Chordate roots of the vertebrate nervous system: expanding the molecular toolkit

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Abstract | The vertebrate brain is highly complex with millions to billions of neurons. During development, the neural plate border region gives rise to the neural crest, cranial placodes and, in anamniotes, to Rohon-Beard sensory neurons, whereas the boundary region of the midbrain and hindbrain develops organizer properties. Comparisons of developmental gene expression and neuroanatomy between vertebrates and the basal chordate amphioxus, which has only thousands of neurons and lacks a neural crest, most placodes and a midbrain–hindbrain organizer, indicate that these vertebrate features were built on a foundation already present in the ancestral chordate. Recent advances in genomics have provided insights into the elaboration of the molecular toolkit at the invertebrate–vertebrate transition that may have facilitated the evolution of these vertebrate characteristics.

Tunicates
The sister group of the vertebrates. Tunicates include the appendicularians or larvaceans, ascidians and the thaliaceans. In phylogenetic analyses, appendicularians typically fall basal to ascidians and thaliaceans, although the branch length is long. The thaliaceans, thought to have evolved from ascidians, include three taxa: doliolids, salps and pyrosomes.

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Over the past two centuries, many groups of invertebrates have been nominated as the immediate ancestors of vertebrates[1]. Recently, however, molecular biology data have supported only one of these older views — that the proximate invertebrate ancestor of the vertebrates most closely resembled a modern amphioxus (or lancelet)[2–4]. Within the chordates, amphioxus is basal to tunicates and vertebrates (FIG. 1)[2,4]. In the past 20 years, comparisons of the expression patterns of developmental genes, together with detailed microanatomy, have indicated that the anterior expanded portion of the amphioxus CNS is probably equivalent to a diencephalic forebrain plus a small midbrain, whereas the remainder of the CNS is homologous to the vertebrate hindbrain and spinal cord. However, amphioxus has a much simpler CNS than vertebrates with approximately 20,000 neurons[5], no telencephalon and no neural crest[6]. Amphioxus has numerous ectodermal sensory neurons (although it is thought that it has homologues of only the adenohypophyseal and olfactory placodes). Even so, expression of genes in the neural plate and those encoding transcription factors that specify the neural plate border and position the midbrain–hindbrain (MHB) boundary are comparable in amphioxus and vertebrates. However, the two groups differ in their expression of late neural crest specifiers and genes encoding proteins that confer organizer properties on the vertebrate MHB. Thus, amphioxus probably reflects the foundation to which a neural crest, most neurogenic placodes and an MHB organizer were added. This raises the question of what were the changes in genetic mechanisms at the invertebrate chordate–vertebrate transition that allowed evolution of such vertebrate-specific structures. Recent advances in genomics and epigenetics are providing some answers to this question.

This Review considers the evolutionary origins of the vertebrate central and peripheral nervous systems emphasizing the genetic and epigenetic mechanisms that may have facilitated the evolution of vertebrate placodes, the neural crest and an MHB organizer. The focus is on the invertebrate chordate amphioxus, but tunicates and hemichordates are also discussed (FIG. 1). The evolution of bilaterian nervous systems from prebilaterian ancestors[7,8] is not covered here.

The neural plate border region

In vertebrates, the neural plate border region gives rise to the neural crest, cranial placodes and, in anamniotes, to Rohon-Beard sensory neurons. Neural crest cells undergo an epithelial–mesenchymal transition and migrate throughout the body differentiating into many cell types including the glia and most of the neurons in the peripheral nervous system[9] (FIG. 2). In the cranial region, additional peripheral neurons develop from neurogenic placodes[10]. Although it has been argued that the neural crest and

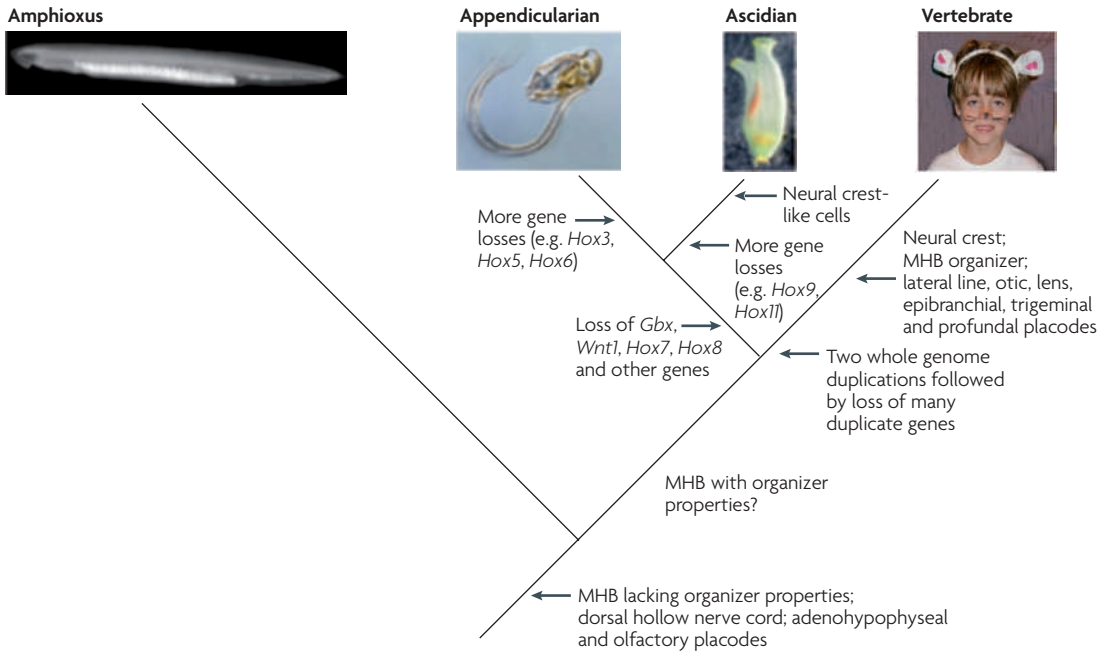

Figure 1 | **Major events in nervous system evolution mapped onto the phylogenetic tree of the chordates.** Although independent gene duplications and losses have occurred in all lineages, the two genome duplications at the base of the vertebrates and the considerable losses of developmental genes in the tunicates (that is, appendicularians and ascidians plus thaliceans) are noted. Nothing is known about developmental genes in the third group of tunicates, the thaliaceans. A third whole-genome duplication occurred at the base of the teleost fish. MHB, midbrain–hindbrain. The amphioxus image is courtesy of M. Dale Stokes; the ascidian image is courtesy of R.W. Zeller; the vertebrate image is courtesy of F. Holland Morris. The appendicularian image is reproduced, with permission, from REF. 131 .

placodes have a common evolutionary origin, as they arise from a population of cells in the neural plate boundary region and give rise to several similar cell types, the prevailing view is that they evolved independently[9,11,12].

Unequivocal homologues of the neural crest and placodes, other than the olfactory and adenohypophyseal placodes, are lacking in amphioxus, although intramedullary sensory cells in the dorsal portion of the amphioxus CNS are thought to be homologous to Rohon-Beard sensory neurons[6]. Amphioxus does have tissues that could be considered harbingers of the neural crest and other placodes — namely, the tissue at the border of the neural plate that detaches from the neural plate, develops lamellipodia and migrates over it as sheets that ultimately fuse in the dorsal midline[13], and the ectodermal sensory cells, which originate from ectodermal cells along the ventral side of the embryo, lose their cilia and migrate dorsally beneath the ectoderm[14]. However, the tissue bordering the amphioxus neural plate remains epithelial, and the amphioxus ectodermal sensory cells develop axons that extend to the CNS, regrow their cilia and microvilli and reinsert themselves into the ectoderm[14]. Amphioxus ectodermal sensory cells do not contribute to ganglia (the dorsal roots of the amphioxus CNS lack ganglia[6]) and do not resemble hair cells of the vertebrate lateral line or otic placode, which lack axons and have a stair-step array of microvilli. Even so, the amphioxus ectodermal sensory cells resemble neuroblasts delaminating from the epibranchial placodes (at least in the chick) in migrating dorsally underneath the ectoderm without undergoing an epithelial–mesenchymal transition[10,15]. In addition, the genetic mechanisms that specify the neural plate border region and migrating ectodermal sensory cells in amphioxus or placodal neuroblasts in vertebrates seem to be comparable (FIG. 3), which suggests a common evolutionary heritage.

Evolutionary origin of the CNS

The genetic basis for initial specification of the neural plate border region is the evolutionarily ancient mechanism that distinguishes neuroectoderm from non-neural ectoderm. This involves a change from a high level of BMP (bone morphogenic protein) signalling on the non-neural side, to a low level on the neural side that is mediated by secreted BMP antagonists. This mechanism is found in all bilaterians with a CNS that have been studied to date (the only known exceptions are some nematodes in the protostomes, and tunicates in the deuterostomes, both of which have modified development)[16–21].

Most data argue for a common evolutionary origin of the CNS in protostomes and chordates[8]. This idea has its roots in the famous drawing by Goeffroy Saint-Hilaire[22], which shows that an upside down lobster has essentially the same dorso–ventral organization as a vertebrate. This principle of unity of organization formed the basis of theories proposing that the ancestral bilaterian had a longitudinal nerve cord and that a dorso–ventral inversion had occurred in either the protostome or deuterostome lineage[23–25]. This view has been supported by data showing that BMP homologues are expressed ventrally in *Xenopus laevis* and dorsally in *Drosophila*

Organizer
An embryonic tissue that, when ectopically transplanted, can redirect the fate of the recipient tissue. Organizers in vertebrate embryos include the dorsal blastopore lip or Spemann's organizer in amphibians and its equivalent in other vertebrate embryos, and the tissue spanning the midbrain–hindbrain boundary.

Rohon-Beard sensory neurons
Large, mechanosensory neurons in the dorsal portion of the spinal cord of larval anamniote vertebrates. They typically degenerate later in development.

Protostomes
One of the two main groups of bilaterally symmetrical animals. The protostomes are divided into the Ecdysozoa, including nematodes and arthropods (insects, spiders, crustaceans and some smaller groups) and the Lophotrochozoa (annelids, molluscs and some smaller groups).

Deuterostomes
One of the two main groups of bilaterally symmetrical animals. The deuterostomes include the Ambulacraria (echinoderms plus hemichordates) and the Chordata or chordates (cephalochordates (amphioxus or lancelets), tunicates and vertebrates).

Direct development
A developmental mode in which the fertilized egg progresses to adult without a drastic metamorphosis. By contrast, the larva of indirect developing organisms is radically different from the adult to which it gives rise to by metamorphosis, often with the loss of most larval tissues.

melanogaster, whereas the BMP antagonist *chordin* and its *D. melanogaster* homologue *sog*, are expressed on the opposite side of both embryos[26]. Moreover, in both *D. melanogaster* and chordates, homologous genes are expressed in similar patterns from the midline to the edges of the neural plate. Thus, in *D. melanogaster, Msh* (muscle segment homeobox), *ind* (intermediate neuroblasts defective) and *vnd* (ventral nervous system defective) are expressed in dorsal, intermediate and ventral columns of neurons, respectively[27]. Correspondingly, in the vertebrate and amphioxus CNS, *Msx* is expressed dorsally, *Gsh*, which is homologous to *ind*, is expressed at an intermediate level, and *Nkx2.1*, which is related to *vnd*, is expressed ventrally[8]. In addition, expression of *Nk2.2* and *Msx* is conserved in the annelid *Platynereis dumerilii*[21].

BMP genes and *chordin* are also expressed on opposite sides of the direct developing hemichordate *Saccoglossus kowalevskii*, which has a diffuse nerve net with dorsal and ventral tracts of axons. However, in ptychoderid hemichordates, the dorsal nerve tract is partly hollow with some probable nerve-cell perikarya[28,29]. Homologues of genes encoding transcription factors that mediate anterior–posterior patterning in the chordate CNS are expressed in comparable patterns in the ectoderm of *S. Kowalevskii*. This led to the suggestion that ancestral bilaterians had a diffuse ectodermal nerve net and, therefore, that the CNS in protostomes and deuterostomes arose independently[30,31]. An alternative interpretation is that *S. kowalevskii* is derived[7,32] and has evolved, perhaps owing to altered BMP levels, a pan-ectodermal nervous system from a CNS. In chordates, whether the embryonic ectoderm gives rise to only non-neural cells, to ectodermal sensory cells or to a CNS seems to depend on BMP levels, with very low levels required for neuroectoderm, intermediate levels for ectodermal sensory cells and very high levels for non-neural cells. As expression of BMP antagonists other than *chordin* (such as *activin/nodal*, *noggin*, *gremlin*, *follistatin*, *BMP3*, *cerberus* and *twisted gastrulation* (*Tsg*)) has not been studied in *S. kowalevskii*, it is not yet possible to compare levels of BMP signalling in *S. kowalevskii* with those in chordates. However, increasing BMP signalling does expand dorsal ectodermal markers in *S. kowalevskii*, whereas decreasing it expands expression of the axon guidance gene *netrin* and the pan-neuronal marker *ELAV* (embryonic lethal, abnormal vision)[31], which indicates that BMP levels regulate the neuronal character of the hemichordate ectoderm.

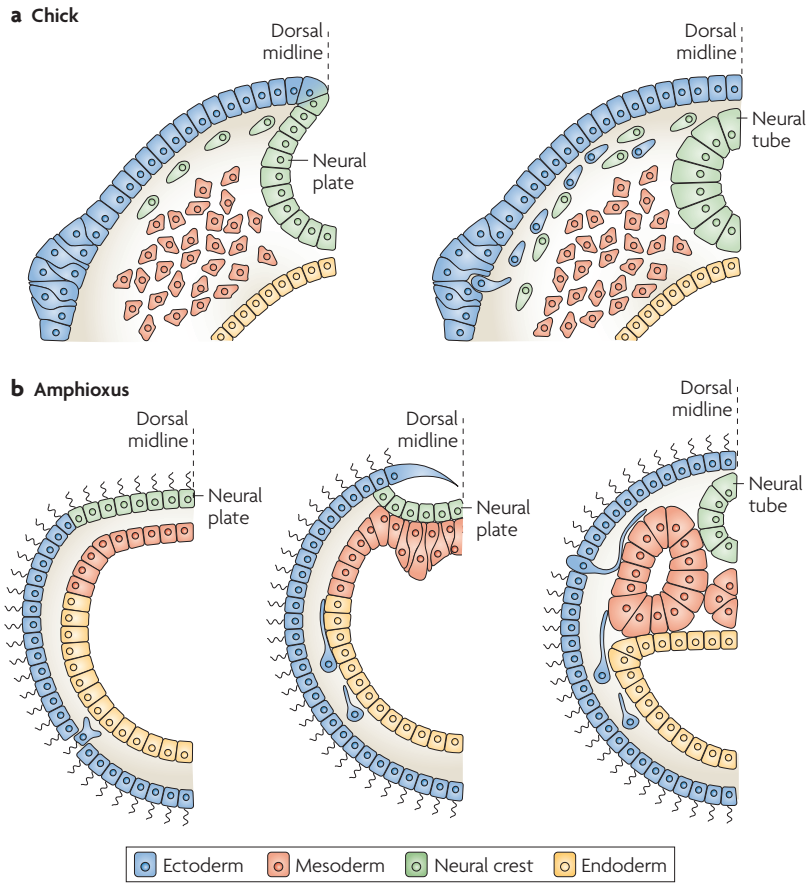

Figure 2 | **Migration of neuroblasts from neurogenic placodes in the chick resembles that of ectodermal sensory cells in amphioxus. a** | In the chick, neural crest cells (green) migrate ventrally from the dorsal edges of the neural tube as it nears closure (left), whereas neuroblasts (blue) following the track of the neural crest cells migrate dorsally from the neurogenic placodes to contribute to cranial ganglia[10] (right). **b** | In amphioxus, ectodermal sensory cells are generated in a 30° arc of ventral ectoderm, lose their cilia and migrate dorsally (left), generate axons (middle), which grow into the CNS, develop a specialized cilium and reinsert into the ectoderm (right). Neural tissues and derivatives are shown in green; ectodermal tissue and derivatives are shown in blue. Neurulation in amphioxus differs from that in all vertebrates in that the ectoderm adjacent to the presumptive neural plate, detaches from it and migrates over it as sheets of ectoderm[13] (middle). Once the sheets of ectoderm have fused in the dorsal midline, the neural plate rounds up to form a neural tube (right).

Specification of the neural plate border region

In vertebrates and amphioxus, levels of BMP signalling are crucial for specification of the neural plate and neural plate border region. In both groups, BMP2/4 and BMP5–8 genes are initially expressed throughout the ectoderm at the onset of gastrulation[33,34], and levels of their encoded proteins are titrated along the dorsal–ventral axis by a wide array of dorsally secreted antagonists[35]. Specification of the neural plate requires very low levels of BMP signalling, whereas a level higher than in the neural plate, but lower than in more ventral regions of the ectoderm, is required for the specification of the neural crest or Rohon-Beard sensory neurons[36]. Thus, knockdown of a single BMP antagonist in vertebrates results only in a mildly ventralized phenotype[37], whereas simultaneous knockdown of *BMP2*, *BMP4*, *BMP7* and the related anti-dorsalizing morphogenetic protein (*ADMP*) in *X. laevis* re-specifies the entire ectoderm as neural[38]. Correspondingly, in both amphioxus and *X. laevis*, overexpression of BMPs ventralizes the embryo with the entire ectoderm being specified as non-neural[17,18,39].

In addition to BMPs, key genes involved in specification of the neural plate border region in vertebrates include WNTs, *distalless* (*DLX*), *MSX*, *BLIMP1* (*prdm1*) and fibroblast growth factors (FGFs) (FIG. 3). DLX genes are co-expressed with *BMP4* and help position the neural plate border region by modulating BMP signalling. Expression of DLX genes is required for the development of Rohon-Beard cells and the trigeminal placode[40–43]. Several WNT

Nerve net
A de-centralized nervous system consisting of interconnected neurons without a brain. Nerve nets occur in cnidarians (for example, sea anemones, jellyfish and corals) echinoderms and hemichordates.

Ptychoderid hemichordates
Hemichordates include the free-living enteropneusts and the sessile pterobranchs. In phylogenetic analyses based on 18s rDNA, enteropneusts are not monophyletic. Instead, two groups of enteropneusts, the Saxipendiidae and the Harrimaniidae (which includes *Saccoglossus kowalevskii*) plus the pterobranchs form one clade, which is sister group to the third group of enteropneusts, the Ptychoderidae.

Derived
A derived character is one occurring in a particular lineage of a larger taxon which was not present in the last common ancestor of the larger group. An organism with a number of derived characters can be said to be derived within the group.

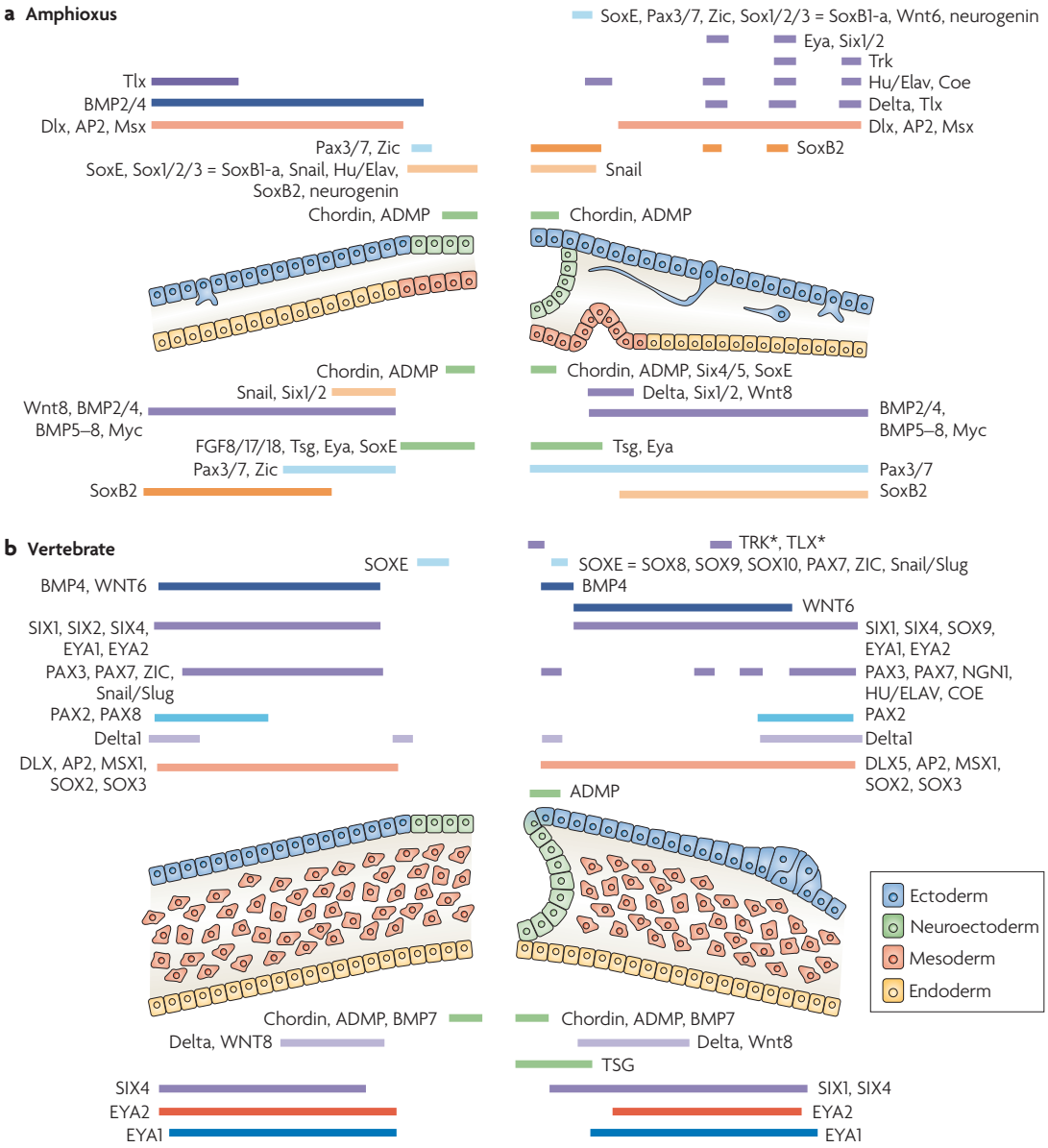

Figure 3 | **Patterns of gene expression in neural plate border region, neural tube and underlying mesendoderm in amphioxus and a generic jawed vertebrate.** The expression of ectodermal genes is shown above, and that of mesendodermal genes below, each diagram. **a** | Amphioxus embryos at the early neurula (left) and mid-neurula (right) stages. At both stages, the entire amphioxus embryo is all future head except for the tissue immediately around the blastopore, which will give rise to the trunk and tail. Embryos are shown in cross-section approximately at the midpoint along the anterior–posterior axis and are split ventrally along the midline and flattened out. In the early neurula, *Sox1/2/3* marks the entire neural plate, *neurogenin* marks the posterior third of the neural plate, whereas *Pax3/7* and *Zic* mark the edges of the neural plate, and *Tlx* marks the ventral ectoderm that will give rise to ectodermal sensory cells. *BMP2/4* (bone morphogenic protein 2/4) is weakly expressed throughout the ectoderm except in the neural plate. The BMP antagonists *chordin* and *ADMP* (anti-dorsalizing morphogenic protein) are expressed in the centre of the neural plate and in the underlying mesoderm. At the mid-neurula stage, the migrating and definitive ectodermal sensory cells express a number of genes, including *Eya*, *Six1/2*, *Trk*, *Hu/Elav*, *Coe*, *Delta*, *Tlx* and *SoxB2*. Expression of several genes that were broadly expressed in the early neural plate (that is, *SoxE*, *Sox1/2/3*, *Snail* and *Hu/Elav*) becomes restricted to the edges of the neural plate together with *Wnt6* and *Pax3/7*. **b** | A consensus of gene expression in the neural plate border region and underlying mesoderm (orange cells) and/or endoderm (yellow cells) in a generic vertebrate. Cross-sections through the head of an embryo at the neural plate stage (left) and late neurula before the onset of migration of neural crest and placodal derivatives (right). Because *TRK* and *TLX* (asterisks) are not expressed in placodal derivatives before their migration, their expression at a later stage is shown. Tissues ventral to the panplacodal region are not shown. Domains along the anterior–posterior extent of the panplacodal region are summed. For details of gene expression in individual placodes see Schlosser[132]. Expression domains are chiefly based on studies of chicks and frogs, but some expression domains from mice, zebrafish and lamprey have been included where those in chicks and frogs are unclear. Vertebrate data derived from REFS 9,44,55,64,113,132–136. Dlx, distalless.

genes, including *WNT3*, *WNT6* and *WNT8*, are expressed in the neural plate border region or underlying mesendoderm[44–46]. In the chick, Wnt–β-catenin signalling induces BMP, which then induces neural plate border cells after Wnt is blocked. The joint action of Wnt and BMP generates non-neural ectoderm[47–49]. MSX genes are expressed in the edges of the vertebrate neural plate and together with *BMP4* and *DLX5* in the pan-placodal region around the anterior neural plate. Rohon-Beard sensory neurons and the neural crest have different requirements for MSX[50]. Whereas knockdown of Msx genes in zebrafish disrupts the neural crest, the development of Rohon-Beard cells is initially normal, although later, half die prematurely[50]. Roles for FGFs in the neural plate border region have also been identified[46,51]. In *X. laevis*, Fgf8 induces development of the neural crest by activating *Wnt8* expression in the paraxial mesoderm[46]. Although these genes are only a few of those involved in development of the neural plate border region, they demonstrate that, starting with a requirement for precise levels of BMP signalling, there is considerable overlap in the gene networks that specify neurogenic placodes, Rohon-Beard sensory neursons and the neural crest.

A comparison between amphioxus and vertebrates indicates that they use broadly similar gene networks to specify the neural plate border region (FIG. 3). It is thus reasonable to assume that a comparable gene network was present in basal chordates although they lacked a definitive neural crest and most placodes. In early amphioxus embryos, BMPs, Wnts, *Dlx*, *Msx* and *Fgf8* are expressed in similar patterns as their vertebrate homologues. *BMP2/4* and *BMP5–8* genes are expressed throughout the early gastrula, and BMP antagonists, including *chordin*, *Nodal* and *Tsg*, are expressed dorsally[18]. Increased levels of BMP block neural development completely[18]. At the onset of gastrulation, *Dlx* is transcribed throughout the ectoderm but is rapidly downregulated in presumptive neuroectoderm[13], whereas *Wnt8* is expressed in the somites, *Fgf8* in the dorsal blastopore lip, *Wnt6* in the edges of the neural plate and *Wnt3* throughout the CNS except for the forebrain[45,52]. At the onset of neurulation, *Msx* is expressed throughout the ectoderm except in the neural plate[53]. Taken together, these patterns indicate that specification of the neural plate border region in vertebrates and amphioxus is probably mediated by a conserved genetic mechanism involving BMPs, Wnts, *Dlx* and *Msx,* among other genes. Expression of *Msx* and *Dlx* throughout the amphioxus ectoderm is consistent with the entire ectoderm of amphioxus at the gastrula and neurula stages being equivalent to the pan-placodal region in vertebrate embryos. The origin of ectodermal sensory cells ventrally in amphioxus embryos supports this idea. Moreover, because amphioxus embryos (and presumably those of the protochordate ancestor of the vertebrates) are much smaller than those of vertebrates, the distance from the ventral to the dorsal midline in an amphioxus embryo is comparable to the width of the pan-placodal region in vertebrate embryos (FIGS 2,3). It may be that ventral ectoderm lacking ectodermal sensory cells arose in the vertebrate lineage in conjunction with a large increase in embryonic size, and as a result, the pan-placodal region became restricted to the neural plate border. Such size differences might correlate with differences in the levels of BMP and/or Wnt signalling along the dorsal–ventral axis. Levels of Wnts and BMPs in neural crest compared with non-neural ectoderm have been measured in *Xenopus* embryos[48], but the techniques required to measure this in the smaller amphioxus and hemichordate embryos are not yet available.

Evolution of the neural crest and placodes

The emerging picture of the evolution of the neural crest and placodes is still unclear because no unique molecular signatures are known for either feature, and because there is no modern organism unequivocally intermediate in organization between amphioxus and vertebrates[54]. The most basal vertebrates (lampreys and hagfish) have a definitive neural crest and placodes, whereas the presence of the neural crest and placodes in tunicates is debatable. However, comparisons between amphioxus and vertebrates show that part of the genetic mechanism for generating these features was present at the base of the chordates (FIG. 1).

In vertebrates, once BMPs, WNTs and other genes map out the non-neural ectoderm, neural plate and neural plate border regions, the cells in the neural plate border develop into the neural crest, the ectoderm and placodes (reviewed in REF. 55). Suites of genes that specify the neural plate, neural plate border and neural crest become active followed by the activation of neural crest effectors[56]. The earliest neural plate specifiers are genes in the SOX1/2/3 class, whereas *ZIC*, *MSX* and *PAX3/7* genes plus *DLX* are early neural plate border specifiers. Expression of all these genes is conserved in amphioxus, lamprey and gnathostomes[57,58]. However, except for *Snail*, expression of neural crest specifiers is not conserved between amphioxus and vertebrates[57,58]. The amphioxus homologues of neural crest specifiers, such as *AP2*, *FOXD3*, *Twist*, *ID*, *MYC* and *SOXE* (also known as *SOX9/10*), are expressed in early development, but not at the edges of the neural plate[53,59]. Thus, in the evolution of the neural crest, a number of old genes have evidently been co-opted for new functions.

Comparatively little is known about differences in the genetic programmes that direct the development of ectodermal sensory cells in amphioxus and placodal neuroblasts in vertebrates. Similar suites of genes are involved in the initial development of the two (FIG. 3). Homologous genes[60], such as *Six1/2*, *Six4/5*, *Eya1/2*, *Tlx* (*Hox11*), *SoxB*, *Trk* (a tyrosine kinase receptor), *Delta* and *Hu/Elav*, are expressed in the nascent ectodermal sensory cells in amphioxus and in the pan-placodal region and/or placodal neuroblasts in vertebrates[14,61–65]. Similarly, one *Irx* gene, *Islet* and *ERR* (oestrogen related receptor) are expressed in particular placodes in vertebrates and in subsets of developing ectodermal sensory cells in amphioxus[66–70]. However, there are also differences. In amphioxus, *Dach*, which typically cooperates with SIX and *EYA* genes, the neural differentiation gene *neurogenin*, *Pax2/5/8* and *Pax3/7* are not expressed in ectodermal sensory cells, whereas their vertebate homologues are expressed in placodes[71–73]. These comparisons

suggest that like the gene network for formation of the neural crest, a conserved gene network initiates development of ectodermal sensory cells in amphioxus and vertebrates, but the more downstream components of the network are divergent. Thus, vertebrate placode evolution, like that of neural crest, seems to have involved conservation of early-expressed genes plus co-option of genes for late functions.

The midbrain–hindbrain boundary organizer

The evolution of the MHB organizer presents a similar story as that of neural crest and placodes. Amphioxus has part, but not all, of the genetic machinery for formation of both. In vertebrates, if the MHB region is transplanted into either more-anterior or more-posterior brain regions it induces midbrain characteristics[74]. The position of the MHB is established by opposition between *GBX2*, which is expressed in the anterior hindbrain, and *OTX2*, which is expressed in the forebrain and midbrain. Subsequently, a suite of genes including *EN1*, *EN2*, *WNT1*, the three PAX genes (*PAX2*, *PAX5* and *PAX8*) and the three FGF genes (*FGF8*, *FGF17* and *FGF18*) confers organizer properties on this region (FIG. 4). The expression patterns of *Gbx* and *Otx* in the amphioxus CNS are comparable to those in vertebrates, which suggests that the division between forebrain–midbrain and hindbrain in amphioxus is equivalent to the vertebrate MHB. However, the genes that confer organizer properties on the vertebrate MHB are not expressed there in amphioxus, suggesting that the amphioxus MHB lacks organizer ability and that organizer properties probably evolved later, chiefly by co-option of old genes to the gene network operating the MHB.

What about tunicates?

Molecular phylogenetic analyses with large data sets consistently place tunicates as the sister group of vertebrates[2–4]. This arrangement initially gave hope that tunicates would give insights into the evolution of the neural crest, placodes and the MHB. Unfortunately, tunicates, in contrast to amphioxus and vertebrates, are evolving rapidly and have diverged both from other chordates and from one another. Therefore, when characters in one tunicate, but not others, are similar to those in vertebrates, it can be difficult to distinguish between convergence or inheritance from a common ancestor. Tunicates have reduced genomes (~160 mb for the ascidian *Ciona intestinalis* and ~60 mb for the appendicularian *Oikopleura dioica* compared with 520 mb for amphioxus and 3 gb for humans) and have lost several developmental genes including *Gbx*, *Wnt1* and several *Hox* genes[75] (FIG. 1).

Consequently, claims that one tunicate or another has homologues of vertebrate hair cells, the neural crest or an MHB organizer have not been universally accepted[12,76,77]. The adenohypophyseal and olfactory placodes probably have homologues in the rostral ectoderm of tunicate larvae and amphioxus. However, although several authors have argued that ectodermal sensory cells inside the atrial siphon of ascidians have homologues in the vertebrate neurogenic placodes[78–80], others maintain that evidence for such homology is insufficient[12,77,81]. Unlike vertebrate hair cells, most ectodermal sensory cells in tunicates and amphioxus have axons, although there are a few exceptions[82]. Perhaps the best candidates for hair-cell relatives in tunicates are the two Langerhans cells in the tail of *O. dioica*. Like hair cells, these are secondary neurons that lack axons. They synapse with a single neuron in the caudal ganglion[83] and express one of two *Pax2/5/8* genes, strengthening a possible evolutionary relationship with the otic or epibranchial placodes[60,84]. However, these cells do not arise from an epithelial thickening and do not have a stair-step array of microvilli. Alternatively, the coronal cells in the siphon of adult ascidians are secondary neurons and, therefore, have been proposed as evolutionarily relatives of hair cells[78]. The difficulty is that there is no one type of ectodermal sensory cells common to all tunicates, and, therefore, conclusions about the kind of sensory cells in the common ancestor of tunicates and vertebrates are problematic. Taken together, it seems likely that hair cells *per se* are a vertebrate innovation, but both hair cells and ectodermal sensory cells of protochordates probably evolved from ectodermal sensory cells in an ancestral chordate.

The evidence for ascidian homologues of the neural crest is also problematic. One neural crest-like feature in *C. intestinalis* is the evident migration of cells from the trunk lateral mesenchyme into the primordia of the adult siphon[85]. Because precursors of these cells express the HNK-1 antigen as well as homologues of 7 of 16 neural crest markers tested (namely *FoxD*, *Ap2*, two *twist*-like genes, *c-myc*, *cadherin-2* and *rhoABC*), they were suggested to be homologous to vertebrate neural crest cells[85]. However, the precursors of these cells do not give rise to CNS cells. Moreover, neither HNK-1 nor any of these seven genes is an exclusive marker of the neural crest. In amphioxus and vertebrates, *FoxD* genes are predominantly expressed in the mesoderm[86] and *AP2* is expressed in the non-neural ectoderm as well as in the lateral neural tube[87], whereas in vertebrates, *Twist, c-myc*, *RhoA*[88] and *N-cadherin* (*CDH2*) are expressed in mesoderm as well as in neural tissues[89,90]. Moreover, these cells do not originate in the same place as the migrating cells that give rise to pigment cells in *Ecteinascidia* (another ascidian), which have also been suggested as related to neural crest[91]. Therefore, it is unclear if the migrating cells in *C. intestinalis* are a general feature of tunicates.

The argument that tunicates may have gone farther than amphioxus in constructing an MHB organizer is better supported. The CNS in *C. intestinalis* has three parts: an anterior sensory vesicle with 215 cells, a neck region of 6 cells and a visceral ganglion of 45 cells[5]. Expression of *Fgf8/17/18* and *Pax2/5/8* in the neck region between anterior *Otx* and posterior *Hox* domains (FIG. 4) led to the suggestion that this region is homologous to the vertebrate MHB organizer. In vertebrates, *FGF8* is essential for organizer activity of the MHB and can mimic its organizer activity when ectopically expressed. Because *C. intestinalis Fgf8/17/18* suppresses *Otx* expression in the posterior sensory vesicle and affects expression of *Pax2/5/8* in the neck region, it has been suggested that *Fgf8/17/18* in the ancestor of vertebrates and tunicates had a role in the establishment of separate midbrain and

Siphon
Tunicates are marine, ciliary feeders. In ascidian tunicates, a current of water is pulled in through the incurrent siphon by cilia around the gill slits. After food particles are filtered out, the water exits through the excurrent siphon.

hindbrain territories[92]. However, there are problems in reconstructing such an ancestor. First, in another ascidian *Halocynthia roretzi*, the neck region, which has been suggested to be homologous to the MHB, is absent. Second, tunicates have lost two key MHB genes (*Gbx* and *Wnt1*). Third, in tunicates, several MHB markers are not expressed in patterns directly comparable with those in vertebrates[76]. Moreover, whereas in both appendicularians and *C. intestinalis*, *Otx* is expressed in the anterior CNS, *Pax2/5/8* is not expressed directly posterior to the *Otx* domain in the appendicularian CNS[76,84] (FIG. 4). Thus, although the MHB in the common ancestor of tunicates and vertebrates might have had some organizer properties, because of the loss of key MHB genes in tunicates, the reduced number of cells in the CNS and differences in expression of MHB genes between appendicularians and *C. intestinalis*, it is unclear whether the expression of homologues of vertebrate MHB markers in the neck region of the CNS of *C. intestinalis* represents inheritance from a common ancestor or convergent evolution.

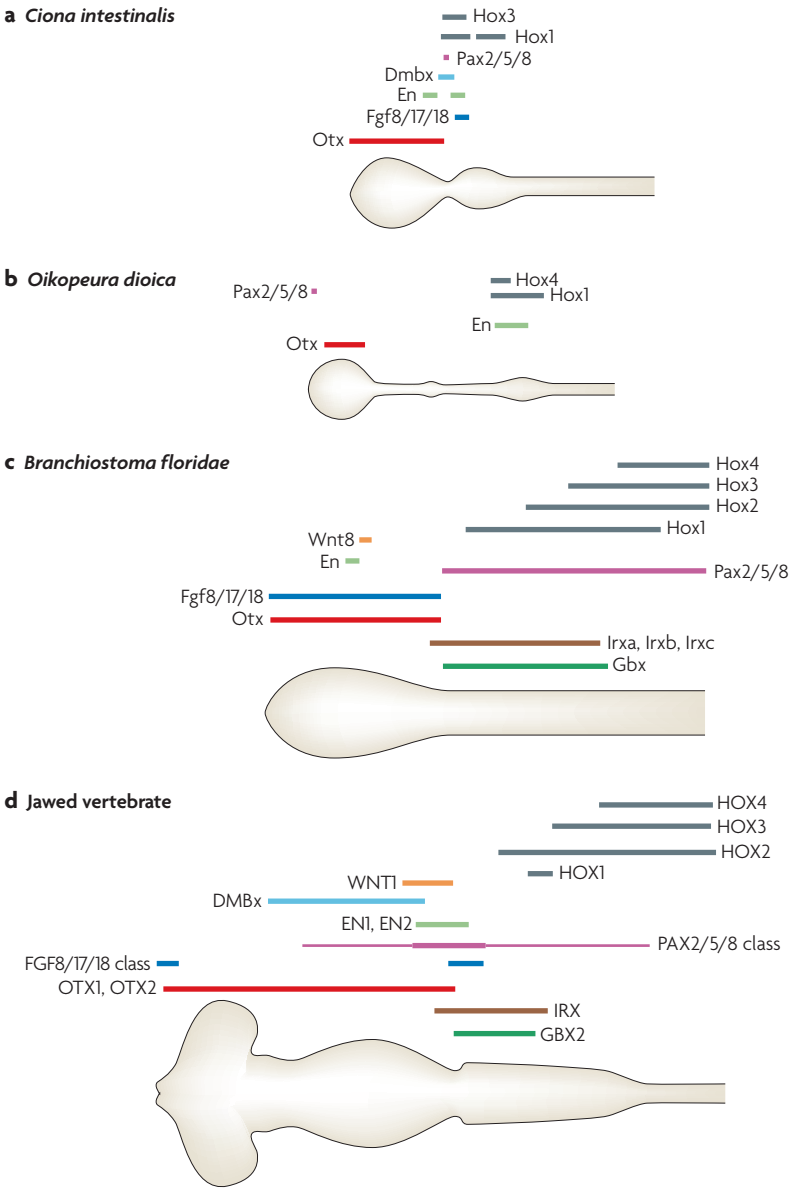

Figure 4 | **Comparison of midbrain–hindbrain gene expression in amphioxus, tunicates, and a generic jawed vertebrate.** Domains of gene expression are shown above dorsal views of the brain. Where the expression domain of a particular gene differs among the vertebrates, that of the chick has been given. In the ascidian tunicate *Ciona intestinalis* (**a**) *Hox2* and *Hox4* are not expressed in the CNS, and *Wnt1* and *Gbx* have been lost. Expression of *Irx* is unknown. The tail nerve cord has no nerve cell bodies and the tail is resorbed at metamorphosis. The appendicularian tunicate *Oikopleura dioica* (**b**) has lost both *Gbx* and *Hox3*. *Hox2* is present, but not expressed in the CNS. Expression of *Dmbx*, *Fgf8/17/18*, *Irx* and Wnts has not been studied. The tail nerve cord, unlike that of *C. intestinalis*, persists in the adult. In the amphioxus *Branchiostoma floridae* (**c**), unlike vertebrates (**d**), neither *Dmbx* nor *Wnt1* are expressed in the CNS.

Evolution of the genomic toolkit

A notable event near the base of the vertebrates was two rounds of whole genome duplication. At least one and possibly both of these duplications occurred before the divergence of hagfish and lampreys[2,93]. Although there has been considerable loss of gene duplicates (humans have only 25% more genes than amphioxus), copies of developmental genes, including transcription factors and signalling proteins, have been preferentially retained[2]. Moreover, gene knockouts in mice have shown that developmental genes and genes resulting from whole genome duplications tend to be more essential to organisms than other duplicates[94]. These data support the idea that the genome duplications gave vertebrates the tools to evolve new structures such as the neural crest, placodes and an MHB organizer.

The classic model for acquiring new gene functions is gene duplication followed by subfunctionalization, in which ancestral gene functions are split between the duplicates, and/or neofunctionalization, in which one or more of the duplicates acquires a new function[95]. This model initially concerned *cis*-regulatory elements. For example, vertebrate FOXD3 has acquired a new domain in the edges of the neural plate, where it functions in specification of neural crest lineages[96]. As none of the other four vertebrate *FOXD* genes or the single amphioxus *FOXD* gene is expressed in neural crest cells, *FOXD3* probably acquired the regulatory elements for expression in the neural crest after genome duplication. This conclusion is supported by the failure of a reporter construct of amphioxus FoxD to direct expression to the neural crest in the chick, although it directed expression in amphioxus to all the domains that normally express the gene and to comparable domains in the chick[53].

New regulatory elements can arise by point mutations, deletions or insertions in regulatory DNA. Transposable elements, which have been referred to as 'genomic parasites' are considered a major source of new regulatory elements[97]. Transposable elements are nearly ubiquitous in eukaryotic genomes and have probably given rise to siRNAs and at least some miRNAs[97,98]. The long terminal repeat (LTR) transposons, long interspersed nuclear elements (LINEs) and some short interspersed nuclear elements (SINEs) have retroviral-like sequences and may be inserted into a gene regulatory region or into an intron, which by exonization can become part of the protein-coding region[99,100]. Transposable elements are concentrated in the regulatory

Cis-regulatory elements
Also known as enhancers. 200–300 bp stretches of non-coding DNA to which *trans*-acting transcription factors bind (along with their co-factors and/or other interacting molecules) in order to upregulate or downregulate the transcription of a gene on the same strand of DNA.

regions of genes and are a source of new regulatory elements[101]. SINEs, which are typically 200–300 bp long, are present in about 50–100 copies in invertebrate genomes, but they can reach up to 500,000 copies in the human genome. Alu elements, a common class of SINEs in primate genomes, have been implicated in mediating DNA rearrangements, gene duplication and alternative splicing. The amphioxus *FoxD* gene exemplifies how transposable elements might give rise to new enhancers. An Alu element was located in the *FoxD* regulatory region in a cosmid derived from one animal[102], however, this element was not present in the comparable position in either allele of the *FoxD* gene in the animal used for gene sequencing, indicating that it has not become fixed in that position in the genome. In other organisms, fixation of such transposable elements, and their function as enhancers, has been shown in a family of sea urchins[103]. It has also been documented that in humans numerous transposable elements near developmental genes undergo purifying selection[104], which indicates that transposable elements are probably major sources of new regulatory elements.

Another example of the acquisition of new domains of expression subsequent to gene duplication is *Wnt1*. Mammals have only one *WNT1* gene due to loss of the other duplicates. The ancestral pattern of *Wnt1* expression, conserved from cnidarians through bilaterians, is around the blastopore. In amphioxus, that is the only region where *WNT1* is expressed. Evidently, subsequent to the genome duplications in the vertebrate lineage, copies of *Wnt1* expressed around the blastopore were lost, and the copy that acquired a new domain of expression in the CNS was retained with a new function: that of maintaining expression of *FGF8*, *EN2* and *PAX2* at the MHB[105,106].

In spite of the acknowledged importance of genome duplication for vertebrate evolution, gene duplication is not the only way genes can acquire new expression domains. For example, roles for *Fgf8/17/18*, *En1/2* and *Pax2/5/8* at the MHB probably arose before duplication, as the three paralogues of *Fgf8* (*FGF8*, *FGF17*, *FGF18*), the two *engrailed* paralogues (*EN1* and *EN2*) and the three *Pax2/5/8* genes (*PAX2*, *PAX5* and *PAX8*) are all expressed at the vertebrate MHB. However, although the expression domains of the vertebrate duplicates of these three genes overlap, they are not congruent, and their functions have diverged. For example, *FGF8b*, but not *FGF8a*, *FGF17b* or *FGF18*, activates *GBX2* expression in the anterior hindbrain[107]. In amphioxus, *Fgf8/17/18*, *En1/2* and *Pax2/5/8* are not expressed at the MHB, but they are all expressed in the CNS with the domains of *Fgf8/17/18* and *Pax2/5/8* abutting the MHB. Thus, the changes in expression of these genes that occurred after amphioxus and vertebrates diverged was not dramatic.

Evolution of protein function

Although the focus of the evolution of gene networks has been on gene duplication followed by the acquisition of new *cis*-regulatory elements, proteins also evolve. Point mutations, exonization and domain recombination, duplication or loss are additional mechanisms that drive changes in protein function[108]. Possibly more important are evolutionary changes in post-transcriptional events, especially alternative splicing and regulation of translation by siRNAs and miRNAs (BOX 1). Many, if not most, developmental genes are alternatively spliced with specific splice forms regulated according to tissue and developmental stage. For example, Pax genes are extensively alternatively spliced. Expression of the single amphioxus *Pax3/7* and *Pax2/5/8* genes is largely, although not entirely, comparable to that of their several vertebrate homologues — amphioxus *Pax3/7* and vertebrate *PAX3* and *PAX7* are expressed in the edges of the neural plate[109]; amphioxus *Pax2/5/8* is expressed in the CNS from the level of the MHB to the tip of the tail and genes in the vertebrate PAX2/5/8 group are expressed in the lateral neural tube in the hindbrain and spinal cord, as well as at the MHB. New domains for the vertebrate homologues of these PAX genes are in placodes — *PAX2*, *PAX5* and *PAX8* are expressed in the otic placode[110,111], and *PAX3* and *PAX7* in the trigeminal placode[112,113]. Some splice forms of the amphioxus Pax genes affect

Box 1 | Epigenetic mechanisms for modulation of gene function

The term epigenetics has been defined as comprising heritable traits not directly encoded by the genome, but can include DNA modifications such as methylation, which are often, but not always, inherited[120]. Importantly, the mechanisms for generating epigenetic traits are encoded by the genome. Although these mechanisms are evolutionarily conserved, the specific end products are much less so, complicating an understanding of the roles of epigenetics in development and evolution. A common DNA modification is cytosine methylation, which in vertebrates is typically global except for CpG islands — clusters of CpG residues often located near transcription start sites[121,122]. Although DNA methylation is typically viewed as irreversibly silencing transcription, it can be dynamic[123]. CpG islands demonstrating tissue-specific methylation are prevalent in developmental genes such as Hox and Pax[121]. By contrast, methylation in invertebrates is usually mosaic, with alternating stretches of methylated and unmethylated DNA[122]. In addition, histone modifications such as reversible acetylation, irreversible methylation, or ATP-dependent mechanisms involving the SWI/SNF, ISWI complexes and Mi-2/NuRD, which mediates histone modification and nucleosome remodelling, can alter the transcriptional activity of chromatin[124,125]. In vertebrates, mutations in the SWI/SNF complex can be early embryonic lethal or cause exencephaly, probably due to neural crest abnormalities[124]. Of all post-transcriptional modifications, alternative splicing and miRNAs and siRNAs have received the most attention. Alternative splicing, which involves exon skipping and the use of alternative splice sites, has been estimated to occur in over 75% of human genes[126]. Alternative splicing can change the ability of transcription factors to transactivate or create subtle changes in the DNA-binding domain. Many splice forms with premature stop codons are predicted to be subject to nonsense-mediated decay, which in turn has been considered a means of regulating the levels of mRNA, although such a role has been controversial[126]. Roles for splice forms in autoregulatory negative-feedback loops have been identified[126]. Evolutionary conservation of some forms with premature stop codons suggests that they may function as competitive inhibitors of other splice forms[114]. miRNAs and siRNAs are transcripts of non-protein coding DNA, which are cut by Dicer proteins into ~20–30 nucleotide pieces that silence target genes. miRNAs typically bind to the 3′UTRs of target mRNAs and inhibit translation. A major difference between them is that nearly all siRNAs silence the same locus from which they originate, whereas miRNAs silence a variety of transcripts from other loci[127]. Moreover, miRNAs, but not endogenous siRNAs, are evolutionarily quite conserved. Not only do miRNAs regulate a wide range of transcripts, but transcription factors such as Twist-1, which is expressed in neural crest cells, can regulate transcription of miRNAs[128]. Post-translational modifications of proteins are myriad. They include methylation and acetylation as mentioned above for histone modifications, phosphorylation of tyrosine, serine and threonine residues, ubiquitylation or sumoylation of lysine residues, prolyl-hydroxylation and glycosylation, among others[129]. Some post-translational modifications may be reversible and/or developmentally regulated[129,130], but the relevance of such modifications to evolution of body plans has not been explored.

Transposable elements
DNA sequences that can move within a genome. They include DNA transposons (rolling-circle transposons or Helitrons, cut-and-paste transposons and Mavericks or Polintrons) and retrotransposons (retrovirus-like LTR (long terminal repeat) transposons and non-LTR retrotransposons including LINEs (long interspersed nuclear elements) and SINEs (short interspersed nuclear elements)), which transpose through an RNA intermediate.

Exonization
The creation of a new exon by incorporation of intronic sequences into the coding region of a gene.

Purifying selection
Also called stabilizing selection, it is natural selection that tends to maintain the status quo.

Locked nucleic acids
Nucleic acids modified by a methylene bridge connecting the 2′ oxygen of ribose with the 4′ carbon, reducing the conformational flexibility of the ribose. Oligonucleotides containing locked nucleic acids have an increased melting temperature and are useful for *in situ* hybridizations with short target sequences as in miRNAs or siRNAs.

the DNA binding ability of these transcription factors, whereas others alter their ability to activate the transcription of target genes. Some, but not all, of these splice forms are conserved in vertebrates, suggesting that they are vital for development[114].

The tissue-specific expression of isoforms of developmental genes has received relatively little attention. The roles of isoforms of the vertebrate genes in the PAX3/7 and PAX2/5/8 groups in the development of the neural crest and placodes have not been studied. However, isoforms of *Pax6* are differentially expressed in the developing retina[115], while isoforms of *Pax1/9* are temporally regulated during amphioxus development[114]. PCR can be used to monitor expression of specific isoforms if the tissues can be dissected, whereas locked nucleic acids, which have been used to detect tissue-specific localization of miRNAs, show promise as isoform-specific probes for *in situ* hybridization.

Of all the genes expressed in the neural crest or the MHB in vertebrates, the roles of specific isoforms of FGF8, FGF17 and FGF18 at the MHB are the most studied. Two isoforms of FGF8 (FGF8a and FGF8b), differing by an additional 11 amino acids at the amino-terminal of FGF8b, are expressed at the MHB together with *FGF17* and *FGF18*. These four proteins have different affinities for FGF receptors (FGFRs), which may account for their different activities in patterning the MHB[116]. For example, of these, only FGF8b can induce *GBX2* expression in the anterior hindbrain[107]. In addition, FGF signalling is post-transcriptionally regulated by an miRNA, miR-9 (REF. 117), which binds to FGF8 and FGFR1 and also regulates the BHLH (basic helix-loop-helix) transcription factors HER5 and HER9 to promote neurogenesis. miR-9 is broadly expressed in the zebrafish CNS except for the MHB, and its overexpression eliminates the MHB. Amphioxus also expresses miR-9, but its only potential target that has been identified by *in silico* analysis is an unknown gene with WD repeats — motifs about 40 amino acids long that often end in Trp-Asp (W-D)[118]. Proteins with WD repeats comprise a very large family mediating a wide range of cellular functions[119]. This raises the possibility that changes in the targets of miRNAs may be yet another set of important factors in the evolution of new gene functions.

Conclusions

Comparative embryology and genomics have begun to answer the question of how the vertebrate brain evolved from that of a chordate ancestor. Comparisons with amphioxus and tunicates have indicated that the vertebrate brain and peripheral nervous system were elaborated from those of an ancestral chordate with a bipartite brain consisting of a diencephalic forebrain–midbrain region and a hindbrain–spinal cord region and migratory ectodermal sensory cells. A division into a distinct forebrain and midbrain may have arisen before tunicates and vertebrates diverged. The neural crest and a midbrain–hindbrain organizer probably arose within the vertebrate lineage by additions to the fundamental gene networks that specify the neural plate boundary and major brain regions, respectively. Comparative studies suggest that vertebrates did not evolve special MHB or neural crest genes, except for a few very far downstream in the neural crest differentiation pathway, but probably used the flexibility afforded by whole genome duplications early in the vertebrate lineage to add more genes to existing gene networks. A better understanding of how this happened will first require a thorough understanding of the gene networks that operate at the neural plate boundary and MHB in both amphioxus and vertebrates, and possibly tunicates as well. However, describing the gene networks may prove to be just a first step. Epigenetics, particularly post-transcriptional controls of the levels of gene expression and tissue-specific alternative splicing, may well turn out to be equally important especially when protein function can be radically different in different contexts. Such epigenetic phenomena are promising to be the next frontier in studies of developmental mechanisms and the evolution of development.

Note added in proof

A recent paper (REF. 137) demonstrates nerve cell bodies in the collar nerve cord of the hemichordates *Ptychodera fava* and *Saccoglossus kowalevskii* and overlying the fibres of the ventral nerve cord in the former, suggesting that the ancestral deuterostome had a CNS. However, whether the collar or ventral nerve cord is homologous to the chordate CNS remains an open question.

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DATABASES
Entrez Gene: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gene
BLIMP1 | BMP2 | BMP4 | BMP7 | *DLX* | *DLX5* | *FGF8* | *FGF17* | *FGF18* | *FOXD3* | *MSX* | *PAX2* | *PAX8* | *WNT3* | *WNT6*

FURTHER INFORMATION
Linda Z. Holland's homepage:
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