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Evolution of the expression and regulation of the nuclear hormone receptor ERR gene family in the chordate lineage

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A B S T R A C T

The Estrogen Related Receptor (ERR) nuclear hormone receptor genes have a wide diversity of roles in vertebrate development. In embryos, ERR genes are expressed in several tissues, including the central and peripheral nervous systems. Here we seek to establish the evolutionary history of chordate ERR genes, their expression and their regulation. We examine ERR expression in mollusc, amphioxus and sea squirt embryos, finding the single ERR orthologue is expressed in the nervous system in all three, with muscle expression also found in the two chordates. We show that most jawed vertebrates and lampreys have four ERR paralogues, and that vertebrate ERR genes were ancestrally linked to Estrogen Receptor genes. One of the lamprey paralogues shares conserved expression domains with jawed vertebrate ERRγ in the embryonic vestibulocochlear ganglion, eye, brain and spinal cord. Hypothesising that conserved expression derives from conserved regulation, we identify a suite of pan-vertebrate conserved non-coding sequences in ERR introns. We use transgenesis in lamprey and chicken embryos to show that these sequences are regulatory and drive reporter gene expression in the nervous system. Our data suggest an ancient association between ERR and the nervous system, including expression in cells associated with photosensation and mechanosensation. This includes the origin in the vertebrate common ancestor of a suite of regulatory elements in the 3' introns that drove nervous system expression and have been conserved from this point onwards.

1. Introduction

The Estrogen Related Receptor (ERR, also sometimes called ESRR) form a small family of transcription factors and encode Nuclear Hormone Receptor (NHR) type DNA binding proteins. There are typically four ERR family paralogues in jawed vertebrates, ERRα, ERRβ, ERRγ and ERRδ, though there is variation between lineages due to extra duplications and/or gene loss. Based on sequence similarity the ERR family is closely related to the Estrogen Receptor (ER) family (Baker, 2008; Bertrand et al., 2004), but there is an important difference in how they function: ER proteins require ligand-binding to activate transcription of target genes, while ERR is an ‘orphan’ NHR that does not need a ligand to bind DNA. ERR proteins act by binding to DNA sequences known as Estrogen Related Receptor Elements (ERREs) and by regulating the expression of target genes (Heard et al., 2000; Tremblay and Giguerre, 2007).

The jawed vertebrate ERR genes have been quite well studied in several species, with embryonic expression identified in multiple tissues including Central and Peripheral Nervous Systems (CNS and PNS), muscle and kidney, with data from enough species to suggest that some and possibly all of these sites of expression could be ancestral for jawed vertebrates (Bardet et al., 2004; Heard et al., 2000; Susens et al., 2000).

Our study was initiated following the development of one ERR gene, ERRγ, as a marker for the neurons of the chicken vestibulocochlear ganglion (VA) (Patthey et al., 2016). ERRγ is also expressed by VA cells in mouse and zebrafish, and the locus is associated with hearing loss in mouse mutants and human genetic conditions (Bardet et al., 2004, 2005; Bertrand et al., 2007; Lorke et al., 2000; Nolan et al., 2013). These data suggest ERRγ functions in the specification of VA cells in a way that is conserved across jawed vertebrates. However, ERRγ expression has also been detected in some other cranial ganglia, including the trigeminal, and is widely expressed in the CNS as well (Bonnelye et al., 1997), complicating inference of the ancestral expression of ERRγ genes. This problem is compounded by limited data from invertebrates, with current studies restricted to the description of expression in the developing Drosophila gut deriving from a large-scale in situ hybridisation screen,

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expression in the larval head of tunicates, and expression in somites, CNS and PNS of amphioxus (Bardet et al., 2005; Gomes et al., 2019; Park et al., 2009; Thurmond et al., 2019).

We studied the molecular evolution, expression and regulation of ERR genes in lamprey and in three invertebrates, a limpet and additional amphioxus and tunicate species to the ones already studied. Lampreys are members of the earliest-diverging extant vertebrate group. They lack hinged jaws and paired appendages but share many developmental processes and structures with other vertebrates (Green and Bronner, 2014; Shimeld and Donoghue, 2012). Evidence from broad genome comparison shows two rounds of whole genome duplication (2R) preceded the radiation of living jawed vertebrate lineages, with at least one of these predating separation of the lamprey lineage (Sacredot et al., 2018; Simakov et al., 2020). Tunicates are the sister lineage to vertebrates and the immediate outgroup for comparison to vertebrates, while amphioxus gives insight into the ancestral chordate condition. Limpets are molluscs with motile planktonic embryos and larvae which may provide a better comparison than amphioxus gives insight into the ancestral chordate condition. Limpets were identified via the same approach. For P. vulgata from Tinside, Plymouth, UK. Both were maintained in a running seawater aquarium at 12–16 °C. Gametes were liberated by dissection and embryos were cultured at 16–18 °C until they reached the desired stage. B. lanceolatum were collected from Banyuls-sur-Mer, France and heat stimulation was used to induce spawning (Fuentes et al., 2004, 2007).

2.2. Embryos and in situ hybridisation

Primers (Supplementary Table S2) were designed to amplify fragments 500-1000bp in length, when possible, from embryonic cDNA. L. planeri embryos were collected from the New Forest, UK, under licence from Forestry England, and staged as described (Tahara, 1988). Adult C. intestinalis adults were collected from Northey marina, Hayling Island, UK, and adult P. vulgata from Tinside, Plymouth, UK. Both were maintained in a running seawater aquarium at 12–16 °C. Gametes were liberated by dissection and embryos were cultured at 16–18 °C until they reached the desired stage. B. lanceolatum were collected from Banyuls-sur-Mer, France and heat stimulation was used to induce spawning (Fuentes et al., 2004, 2007).

L. planeri, C. intestinalis and B. lanceolatum embryos were fixed with 4% paraformaldehyde (PFA) and stored at –20 °C after gradual transfer to Methanol (L. planeri) or Ethanol (invertebrate embryos). Lamprey in situ hybridisation was performed as described (Lara-Ramirez et al., 2015), C. intestinalis and B. lanceolatum in situ hybridisation was performed as described (Boorman and Shimeld, 2002). P. vulgata in situ hybridisation was performed as described (Shimeld et al., 2010). Scanning electron microscopy of P. vulgata embryos was performed as described (Thompson and Shimeld, 2015). Lamprey embryo clearing was either by transferring to 25% then 50% glycerol in PBS, or by dehydrating with 25%, 50%, 75% and 100% ethanol and transferring to benzyl benzoate/benzyl alcohol 2:1 (BBAB). Differential Interference Contrast (DIC) microscopy was used to observe and photograph embryos. The Ciona ERR probe was derived from the Ciona release 1 gene collection library, clone coordinate R1GIG030605 (gene model KH2012.18.21).

Adult sea lamprey (Petromyzon marinus) were supplied by the US Fish and Wildlife Service and Department of the Interior. Embryos obtained by in vitro fertilisation were grown to the desired stage in compliance with California Institute of Technology Institutional Animal Care and Use Committee protocol #1436.

2.3. CNE cloning and transgensics

Human and Zebrafish CNE sequences from the Conserved Non-coding Orthologous Regions (CONDOR (Woofe et al., 2007):) database were searched against the Lethenteron camthacitum genome using BLAST, providing evidence that shared CNEs were present between the 3’ most introns of ERR1 and ERRa. The last two introns of L. camthacitum ERRa and ERRy from Human, Mouse, Chicken, Anolis, Xenopus, Zebrafish and elephant shark were subsequently aligned using BLAST, and VISTA plots were built using mVISTA (Frazer et al., 2004) to reveal conservation peaks. Sequences in each identified peak were extracted for each species and alignments were built using MUSCLE. CNEs were cloned from the second to last intron of ERRa from L. planeri genomic DNA. CNE-containing fragments were cloned using the InFusion system ( Takara Biosciences) into respective GFP reporter vector for testing in chicken (barcode 1 tagged pTK vector (Chen and Streit, 2015)) and lamprey (Parker et al., 2014). More specifically, for
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2.4. Immunohistochemistry

Fixed embryos were initially washed 3 times in PBS-TritonX100 (PBS with 0.5% Triton ×100) for 30 min, washed 2 times in block solution (PBS-TritonX100 with 10% heat-treated sheep serum) for 30 min and blocked in block solution for longer than 2 h, and up to overnight. Embryos were then incubated overnight with the primary antibody solution followed by 5 PBS-TritonX100 and 2 block solution washes for 30 min. Secondary antibody solution incubation was then carried out for longer than 4 h up to overnight, followed by 5 PBS-TritonX100 washes for 30 min each. Embryos were finally transferred to 25% and then 50% glycerol in PBS to clear and visualised in an optical fluorescent (Zeiss Axioskop) or confocal (Olympus FV1000) microscope. Sections were initially incubated in PBS at 45 °C for 30 min to remove gelatin. Then 5 washes in PBS-TritonX100 for 15 min were followed by blocking for 1 h at 4 °C and overnight incubation in primary antibody, 5 washes in PBS-TritonX100 for 15 min before transferring to glycerol and sealing. Antibodies used were: Chicken Polyclonal Anti-GFP antibody (Abcam AB13970), Goat Anti-Chicken IgG H&L Alexa Fluor® 488 (Abcam AB150169), Mouse Hu/ELAV Monoclonal Antibody (Invitrogen 16A11), anti-Mouse alexa594 (Abcam AB150116), mouse anti-GFP (Invitrogen A-11120) (used in chicken) visualised with Goat anti-Mouse IgG H + L Alexa Fluor® 488 (Invitrogen A-11001).

2.5. Image analysis

Analysis and processing of fluorescence microscopy data were carried out using ImageJ v. 1.52g. Confocal or optical z stacks and 3D projections were created using maximum intensity projection. Some images are composites of multiple individual images of the same embryo focused at slightly different levels to follow expressing cell populations as the embryo moved in and out of focus. These were assembled in PowerPoint by overlapping individual images. This applies to images shown in Fig. 7A, B, G, and H.

2.6. Transcriptome assembly and analysis

RNA-seq data from dissected eyes from lamprey, hagfish, gar and shark (Lamb et al., 2016) were downloaded from the NCBI (Bioproject PRJNA292033). Libraries for each species were separately assembled using Trinity with the integrated Trimomatic option for trimming (Haas et al., 2013). These assemblies were then searched for ERR genes using BLAST. Read counts for each transcript were then generated using Kalisto (Bray et al., 2016).

2.7. Single-cell RNAseq data analysis

Ciona robusta and amphioxus single-cell RNAseq (scRNAseq) data have been previously published (Cao et al., 2019; Ma et al., 2022). Data were accessed at https://singlecell.broadinstitute.org/single_cell/study/SCP454/comprehensive-single-cell-transcriptome-lineages-of-a-proto-vertebrate and https://lifeomics.shinyapps.io/shinyapp_multi/respectively. In each case, we first used the website interface to search for the profiles of selected genes across cell clusters. For the Ciona robusta data we were able to identify two well-defined neural cell clusters expressing ERR (gene ID KH2012: KH. L8.21). To gain insight into the nature of ERR expressing cells we extracted the lists of genes significantly enriched in these cell clusters (as defined by the original scRNAseq study (Cao et al., 2019)), and retrieved Gene Ontology (GO) terms for C. robusta genes from the Aniseed database (https://www.aniseed.fr/ (Brozovic et al., 2018)). These data were then analysed with gProfiler (Raudvere et al., 2019) to identify functional enrichment with a threshold of 0.05. We also directly searched genes in these cell clusters for those annotated with GO terms related to photo-reception.

3. Results

3.1. Evolutionary history of the ERR gene family

We first sought to describe the evolutionary history of chordate ERR genes. We identified ER and ERR genes from three lamprey species (Lampeatra planeri, Petromyzon marinus, Lethenteron camtschaticum), one hagfish species (Eptatretus burgeri) and a range of other vertebrate and invertebrate taxa. Molecular phylogenetic analysis of encoded protein sequences using the Retinoid X Receptors as outgroups (Mangelsdorf et al., 1992) showed ER and ERR sequences formed well supported groups, each including vertebrate and invertebrate sequences (Fig. 1). Most invertebrates (including the invertebrate chordates) had a single ERR gene, while lampreys and hagfishes had four ERR genes. As expected, jawed vertebrate ER (ER1, ER2) and ERR paralog sequences formed separate well-supported groups. Lamprey and hagfish ERR and ER sequences grouped within the ERR and ER families respectively. However, while lamprey and hagfish sequences generally grouped, the tree did not resolve sufficiently to determine the orthology between lamprey and jawed vertebrate sequences.

While extracting lamprey ERR and ER sequences, we noted one ERR paralogue and one ER paralogue were about 600 kb apart in the same genomic scaffold. This prompted us to examine ERR and ER gene localisation in other genomes, identifying additional ERR-ER gene linkages (Fig. 2A and B). The distance between ERR and ER genes is small in some genomes, for example around 40 Kb in the hemichordate Saccoglossus kowalevskii, but in jawed vertebrates is usually in the order of several Mb. Vertebrate paralogue pairs ERRγ-ER1 and ERRδ-ER2 often appear in linkage, while the ERRαs and ERRδ loci appear unlinked to ER loci in all jawed vertebrates examined, except for Xenopus where ERRδ is found close to ER2 (Fig. 2).

3.2. ERR gene expression in tunicate, amphioxus and mollusc embryos

Previous studies of embryonic ERR spatial expression in invertebrates are limited to Drosophila (developed as part of high throughput in situ hybridisation screening (Thurmond et al., 2019)), the amphioxus Branchiostoma floridae (Bardet et al., 2005) and the larval head of an ascidian, Phallusia mammilatata (Gomes et al., 2019a, 2019b). Since these data were insufficient for useful comparison to vertebrates, we examined embryonic gene expression in additional invertebrates: a second amphioxus species, Branchiostoma lanceolatum, another tunicate (Ciona intestinalis) and a gastropod mollusc (the common limpet Patella vulgata). ERR expression in B. lanceolatum localised to both CNS and PNS, and to axial muscles (Fig. 3A–D), essentially as reported in B. floridae (Bardet et al., 2005). As seen in B. floridae, expression in the
PNS was confined to a subset of PNS cells. We also searched published scRNAseq data from amphioxus (Ma et al., 2022). This identified expression in mesoderm and neural tissue (Supplementary Fig. S1) but was insufficient to identify specific PNS cell types.

In C. intestinalis ERR was expressed in axial muscles and in the CNS, in a structure called the sensory vesicle (Fig. 3E and F). Sensory vesicle cells expressing ERR form part of the ocellus (the visual organ of the larva) and also include two cells in the lining of the vesicle in which the otolith, the larval inertial/gravity sensor, lies. From their position, we considered that these cells are possibly those known as the 'antenna cells', which connect the otolith to the brain, probably relaying information about its physical displacement and thus allowing inertial sensing (Bostwick et al., 2020; Esposito et al., 2015; Ryan et al., 2017). Ciona spcs. also have several types of PNS cells at the stages we examined (Liu and Satou, 2020), but we did not detect ERR expression in any of these by in situ hybridisation.

To gain additional insight into the cell types expressing ERR we mined scRNAseq data from Ciona robusta (Cao et al., 2019; Horie et al., 2018b). The Ciona robusta ERR gene (KH2012:KH.L8.21) showed no evidence of expression in peripheral neural cell types, confirming in situ hybridisation results. However, ERR was prominently expressed in two central neural cell clusters, ‘Rx+ anterior sensory vesicle (Rx+aSV)’ cells and ‘Lox5+ anterior sensory vesicle (Lox5+aSV)’ cells (here we use the names given to these clusters by the authors of the scRNAseq study) (Supplementary Fig. S1). Rx+aSV cells express the Rx (Retinal homeobox) gene and are hence likely to be visual system cells (Mathers et al., 1997). To examine this, we extracted the list of genes significantly expressed in the Rx+aSV cluster and assessed GO term enrichment,
finding terms related to photosensation to be significantly enriched in this gene set (25 genes including an Opsin gene: Supplementary Fig. S1). This indicates these cells are likely to be the ERR expressing cells adjacent to the ocellus, in turn implying the Lox5\(^+\)aSV cells may be the cells adjacent to the otolith. Genes significantly expressed in the Lox5\(^+\)aSV cells did not show informative GO term enrichment, though do include several genes with visual system GO annotations (19 genes, 8 of which overlap with the 25 genes from the Rx\(^+\)aSV cluster analysis: Supplementary Fig. S1). If these cells are antenna cells, they should also express the vesicular glutamate transporter VGlut (KH2012:KH.C3.324) (Bostwick et al., 2020; Kourakis et al., 2021) while Rx\(^+\)aSV cells should not. The scRNAseq data confirmed this was the case (Supplementary Fig. S1).

In *P. vulgata* ERR was expressed in trochophore larvae by cells of the mantle edge surrounding the shell field, and by cells of the apical organ (Fig. 3G, I, J). The apical organ is a ciliated sensory organ with a central tuft of long cilia, and comparison to scanning electron microscope images of embryos at the same developmental stage (Fig. 3H, K) shows expression is not in the central cells with elongated cilia, but in cells with shorter cilia lying next to them.

### 3.3. ERR gene expression in lamprey embryos

We named the four lamprey ERR genes ERR\(_a\) to ERR\(_d\) to avoid direct attribution to jawed vertebrate ERR\(_a\) to ERR\(_b\), since this was not supported by molecular phylogenetic analysis. Sequences for ERR\(_a\), ERR\(_b\) and ERR\(_c\) were retrieved from embryo transcriptome data for three lamprey species and cloned from *L. planeri* embryo mRNA by reverse transcription PCR (RT-PCR). ERR\(_d\) was identified in the *P. marinus* and *L. camtschaticum* genomes (Mehta et al., 2013; Smith et al., 2018), but was not present in the embryo transcriptomes and we were unable to amplify this gene from *L. planeri* embryo mRNA by RT-PCR. In situ hybridisation showed ERR\(_c\) was only expressed in the hindbrain and part of the mandibular arch (Supplementary Fig. S2), while embryonic expression of ERR\(_b\) was not detected by this method. It also showed ERR\(_a\) expression in the CNS including the midbrain, hindbrain, epiphysis, olfactory epithelium and spinal cord, as well as in the VA (Fig. 4). ERR\(_a\) expression commenced at Tahara stage 24 (Tahara, 1988) in the epiphysis, midbrain and the VA (Fig. 4A). By stage 25 expression was also seen in the olfactory system, forebrain, hindbrain and in the spinal cord (Fig. 4B). From stage 27 onwards, expression persisted in all these domains, but with more widespread and stronger signal in the hindbrain and spinal cord (Fig. 4C). Expression in some cells in the eye could also be seen from stage 28 onwards (Fig. 4D, Supplementary Fig. S3). Lamprey eye development is delayed relative to other vertebrates, with full vision only developing at metamorphosis after some years as a larva. To see if ERR expression persisted we examined published RNA-seq data from adult lamprey (*Geotria australis*, *Mordacia mordax*) and hagfish (*Eptatretus cirrhatus*) eyes (Lamb et al., 2016). We assembled transcriptomes for each species and searched for transcripts coded by ERR loci using reciprocal BLAST matches to *P. marinus* transcripts.
identifying ERRα, ERRβ and ERRγ transcripts in all three species and ERRd in two species. Based on transcript per million (TPM) scores, we concluded that ERRα, ERRβ and ERRγ transcripts are expressed in adult lamprey and hagfish eyes (Supplementary Table S1).

3.4. Lamprey ERRα and jawed vertebrate ERRγ share conserved synteny and non-coding elements driving CNS and PNS expression

It is notable that while lamprey ERRα and jawed vertebrate ERRγ do not appear as orthologues in our molecular phylogenetic analysis they share similarity in expression in the brain, spinal cord and VA. Synteny can provide parallel evidence to molecular phylogenetics for gene relationships between species. We compared neighbouring genes between lamprey and jawed vertebrate ERR loci, revealing they all evolved by block duplication of a single ancestral ERR locus (Fig. 5A; Supplementary Fig. S4). This is consistent with the origin of vertebrate ERR paralogues by chromosome and genome scale duplications proposed to have occurred early in vertebrate evolution (Putnam et al., 2008; Simakov et al., 2020). Synteny was most similar between lamprey and hagfish eyes (Supplementary Table S1). We hypothesised that the ancestral vertebrate CNEs are regulatory elements responsible for expression in territories shared between the genes in jawless and jawed vertebrates. To investigate this, we selected a region with multiple CNEs nearby (named ERRβt12 and containing CNE6, CNE7 and CNE9; Fig. 5C), which was cloned from lamprey genomic DNA and tested for its ability to drive reporter expression in transgenic lamprey embryos generated by i-SceI meganuclease integration into zygotes (Fig. 6, Supplementary Fig. S5). Reporter expression viewed in live embryos overlapped with domains of the endogenous ERRα expression, with 36.3% of embryos showing expression in the brain, and 40.3% in more posterior neural tube (Fig. 6A, Supplementary Fig. S5). We also saw expression of the reporter in the branchial arches and head muscles in many embryos (Supplementary Fig. S5). These sites do not map to sites of ERRα expression and are common ectopic sites when using this plasmid vector (Papadogiannis et al., 2022).

Since precise sites of expression in the CNS were difficult to see in live embryos and cranial ganglia difficult to distinguish from underlying CNS, we stained fixed embryos using antibodies against GFP and the neural differentiation marker Hu/ELAV and recorded detailed expression via optical fluorescence microscopy (20 embryos) and confocal microscopy (3 embryos) (Fig. 6C–E). This gave single cell level resolution via optical fluorescence microscopy (20 embryos) and confocal microscopy (3 embryos) (Fig. 6C–E).
resolution and revealed GFP in sites that could not be seen in live embryos. It confirmed CNS expression mirrored the expression of ERRα, with reporter expression in the olfactory system (43.5% of embryos) and epiphysis (30.5% of embryos) (Fig. 6C and D), midbrain (69.5% of embryos) (Fig. 6C) and spinal cord (56.5% of embryos) (Fig. 6E). Activity was also seen in the eye (56.5% of embryos), in a pattern similar to the ERRα in situ hybridisation signal (Supplementary Fig. S3). However, GFP staining in the cranial ganglia (including the VA) (21.5% of embryos) was not detected above the level seen in transgenic vector-only transgenic control embryos (Papadogiannis et al., 2022), and as viewed by confocal microscopy showed signal in only a few cranial ganglia cells in the two embryos screened (Supplementary Fig. S6).

Since detecting weak reporter signal in lamprey embryos was hampered by both background and the need to fix and stain transgenic embryos, we sought an alternative approach to testing for reporter expression. We used Imaging and sectioning of embryos showed that lamprey ERRα reporter activity in transgenic chicken embryos (Papadogiannis et al., 2022), and as viewed by confocal microscopy showed signal in only a few cranial ganglia cells in the two embryos screened (Supplementary Fig. S6).

4. Discussion

4.1. ERR gene family evolution

The ER and ERR genes were proposed to have originated from an ancestral NHR gene early in animal evolution (Bertrand et al., 2004). Our data suggest that this occurred by tandem gene duplication, as the genes are linked in many species. Molecular phylogenetic analysis resolved ER and ERR gene families well, as seen previously (Bertrand et al., 2004; Filowitz et al., 2018). Most invertebrates have a single ERR gene, while vertebrates had three or more ERR genes. Lamprey and hagfish ERR genes were placed with other vertebrate ERR genes as expected, but not as clear orthologues of ERRα, ERRβ, ERRγ or ERRδ. Comparing synteny between ERR loci in lamprey and jawed vertebrates showed they evolved by block duplication of a single ancestral locus. This is consistent with the evolution of lamprey and jawed vertebrate genomes by chromosome and genome level duplications (Simakov et al., 2020). It also showed that the lamprey ERRα and jawed vertebrate ERRγ loci are most similar to each other, as they share proximity with several single copy genes. This, along with similarities in expression and CNE content (discussed below) mean ERRα and ERRγ are probably orthologues.

4.2. ERR gene expression in invertebrates and vertebrates

While ERR expression in jawed vertebrates like zebrafish and mice has been quite well described, expression in invertebrate embryos has been poorly documented. We evaluated ERR expression in the embryos of three additional invertebrates, plus lampreys. Comparison between these species and jawed vertebrates allows us to infer conserved and derived aspects of ERR expression.

4.2.1. ERR expression in the nervous system

All species examined showed ERR expression in the nervous system. Lamprey ERRα was expressed in several sites in the developing CNS, including the eye, epiphysis, olfactory system, hindbrain and spinal cord. Many of these sites are shared with jawed vertebrate embryos, suggesting they are conserved. ERR appears to be confined to the endoderm of Drosophila (Thurmond et al., 2019), however we observed ERR in the nervous systems of all three invertebrates we studied. The limpet produces a typical planktonic trochophore larva and we observed ERR expression in the cells of its apical organ, a ciliated sensory structure situated at the animal pole. In some species, the apical organ mediates multiple stimuli including mechanosensation and chemosensation.
Different cell morphologies in the limpet apical organ (Fig. 3H, K) suggest that it may also mediate multiple stimuli, although this has not been experimentally validated.

Both species of amphioxus show ERR expression in CNS and PNS (this study (Bardet et al., 2005; Ren et al., 2020)). CNS ERR expression includes the frontal eye (part of the anterior CNS and a homolog of the vertebrate eye (Vopalensky et al., 2012)), plus the photoreceptor cells of Hesse, scattered light-sensitive cells found more posteriorly in the CNS (Bardet et al., 2005). This association with the visual system may be conserved across chordates, given ERR expression in lamprey and jawed vertebrate eyes and the tunicate ocellus (discussed further below). Amphioxus ERR is also expressed in some other cells along the length of the anterior CNS whose precise identity is unknown (Ren et al., 2020). In mice, single cell sequencing has identified ERRγ as expressed in neurons in the dI2 region of the spinal cord (which houses commissural neurons) as well as in ventral interneuron populations (Delile et al., 2019).
Embryonic lamprey spinal cord neurons have not been sufficiently well described to determine if ERRα is expressed by equivalent cells, though the activity of ERR8t12::GFP shows there is probably conservation of regulation. It is likely at least some of the ERR positive cells in lamprey and jawed vertebrate spinal cords are of the same type, though proving this will require further study.

Amphioxus ERR expression is also found in epidermal sensory neurons, part of the PNS. Some amphioxus epidermal sensory neurons delaminate to lie between the epidermis and underlying mesendoderm, leaving their sensory cilia projecting through the epidermis to the outside world (Mazet et al., 2004; Schubert et al., 2004). Their function has not been tested, though it is likely some are mechanosensory and some chemosensory (Parker, 1908; Satoh, 2005). Their developmental history is distinct from vertebrate cranial ganglia neurons as they are born in the ventral embryonic ectoderm as scattered precursors (Lu et al., 2012) rather than in dorsal placodes. However, they do express some genes characteristic of vertebrate placodes such as Eya and Six1/2 (Kozmik et al., 2007). One possibility that accounts for these conflicting data is that the cells are comparable to vertebrate cranial ganglia cells at the level of the cell types concerned, but not at the level of the placode or ganglion morphogenesis (Patthey et al., 2014). ERR is restricted to a subset of epidermal neurons in the middle of the animal, alongside the ‘rhombospinal’ part of the amphioxus neural tube which is homologous (based on Hox expression) to the hindbrain and anterior spinal cord region of vertebrates (Albuixech-Crespo et al., 2017; Ferran and Puelles, 2019). If ERR expression in these amphioxus cells is conserved with vertebrates it would imply homology to vertebrate VA ERR-expressing cells. Our data neither support nor conflict with this possibility.

Tunicates are the sister group to vertebrates. A previous study has described spatial ERR expression in tunicate development, revealing expression in parts of the sensory vesicle of the ascidian P. mammillata but not defining the precise cells. In the tunicate Ciona, we identified ERR expression in the CNS associated with cells of two sensory organs, the ocellus and the otolith. Expression in the ocellus is interesting, as the organ has been suggested to be homologous to the vertebrate retina (Kusakabe et al., 2001) and our mining of scRNAseq data also suggests these cells have a visual system function. This adds support to the contention discussed above that ERR expression in the visual sensory system of the anterior CNS is an ancestral chordate character still shared by amphioxus, tunicates, lampreys and jawed vertebrates.
The Ciona otolith system functions as a gravity sensor (Tsuda et al., 2003), helping the swimming larva swim downwards to settle and metamorphose. The system is thought to work as an inertial sensor, detecting displacement of the otolith pigment cell. Our data suggest Ciona ERR expression near the otolith is in cells known as ‘antenna cells’, based on their position and on ERR co-expression with Vglut in scRNAseq data. Antenna cells are neurons that relay sensation of otolith displacement to the processing part of the brain (Ryan et al., 2017). This has functional equivalence to inertial sensing by the otic/VA system of vertebrates but does not appear to be homologous in the classical sense, as in Ciona these cells develop from within the CNS and not from sensory placodes and cranial sensory ganglia. Furthermore, good candidates for placode homologs are present in Ciona and produce some sensory neurons (Horie et al., 2018a; Mazet et al., 2005; Papadogiannis et al., 2022), but we did not detect ERR at all in the Ciona PNS either by in situ hybridisation or through examining published scRNAseq data. We therefore suggest that if these ERR expressing systems do have shared ancestry it is also at the level of the cell type. We can speculate that the common sensory theme is mechanosensation: in the VA/otic system of vertebrates, the otic system of Ciona and the epidermal sensory cells of amphioxus. This could be tested by establishing the downstream targets of ERR genes in the respective cells in these different species.

4.2.2. Mesoderm ERR expression in amphioxus, Ciona and vertebrates

In both Ciona and amphioxus ERR genes are expressed by segmented muscle cells (Bardet et al., 2005). In amphioxus these cells derive from multicellular somites, while in Ciona these are individual muscle cells arranged segmentally on either side of the notochord. In zebrafish ERR expression in somites and other mesoderm derivatives has also been reported (Bardet et al., 2004; Bertrand et al., 2007), which could point to somite expression as conserved across the chordates. However, we did not identify ERR expression in these tissues in lamprey. If mesodermal ERR expression is ancestral for chordates it has either been lost by lampreys or occurs at a later stage in the life cycle than those analysed here.

4.2.3. A summary of the evolution of ERR expression

Neural and possibly mesodermal expression appear to be ancestral for chordate ERR genes. The visual system expresses ERR genes in all the chordate lineages examined. Cell type expression also suggests a connection to mechanosensation, albeit speculative as there are also substantial differences here between amphioxus, Ciona and vertebrates. Within vertebrates, expression of ERRα in lamprey and ERRγ in other vertebrates show similarity in the eye, other parts of the CNS and VA. This suggests the presence of a conserved regulatory network in the vertebrate common ancestor, driving ERR expression in these tissues, inherited by ERRα/ERRγ. Mapping and testing of potential regulatory elements allowed us to examine this further.

4.3. The evolution of vertebrate ERR regulation

Lamprey ERRα and jawed vertebrate ERRγ share CNEs with the same order, orientation and intron location. We did not find similar CNEs around other ERR paralogues. These features indicate ERRα and ERRγ are probably orthologues, and we can definitively conclude that the vertebrate common ancestor had at least one ERR gene with this
While low level ERR812 reporter expression was observed in the cranial ganglia of transgenic lampreys, the weak activity was similar to that previously observed in vector-only control transgenics (Papadogiannis et al., 2022). However, ERR812 activity in the otic and VA system of transgenic chicken embryos provides some evidence for a conserved role in these tissues. These results make the lack of clear ERR812 activity in the VA of transgenic lampreys surprising, as we might expect element activity to be more robust in a same-species environment than when tested cross species. One possibility is that ERR812 drives lamprey VA activity at different developmental stages than the ones analysed here. This is possible as detecting some aspects of transgene expression required fixation and antibody labelling, limiting the stages we could census.

Combined, these data show that the expression of this subset of vertebrate ERR genes was inherited from the expression of one ancestral ERR gene and that this was regulated by conserved regulatory elements in the 3’ introns. This shows the gene regulatory network driving ERR expression in these conserved sites evolved before the separation of lamprey and jawed vertebrate lineages. This network presumably came under sufficient constraint to be maintained from that point onwards. Similarly, the absence of conservation outside of vertebrates suggests it is vertebrate specific. This last point will be difficult to test directly, though it might be insightful to determine how ERR is regulated in one or more invertebrate outgroups, as we would not expect it to be conserved with vertebrates.

Data availability

Data will be made available on request.

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References


