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Developmental genetic evidence for a monophyletic origin of the bilaterian brain

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The widely held notion of an independent evolutionary origin of invertebrate and vertebrate brains is based on classical phylogenetic, neuroanatomical and embryological data. The interpretation of these data in favour of a polyphyletic origin of animals brains is currently being challenged by three fundamental findings that derive from comparative molecular, genetic and developmental analyses. First, modern molecular systematics indicates that none of the extant animals correspond to evolutionary intermediates between the protostomes and the deuterostomes, thus making it impossible to deduce the morphological organization of the ancestral bilaterian or its brain from living species. Second, recent molecular genetic evidence for the body axis inversion hypothesis now supports the idea that the basic body plan of vertebrates and invertebrates is similar but inverted, suggesting that the ventral nerve chord of protostome invertebrates is homologous to the dorsal nerve cord of deuterostome chordates. Third, a developmental genetic analysis of the molecular control elements involved in early embryonic brain patterning is uncovering the existence of structurally and functionally homologous genes that have comparable and interchangeable functions in key aspects of brain development in invertebrate and vertebrate model systems. All three of these findings are compatible with the hypothesis of a monophyletic origin of the bilaterian brain. Here we review these findings and consider their significance and implications for current thinking on the evolutionary origin of bilaterian brains. We also preview the impact of comparative functional genomic analyses on our understanding of brain evolution.

Keywords: *Hox* genes; *Otx*; brain development; brain evolution; *Drosophila*; Urbilateria

1. INTRODUCTION

Until recently it has been assumed that the brains of deuterostomes such as vertebrates and protostome invertebrates are phylogenetically unrelated; in consequence, an independent evolutionary origin has been proposed for the brains of these two bilaterian groups. There are several observations that support this widely held assumption. First, classical phylogeny has led to the notion that certain extant animal groups such as the acoelomate platyhelminths (flatworms) exemplify the organization of basal protostome or deuterostome body plans (Barnes *et al.* 1993). Since these animal groups all have very simple nervous systems, some with a diffuse, nerve-net like structure, it has been considered unlikely that the last common ancestor of protostomes and deuterostomes had already evolved a more complex brain-like structure. Second, there are striking differences in the overall anatomy of vertebrate versus invertebrate brain types both in terms of identifiable neuronal substructures and in the relation of the brain to body coordinates. The central nervous system (CNS) of vertebrates is a dorsally located nerve cord; the CNS of invertebrates is typically a ventrally located ganglionic chain (Bullock & Horridge 1965; Nauta & Feirtag 1986). Third, the embryological processes that give rise to vertebrate and invertebrate brains are different in several respects. The brain of vertebrates derives primarily from a neural tube that invaginates from the dorsal neuroectoderm; the brain of invertebrates derives from the ventral neuroectoderm (Wolpert *et al.* 1998).

In the last decade, a large amount of comparative molecular, genetic and developmental evidence has accumulated that makes it necessary to reconsider the validity of these observations and assumptions. Modern molecular systematics dictates a new interpretation of the bilaterian phylogenetic tree which leaves no evolutionary intermediates between the protostome and deuterostome lineages and thus necessitates a re-evaluation of ideas on the phylogenetic generation of bilaterian complexity (Adoutte *et al.* 1999, 2000). A molecular genetic revival of the body axis inversion hypothesis currently gives strong support to the idea that the basic body plan of vertebrates and invertebrates is similar but inverted, in which case the ventral nerve chord of protostome invertebrates would correspond to the dorsal nerve cord of deuterostome chordates (De Robertis & Sasai 1996; Arendt & Nübler-Jung 1999). Finally, a developmental genetic analysis of the molecular control elements involved in early embryonic brain patterning is uncovering the existence of structurally and functionally homologous genes that have comparable, and indeed interchangeable, function in key aspects of brain development in invertebrates and vertebrates (Chan & Jan 1999; Reichert & Simeone

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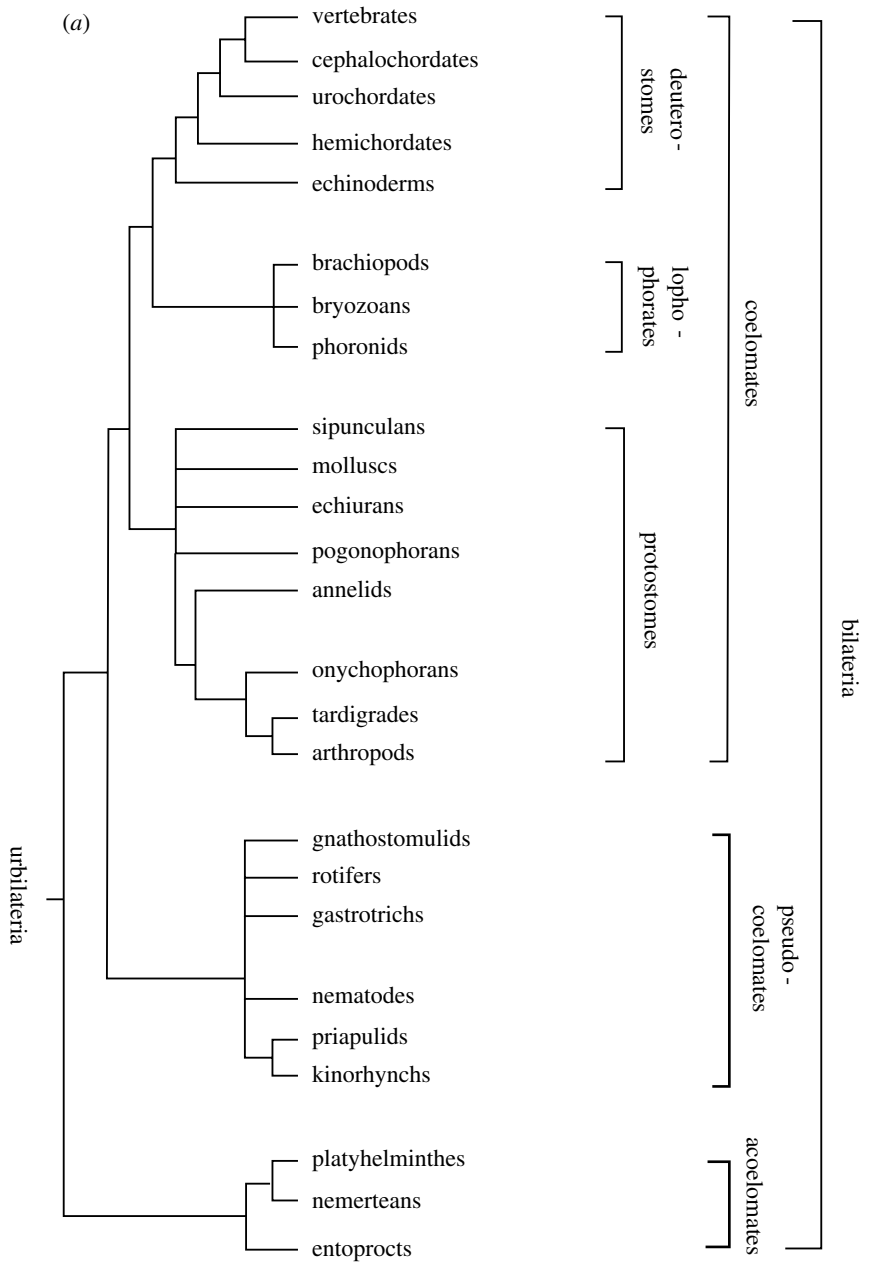

Figure 1. Bilaterian phylogenies. Only a representative subset of the metazoan phyla are indicated. (*a*) A classical phylogeny of the bilaterian animals based mainly on morphology and embryology. In this phylogeny, extant acoelomates such as the platyhelminths and extant pseudocoelomates such as the nematodes have generally been considered to be prime representatives of 'primitive' bilaterian lineages and were, thus, thought to be at the base of the bilaterian tree. (*b*) A new molecular phylogeny of metazoans based on small subunit rRNA gene sequence analysis. Several 'primitive' invertebrate lineages such as the platyhelminths and the nematodes are now placed next to protostome groups that have highly complex body morphologies and brain structures such as the molluscs and the arthropods. (Adapted from Adoutte *et al*. 2000).

1999). All three of these fundamental findings are compatible with the hypothesis of a monophyletic origin of the bilaterian brain. Here we review these findings and consider their significance and implications for current thinking on the evolutionary origin of bilaterian brains.

2. MOLECULAR PHYLOGENY AND THE DEMISE OF A 'PRIMITIVE' EXTANT BILATERIAN BRAIN

Classical animal phylogeny is pervaded by the assumption that the evolution of bilaterians went exclusively from simple to complex and that extant bilaterians represent grades of intermediate complexity that reflect the organizational levels of their ancestors. An example of this type of traditional animal phylogeny is given in figure 1*a*. Accordingly, the classical textbook view of animal evolution is one in which a primitive acoelomate-like ancestor progressively acquired more derived and advanced features such as a coelom, segmentation and differentiated internal organs that characterize some of the more complex protostomes and deuterostomes living today (Hyman 1940; Barnes *et al.* 1993; Campbell *et al.* 1999). Thus, extant acoelomates such as the platyhelminths and extant pseudocoelomates such as the nematodes have

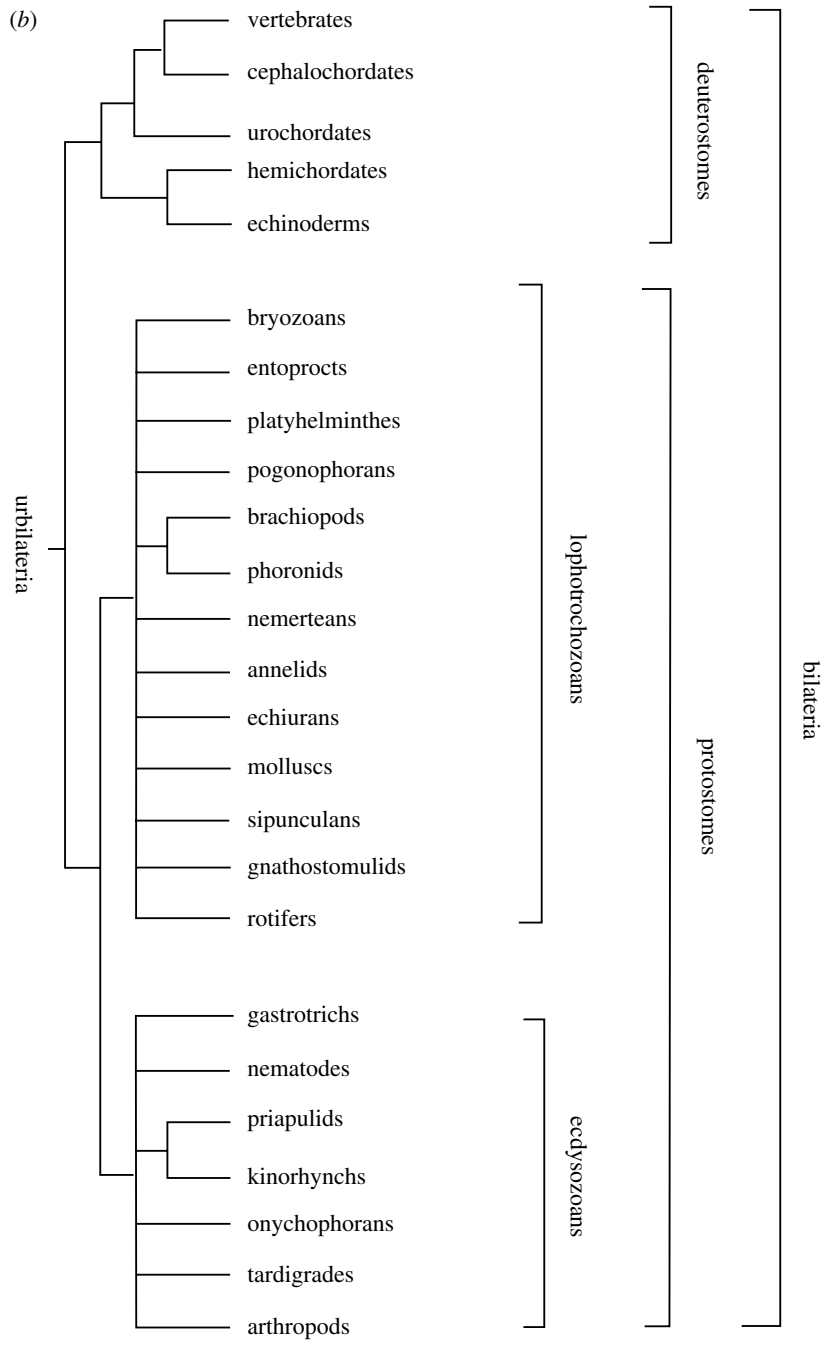

Figure 1. (*Continued*.)

generally been considered to be prime representatives of primitive bilaterian lineages. Given that the CNS of platyhelminths like *Planaria* and nematodes such as *Caenorhabditis elegans* are very simple, and are often organized in diffuse networks with only modest signs of cephalization (Bullock & Horridge 1965), the acceptance of a classical animal phylogeny would imply that the CNS of the putative last common bilaterian ancestor, the Urbilateria, was also simple and diffuse, and probably had evolved little in the way of a brain-like structure. Similarly, classical views of the deuterostome lineage suggest that extant hemichordates as well as the (generally less widely known) lophophorate brachiopods, bryozoans and phoronids are basal deuterostomes that have features that might be representative of primitive deuterostomes (Purves *et al.* 1997; Adoutte *et al.* 2000). Given that the CNS of these deuterostome groups is also simple and often diffuse, this would imply that the brains of the more advanced chordates evolved from the primitive CNS of these deuterostomes. This, in turn, would imply that brains evolved independently in the protostome and deuterostome lineages.

Molecular phylogeny based on small ribosomal subunit RNA analysis as well as on *Hox* gene analysis has led to several major revisions of the classical bilaterian phylogeny (Aguinaldo *et al.* 1997; Knoll & Caroll, 1999; de Rosa *et al.* 1999; Adoutte *et al.* 2000; Graham 2000). The most important consequence of these revisions is that there are no longer any living evolutionary intermediates between the protostomes and the deuterostomes. Thus, several seemingly 'primitive' invertebrate lineages, such as the acoelomate

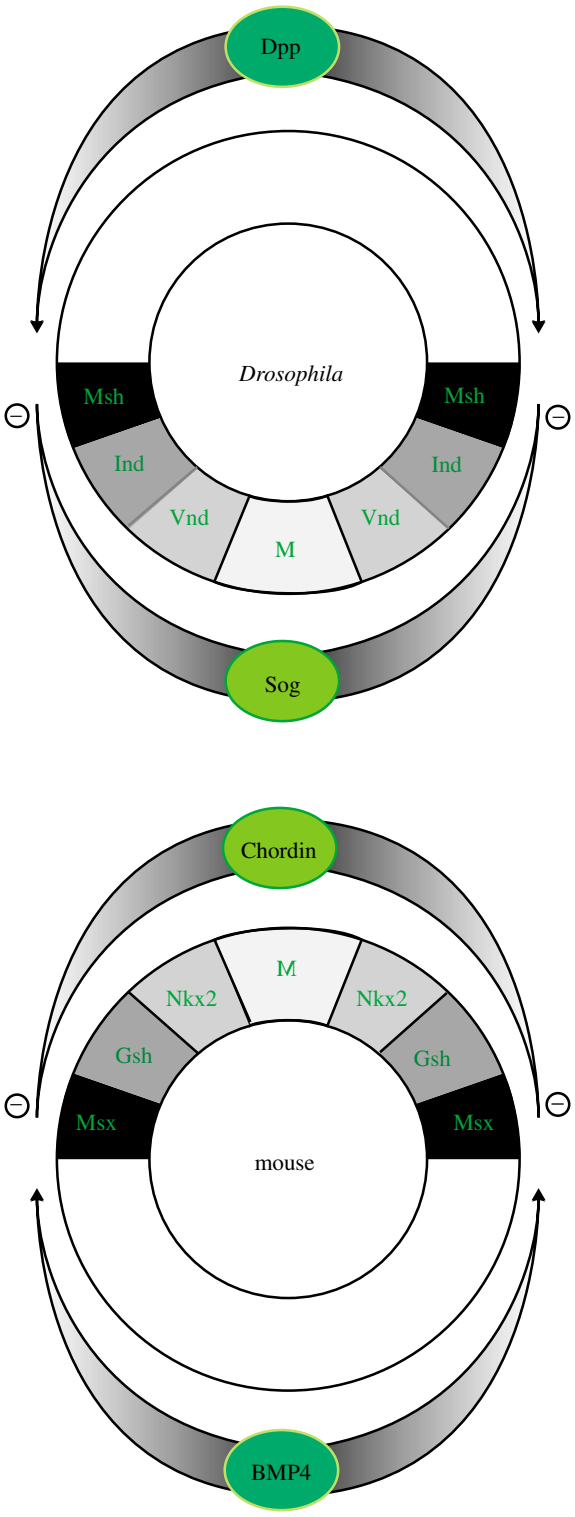

Figure 2. Establishment of the dorsoventral axis through interactions between the Dpp/BMP4 and Sog/Chordin signalling pathways and organization of the neuroepithelium into three columns of neuronal precursors in *Drosophila* and the mouse. The two groups of interacting signalling molecules, Dpp/BMP4 and Sog/Chordin, work from opposing dorsoventral poles in both insect and vertebrate embryos. The developing *Drosophila* embryonic CNS and the vertebrate neural plate are both organized into three columns and similar homeobox genes are expressed in the same medial–lateral order in both cases with Vnd/Nkx2 proteins expressed most medially, Ind/Gsh proteins expressed in an intermediate position and Msh/Msx proteins expressed most laterally. M, midline.

platyhelminths and the pseudocoelomate nematodes, which were classically thought to be at the base of the bilaterian tree, are now placed next to protostome groups that have highly complex body morphologies and brain structures, such as the molluscs and the arthropods (figure 1*b*). This means that these most 'primitive' bilaterian lineages are in fact secondarily simplified in their morphology. For example, the flatworms, which have classically been regarded as very primitive bilaterians, appear now to be anatomically degenerate, dispensing with many morphological features such as an anus. Similarly, the unusually simple body plan of nematodes such as *Caenorhabditis elegans*, which has contributed to the 'model organism' role of this species in modern molecular science, now appears to be highly derived. Comparable considerations hold for the seemingly 'primitive' deuterostome lineages. The hemichordates and echinoderms have been joined in the new molecular phylogeny and are now considered to be a sister group of the chordates (Wada & Satoh 1994). Plausible functional intermediates between the echinoderms and hemichordates, and the chordates are lacking (Gee 1996). More strikingly, the morphologically simple lophophorate lineages, which were classically attributed to the deuterostomes, have been removed from the deuterostome lineage altogether and are now firmly embedded within the lophotrochozoan protostomes (Halanych *et al.* 1995). Thus, based on current molecular phylogeny, we lack extant examples of both basal protostomes and basal deuterostomes.

As a result of this phylogenetic revision, we are now faced with a major information gap for reconstructing features of the urbilaterian ancestor (Adoutte *et al.* 1999). Since we no longer have access to a *bona fide* primitive bilaterian, we cannot obtain insight into the organization of the primitive urbilaterian brain based on comparative morphological analysis of extant animals. A corollary of the above considerations is that the urbilaterian brain may not have been primitive at all, as compared with an extant planarian brain, for example. Indeed, if we accept the notion that all extant bilaterians descend from the same ancestral urbilaterian, we may also have to accept the notion that this ancestral urbilaterian had already attained an unexpectedly complex organizational level. At the genomic level, there is now increasing evidence that the basal bilaterian genome is made up of 12 000–18 000 genes, most of which were retained in homologous form after the twofold duplications that are likely to have occurred in the vertebrate lineage (Rubin *et al.* 2000). This indicates that the complex basal array of genes was already present in the urbilaterian animal before the protostome–deuterostome split. It is not unreasonable to assume that this complex genetic array could control the development of a complex brain. In conclusion, we posit that the current modern interpretation of animal phylogeny no longer argues against a monophyletic origin of the bilaterian brain.

3. THE DORSOVENTRAL AXIS INVERSION HYPOTHESIS: A COMMON ORIGIN OF THE VENTRAL CNS IN INSECTS AND THE DORSAL CNS IN VERTEBRATES

Classical descriptive analyses of nervous system embryogenesis and morphology had led to the subdivision of animals into two large groups termed the gastroneuralia

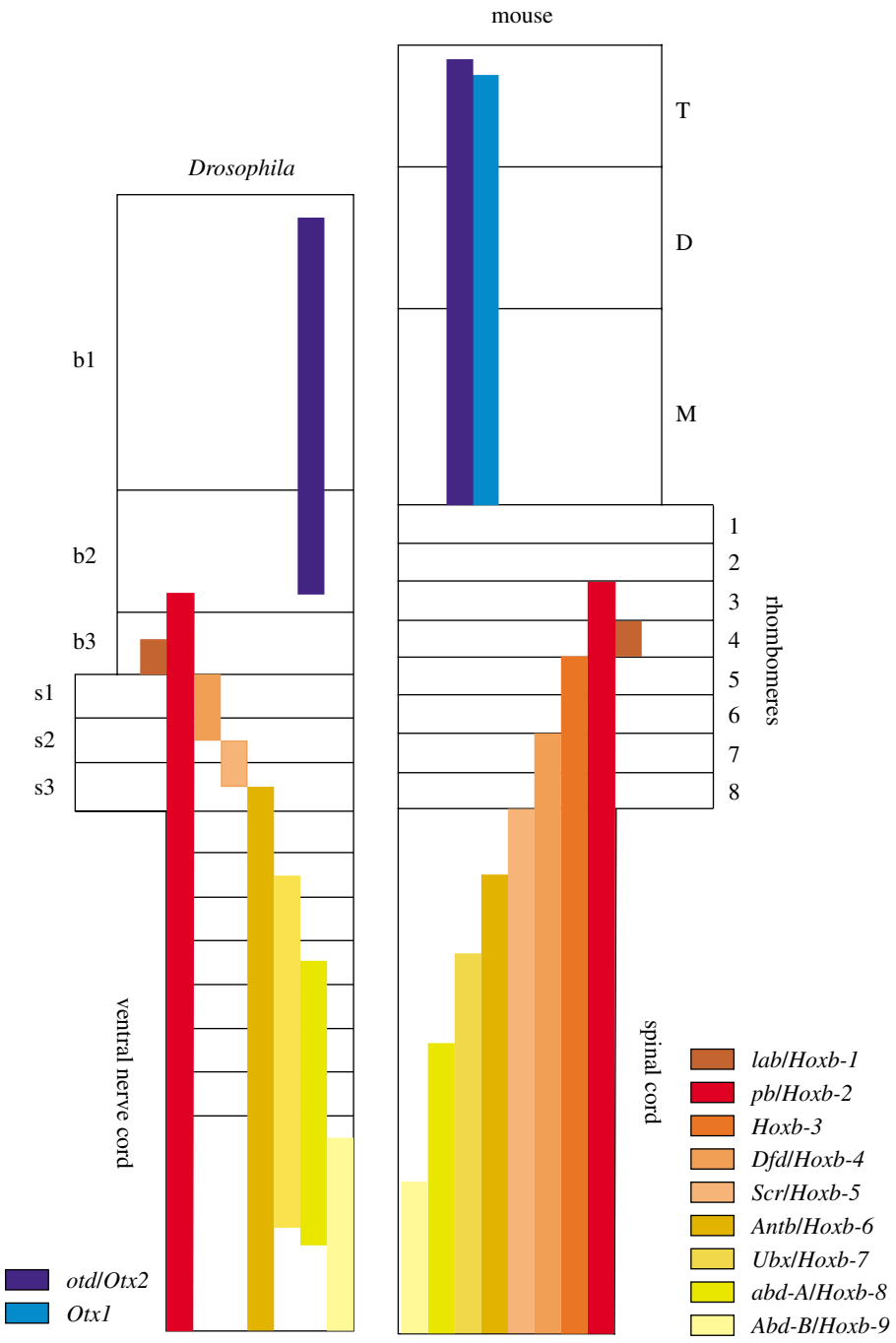

Figure 3. Conserved anteroposterior order of gene expression in embryonic brain development. Schematic of homeotic (*Hox*) and *otd/Otx* gene expression patterns in the developing CNS of *Drosophila* and the mouse. Expression domains are colour coded. Anterior is towards the top. For *Drosophila*, gene expression corresponds to a stage 14 embryo; for the mouse, gene expression corresponds to a stage 9.5–12.5 embryo. The *Drosophila* brain is composed of an anterior supra-oesophageal ganglion and a posterior sub-oesophageal ganglion; the supra-oesophageal ganglion is subdivided into the protocerebrum (b1), deutocerebrum (b2) and tritocerebrum (b3), and the sub-oesophageal ganglion is subdivided into the mandibular (s1), maxillary (s2) and labial (s3) neuromeres (Therianos *et al*. 1995; Younossi-Hartenstein *et al*. 1996; Reichert & Boyan 1997). The mouse brain is divided into a rostral region that comprises the telencephalon (T) and diencephalon (D) (prosencephalon) as well as the mesencephalon (M), and into a caudal hindbrain region which has a metameric organization based on rhombomeres (Lumsden & Krumlauf 1996).

and the notoneuralia (Hatschek 1891). The gastroneuralia included protostomes such as arthropods, annelids and molluscs that are characterized morphologically by a ventral nerve cord. The notoneuralia included all chordates and are characterized by a dorsal nerve cord. Based on differences in topography and morphogenesis of the nervous system, an independent evolutionary origin was proposed for the nervous systems of these two bilaterian groups (gastroneuralia–notoneuralia concept, e.g. Siewing 1985; Brusca & Brusca 1990; Nielsen 1995). Recently, the validity of this proposal has been challenged by a wealth of molecular, developmental and genetic evidence that indicates that the ventral body side of invertebrates such as insects is equivalent to the dorsal body side of vertebrates due to an ancestral inversion of the body axis in these two animal groups (Arendt & Nübler-Jung 1999; Holley *et al.* 1995; De Robertis & Sasai 1996). These data, which reinforce the original body axis inversion hypothesis originally by Geoffroy St Hilaire (1822), imply that evolutionarily equivalent neural body sides give rise to the brain and nerve cords in both invertebrates and vertebrates and, thus, suggests that the CNS of both animal groups might be homologous.

Among the significant molecular control elements involved in the embryonic establishment of dorsoventral body axis, which appear to be conserved in insects and vertebrates, are signalling proteins of the transforming growth factor β (TGFβ) class such as Dpp, studied most extensively in *Drosophila*, and BMP4, one of the vertebrate homologues of Dpp (Sasai & De Robertis 1997). These proteins establish dorsoventral polarity in the embryo and are restricted in their spatial action by antagonistically acting extracellular signalling proteins. These antagonists are Sog in *Drosophila* and its homologue Chordin in vertebrates. The two groups of interacting signalling molecules, Dpp/BMP4 and Sog/Chordin work from opposing dorsoventral poles in both insect and vertebrate embryos (Holley *et al.* 1995). Strikingly, and in support of the body axis inversion hypothesis, in *Drosophila* Dpp exerts its actions on dorsal cells and Sog acts on ventral cells, whereas in vertebrates BMP4 acts on ventral cells and Chordin activity is found in dorsal cells. In both cases it is the region of the embryo, in which Sog/Chordin is expressed and inhibits the action of invading Dpp/BMP4 signals, that attains neurogenic potential and forms neuroectoderm. Similar considerations hold for the *Drosophila tsg* gene, which is expressed in the dorsal-most blastoderm, and its vertebrate homologue *xTsg*, which is expressed in the ventral-most tissues of *Xenopus* (Oelgeschläger *et al.* 2000). Since the interactions between all of these extracellular signalling molecule types control the initial formation of the neuroectoderm in both insects and vertebrates (figure 2), this pervasive equivalence in gene structure, expression and action points to an essential homology of CNS induction/specification in insects and vertebrates.

Beyond the mechanisms of early neuroectoderm formation, a further set of key genetic elements involved in early dorsoventral patterning of the CNS appears to be evolutionarily conserved (Chan & Jan 1999; Cornell & Von Ohlen 2000; Scott 2000). These also have an inverted expression topology in embryos of insects and vertebrates. These genetic regulatory elements are three sets of homeobox genes that control the formation of columnar dorsoventral domains in the ventral neuroectoderm of *Drosophila*; their homologues may act in similar fashion in dorsoventral patterning in the neural plate of vertebrates (figure 2). In *Drosophila*, the homeobox genes are *vnd*, *ind* and *msh* and they are expressed in ventral (*vnd*), intermediate (*ind*) and dorsal (*msh*) columns in the neuroectoderm (McDonald *et al.* 1998; Chu *et al.* 1998; Weiss *et al.* 1998). In each column, expression of the appropriate homeobox gene is required for neuroblast formation and for specification of columnar identity. In the vertebrate neural plate, the homologous genes are members of the *Nkx2* (*vnd*), *Gsh* (*ind*) and *Msx* (*msh*) gene families. The murine *Nkx2.2* gene has been shown to be required for the specification of cell fate in the column in which it is expressed, implying functional equivalence to its homologue *vnd* in the fly (Briscoe *et al.* 1999). The order of expression for these vertebrate genes in the ventral (*Msx*), intermediate (*Gsh*) and dorsal columns (*Nkx2*) is inverted in dorsoventral polarity compared with that of their homologues in *Drosophila*. (Following invagination of the neural plate to form the neural tube, this dorsoventral order is inverted and then corresponds with that seen in the fly.)

While an extreme case of evolutionary convergence cannot be ruled out, the most parsimonious explanation for the striking similarities in dorsoventral patterning genes, their relative topographical expression patterns and their functional roles in the CNS of different animal phyla is that the longitudinal nerve cords of chordates and arthropods are of common evolutionary origin, i.e. homologous, and that their opposite location relative to the body axis is a consequence of a general dorsoventral body axis inversion.

4. EVOLUTIONARILY CONSERVED MOLECULAR GENETIC MECHANISMS FOR PATTERNING THE EMBRYONIC BRAIN

Recently, a wealth of molecular genetic data on conserved mechanisms of CNS development has been obtained, which is in accordance with the notion that the CNS of vertebrates and invertebrates has a common evolutionary origin. Homologous regulatory genes have been identified which control polarity, regionalization, proliferation, identity, process outgrowth and patterning of the embryonic nervous system in a comparable manner in insects and vertebrates. Remarkable examples of evolutionary conservation in the genetic control of CNS development include the proneural genes (Lee 1997), the neurogenic genes (Chan & Jan 1999), the homeotic genes (Lumsden & Krumlauf 1996), the cephalic gap genes (Hirth & Reichert, 1999; Reichert & Simeone 1999), the *ey/Pax6* genes (Callaerts *et al.* 1997), and the *en/En* genes (Hanks *et al.* 1998).

Two of these gene families, the homeotic genes and the cephalic gap genes, are intimately involved in anteroposterior regionalization and patterning of the neuraxis, and their action is, thus, directly linked to the formation of the structures at the anterior pole of the CNS that give rise to the brain. A comparison of expression and function of these two gene families in embryonic brain

development in invertebrates and vertebrates is, thus, of central importance to the hypothesis of a monophyletic origin of the brain. If the brains of invertebrates and vertebrates do have a monophyletic origin, one might expect that these gene families might have similar conserved expression patterns and functional roles in the different animal groups. This appears to be the case.

The homeotic *Hox* genes encode homeodomain transcription factors that were first identified in *Drosophila* and have subsequently been found in homologous forms in all other bilaterian animals, including mammals (Lewis 1978; McGinnis & Krumlauf 1992; Lumsden & Krumlauf 1996; Gellon & McGinnis 1998). In both invertebrates and vertebrates, *Hox* gene expression is especially prominent in the developing CNS. In the CNS of *Drosophila*, they are expressed in the posterior regions of the developing brain and in the ventral nerve cord (Kaufman *et al.* 1990; Hirth *et al.* 1998). In the CNS of the mouse, they are expressed in the developing hindbrain and spinal cord. In both cases the *Hox* gene expression patterns form an ordered set of domains along the neuraxis that have a remarkably similar anteroposterior order (figure 3). Comparative expression data suggest that the *Hox* genes may play a fundamental role in nervous system patterning in all animals. For example, in the urochordate ascidians and in the cephalochordate *Amphioxus*, *Hox* genes are expressed in specific domains of the CNS, suggesting that regionalized *Hox* gene expression in the nervous system is an ancient characteristic of all chordates (Katsuyama *et al.* 1995; Gionti *et al.* 1998; Holland & Garcia-Fernandez 1996).

The function of the *Hox* genes in embryonic brain patterning has been studied through loss-of-function experiments. In *Drosophila*, loss-of-function of two homeotic genes, *labial* and *Deformed*, results in severe axonal patterning defects in the brain (Hirth *et al.* 1998). These axonal projection defects arise because the postmitotic cells generated in the mutant domain do not extend axons or dendrites and are not contacted by axons from other parts of the brain. Moreover, these cells do not express any of the numerous neuronal molecular markers that are expressed by positionally equivalent neuronal cells in the wild-type, indicating that the mutant cells in the brain do not acquire a neuronal identity. Thus, the expression of these homeotic genes appears to be necessary for proper neuronal differentiation and correct establishment of regionalized neuronal identity in the posterior *Drosophila* brain.

Comparable loss-of-function experiments have been used to study *Hox* gene function in the mouse (Studer *et al.* 1998; Gavalas *et al.* 1998). The *labial* homologues *Hoxa1* and *Hoxb1* are expressed in the developing murine hindbrain where they reach a sharp anterior boundary coinciding with the anterior rhombomere 4 (r4) border. In $Hoxa1^{-/-}$; $Hoxb1^{-/-}$ double mutants, a region corresponding to r4 is formed, but r4-specific markers fail to be activated, indicating the presence of a territory between r3 and r5 with an unknown identity. $Hoxa1^{-/-}$; $Hoxb1^{-/-}$ double mutants also have a reduced number of facial motor neurons that appear to exit randomly from the neural tube without fasciculating. These results suggest that *Hoxa1* and *Hoxb1* act together in the specification of r4 neuronal identity and in the patterning of nerves during vertebrate hindbrain development. This mode of action is remarkably similar to that of *labial* in specifying segmental neuronal identity during brain development of *Drosophila* (Hirth *et al.* 1998).

Evidence that individual *Hox* gene action is sufficient for the specification of distinct neuronal fate comes from gain-of-function experiments carried out in the chick (Bell *et al.* 1999; Jungbluth *et al.* 1999). Retroviral-mediated misexpression of either *Hoxa2* or *Hoxb1* in r1 of avian embyros leads to the generation of ectopic branchiomotor neurons, even though r1 is normally lacking this cell type. Gain-of-function experiments also demonstrate a role for *Hox* genes in the establishment of appropriate connectivity between the hindbrain and its peripheral targets. Misexpression of *Hoxb1* in basal r2 results in a reassignment of motor neuron identity and a re-routing of motor axon projections. Conversely, misexpression of the inappropriate *Hoxb1* gene in the first branchial arch target tissue results in truncation of axon projections from a normal r2. These results show that a classical homeotic transformation can be induced in defined neuronal populations by the ectopic expression of a single *Hox* gene, and are further evidence that in vertebrates, as in flies, *Hox* genes confer positional identity on metameric neuronal structures of the developing CNS.

The cephalic gap genes *otd* and *ems* were first identified in *Drosophila* as homeobox genes that act in the embryo to specify adjacent head segments during early embryogenesis (Cohen & Jürgens 1990). Later in development both genes are expressed in the anterior embryonic brain anlage (Hartmann & Reichert 1998). Thus, during neuroectoderm formation, *otd* expression occurs throughout most of the protocerebrum (b1) and adjacent deutocerebrum (b2), and *ems* expression occurs in domains of the deutocerebrum (b2) and tritocerebrum (b3) (Hirth *et al.* 1995; Younossi-Hartenstein *et al.* 1997). In mammals such as the mouse there are two *otd* homologues *Otx1* and *Otx2* and two *ems* homologues *Emx1* and *Emx2* (Simeone *et al.* 1992; Simeone 1998; Reichert & Simeone 1999). In the embryonic CNS the *Otx* and *Emx* genes have nested expression domains that include the developing forebrain and midbrain. Expression data also suggest an involvement of the *Emx* genes in dorsal telencephalic development, of the *Otx1* gene in corticogenesis, and of the *Otx2* gene in the early specification of the rostral neural plate. Thus, both in the mouse and in *Drosophila*, expression of the *otd/Otx* and *ems/Emx* genes occurs in specific domains of the anterior embryonic brain. Strikingly, in both cases, expression of the *otd/Otx* genes extends throughout most of the anterior brain regions (figure 3).

Loss-of-function of either *otd* or *ems* results in dramatic effects on embryonic brain development in *Drosophila*. In *otd* mutants the protocerebrum is deleted and in *ems* mutants the deutocerebrum and tritocerebrum are deleted. Deletion of specific brain anlagen is due to defective neuroectoderm specification and the subsequent failure of neuroblast formation in the affected domains. The regionalized absence of brain neuroblasts in the mutants correlates with the loss or reduction in expression of proneural genes, which are thought to be required for neuroectodermal cells to acquire the competence to form neuroblasts, in the mutant domain (Younossi-Hartenstein *et al.* 1997; Hartmann *et al.* 2000).

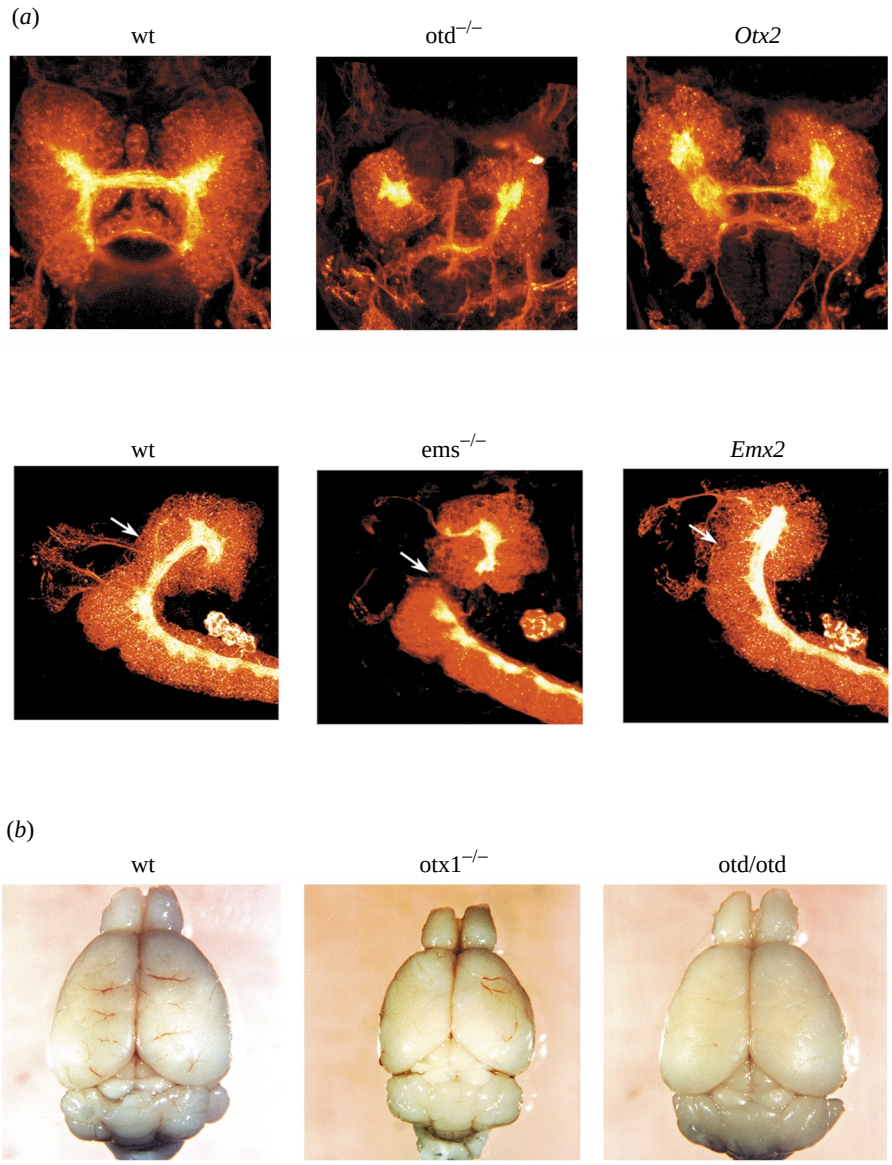

Figure 4. Cross-phylum rescue experiments in *Drosophila* and the mouse. In genetic rescue experiments, the human *Otx2* gene was overexpressed in *Drosophila otd* mutants, the murine *Emx2* gene was overexpressed in *Drosophila ems* mutants and the murine *Otx1* coding sequence was replaced with the *Drosophila otd* gene. (*a*) In *Drosophila*, the embryonic wild-type brain (wt) shows prominent anterior lobes interconnected by an anterior brain commissure; these structures are lost in the *otd* null mutant ($otd^{-/-}$), but are restored by overexpression of the human *Otx2* gene in the *otd* null mutant (*Otx2*). (*b*) In the *Drosophila* embryonic wild-type brain (wt), prominent descending connectives interconnect anterior and posterior brain regions (lateral view). These longitudinal connectives are interrupted and a gap appears in the brain of *ems* null mutant ($ems^{-/-}$). The connectives are restored and the gap disappears following overexpression of the murine *Emx2* gene in the *Drosophila ems* null mutant (*Emx2*). (*c*) In the mouse, the normal size of the wild-type aduilt brain (wt) is markedly reduced in the *Otx1* null mutant ($Otx1^{-/-}$) but is largely restored by gene replacement (knock in) with the *Drosophila otd* gene (*otd/otd*). (Modified after Leuzinger *et al*. 1998; Acampora *et al*. 1998; and Hartmann *et al*. 2000.)

Loss-of-function analyses for the *Otx* and *Emx* genes carried out in the mouse show that these genes are also critically required at different stages of embryonic brain development (Acampora *et al*. 1995, 1996; Matsuo *et al*. 1995; Ang *et al*. 1996). *Otx2* null mice are early embryonic lethal and lack the rostral neuroectoderm fated to become the forebrain, midbrain and rostral hindbrain due to an impairment in early specification of the anterior neuroectoderm by the visceral endoderm. *Otx1* null mice have spontaneous epileptic seizures and abnormalities affecting the telencephalic dorsal cortex and the mesencephalon, as well as parts of the cerebellum and certain components of the acoustic and visual sense organs. Similarly, studies carried out on null mice for the *Emx* genes demonstrate that these genes are necessary for the establishment of discrete regions of the telencephalon (Pellegrini *et al*. 1996; Yoshida *et al*. 1997; Qui *et al*. 1996). Mutation of *Emx2* leads to a deletion of the dentate gyrus and to a reduction in size of the hippocampus and medial limbic cortex. Mutation of *Emx1* results in the disruption of the corpus callosum as well as more subtle defects in the forebrain. Taken together, investigations of early morphogenesis and patterning in the embryonic brains of *Drosophila* and

mouse reveal developmental mechanisms that are strikingly similar, and suggest an evolutionary conservation of *Hox* and *otd/Otx* genes in embryonic brain development that extends beyond gene structure to patterned expression and function.

5. CROSS-PHYLUM GENETIC RESCUE EXPERIMENTS: BRIDGING THE PROTOSTOME–DEUTEROSTOME GAP

Genes related to *otd* have been found in the anterior CNS of all invertebrates examined, including animals as 'primitive' as planarians (Umesono *et al*. 1999). Moreover, comparative studies reveal the existence of *otd*-related genes in all chordates (Simeone *et al*. 1992; Bally-Cuif *et al*. 1995; Li *et al*. 1994; Mercier *et al*. 1995; Pannese *et al*. 1995) including urochordates (Wada *et al*. 1996), cephalochordates (Williams & Holland 1998) and agnates (Ueki *et al*. 1998), where they are expressed in the rostral-most CNS. In addition to the extensive similarities in expression patterns, cross-phylum *in vivo* genetic rescue experiments carried out for members of the *otd/Otx* gene family provide remarkable and direct evidence for the striking evolutionary conservation of functional properties of these control genes in patterning the brain (figure 4).

In these cross-phylum replacement experiments, human *Otx1* and *Otx2* genes were over-expressed in *Drosophila otd* mutants (Leuzinger *et al*. 1998; Nagao *et al*. 1998) and, conversely, the murine *Otx1* coding sequence was replaced with the *Drosophila otd* gene (Acampora *et al*. 1998). In *Drosophila*, both human *Otx* genes, like the endogenous fly *otd* gene, are able to rescue the defects in the protocerebral brain anlage. Similarly, the *Drosophila otd* gene is able fully to rescue corticogenesis impairment and epilepsy, and also partially to restore eye defects and brain patterning abnormalities seen in $Otx1^{-/-}$ embryos. Interestingly, the rescue is less efficient nearer to the midbrain–hindbrain boundary, in that the mesencephalon is never completely normal and cerebellar foliation remains abnormal. Moreover, the defective lateral semicircular duct of the inner ear of $Otx1^{-/-}$ mice is never rescued by the *Drosophila otd* gene, thus suggesting that the ability to direct the development of this structure correctly is an *Otx1*-specific property. *Drosophila otd* is also able to replace *Otx1* partially in its cooperative interactions with *Otx2* for correct brain patterning.

Drosophila and vertebrate *otd/Otx* gene products share structural homology that is confined mainly to the homeodomain; the 60 amino acid residues of the fly Otd homeodomain differ from the homeodomains of the human OTX1 and OTX2 protein in only three and two amino acids, respectively. This implies that the extensive functional equivalence of the *otd/Otx* genes may be due to conserved developmental genetic circuits with common functional features that are controlled by the homeodomain. Thus, the *otd/Otx* gene family might be part of a general developmental genetic control system that operates in vertebrate and invertebrate brains to specify segmental identities in anterior brain and head regions. In this sense it would complement the developmental genetic control system encoded by the *Hox* genes that control posterior brain and CNS regions in trunk and tail structures of vertebrates and invertebrates.

Although cross-phylum gene replacement experiments cannot formally rule out the possibility that the functional equivalence of *otd* and *Otx* might have been independently acquired through convergent evolution, they argue quite strongly for an evolutionary conservation of gene function. This, in turn, suggests that common genetic mechanisms for brain development evolved in the common ancestor of flies and mice, where they were involved in the developmental control of the ancestral urbilaterian brain. If this is the case, one would expect that cross-phylum rescue experiments could also be carried out with other developmental control genes involved in patterning the embryonic brain. Recent experiments carried out on the *ems/Emx* genes confirm this expectation.

To determine whether the murine homologues of *ems* are capable of restoring the brain phenotype of *ems* mutant flies, genetic rescue experiments involving ubiquitous overexpression of the mouse *Emx2* gene were carried out in *Drosophila* (Hartmann *et al*. 2000). When the *Emx2* transgene was overexpressed in the *ems* null mutant, substantial restoration of brain morphology was observed (figure 4). Thus, in over one quarter of the cases, the cellular gap in the deutocerebral and tritocerebral anlagen was restored. This suggests that a functional murine *Emx2* gene can replace the *ems* gene to a large degree in the development of the anterior part of the *Drosophila* brain. It will now be interesting to carry out reciprocal genetic rescue experiments in the mouse to see if and to what extent the *Drosophila ems* gene can rescue these defects in the embryonic murine brain.

6. RECONSTRUCTING URBILATERIAN BRAIN DEVELOPMENT THROUGH EVOLUTIONARY FUNCTIONAL GENOMICS: FROM CONSERVED GENES TO CONSERVED GENE NETWORKS

The identification and investigation of specific families of developmental control genes, which play central and evolutionarily conserved roles in patterning the embryonic brain, represent important steps towards a comprehensive understanding of the molecular genetic networks involved in brain morphogenesis in animals as diverse as *Drosophila* and the mouse. The advent and implementation of powerful new genomic technology in these two model systems is currently extending these studies in a spectacular manner. In *Drosophila*, where the full genome sequence is now available, it is already possible to combine extensive manipulative molecular genetic technology and large scale functional genomics with the long-term goal of identifying all of the control genes involved in brain development.

One useful strategy in this respect is to manipulate, genetically and in the developing organism, high-order developmental control genes and genetic switches that regulate specific aspects of brain development, and then use full genome DNA microarrays (gene chips) to identify all of the gene transcripts that are influenced by the corresponding genetic manipulation. Once the entire set of genes that are involved in specific aspects of brain formation is known, manipulative genetics can once again be used to investigate individual gene expression and function *in vivo* and, thus, reconstruct the genetic network

that controls these processes. Recent functional genomic experiments in *Drosophila* indicate that the use of large scale oligonucleotide microarrays permits the simultaneous identification and quantification of hundreds of gene transcripts that are expressed in the embryo (Leemans *et al.* 2000). These functional genomic analyses are currently being coupled with genetic overexpression studies of the cephalic gap and homeotic genes in order to identify the complement of downstream target genes in the embryo (Leemans *et al.* 2001). Many of these identified genes are likely to represent novel target genes which are part of the genetic network that directs embryonic patterning of the fly brain. Since similar experiments should soon, in principle, also be possible in the mouse, it will be important to use comparative functional genomics to identify similarities and differences in the endogenous genes and gene networks that control brain development in *Drosophila* and in the mouse, as well as in other evolutionarily relevant animal organisms.

Comparative functional genomics coupled with interphylum transgenics may also lead to profound insight into the degree of evolutionary conservation of the gene networks that control brain development. Current functional genomic experiments in which high-order vertebrate developmental control genes such as *Otx2* are expressed in *Drosophila* are identifying a large number of genetic targets of the endogenous *Drosophila* gene that are also targets of the homologous vertebrate gene. Through this type of approach, it should be possible to determine which elements in the overall control network for brain development are conserved and remain genetically accessible across the interphylum gap that separates insects and mammals. Moreover, this type of approach should also reveal which elements of the developmental control network have been evolutionarily modified in different organisms and may be responsible for generating morphological differences. From a genomic perspective, evolutionary constancy and diversity are two sides of the same coin.

Taken together, these comparative functional genomic approaches should lead to unprecedented insight into the degree of conservation versus divergence of the overall molecular genetic programme for brain development in vertebrates and invertebrates. We expect that the results of these novel genomic investigations will provide a rigorous test of the hypothesis of a monophyletic origin of the bilaterian brain.

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