract them to the hydrophobic cores of NRs. The affinity of novel ligands for receptors is often low, but pharmacokinetic differences and simultaneous exposure to many EDs may make environmental exposures significant [21].

DIVERSIFICATION OF ORGANISMAL FUNCTIONS

The regulatory roles played by NRs at the organismal level also reveals considerable evolutionary plasticity. The EcR of arthropods, the master regulator of insect metamorphosis, is orthologous to a group of three hepatic receptors (FXR, LXR α , and LXR β), which regulate cholesterol homeostasis in vertebrates. It is not closely related to the thyroid hormone receptor, which regulates metamorphosis in amphibians. Similarly, the SEX-1 receptor of nematodes is involved in sex determination, but its ortholog E78a functions in insect metamorphosis [22].

It is, therefore, not possible to predict from distantly related mammalian models the phenotypic consequences that will ensue when a receptor is activated or disrupted by endocrine-disrupting chemicals. This makes the design of *in vivo* assays for environmental disruption of nuclear receptors difficult in taxa for which we do not yet know the natural functions of nuclear receptors.

BRIEF SURVEY OF NUCLEAR RECEPTOR DIVERSITY

In the following sections, I review the distribution of NRs in major nonmammalian taxa (Fig. 2). (Birds, reptiles, and teleosts are treated only briefly, because they are discussed in detail by Crews and colleagues in this volume.) For each group, I describe the identified NRs, based on taxonomically restricted protein BLAST searches of the National Center for Biological Information's nonredundant (NR) sequence database [23]. With the exception of species whose genomes have been completely sequenced, these descriptions are almost certainly incomplete: the methods used to isolate NRs—PCR and nucleic acid hybridization—are subject to false negatives, particularly when sequences have diverged greatly since an ancient common ancestor. Identified receptors therefore give a lower bound on

the actual NR set in any taxon. I also describe what is known about the function of these receptors, emphasizing evolutionary implications, and briefly discuss knowledge of and potential for endocrine disruption in each taxon.

CNIDARIA

Cnidarian genomes have been found to contain 11 nuclear receptors, including phylogenetic orthologs of the vertebrate receptor groups RXR, COUP, tailless (TLL), the testicular receptors TR2 and TR4, and SF-1 [10,24]; several of the other Cnidarian genes are so divergent that they cannot be clearly assigned to ortholog groups. Only one of the Cnidarian receptors, the RXR of the jellyfish *Tripedalia cystophora*, has been functionally analyzed: like its mammalian ortholog, it activates gene expression in the presence of very low concentrations of 9-cis retinoic acid [11], although its organismal role remains unknown. This receptor is therefore a candidate for endocrine disruption; if the others are also ligand-regulated, they may also be ED targets.

There may be other ligand-binding receptors in Cnidaria yet to be discovered. Vertebrate hormone-type steroids have been identified in mass coral spawns in concentrations that correlate with reproductive/developmental function [25], suggesting the possibility that these hormones are playing signaling functions in Cnidaria and that receptors that transduce their signals may yet be discovered. No studies of endocrine disruption in Cnidaria have been conducted, despite the fact that these animals play important roles in some marine food webs.

ECDYSOZOANS: ARTHROPODS, NEMATODES, AND OTHER PHyla

Bilaterally symmetric animals are divided phylogenetically and taxonomically into two major groups: the protostomes (in which gut development occurs mouth first) and deuterostomes (in which the anus develops first). The protostomes are in turn divided into two great clades: the Ecdysozoa (organisms that molt, including arthropods, nematodes, priapulids, and numerous others) and the Lophotrochozoa (organisms with specific kinds of larvae or mouth structures, including annelids, mollusks, kinorhynchans, sipunculans, and numerous other marine phyla) (Fig. 2).

The ecdysozoans include the model organisms *D. melanogaster* and *C. elegans*, the genomes of which have been completely sequenced. The fruit fly contains 21 nuclear receptors, most of which are involved in molting and metamorphosis, embryonic development, or behavior [26]. Several of these genes appear to encode arthropod or dipteran-specific receptors—including three developmental NRs that have no ligand-binding domains (Knirps, Kruppel, and Egon), as well as duplicates of the SF-1 gene and of the photoreceptor nuclear receptor gene (PNR).

The nematode *C. elegans* presents perhaps the most radical example of lineage-specific NR diversity. *C. elegans* possesses orthologs of most of the NRs identified in arthropods, with the notable exception of EcR and RXR [27], although an RXR ortholog has been detected in the nematode *Diriofilaria immitis* [28]. In addition to the previously recognized NRs, however, the *C. elegans* genome contains an additional >200 nuclear receptors, and a large number are also present in *Caenorhabditis briggsae* [27]. Phylogenetic analysis and gene mapping data show that the vast majority of these genes cluster together, indicating that the excess NRs are largely due to tandem gene duplications that occurred within the nematode lineage. Few of these receptors have been functionally assayed, but most have intact ligand-binding domains with hydrophobic pockets, suggesting that they may be regulated by ligands.

No information on the nuclear receptor content of other ecdysozoan phyla are available, and little research has been carried out on endocrine disruption in any ecdysozoans. It is likely, however, that signaling via some ecdysozoan receptors can be disrupted by synthetic chemicals: polycyclic aromatic hydrocarbons potentiate ecdysone signaling through the EcR, and numerous phytochemicals are EcR antagonists [29,30]. In addition, metabolites of the insect control chemical methoprene disrupt the ac-

tion of juvenile hormone—another master regulator of moulting and metamorphosis, the receptor for which has not been identified—and are also potent agonists of the mammalian RXR [31]. Insects, crustaceans, spiders, nematodes, and other ecdysozoans play major roles in terrestrial and marine food webs, and some species in these groups—from butterflies to lobsters—are of considerable economic and cultural importance.

LOPHOTROCHOZOANS: MOLLUSKS, ANNELIDS, ETC.

The nuclear receptors of lophotrochozoans have not been well studied. Published complete or partial sequences are available only for a COUP of an annelid and a COUP-TF, an RXR, and an NGFI-B ortholog from a mollusk. No studies whatsoever are available on other lophotrochozoans, such as sipunculans, bryozoa, or echiura.

Recently, however, it has been discovered that one major group of receptors previously thought to be unique to vertebrates is in fact ancient and widespread. Thornton, Need, and Crews [56] have found that mollusk neural and reproductive tissues express a nuclear receptor gene that is unambiguously orthologous to the vertebrate estrogen receptor. This finding indicates that the gonadal and adrenal steroid receptor family—the estrogen, androgen, progestin, and corticoid receptors—are far more ancient than previously thought, with their origin predating the protostome-deuterostome divergence. The ER must have been lost in the ecdysozoan lineage leading to flies and nematodes, presumably due to evolutionary changes that made the receptor's functions dispensable. We can therefore predict that all lophotrochozoans and all deuterostomes—and possibly some ecdysozoans, as well—will possess estrogen receptor orthologs. Their endocrine systems may thus be subject to endocrine disruption by ER agonists and antagonists.

The discovery of an ER in mollusks provides a potential mechanism for the previously unexplained findings of vertebrate-type steroids, steroid-binding activity, and steroid-induced reproductive changes in mollusks (reviewed in ref. [32]). Further, endocrine disruption and reproductive impairment has been documented in mollusks after exposure to pollutants that are vertebrate estrogens [33]. If steroids do play a role in mollusk reproduction via the mollusk ER, then some of the xenobiotic estrogens and antiestrogens now circulating in the environment have the potential to cause significant damage to populations of mollusks and other ER-containing lophotrochozoan phyla, which are important in aquatic and terrestrial ecosystems.

INVERTEBRATE DEUTEROSTOMES

Deuterostomes are composed of two major phyla: the echinoderms (sea stars and urchins) and the chordates (tunicates, arrow worms, branchiostomes, and vertebrates). Very little work has been done to characterize the diversity, function, or disruption of NRs in echinoderms. Only three nuclear receptors—the orphans SF-1, COUP, and TR2/4—have been identified in echinoderms. Steroids, including testosterone and estradiol, however, have been found to be produced by sea stars and to vary with the animal's reproductive cycle, suggesting a signaling role in these organisms (reviewed in ref. [31]). The finding of an ancient origin for the estrogen receptor indicates that echinoderms are likely to possess ER orthologs. If they regulate the response to these steroids, these receptors may be targets for endocrine disruption.

A bit more information is available on NRs in urochordates, the most basal chordates. A preliminary study identified fragments that are apparently orthologs of RXR, SF-1, and estrogen-related receptors (ERR1) in urochordates [10]. In addition, full-length RXR and RAR sequences have been identified in the tunicate *Polyandrocarpa misakiensis*, and the expressed proteins, like their vertebrate orthologs, have been shown to activate transcription in the presence of retinoic acid [34]. In addition, a thyroid hormone receptor has been identified in the tunicate *Ciona intesimalis*. Surprisingly, it did not activate transcription in the presence of triiodothyronine (T3), the vertebrate ThR ligand, despite the

fact that thyroxine, the precursor of T₃, plays a well-established role in *Ciona* metamorphosis [35]. If this result is not an artifact, it suggests a possible shift during urochordate or vertebrate evolution in the receptor's affinity for different metabolites of thyroxine.

Tunicates are the most basal taxa in which RAR and ThR have been identified, indicating that these receptor groups first emerged before tunicates split from the rest of the chordate lineage. Only a single member of each group has been recovered from species in this subphylum, a pattern consistent with the serial genome duplication hypothesis. RARs and ThRs are, therefore, expected to be present in all chordates—including hemichordates, urochordates, and cephalochordates—and these receptors may be targets for disruption by synthetic chemicals.

The cephalochordate genus *Branchiostoma*—including the species known as amphioxus—are the invertebrates most closely related to vertebrates. Single members of the NR groups SF-1, RXR, COUP, RAR, ERR, PPAR, FXR, and TR2/4 have been identified and sequenced in branchiostomes. Like its vertebrate ortholog, the amphioxus RAR activates transcription in the presence of retinoic acid [36]. Each receptor appears to be present in amphioxus in a single copy—again consistent with serial genome duplications, with both duplications occurring after the amphioxus-vertebrate divergence.

JAWLESS AND CARTILAGINOUS FISHES

Hagfish, lamprey, and elasmobranchs are the most basally branching vertebrates. In hagfish, a PCR screen found one ortholog each of COUP-TFs, PPARs, ERRs, and an unidentified steroid receptor [10]; other nuclear receptors are likely present but were not identified.

Lamprey have been studied in more detail. One RXR, three PPARs, two ERRs, one NGFI-B and a COUP-TF ortholog were found in a preliminary screen [10]. Subsequently, an estrogen receptor, a progesterone-like receptor, and a corticoid receptor were identified in the sea lamprey *Petromyzon marinus*. Phylogenetic analysis indicated that lampreys are not expected to possess orthologs of the androgen, mineralocorticoid, and estrogen receptor beta, because these NRs were produced by duplications in the jawed vertebrate lineage after its divergence from jawless fishes about 435 million years ago [37]. This picture is consistent with the vertebrate serial genome duplication hypothesis, with one duplication occurring before the divergence of lamprey from jawed vertebrates and the other occurring after. The ancestral steroid receptor was inferred by phylogenetic methods and structure–function prediction to have functioned as an estrogen receptor, despite the fact that estrogens are the terminal hormones in the steroid biosynthetic pathway. The PR and AR are therefore duplicates of an ancient ER gene, which subsequently evolved high affinity for progestins and androgens, thereby turning intermediates in the estrogen synthesis pathway into bona fide hormones.

Very limited research has been conducted on the NRs of cartilaginous fishes. Sharks have an estrogen receptor, and also apparent orthologs of the androgen and glucocorticoid receptors, although the sequences of the latter are so short that orthology to other steroid receptors cannot be ruled out. In addition, two PPARs, two Reverbs, two ERRs, and two COUPs have been identified in a preliminary screen of shark cDNA [10]. The likely presence of orthologs to a variety of ligand-binding receptors indicates that cartilaginous fishes may be targets for a wide range of endocrine disruptors. It is also clear that some receptors in sharks have evolved lineage-specific ligand preferences. For example, the principle mineralocorticoid is aldosterone in mammals, cortisol in teleosts, but 1 α -hydroxycorticosterone in cartilaginous fishes [38].

TELEOST FISHES

Teleosts, the major group of aquatic bony fishes, appear to possess all the receptor types found in mammals, although some NRs have not been specifically studied. For example, teleosts have orthologs of all six steroid receptors found in humans and mice, and they have a full complement of RARs, RXRs, COUPs, and thyroid hormone receptors. In fact, due to an apparent whole-genome duplication that

caused the expansion of a large number of gene families deep in the teleost lineage, followed by the retention of some duplicates and the apparent loss of others [39], bony fish have more NRs than most mammals, birds, and reptiles do. For example, several teleost species have been found to have two androgen receptors [40], two progesterone receptors [40], and three estrogen receptors [41] in contrast to the one, one, and two members of each group, respectively, that are found in tetrapods. As sequencing of teleost genomes continues, it is likely that most teleosts will have a set of NRs larger than tetrapods' by a factor of up to two.

There has been functional diversification of teleost NRs at both organismal and molecular levels. Estrogens and estrogenic pesticides, for example, can reverse the primary sexual differentiation of male fish, a phenomenon unknown in mammals [42]. Like the teleost androgen receptor, which has unique ligand affinity (see above), teleost progesterone receptors are activated primarily by $17\alpha,20\beta$ -progesterone and $17\alpha,20\beta,21$ -trihydroxyprogesterone, not progesterone as they are in tetrapods [43,44]. Teleost receptors also have quantitatively different relationships with endocrine disruptors than their mammalian orthologs: endosulfan, dieldrin, and methoxychlor bind to the ER α of trout but not to those of human, mouse, chicken, or lizard [45].

BIRDS, REPTILES, AND AMPHIBIANS

The proliferation of nuclear receptors largely stopped before the divergence of tetrapods into its major classes, so most birds, reptiles, and amphibians have the same complement of NRs as mammals do. There are exceptions, however: the frog *Xenopus laevis*, an apparent tetraploid species, has retained duplicates of some mammalian NRs, such as the RARs, SF-1, and ER α [46,57].

Although the number of NRs has remained more or less stable, there has been considerable functional diversification of receptors in these vertebrate lineages. At the organismal level, thyroid hormone regulates metamorphosis in amphibians, but it regulates basal metabolism, behavior, and neurological development in nonmetamorphic vertebrates. At the molecular level, the benzoate X receptors (BXR) of *X. laevis* are activated by benzoic acids, but their mammalian ortholog PXR/SXR binds steroids with no affinity for benzoates [47]. The potency of synthetic endocrine disruptors also varies considerably from that in mammals: chicken estrogen receptors, for example, have affinity for the pesticide chlordane 100-fold higher than in the rat [3]. Endocrine disruption has already been documented in populations of nonmammalian vertebrates at environmental concentrations (i.e., refs [48,49]).

IMPLICATIONS FOR ENDOCRINE DISRUPTION

The evolutionary insights discussed in this paper have two major implications for endocrine disruption assessment and policy, which currently rely largely on testing in model vertebrates to identify individual EDs that interact with a few NRs. For example, U.S. EPA's Endocrine Disruptor Screening Program is using assays on mammals, amphibians, and teleosts to identify disruptors of the estrogen, androgen, and thyroid disruptors only [20].

The first insight is this: NRs have diversified so extensively in the many metazoan lineages that scientific and policy efforts focused on a few receptors in a few vertebrate classes cannot be expected to protect all species from all kinds of endocrine disruption. Since their origin, NRs have been diversifying independently in all the major metazoan lineages over many billions of years of parallel evolutionary time. Each receptor in each taxonomic lineage has the potential, often realized, to be functionally unique. It is therefore not possible to predict with confidence the existence, ligands, and functions of NRs in taxa that have not been specifically and empirically evaluated. The more distantly related a species is from mammalian models, the longer the evolutionary time over which its receptors have been diversifying, and the less reliable extrapolations from mammals will be. Programs to identify EDs using vertebrate models are therefore likely to falsely judge as non-EDs chemicals that are in fact ligands for nonmammalian NRs. The broad diversity of ligands for which NRs have evolved affinity implies that a

nontrivial number of the many thousands of synthetic chemicals now in production are likely to have the potential to disrupt NR-mediated signaling.

Endocrine disruptor screening and testing programs should be expanded to evaluate a broader array of nonmammalian taxa. Such programs might be expanded to include, for example, testing of several classes each of arthropods, mollusks, annelids, cnidarians, echinoderms, and nonmammalian vertebrates. Such a program would be very demanding of time and resources, and it would leave numerous phyla and taxonomic classes empirically unevaluated. Still, it is the only way to gain even preliminary insight into the effects of synthetic chemicals on the endocrine systems of nonmammalian taxa and avoid false negative judgments of chemical safety.

The second insight relates to the impact of limited knowledge on ED science and policy. Current understanding of NRs and their functions throughout the Metazoa barely scratches the surface of their actual diversity. The full nuclear receptor complement is known for only two model invertebrates, and these are unfortunately relatively closely related to each other. In poorly studied taxa like echinoderms, mollusks, and annelids, our understanding of the NRs present and their functions ranges from very little to absolutely nothing. Even in such well-studied model organisms as humans, mice, and flies, we remain uncertain of the ligands and physiological functions of many receptors. To some extent, knowledge gaps can be repaired with more research. But the gaps are so great that we cannot even design an adequate program to identify EDs and their impacts in most nonmammalian taxa. The physiological/developmental roles of most NRs are unknown in the majority of invertebrate taxa; these roles have diversified so thoroughly that the endpoints used as indicators of endocrine disruption based on their function in mammals are seldom appropriate in invertebrates. At present, then, we lack the background knowledge to develop *in vivo* assays for endocrine disruption in most nonmammalian taxa, and extrapolation across phyla, classes, and receptors is often unreliable.

Efforts to identify as many endocrine disruptors as possible through broader testing are undoubtedly valuable for scientific and regulatory purposes. But we cannot be confident that any feasible testing program will be able to identify all—or even the majority—of EDs in animals. EDs that interact with receptors in bacteria, fungi and plants are even further beyond the ken of such a program. The likely outcome of current testing programs' focus on vertebrates and a small number of nuclear receptors is that a nontrivial number of EDs will be judged safe, though they may be capable of causing significant ecological damage. This situation will not be remedied soon, and exposure to endocrine-disrupting chemicals will continue as long as policies require each and every endocrine disruptor to be individually and positively identified before action can be taken to reduce its production or use.

To cope with our lack of knowledge on metazoan endocrinology and endocrine disruption, environmental policies can be based on the precautionary principle: action should be taken to prevent plausible environmental hazards even in the absence of specific and definitive evidence of damage [50]. Rather than requiring evidence of endocrine disruption for each and every chemical, as current policies do, a precautionary framework would prioritize classes of substances that are particularly likely to contain EDs, based on their structure, size, hydrophobicity, and persistence. Policies would then focus on avoiding the production and use of members of these classes by developing and implementing safer materials and processes [51], based on the principles of sustainability and green chemistry [52].

CONCLUSIONS

Over the course of billions of years, receptors and the organisms in which they function have been evolving endocrine systems that are astonishing in their complexity, diversity, and biological importance. Protecting the life forms and ecosystems that have emerged from this evolutionary process will require that our policies take account of these characteristics. Expanding testing programs is useful, but true prevention of endocrine disruption will require a new approach to chemicals management that begins with a recognition of the limits as well as the strengths of current scientific tools and knowledge in the face of nature's diversity and complexity.

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