Published in final edited form as:
Nature. ; 483(7389): 289–294. doi:10.1038/nature10838.

Deep deuterostome origins of vertebrate brain signalling centres

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Abstract

Neuroectodermal signalling centres induce and pattern many novel vertebrate brain structures but are absent, or divergent, in invertebrate chordates. This has led to the hypothesis that signalling centre genetic programs were first assembled in stem vertebrates, which potentially drove morphological innovations. However, this scenario presumes that extant cephalochordates accurately represent ancestral chordate characters, which has not been tested using close chordate outgroups. Here, we report that genetic programs homologous to three vertebrate signalling centres; the anterior neural ridge, zona limitans intrathalamica, and isthmic organizer are present in the hemichordate *Saccoglossus kowalevskii. Fgf8/17/18, sfrp1/5, hh*, and *wnt1* are expressed in vertebrate-like arrangements in hemichordate ectoderm, and homologous genetic mechanisms regulate ectodermal patterning in both animals. We propose these genetic programs were components of an unexpectedly complex, ancient genetic regulatory scaffold for deuterostome body patterning that degenerated in amphioxus and ascidians, but was retained to pattern divergent structures in hemichordates and vertebrates.

During vertebrate development, the brain arises from coarsely patterned planar neuroectoderm through successive refinement of regional identities, resulting after morphogenesis and growth, in a complex structure composed of highly specialized areas[1-3]. In contrast, invertebrate chordates have relatively simple nervous systems that lack unambiguous homologs of many vertebrate brain regions [4-6]. These clear disparities in nervous system complexity indicate that key innovations in patterning mechanisms have

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Author contributions

A.M.P., C.J.L., and J.A. conceived the project. A.M.P. and C.J.L. performed the hemichordate experiments and wrote the paper. E.E.M. and S.A. performed mouse experiments, and E.A.G. edited the paper. All authors discussed and commented on the data.

Author information

S. kowalevskii gene sequences have been deposited in Genbank, and accession numbers are provided in Supplementary Table 2. Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests.

Supplementary information is linked to the online version of the paper at www.nature.com/nature.

attended the evolution of the vertebrate brain from a simpler CNS. However, identifying the presumed genetic regulatory novelties has been elusive, and comparative studies have largely revealed similarities, rather than differences, in the early transcriptional architectures of diverse bilaterian nervous systems[7-12]. Notable exceptions are CNS signalling centres, which have emerged as strong candidates for vertebrate genetic regulatory novelties involved in early vertebrate brain evolution[8-10]. These centres act as secondary organizers that mediate regional patterning in the CNS and are often necessary and sufficient for the establishment of vertebrate-specific brain structures[1-3,13-18].

The anterior neural ridge (ANR), zona limitans intrathalamica (ZLI) and isthmic organizer (IsO) are the three primary signalling centres that direct anteroposterior (AP) patterning in the vertebrate anterior neural plate and then brain[1-3]. Homologous signalling centres and their molecular signatures are absent, or divergent, in amphioxus and ascidians[8-10,19-25], consistent with the hypothesis that they are vertebrate novelties whose origins could have driven CNS innovations in stem vertebrates[8-10]. However, this hypothesis depends on amphioxus adequately representing ancestral states for chordate developmental genetic characters, and does not account for the possibility of secondary losses in cephalochordates. Here, we present developmental data from the hemichordate *Saccoglossus kowalevskii* to test an alternative scenario for signalling centre origins, namely, that ANR, ZLI, and IsO genetic programs predate chordate origins and were secondarily simplified or lost along the lineages leading to the invertebrate chordates.

Hemichordates are a deuterostome phylum closely related to chordates[26] and are a promising outgroup for investigating chordate evolution. Previous studies established that despite significant body plan divergence between the two groups, hemichordates and vertebrates share a broadly conserved transcriptional regulatory architecture during early body patterning[7,27-29], revealed by their similar maps of expression domains for genes involved in early AP patterning. This combination of morphological divergence and developmental genetic similarity makes hemichordates an informative outgroup for testing the proposed coupling of vertebrate morphological and developmental genetic innovations. Here, we present descriptive and functional evidence that genetic programs homologous to the ANR, ZLI, and IsO are present in *S. kowalevskii*, suggesting that they were elements of an ancient genetic and developmental toolkit for deuterostome body patterning that were subsequently modified and elaborated to regulate vertebrate brain development.

An ANR-like regulatory program in hemichordates

The ANR is located in the anterior neural plate of vertebrates and is a source of fibroblast growth factors (FGFs) and secreted frizzled-related proteins (SFRPs), which establish and pattern the telencephalon [1,2,15,16,30,31]. Topologically consistent with vertebrate CNS expression domains, *sfrp1/5* and *fgf8/17/18* are expressed in *S. kowalevskii* anterior proboscis ectoderm (Fig. 1a-d). In vertebrates, FGFs and Wnt antagonists mediate ANR function and telencephalon patterning[15,16,30-32]. In mice, conditional *fgfr1,2,3* knockout abolishes the telencephalon[16], while *fgf8* mutants have a posteriorized neocortex[33]. Similarly, treating *S. kowalevskii* embryos with the FGF receptor inhibitor SU5402[34] from late gastrula through double-groove stages (28-36 hrs) eliminated the morphological boundary between the proboscis and collar and resulted in altered gene expression domains indicating loss and/or posteriorization of anterior proboscis (Fig. 1j-m). Expression of *foxg*, which is an FGF target in vertebrates and marks *S. kowalevskii* proboscis ectoderm, was completely eliminated (Fig. 1j, l), while *rx*, which is normally excluded from the anterior proboscis, was expressed up to the anterior limit of the embryo (Fig. 1k, m).

Wnt antagonists expressed in the ANR and/or telencephalon are also critical for forebrain development in vertebrates[2,15,35]. To explore potential similarities in Wnt functions between the telencephalon and proboscis, we first suppressed Wnt activity in the developing proboscis by utilizing RNAi to knock down the Wnt receptor *fz5/8*, which is expressed with a sharp boundary at the proboscis base (Fig. 1h). *Fz5/8* siRNA expanded apical identity at the expense of the posterior proboscis as demonstrated by expansion of the apical marker *fgf-Sk1* (Fig. 1n, p) and contraction of *rx* expression to a more posterior domain (Fig. 1o, q). Embryos injected with a scrambled control siRNA were indistinguishable from wild type (Fig. 1p, q), and the effects of *fz5/8* knockdown were limited to its endogenous expression domain (Supplementary Fig. 1). Second, we suppressed Wnt signalling in small patches of proboscis ectoderm by injecting β-*catenin* siRNA into single animal blastomeres at early cleavage stages. *β-catenin*-deficient clones in the posterior proboscis ectoderm expressed the apical marker *fgf-Sk1* (Fig. 1r-t), but not the more posterior proboscis marker *rx* (Fig. 1u-w), which indicates a transformation of posterior to apical fate in the absence of local Wnt signalling.

Although not a genetic component of the ANR itself, *shh* is expressed in the nearby medial ganglionic eminence[1,36] (see Fig. 2c, d) and interacts with FGFs to regulate telencephalon patterning[37]. The apical *S. kowalevskii* proboscis ectoderm expresses *hh* in a domain partially overlapping *fgf-Sk1*, with *hh* more broadly expressed dorsally and laterally (Fig. 1g). Embryos injected with *hh* siRNA lacked apical *fgf-Sk1* expression (Fig. 1x, y) indicating that *hh* at least partially regulates anterior FGF signalling and apical/ventral patterning. This finding raises the hypothesis that an anterior signalling domain including FGFs, Wnt antagonists, and *hh* was present in stem deuterostomes and was spatially partitioned during vertebrate evolution.

A ZLI-like regulatory program in hemichordates

The ZLI is located in the vertebrate diencephalon and specifies the flanking prethalamus and thalamus[1,17,18,23]. The molecular signature of the ZLI is a narrow, transverse domain of *shh* expression (Fig. 2C, D) that patterns the mid-diencephalon along its AP axis[17,18,23]. A homologous *hh* expression domain is absent in invertebrate chordates, supporting the hypothesis that the ZLI is a vertebrate genetic innovation, possibly associated with forebrain origins[10,21-23]. Surprisingly, we found that *S. kowalevskii hh* is expressed in a circumferential ectodermal band at the proboscis/collar boundary (Fig. 2a) in a transcriptional context of expression domains similar to the posterior vertebrate forebrain[7]. Broader *ptch* expression indicates that *hh* signals to adjacent ectoderm (Fig. 2b) similar to the ZLI (Fig. 2e, f). The proboscis/collar boundary region also expresses *fng, otx, wnt8* (Fig. 2g-m, v) and *six3*[7] in relative spatial domains characteristic of the mid-diencephalon and ZLI. In vertebrates, *lfng* is expressed broadly in the diencephalon except for the ZLI[38], while *wnt8b* and *otx* genes mark the ZLI itself in a combinatorial pattern that is unique to this region[18] (Fig. 2i, j, m). *S. kowalevskii hh* is also expressed in a *fng*-negative, *otx* and *wnt8*-positive territory posterior to *six3*[7] (fig. 2 g, h, k, l, v). In vertebrates, *otx* genes are expressed in restricted domains in the mid-diencephalon at the stage when the ZLI forms (Fig. 2i, j), and *otx1l* and *otx2* knockdown eliminates *shh* expression at the zebrafish ZLI[39]. Similarly, in *S. kowalevskii, hh* and *otx* are co-expressed in the presumptive proboscis/collar boundary ectoderm shortly after gastrulation (Fig. 2k), and *otx* siRNA knockdown reduced *hh* expression at the proboscis/collar boundary (Fig. 2w, x).

Homologs of diencephalic markers regulated by *shh* at the vertebrate ZLI are also expressed in similar topological arrangements at the *S. kowalevskii* proboscis/collar boundary. In vertebrates, *pax6* and *dlx2* are expressed anterior to the ZLI in the prethalamus (Fig. 2p, s), where their expression requires *shh*[17,18]. Similarly, *S. kowalevskii pax6* and *dlx* are

expressed anterior to *hh* at the proboscis base (Fig. 2n, o, q, r, v). In vertebrates, *foxa2* is expressed in the ZLI itself[17], and in *S. kowalevskii, foxa* is expressed at the proboscis/collar boundary (Fig. 2t, u). Targeting *hh* function in *S. kowalevskii* by injecting *hh* siRNA downregulated *dlx* at the proboscis/collar boundary (Fig. 2y, z). However, these embryos had other strong defects in AP and DV patterning, likely due to additional roles of *hh*, complicating the assessment of *hh* function at the proboscis/collar boundary (Supplementary Fig. 2). To reduce early pleiotropic effects, we treated embryos with the Hh signalling inhibitor cyclopamine[40] from the end of gastrulation through double-groove stage. 50 μM cyclopamine downregulated *dlx* at the proboscis/collar boundary with limited effects on midline expression and general morphology (Fig. 2ai, bi), suggesting that Hh signalling from the proboscis/collar boundary regulates *dlx*. The prominent similarities in expression of ZLI marker homologs in *S. kowalevskii* and vertebrates and the conserved functions for *otx* and *hh* suggest that an ancestral signalling centre homologous to the ZLI was present in early deuterostomes and was independently lost in amphioxus and ascidians.

An IsO-like regulatory program in hemichordates

The IsO is located at the midbrain/hindbrain boundary (MHB) and is defined molecularly by abutting domains of FGF8 and WNT1[1,3], which induce and pattern adjacent neural structures[1,3,13,14]. The search for a pre-vertebrate IsO has focused on expression patterns of these ligands along with the transcription factors *otx, gbx, en*, and *pax2/5/8*, whose combinatorial expression patterns in vertebrates define a molecular territory unique to the MHB (Fig. 3b, d, g, i, m, p-r). In amphioxus, CNS expression of *fgf8/17/18* is restricted to the anterior cerebral vesicle[19,24], and *wnt1* is not expressed in the CNS[25]. In *C. intestinalis, fgf8/17/18* is expressed in larval visceral ganglion where it regulates *en* and *pax2/5/8* to delineate the sensory vesicle and neck regions[20] suggesting that at least a partial IsO-like signalling centre predates vertebrates. However, *wnt1* is absent in the *C. intestinalis* genome, making it difficult to infer the full extent of this ancestral centre. In *Drosophila melanogaster, otx* and *gbx* orthologs are expressed in patterns similar to the MHB, but the absence of compelling similarities in the expression of *fgf8/17/18*-related genes and repeated expression of *wnt1* and *en* at parasegmental boundaries[12] weakens hypotheses of homology with the vertebrate IsO. To assess the presence of an IsO-like region in hemichordates, we investigated expression patterns for *S. kowalevskii* homologs of vertebrate MHB markers (Fig. 3a-t). We found that at double-groove stage (36 hrs), *wnt1* and *fgf8/17/18* are expressed in adjacent ectodermal bands in the anterior trunk with *wnt1* expressed anterior to *fgf8/17/18* (Fig. 3a, c, e); a topology similar to the vertebrate IsO (Fig. 3b, d). In vertebrates, abutting domains of *otx* and *gbx* genes position the IsO[3], and opposing *otx* and *gbx* domains are also found in protostomes[12] and amphioxus[8,9] suggesting that this pattern is ancestral to bilaterians. A reassessment of *otx* and *gbx* expression patterns in *S. kowalevskii*[7] revealed that they are also expressed in adjacent domains at the collar/trunk coelom boundary with *gbx* expressed in the ectoderm between the most posterior *otx* domains (Fig. 3f, h, j). However, the spatial arrangements of *otx/wnt1* and *gbx/fgf8/17/18* are reversed in *S. kowalevskii* and vertebrates (Fig. 3t) implying that divergent mechanisms position homologous IsO-like regions in these groups. In vertebrates, *pax2,5,8* and *en1,2* are co-expressed at the MHB (Fig. 3m, p-r). However, *pax2/5/8* and *en* do not overlap in *S. kowalevskii* (Fig. 3n, o) or invertebrate chordates[9,20], which suggests that regulation of *en* genes by *pax2/5/8* genes is a novel feature of vertebrate development. Beyond similarities in expression of signalling molecules and transcription factors, we found that the catecholaminergic neuron marker *th* (*tyrosine hydroxylase*) is co-expressed with *en* in the *S. kowalevskii* posterior collar (Fig. 3s), raising the possibility that this neuronal population is homologous to vertebrate midbrain dopaminergic neurons.

Functional assays further support the deep deuterostome ancestry of an MHB-like genetic module. In vertebrates, *fgf8* mediates organizing abilities of the IsO and maintains expression of other MHB markers[3,13,14]. To assess similar requirements for FGFs in hemichordates, we first treated embryos with 100 μM SU5402 at the end of gastrulation to suppress FGF signalling without perturbing earlier patterning events. *En* expression was strongly reduced in SU5402 treated embryos (Fig. 3u, v), while *pax2/5/8* was unaffected (Fig. y, z) compared to DMSO treated embryos. To specifically test for a role of *fgf8/17/18*, we injected fertilized oocytes with *fgf8/17/18* siRNA. *Fgf8/17/18* knockdown reduced *en* expression (Fig. 3w, x), but had no effect on *pax2/5/8* (Fig. 3ai, bi) relative to embryos injected with a scrambled control siRNA. The absence of any effect on *pax2/5/8* expression in these experiments highlights differences in gene regulation downstream of *fgf8/17/18* between hemichordates and chordates[3,20].

In vertebrates, *wnt1* is required to maintain *en* expression at the MHB[3,41]. To assess local Wnt functions at the *S. kowalevskii* collar/trunk boundary, we injected single blastomeres with $\tilde{\beta}$*catenin* siRNA at early cleavage stages to suppress Wnt signalling in small patches of collar/trunk ectoderm. Clonal descendants of injected blastomeres failed to express *en* (Fig. 3ci-ei), demonstrating a similar role for Wnts in regulating *en* genes at the MHB and collar/trunk boundary.

Discussion

Extensive similarities in expression patterns of signalling centre markers and conserved functions for FGF, Wnt, and Hh signals in *S. kowalevskii* and vertebrates provide compelling evidence that signalling centres homologous to the ANR, ZLI, and IsO were parts of an ancient genetic regulatory scaffold that predate the morphological innovations of vertebrates (Fig. 4). Therefore, assembly of these genetic networks did not trigger morphological novelties of the brain. Rather, early vertebrate brain evolution involved modifying and elaborating existing signalling centres to pattern novel structures within a highly conserved gene regulatory framework for AP ectodermal patterning[7].

Widespread losses of signalling centre components in invertebrate chordates and the unresolved nature of the ancestral deuterostome nervous system present a challenge for inferring when the ANR, ZLI, and IsO genetic circuits were first deployed to regulate CNS patterning specifically, and to what extent these integrations could have been associated with origins of vertebrate novelties. In *S. kowalevskii*, signalling centre programs are deployed at stages when the nervous system is still circumferentially organized[7] and in body regions that have been described as containing components of the adult peripheral, rather than central, nervous system in the hemichordate *Ptychodera flava*[42]. Notably, similar to their roles in the vertebrate brain, the ZLI and IsO-like signalling centres are located at morphological boundaries in *S. kowalevskii*, suggesting ancient roles in demarcating ectodermal divisions. We propose that rather than having evolved for CNS patterning, the most ancient role for signalling centres was in general body plan regionalization.

This work also highlights that basal chordates have not retained all ancestral chordate characters and have undergone substantial independent evolutionary changes. This is generally accepted for urochordates, but based on available data, cephalochordates are often considered to be the most informative extant group for reconstructing ancestral chordate characters[8,9,24]. Our data provide clear evidence that secondary losses of complex developmental mechanisms have occurred in cephalochordates, and that overlooking this possibility can inflate the numbers of putative vertebrate novelties. Although difficult to test, divergence of ANR, ZLI, and IsO-like genetic programs in extant invertebrate chordate lineages may have been associated with the loss or modification of anterior ectodermal

structures of the CNS and head. Based on these new observations, reanalyses of early chordate fossils accommodating scenarios of greater anterior complexity in stem chordates may be informative. However, in the absence of additional fossil data, predictions of the morphological consequences of developmental genetic losses based on molecular data alone are problematic.

This study provides a compelling example of the challenges associated with identifying key developmental genetic innovations responsible for morphological innovations at macroevolutionary scales. The unexpected presence of ANR, ZLI, and IsO-like programs in *S. kowalevskii* highlights the point that basing outgroup choice primarily on morphological criteria can lead to erroneous conclusions about links between morphological and developmental genetic characters: although by almost all morphological criteria amphioxus shares more similarities with vertebrates than do hemichordates, our data support the hypothesis that in certain cases hemichordates will be a more informative group than basal chordates for reconstructing stem chordate characters and understanding the origins of vertebrate developmental genetic processes. Additional data from protostomes, especially lophotrochozoans, will be required to assess whether the ANR, ZLI, and IsO genetic programs are unique deuterostome features or have even deeper bilaterian origins.

Our findings stress the importance of broad phylogenetic sampling, including morphologically divergent outgroups, to identify gene regulatory innovations responsible for evolutionary changes in body plans. With the expanding use of novel model organisms, it appears that secondary losses of complex developmental regulatory characters may occur commonly[43-45] with as yet poorly understood consequences for morphological evolution. Conversely, the presence of complex developmental modules that regulate morphologically disparate structures in distantly related lineages suggests a loose coupling between morphological and gene regulatory evolution over macroevolutionary timescales, and highlights the difficulties of reconstructing ancestral morphological characters from molecular genetic data.

Methods

Embryo procurement

Gravid *S. kowalevskii* adults were collected from the intertidal near Woods Hole, MA and maintained in flow-through seawater tables at the Marine Biological Laboratory. Spawning and embryo rearing used established techniques[7]. Mouse embryos were obtained using standard protocols approved by the University of Chicago Institutional Animal Care and Use Committee.

Experimental manipulations

SU5402 (Calbiochem) and cyclopamine (Calbiochem) were resuspended in minimal volumes of DMSO and diluted in 0.2 μM filtered seawater for treatments. Embryos were raised at room temperature (RT). Control embryos were obtained from the same batches and raised in an equivalent concentration of DMSO. siRNA microinjections were performed as described previously[27,29]. siRNAs were resuspended at 100 mM and injected at 1/10 concentration in a suspension buffer[27] including either calcein or lysinated 10,000 MW tetramethylrhodamine dextran (Molecular Probes). Unsuccessfully injected embryos, or embryos with abnormal cleavage patterns, were discarded. siRNAs were ordered from Ambion or Qiagen, and sequences are provided in Supplementary table 1.

In situ hybridizations

Colorimetric *in situ* hybridizations were performed as described previously[7,31]. Clonal descendants of injected blastomeres were identified after *in* situ hybridization using α-tetramethylrhodamine (Molecular Probes) immunofluorescence. For fluorescence *in situ* hybridization (FISH), pre-hybridization steps were performed using our standard protocol. For double FISH, embryos were simultaneously hybridized with antisense RNA probes for both genes labeled separately with digoxigenin-11-UTP (Roche) or fluorescein-12-UTP (Roche). Due to reduced sensitivity of FISH, we typically utilized two to three times more probe compared to colorimetric methods. Post-hybridization washes and blocking steps utilized our standard protocol. Embryos were then incubated in 1:200 α-digoxigenin-POD or α-fluorescein-POD antibodies (Roche) in blocking solution (2% Roche blocking reagent, 1x MAB) for four hours at RT on a rotary shaker. Embryos were washed 4 times in 1x MAB for 30 minutes and once for 1 hour at RT, or overnight at 4°C. Embryos were then washed in 0.1 M imidazole (Sigma) in 1x PBS for 10 minutes at RT and probes detected using a Tyramide Signal Amplification (TSA) Plus kit (Perkin-Elmer). Embryos were rinsed in 1x amplification diluent and incubated in cyanine-3 or cyanine-5 tyramide diluted 1:50 in 1x amplification diluent for 45 minutes on a rotary shaker at RT. Embryos were washed three times in detergent solution (1% triton x-100, 1% SDS, 0.5% sodium deoxycholate, 50 mM Tris-HCL pH 8.0, 1 mM EDTA pH 8.0, 150 mM sodium chloride) for 20 minutes at 60°C. If detecting a second gene, embryos were washed once for 20 minutes in solution X (50% formamide, 2x SSC, 1% SDS) at 60°C. Embryos were then washed three times in 1x MAB for five minutes and returned to the blocking step.

For genes with low signal using standard FISH methods, we performed an additional DNP amplification step. After primary antibody incubation and 1x MAB washes, embryos were incubated in DNP amplification reagent diluted 1:50 in 1x amplification diluent (Perkin-Elmer) for 5-10 minutes at RT on a rotary shaker. Embryos were washed 4 times in 1x MAB for 30 minutes and once for 1 hour at RT, re-blocked for one hour, and incubated in α-DNP-HRP antibody (Perkin-Elmer) diluted 1:200 in blocking solution for four hours at RT on a rotary shaker. Embryos were washed 4 times in 1x MAB for 30 minutes and once for 1 hour at RT. Expression was then detected using a TSA Plus kit (Perkin-Elmer) as described above.

Photography

Embryos were photographed using Zeiss Axiocam MRc5 or MRm cameras on Zeiss AxioImager.Z1 or Discovery.V12 microscopes. For optical clearing, *S. kowalevskii* embryos were dehydrated into methanol, cleared using Murray's clearing reagent (1:2 benzyl alcohol to benzyl benzoate), and mounted in permount (Fisher Scientific). Cleared embryos were imaged under DIC optics, and uncleared embryos were photographed in 1x PBS or 1x MAB on agarose-coated dishes. Images were acquired using Zeiss Axiovision 4.8 software and adjusted for color balance and/or levels or gamma using Zeiss Axiovision 4.8 or Adobe Photoshop CS3-5 software. Images of experimental and control embryos were processed utilizing the same parameters.

Gene identification

S. kowalevskii homologs of vertebrate genes were identified in an EST library screen[46], Amino acid sequences were aligned using ClustalW[47], and putative homologies were confirmed by constructing gene trees using MrBayes 3.1.2[48,49] with parameters optimized for each gene (Supplementary Figs. 3, 4). See Supplementary Table 2 for accession numbers.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors would like to acknowledge John Gerhart and Marc Kirschner for assistance and support, Robert Freeman for bioinformatics assistance, Ellyn Farrelly, Mark Terasaki, and Sebastien Darras for technical guidance, the members of the Lowe lab for stimulating discussions, and Gregory Wray and John Gerhart for comments on drafts of the manuscript. We also thank the staff of the Marine Biological Laboratory, the Waquoit Bay National Estuarine Research Reserve, Carl Zeiss, and Nikon, USA for assistance during our field season. This work was funded by the Searle Kinship Foundation (C.J.L), Brain Research Foundation (C.J.L.), NSF grant 1049106 (C.J.L), National Institutes of Health grant R01 HD42330 (E.A.G.), and The University of Chicago Hinds Fund (A.M.P). A.M.P. was supported by a Marine Biological Laboratory Frank R. Lillie Fellowship, National Institute of Child Health and Development institutional training grant 1T32HD055164-01A1, and National Institute of Neurological Disorders and Stroke predoctoral fellowship 1F31NS074738-01A1.

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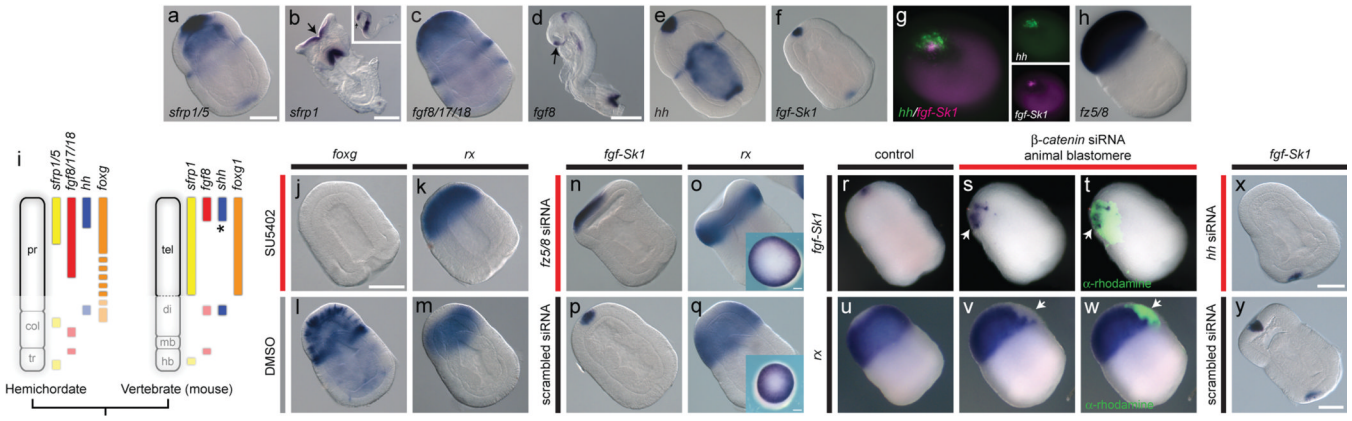

Figure 1. An ANR-like signalling centre in *S. kowalevskii*

a-h, *S. kowalevskii* and mouse *in situ* hybridizations for markers of ANR and telencephalon. *S. kowalevskii* embryos are at double groove stage (36 hrs), and are shown in dorsal view with anterior to top left, except where noted. Embryos are optically cleared except in **o, q** insets and **r-w**. Mouse embryos are at stage ~E8.5. **a**, *S. kowalevskii sfrp1/5* expression. **b**, frontal view of mouse *sfrp1* expression, arrow denotes ANR. Inset shows lateral view. **c**, *S. kowalevskii fgf8/17/18* expression. **d**, mouse *fgf8* expression, arrow denotes ANR. **e**, *S. kowalevskii hh* expression. **f**, *S. kowalevskii fgf-Sk1* expression. **g**, frontal view of double fluorescence *in situ* hybridization for *hh* and *fgf-Sk1*. **h**, *fz5/8* expression. **i**, AP expression topologies in *S. kowalevskii* and mouse embryos. Anterior to top. **j-k**, *foxg* (**j**) and *rx* (**k**) expression in embryos treated with SU5402. **l, m**, *foxg* (**l**) and *rx* (**m**) expression in embryos treated with DMSO. **n**, expanded apical *fgf-Sk1* expression in an embryo injected with *fz5/8* siRNA. **o**, retracted *rx* expression in an embryo injected with *fz5/8* siRNA. **p**, *fgf-Sk1* expression in a siRNA control embryo. **q**, *rx* expression in a siRNA control embryo. Insets in **o, q** show frontal views of uncleared embryos. **r**, wild type *fgf-Sk1* expression. **s**, *fgf-Sk1* expression in descendants of a blastomere injected with *β -catenin* siRNA. **t**, merged darkfield/fluorescence images showing clonal descendants of injected cell (green). **u**, wild-type *rx* expression. **v**, *rx* is not expressed in descendants of a blastomere injected with *β -catenin* siRNA. **w**, merged darkfield/fluorescence images showing location of the *β-catenin* deficient clone (green). **x**, *fgf-Sk1* expression in an embryo injected with *hh* siRNA. **y**, *fgf-Sk1* expression in a siRNA control embryo. Scale bars = 100 μm in *S. kowalevskii*, and for mice, 200 μm in (**b**) and 500 μm in (**d**). Abbreviations: col, collar; di, diencephalon; hb, hindbrain; mb, midbrain; pr, proboscis; tel, telencephalon; tr, trunk. *, note *shh* is expressed in the medial ganglionic eminence, near to the ANR.

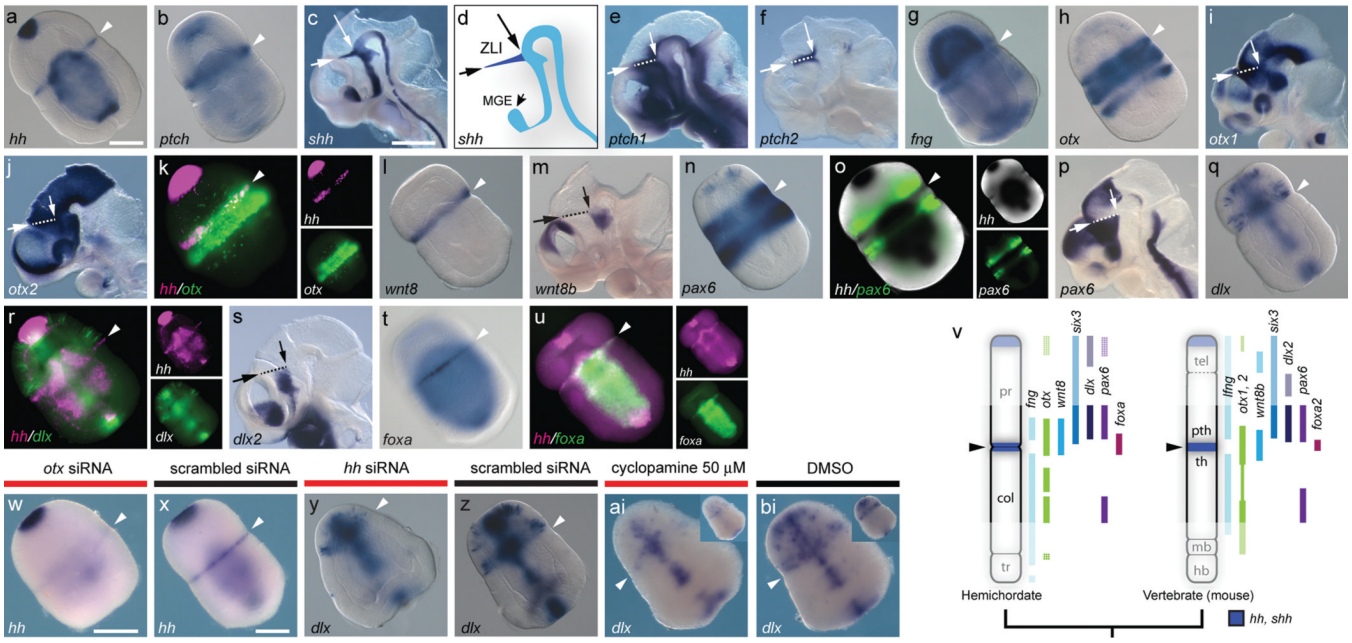

Figure 2. A ZLI-like signalling centre in *S. kowalevskii*

a-u, *in situ* hybridizations for *S. kowalevskii* and mouse homologs of ZLI and diencephalon markers. Arrowheads mark the proboscis/collar boundary in *S. kowalevskii*. Mouse images show hemisected heads at E10.5, and dashed lines indicate the ZLI with arrows denoting its extent. **a**, *S. kowalevskii hh* expression. **b**, *S. kowalevskii ptch* expression (also see Supplementary fig. 2). **c**, mouse *shh* expression. **d**, diagram of *shh* expression showing ZLI in dark blue. **e**, mouse *ptch1* expression. **f**, mouse *ptch2* expression. **g**, *S. kowalevskii fng* expression. **h**, *S. kowalevskii otx* expression. **i**, mouse *otx1* expression. j, mouse *otx2* expression. **k**, *hh* (magenta) and *otx* (green) are co-expressed at the presumptive proboscis/collar boundary. **l**, *S. kowalevskii wnt8* expression. **m**, mouse *wnt8b* expression. **n**, *S. kowalevskii pax6* expression. **o**, double *in situ* hybridization showing *pax6* expression (fluorescence, green) anterior to *hh* (colorometric, black). **p**, mouse *pax6* expression. **q**, *S. kowalevskii dlx* expression. **r**, double FISH showing *dlx* expression (green) in the proboscis base anterior to *hh* (magenta). **s**, mouse *dlx2* expression. **t**, *S. kowalevskii foxa* expression. **u**, double FISH showing *foxa* (green) and *hh* (magenta) expression. **v**, diagram of AP expression topologies of ZLI and forebrain marker homologs in *S. kowalevskii* and mice. *Six3* expression based on previous data[7]. Anterior to top. **w, x**, *otx* siRNA downregulates *hh* expression at the proboscis/collar boundary (**w**) relative to a control siRNA (**x**). **y, z**, *hh* siRNA reduces *dlx* expression in the proboscis base (**y**) relative to a scrambled siRNA (**z**). **ai, bi**, cyclopamine treatment reduces *dlx* expression in the proboscis base (**ai**) relative to a control embryo treated with DMSO (**bi**). Insets show ventral views. Scale bars = 100 μm in *S. kowalevskii* embryos and 1 mm in mice. Abbreviations: mge, medial ganglionic eminence; pth, prethalamus; th, thalamus; others as described previously.

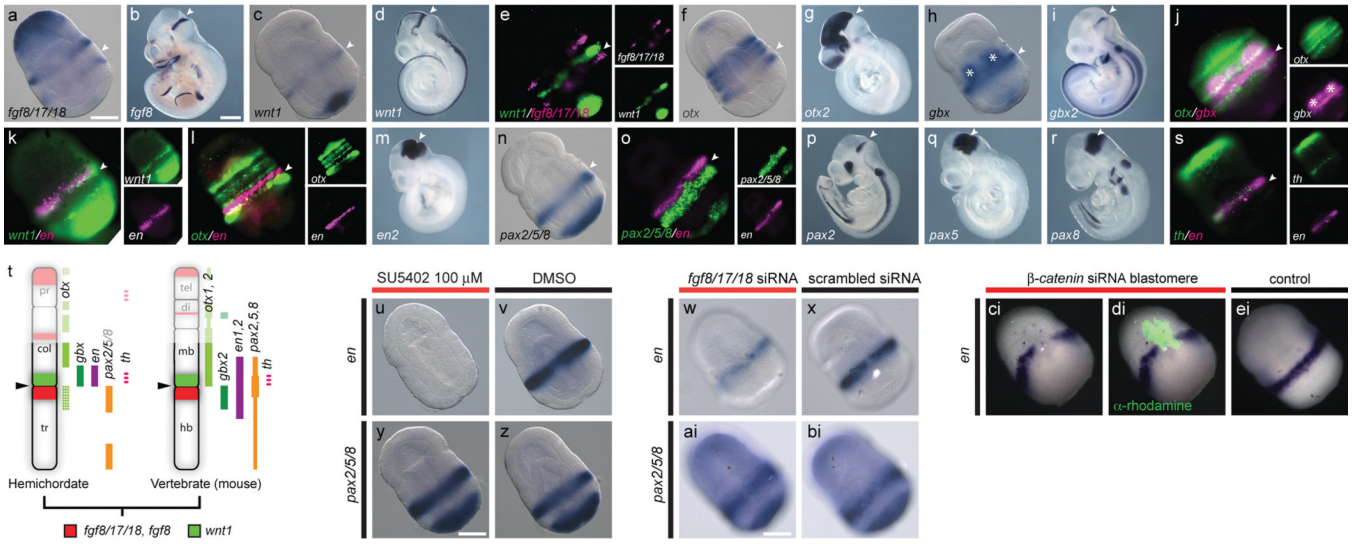

Figure 3. An IsO-like signalling centre in *S. kowalevskii*

a-s, *in situ* hybridizations for *S. kowalevskii* and mouse homologs of MHB markers. Arrowheads mark the *S. kowalevskii* collar/trunk coelom boundary the mouse IsO. **a**, *S. kowalevskii fgf8/17/18* expression. **b**, mouse *fgf8* expression. **c**, *S. kowalevskii wnt1* expression. **d**, mouse *wnt1* expression. **e**, double FISH showing *S. kowalevskii wnt1* (green) expressed directly anterior to *fgf8/17/18* (magenta). **f**, *S. kowalevskii otx* expression. **g**, mouse *otx2* expression. **h**, *S. kowalevskii gbx* expression. Asterisks denote endodermal domains. **i**, mouse *gbx2* expression. **j**, double FISH for *S. kowalevskii otx* (green) and *gbx* (magenta). **k**, double FISH for *S. kowalevskii wnt1* (green) and *en* (magenta). **l**, double FISH for *S. kowalevskii en* and *otx*. **m**, mouse *en2* expression. **n**, *S. kowalevskii pax2/5/8* expression. **o**, double FISH showing *S. kowalevskii pax2/5/8* and *en* expression. **p-r**, expression of mouse *pax2* (**p**), *pax5* (**q**), and *pax8* (**r**). **s**, double FISH for *S. kowalevskii th* (green) and *en* (magenta). **t**, summary of AP expression topologies in hemichordates and mice. Anterior to top. **u**, *en* expression is reduced in an embryo treated with SU5402. **v**, *en* expression in a DMSO-treated control embryo. **w**, *en* expression in an embryo injected with *fgf8/17/18* siRNA. **x**, *en* expression in an embryo injected with a control siRNA. **y**, *pax2/5/8* expression in an embryo treated with SU5402. **z**, *pax2/5/8* expression in a DMSO-treated control embryo. **ai**, *pax2/5/8* expression in an embryo injected with *fgf8/17/18* siRNA. **bi**, *pax2/5/8* expression in an embryo injected with a control siRNA. **ci**, *en* is not expressed in descendants of a blastomere injected with *β -catenin* siRNA. **di**, merged darkfield/ fluorescence images showing location of the *β-catenin* deficient clone (green). **ei**, wild-type *en* expression. Scale bars = 100 μm in *S. kowalevskii* embryos and 1 mm in mice. Abbreviations as described previously.

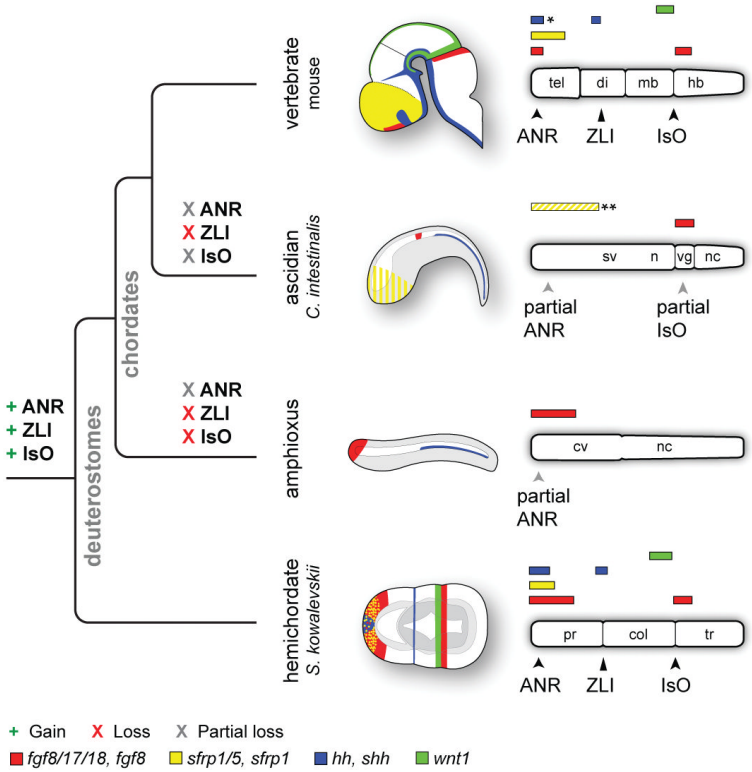

Figure 4. Evolutionary gain and loss of ANR, ZLI, and IsO-like genetic programs

Schematic diagrams depicting expression of *fgf8, sfrp1, shh*, and *wnt1* homologs in the mouse brain and ectoderm of *C. intestinalis*, amphioxus, and *S. kowalevskii*. Embryos are oriented with anterior to left and dorsal to top. Bar diagrams are oriented with anterior to the left. Diagrams depict only expression domains related to signalling components of vertebrate CNS signalling centres. Abbreviations: cv, cerebral vesicle; n, neck; nc, nerve cord; sv, sensory vesicle; vg, visceral ganglion; others as described previously. Diagrams not to scale. *Note that *shh* is expressed in the medial ganglionic eminence, adjacent to the ANR.** *Sfrp1/5* is expressed in the *C. intestinalis* anterior ectoderm from 64-cells up to neurulation, but is then downregulated in the anterior ectoderm and CNS (depicted as yellow stripes).