Deiodinases: how non-mammalian research helped shape our present view.

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Abstract

Iodothyronine deiodinases are enzymes capable of activating and inactivating thyroid hormones (THs) and have an important role in regulating TH action in tissues throughout the body. Three types of deiodinases (D1, D2 and D3) were originally defined based on their biochemical characteristics. Cloning of the first cDNAs in the 1990s (Dio1 in rat and dio2 and dio3 in frog) allowed to confirm the existence of three distinct enzymes. Over the years, increasing genomic information revealed that deiodinases are present in all chordates, vertebrates and non-vertebrates, and that they can even be found in some mollusks and annelids, pointing to an ancient origin. Research in non-mammalian models has substantially broadened our understanding of deiodinases. In relation to their structure, we discovered for instance that biochemical properties such as inhibition by 6-propyl-2-thiouracil, stimulation by dithiothreitol and temperature optimum are subject to variation. Data from fish, amphibians and birds were key in shifting our view on the relative importance of activating and inactivating deiodination pathways, and in showing the impact of D2 and D3 not only in local but also whole body $T_3$ availability. They also led to the discovery of new local functions such as the acute reciprocal changes in D2 and D3 in hypothalamic tanycytes upon photostimulation, involved in seasonal rhythmicity. With the present possibilities for rapid and precise gene silencing in any species of interest, comparative research will certainly further contribute to a better understanding of the importance of deiodinases for adequate TH action, also in humans.

Key words: deiodinase, vertebrates, evolution, function, development
Introduction

Iodothyronine deiodinases are enzymes that are present throughout the body and are essential in the metabolism of thyroid hormones (THs). They either activate 3,5,3',5'-tetraiodothyronine (T₄), the major hormone secreted by the thyroid gland, to the more active 3,5,3'-triiodothyronine (T₃) by outer ring deiodination (ORD), or inactivate T₄ and T₃ by inner ring deiodination (IRD) to 3,3',5'-triiodothyronine (rT₃) and 3,3'-diiodothyronine (3,3'-T₂), respectively. Vertebrates typically possess three types of deiodinases: deiodinase type 1 (D1) with both ORD and IRD activity and low substrate affinity (high Km), type 2 (D2) with only ORD activity and high substrate affinity (low Km), and type 3 (D3) with only IRD activity and also high substrate affinity [1, 2]. Within a given species, these deiodinases show a highly dynamic spatiotemporal expression pattern. In combination with differences in expression of transmembrane TH transporters, this allows different tissues and even specific cell types to regulate intracellular TH levels at least in part independently from the levels present in the general circulation [2, 3]. Although biochemical proof for the existence of three different types of deiodinases was originally obtained by in vitro studies on mammalian (mostly rat) tissues in the late 1970s and early 1980s (see e.g. [4]), much about what we presently know about their structure and function was discovered first in non-mammalian vertebrates. Over the years, studies in fish, amphibians, birds, a few reptiles, and more recently also a number of invertebrates, have provided essential windows for our view on deiodinases, their origin, evolution and function.

The present review aims to highlight a few of these findings.

Evolution of deiodinases

Structure and function of vertebrate deiodinases

Vertebrate deiodinases are integral membrane proteins, which normally function as homodimers. The hydrophobic transmembrane region of the enzymes is located in the N-terminal domain. Their catalytic center is oriented towards the cytoplasm and contains the rare amino acid selenocysteine (Sec), which is essential for their catalytic activity. Since Sec is encoded by UGA that normally...
functions as a stop codon, their mRNAs contain a selenocysteine insertion sequence (SECIS) element in the 3′-UTR [2].

Before any deiodinase was cloned, biochemical studies in a variety of species already indicated the presence of TH deiodinating enzymes in both mammals and non-mammalian vertebrates. While these studies showed important similarities, they also suggested some functional variability, especially in fish (e.g. [5, 6]). The first deiodinase sequence information became available with the cloning of Dio1 cDNA from rat liver in 1991 [7]. The first cDNAs for dio2 and dio3 were identified in frogs with the cloning of dio3 cDNA from Xenopus laevis tadpole tails in 1994 [8] and dio2 cDNA from bullfrog Rana catesbeiana skin and hindlimbs in 1995 [9]. By now, cDNA sequences are available for one or more deiodinases in almost two hundred vertebrate species [10] and for some species also the complete gene structure has been elucidated. Until a few years ago, this sequence information suggested that all vertebrate dio1 and dio2 genes consist of multiple exons while dio3 genes contain no introns, a characteristic that is rather unusual in the eukaryotic kingdom [11]. In 2014, however, a multi-exon dio3 gene was identified in zebrafish [12]. Teleosts, the ‘modern’ bony fishes (Fig. 1), underwent a whole-genome duplication during evolution and as a result they express two paralogs of many genes, including dio3. Both paralogs encode functional D3 enzymes but in contrast to the dio3a gene, the dio3b gene contains a large intron separating its open reading frame from its SECIS element [12, 13]. It remains to be studied if this characteristic is specific for zebrafish dio3 or whether it is also found in other, related teleosts.

The large number of sequences available allowed to construct comprehensive phylogenetic trees, showing that the D1 cluster shows most sequence variation and suggesting that D1 is the oldest vertebrate deiodinase, while the D2 and D3 paralogs appeared more recently [11]. A well-known example of functional variability of D1 is its sensitivity to inhibition by the thyrostatic drug 6-propyl-2-thiouracil (PTU). This characteristic has long been considered an important criterion to distinguish PTU-sensitive D1 activity from the PTU-insensitive D2 and D3 activities. This was one of the main reasons why it was originally thought that amphibians and fish do not possess D1, as addition of PTU
did not inhibit deiodination in these species. However, a study in bullfrog tadpoles in 1988 already mentioned the presence of a high Km ORD activity in the gut during metamorphosis [14], and biochemical characterization of the ORD activity in tilapia kidney and liver in 1993 showed the presence of two different enzymes: a high Km (μM) enzyme with substrate preference rT₃>T₄, and a low Km (nM) enzyme with substrate preference T₂>rT₃, resembling mammalian D1 and D2 activity, respectively. The high Km enzyme was, however, insensitive to inhibition by PTU [15]. Subsequent cloning of dio1 from tilapia and killifish [16, 17] as well as Xenopus [18] confirmed that both fish and amphibians express a PTU-resistant D1. Sequence comparison with PTU-sensitive D1s from mammals and birds showed the presence of a Pro residue instead of a Ser residue two positions downstream of the Sec in the catalytic site, similar to the situation in known PTU-insensitive D2 and D3 enzymes. Site-directed mutagenesis in Xenopus D1 (Pro132Ser) restored PTU sensitivity, suggesting that this amino acid is essential for PTU sensitivity [18]. However, it also increased Km and V_max for ORD of T₄. Further comparative work in killifish and sea bream revealed that rather than a change of one specific amino acid, a combination of changes resulting in an increase in substrate turnover rate are probably determinative in the efficiency of PTU to block D1 activity [17, 19].

Another surprising finding in teleosts was related to the efficiency of dithiothreitol (DTT) to stimulate deiodinase activity. As the nature of the natural cofactor for deiodinase activity remains elusive, this reducing agent is typically used as cofactor in in vitro kinetic tests for all three types of deiodinases. However, in 2005 a cDNA was cloned for sea bream dio1, coding for a D1 enzyme that proved to be inhibited by DTT [19]. This apparently was also the case for rT₃ ORD activity in killifish and in the flatfish Solea senegalensis [20, 21], but not in several other species including tilapia, African catfish, rainbow trout and turbot, where all deiodinases were stimulated by DTT [22]. A later study in common carp, where DTT potently inhibited ORD in kidney homogenates but stimulated ORD in liver homogenates, continued to investigate this problem. Although the exact reason for the discrepancies in DTT action between and even within species remains unclear, the study at least
suggests that the present view of DTT as a strictly reducing agent of a selenyl-iodide intermediate of the enzyme may be too simple, and that the endogenous cofactor may have different sites of action on the deiodinase protein [23].

Comparative studies in tissue homogenates of exothermic vertebrates also revealed variability in the temperature optimum of catalytic activities between species. In general, the optimum tends to be lower (20-30 °C versus 30-37 °C) in exothermic species living at a low ambient temperature, such as trout, turbot, and red drum, although it may also vary between tissues [22, 24]. Optima can apparently also shift with acclimation to different rearing temperatures as found in red drum [24], pointing to shifts in the expression of different isozymes, but this was not the case in a study in rainbow trout [25]. Interestingly, ORD activity in liver of the turtle *Trachemys scripta* and the lizard *Sceloporus grammicus* remained fairly constant over the range of 18-37 °C and 15-42 °C, respectively [26, 27], suggesting that deiodinases in reptiles are well adapted to a broad range in body temperature.

**Deiodinases in invertebrates**

Over the years an increasing number of papers have reported on the presence of deiodinases in invertebrate chordates (Fig. 1). One of the first reports, from 1989, showed *in vivo* deiodination of T₄ in the ascidian *Phallusia mammillata* [28]. Twelve years later an *in vitro* study in different tissues of hagfish showed the presence of T₄ ORD, T₄ IRD as well as T₃ IRD activities [29]. Interestingly, both ORD and IRD of T₄ in hagfish were inhibited by PTU, suggesting this primitive chordate, situated in the evolutionary tree just before the transition to vertebrates (Fig. 1), expresses a D₁-like deiodinase that is PTU-sensitive. This adds additional complexity to the question as to why D₁ in teleost fish and amphibians is resistant to PTU inhibition.

More recently, the rapid increase in sequence information allowed to identify deiodinase cDNAs in several invertebrate chordates, including both cephalochordates and urochordates [3]. Full cloning and characterization of some of these sequences revealed a few puzzling observations. The ascidian
Halocynthia roretzi expresses a Sec-containing deiodinase that shows a mix of mammalian D1 and D2 characteristics: a high Km for ORD of rT₃ and T₄ and ping-pong kinetics, but negligible IRD activity and resistance to PTU [30]. Partial sequences available from several other ascidian species suggest that urochordates possess at least two deiodinase homologs [30]. In the cephalochordate Branchiostoma floridæ, at least three deiodinase homologs have been identified. Two of them have Sec in their catalytic site, but a third one instead has a Cys residue [31]. This deiodinase does not deiodinate T₄, T₃ or rT₃ but instead catalyzes IRD of 3,5,3’-triiodothyroacetic acid (Triac) and 3,5,3’,5’-tetraiodothyroacetic acid (Tetrac). It has a low nM Km and is stimulated by DTT up to 10 mM. Taking into account that the ligand of the TH receptor (TR) in Branchiostoma is neither T₃ nor T₄ but Triac, this non-selenodeiodinase may be an important regulator of TH signaling in this species. The deiodinases cloned from Halocynthia and Branchiostoma both have a temperature optimum around 20-30 °C while activity is strongly decreased at 37 °C [30, 31]. In an evolutionary context, this could mean that the typical optimum around 37 °C found in endothermic mammals and birds evolved later as an adaptation to their high body temperature.

Sequences for putative selenoproteins resembling iodothyronine deiodinases have in the meantime also been found in a wide variety of non-chordate invertebrates, including mollusks, annelids and even sponges and sea anemones [32]. So far, no iodothyronine deiodinases have been reported in arthropods and nematodes. This is in line with the fact that in these phyla also no TR orthologs have been identified and hence classical TH signaling may be absent. In contrast, annelids and mollusks contain TR orthologs and TH signaling, although not necessarily TR-mediated, has been demonstrated in some species [33]. Three full length deiodinase-like cDNAs have been cloned in mollusks: one from a scallop and two from the Pacific oyster. All three of them encode a selenoprotein [34, 35]. In vivo expression of the scallop deiodinase increased following stimulation of the immune system with lipopolysaccharide, while suppression of its expression by dsRNA injection was followed by an increase in the T₄/T₃ ratio in the haemolymph [34]. Exposure of Pacific oysters to T₄ increased expression of both deiodinases [35]. Changes in haemolymph T₄ and T₃ levels
and in deiodinase mRNA expression were also observed in response to exposure to polybrominated diphenyl ether, a well-known disruptor of mammalian TH homeostasis, in the Manila clam *Ruditapes philippinarum* [36]. This indicates that iodothyronine deiodinases may have a regulatory role under changing physiological conditions in mollusks and possibly also in some other phyla of non-chordate invertebrates where THs have been shown to influence development and life stage transitions.

**Local and whole body impact of D2 and D3**

Based on the characteristics and tissue distribution of deiodinases in mammals, mainly studied in rodents, the original view on deiodinase action was that D1 in liver, kidneys and thyroid was the enzyme responsible for regulating the T3 level in circulation, while D2 and D3 only acted locally to regulate intracellular T3 levels at the site of expression. Emphasis of the research was also mainly on the ORD pathway, producing T3 from T4. Less attention was given to the IRD pathway, responsible for T4 and T3 degradation, as D3 was thought to be almost absent in postnatal life. Research in non-mammalian vertebrates helped to discover some specific local actions of D2 and D3, but it also demonstrated their more general impact and was key in gradually shifting our view on the relative importance of the different deiodinases. It is now generally accepted that in mammals too, D2 activity in various tissues contributes to circulating T3 levels and that D3 is expressed throughout life, where increased T3 degradation is essential to cope with challenges such as food restriction, infection and tissue damage [1, 37-40].

**Deiodinases in development**

Chicken embryos and amphibian tadpoles are two long-standing models in developmental biology and their deiodination pathways have been studied already extensively in the 1980s and early 1990s. Data from chicken embryos showed that high Km, D1-like activity in liver increased 2- to 3-fold during the last (3rd) week of embryonic development while low Km, D3-like activity was highest around embryonic day 15-16 and decreased by more than 90 % during the last 3 days prior to
hatching (Fig. 2). It was suggested that this rapid drop in hepatic IRD activity was the main reason for the concurrent sharp increase in plasma T₃ [41, 42]. Following the cloning of chicken DIO1 and DIO3, these changes were confirmed at the level of mRNA expression [43]. In the same period, studies in bullfrog tadpoles showed high IRD, D3-like activity in liver of pre- and prometamorphic tadpoles that decreased to undetectable levels during metamorphic climax. ORD, D2-like activity was absent from liver at all stages but present in gut and skin, and increased significantly in skin during metamorphic climax. Rapid degradation of T₃ by hepatic D3 was suggested to be the reason for the failure to demonstrate in vivo T₄ to T₃ conversion in tadpoles prior to metamorphic climax [44, 45]. Again the changes were confirmed later at the mRNA level when dio2 and dio3 expression could be quantified [46]. In turn, investigations in fish showed that in several teleosts D1 activity was low or absent in the liver of euthyroid animals and D2 was the major enzyme regulating plasma T₃ [15, 22, 47]. These are just a few examples of how early data from non-mammals helped to challenge and subsequently change the old view on the relative impact of different deiodinases on the T₃ level in circulation.

Throughout the years, non-mammalian vertebrate models have contributed substantially to reveal the importance of divergent spatiotemporal expression of deiodinases in order to ensure the correct sequence in tissue development. A typical example is the metamorphosing tadpole where development of the limbs has to precede shrinkage of the tail to ensure continuous mobility. D2 and D3 activity and expression were analyzed in these tissues throughout metamorphosis [48]. While D2 expression and activity peaked in hindlimbs during late premetamorphosis and prometamorphosis, levels only started to increase in the tail during metamorphic climax. D2 activity in forelimbs, which develop later than hindlimbs, was still low during prometamorphosis and only increased during metamorphic climax. Interestingly, D3 activity levels in these tissues varied more or less in parallel with those of D2. This may have been one of the first clear examples showing that although D2 and D3 have opposite activities, their expressions do not necessarily change in an opposite way. Instead, their combined increase in the same tissue indicated a potential for tight control of local T₃ levels during important phases of differentiation [48].
Non-mammalian, externally developing model species have also been very helpful in demonstrating the presence of deiodinases and the need for TH signaling at early stages of embryonic development. *Dio2* and *dio3* mRNA of maternal origin were detected by whole mount in situ hybridization (WISH) in *Xenopus* embryos at the two cell stage and disappeared during gastrulation. Subsequent embryonic expression of all three deiodinases was shown by WISH and qPCR around the onset of neurulation [49, 50]. Exposure to T$_3$ starting at the tailbud stage increased expression of *dio2* and *dio3*, indicating their responsiveness to thyroid status [51]. In zebrafish, *dio1* and *dio2* mRNA of maternal and later embryonic origin was visualized by WISH from the two cell stage throughout embryonic development. Both deiodinases were colocalized at first but their localization started to diverge around 24 hours post-fertilization (hpf). *Dio3* mRNA was first detected at the 6-somite stage (12 hpf) [52]. *Dio1* and *dio2* expression was also confirmed by qPCR from the first stage studied (8 hpf) [53]. Because of the convenience of rapid and external development, these species are now also increasingly used in high-throughput screening of potentially endocrine disrupting compounds, where expression of deiodinases is one of the biomarkers for harmful interaction with thyroid function [54, 55]. The relatively large chicken embryo, in turn, has been very useful to study the dynamic and cell-specific expression pattern of deiodinases in the early developing brain, highlighting their role in regulating T$_3$ availability in structures such as the brain barriers (Fig. 3), the cerebellum, and the retina [56-60].

One of the obvious ways to investigate deiodinase function is to silence their expression. While transgenic mice deficient in D1, D2 or D3 became available between 2001 and 2006 [61-63], methodological limitations delayed deiodinase knockout in other species for many years. The first alternative deiodinase knockout vertebrate, a D2-deficient zebrafish, was finally reported in 2016 [64]. Ten years earlier however, knockdown by antisense morpholinos already allowed to efficiently block expression of one or more deiodinases in zebrafish throughout embryonic and early larval development. This revealed amongst others that *dio2* knockdown disrupted normal development but that *dio1* knockdown was only detrimental when T$_3$ availability was already low due to combined
dio2 knockdown [65, 66]. It also proved that both D3 paralogs are active in zebrafish but that dio3b is the predominant one [13]. In agreement with the observations in deiodinase knockout mice, dio3 knockdown was more detrimental for zebrafish development than knockdown of the TH activating deiodinases (Fig. 4) [67, 68]. Nevertheless, recent results in dio2 knockout zebrafish showed that D2 is not only needed for normal development but that permanent D2 deficiency has long-lasting effects, including disruption of male and female reproduction and changes in glucose homeostasis [64, 69, 70].

Deiodinases and seasonal rhythms

A clear and fairly recent example of a local action of deiodinases that was first discovered in non-mammals is the role of D2 and D3 in photoperiodism and seasonal reproduction. This time the model organism was Japanese quail. They are long day breeders and their photoperiodic clock is located in the mediobasal hypothalamus. It was found that even a short light pulse in the photo-inducible phase resulted within hours in a sharp increase in Dio2 expression and a sharp decrease in Dio3 expression in this brain area [71, 72]. It was shown that Dio2 and Dio3 are co-expressed in the tanycytes lining the 3rd ventricle and that the reciprocal switch in D2 and D3 activity results in a local increase in T3 levels. This in turn induces morphological changes in the terminals of the gonadotropin-releasing hormone (GnRH)-producing cells in the median eminence, facilitating GnRH release and hence stimulation of gonadotropin secretion from the pars distalis [73]. Interestingly, the increase in Dio2 expression appears to be triggered by thyrotropin (TSH) that is produced locally within the pars tuberalis and binds to TSH receptors within the ependymal cells [74]. The same mechanism has also been found in other birds including chickens and buntings [75-77]. Although there are some differences in the location of the photosensitive structures and the molecules involved in transmitting light information, TSH signaling and reciprocal changes in hypothalamic D2 and D3 activity have in the meantime also been identified as crucial in seasonal rhythmicity in several mammals as well as some fish (reviews e.g. [78-81]).
Conclusion

Research in non-mammalian species has contributed greatly to our present knowledge of the origin and evolution of iodothyronine deiodinases. Moreover, it has been essential in studying their function. Several findings in non-mammalian vertebrates have pointed to new roles that were later found to exist also in mammals, including humans. It is clear that comparative research has broadened our view on the impact of these important enzymes on TH action throughout life. With the present possibilities for rapid and precise gene silencing in any species of interest, it will also continue to do so in the future.

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**Figure legends**

**Figure 1**

Phylogenetic tree showing the evolutionary relationship between the different groups of chordates mentioned in the text.

**Figure 2**

Changes in plasma $T_4$ and $T_3$ and *in vitro* hepatic D1 and D3 activity during the last week of chicken embryonic development. Graphs show average values of data taken from two studies (references [41, 43]). E14-E19: embryos of 14-19 days old, H: day of hatching, C1: 1-day-old chick.

**Figure 3**

Expression of deiodinases at the brain barriers in 8-day-old chicken embryos. *In situ* hybridization shows *Dio2* expression in the developing capillaries of the blood-brain barrier while *Dio3* is highly expressed in the developing choroid plexus at the blood-cerebrospinal fluid barrier. Scale bar 100 $\mu$m for both pictures.

**Figure 4**

Knockdown of the TH activating (D1+D2) or the inactivating (D3) pathway in developing zebrafish by antisense morpholino (MO). Both knockdown conditions disturb normal development, but the impact of blocking D3 activity is more severe. (A) Larvae at 4 days post fertilization (dpf). Arrows point to impact on eye, ear and swim bladder development. (B) Free swimming behavior and
reaction to a light pulse at 3 dpf. D1D2MO: combined injection of dio1- and dio2-blocking MOs, D3MO: injection of dio3b-blocking MO, SCMO: injection of standard control MO.
Figure 2

plasma $T_4$ and $T_3$

hepatic D1 and D3 activity
Figure 3

Dio2

Dio3
Figure 4

A  4 dpf larvae

SCMO
D1D2MO
D3MO

B  free swimming at 3 dpf

light
dark

0 10 20 30 40 50 60 70 80 90 100 110 120 130 140 150 160 170 180 190 200 210 220 230 240 250 260 270

minutes

D3MO  blue  green
D1D2MO  red  blue
SCMO  green  blue
