Evolution and homology of the nervous system: cross-phylum rescues of *otd/Otx* genes

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The homeotic or *Hox* genes were the first gene family to be shown to act in similar, probably homologous, ways in insect and mammalian development. They are thought to form a combinatorial code specifying the identity of different segments. They are clustered in the genome, and expressed in a nested pattern along the anterior–posterior axis in an order that is colinear with their chromosomal order[1].

Since then, perhaps a surprisingly high number of developmental gene families have also been shown to have conserved expression patterns over several phyla. One such family is the *Otx* genes, which like the *Hox* genes encode homeodomain-containing DNA-binding proteins[2,3]. Flies and amphioxus have a single gene in the family, called *orthodenticle* (*otd*) in *Drosophila*, while vertebrates typically have two *Otx* genes. Mouse *Otx1* and *Otx2*, together with another family of homeobox genes, *Emx1* and *Emx2*, are expressed in nested patterns in the fore- and mid-brain[3], while their fly homologues *otd* and *ems* are expressed in the most anterior segments[4–6]. It has been suggested[3,7] that the *Otx* and *Emx* genes specify segmental identity in insects and vertebrates, fulfilling a role for the anterior brain similar to that of the *Hox* genes in the hindbrain and spinal cord. This hypothesis is supported by knockout phenotypes and mutations[8–12]: in $Otx2^{+/-}$ $Otx1^{-/-}$ mice the midbrain and posterior diencephalon are completely missing (interpretation of single *Otx* mutant phenotypes is complicated in mice by variability, an early role for *Otx2* in gastrulation and by redundancy between *Otx1* and *Otx2*). It also appears that in mammals, *Otx* function influences development of the midbrain–hindbrain boundary, known to be an organizer of cell fate in the midbrain and anterior hindbrain[8–13]. In *Emx* knockouts discrete parts of the forebrain are missing[14,15].

So expression and mutation of *Otx/otd* and *Hox* genes show that the anterior–posterior (AP) patterning mechanisms in insects and vertebrates share many features. Does this mean that these mechanisms are homologous, that is, that the common ancestor had these mechanisms too? The fact that amphioxus, a primitive chordate, also has anterior *Otx* expression[2] indicates that the mechanism is quite widespread, although *otd* expression in echinoderms[16] is quite different and variable. It is first of all necessary to test whether the genes are acting in the same way in both groups of animals. This can be done experimentally by expressing vertebrate genes in flies mutant for their homologues, or vice versa, and asking whether the foreign gene can rescue the mutant phenotype. This has already been done for some *Hox* genes[17–19], showing that they can function in an equivalent manner, but it has not until now been tried for the *Otx/otd* family.

A second factor also needs to be considered. The nervous system of vertebrates has been compared to the early ectoderm of insects, at a stage long before the central nervous system (CNS) is formed. A question mark has hung over interpretations of AP patterning similarities: could these two systems really be homologous, when they referred to such different stages of development?

Two responses have been given towards the latter question. One (called the auricularia hypothesis) was to propose that the nervous system of chordates is homologous to the entire outer ectoderm of insects while the insect nervous system and the chordate outer ectoderm have arisen independently[20]. This was supported by studies of the anatomy of larval echinoderms (particularly auricularia larvae) and urochordates, which are proposed to be intermediates in the transition between the arthropod- and chordate-type body plans, and by the fact that insect *Hox* genes are expressed in the surface ectoderm, whereas they are not in vertebrates. The nervous systems of insects and vertebrates have long been assumed to have evolved independently, because they are organized differently and are on opposite sides of the embryo[21].

The other response grew out of recent work showing that the dorsoventral axis is in fact patterned by the same set of genes in similar relationships in insects and in vertebrates, but with the dorsoventral axis inverted[21,22]. Many developmental genes, such as the *achaete/scute* homologues, *Nkx2*, *Msx* and *Hox* homeobox genes and *netrins* are expressed in similar patterns in insect and vertebrate nervous systems[21,23]. It is a reasonable hypothesis that the dorsoventral axis inverted at some point in evolution, and thus that the nervous systems of insects and vertebrates are homologous (Fig. 1).

Which of these two theories is correct? Four recent papers[24–27] address the question of *Otx* functional equivalence, and supply further evidence for homology of the insect and vertebrate nervous systems. Three of them[24–26] show that the *Otx* and *otd* genes have a conserved function, as well as conserved expression patterns. Previously, the expression of *otd* and *ems* in the fly nervous system was determined (Ref. 6 and Fig. 2), and the fourth paper[27] finds that *Hox* gene expression there is more similar to expression of vertebrate *Hox* genes than was previously suspected.

Functional equivalence of *Otx* and *otd* genes

Two papers describe the overexpression of human *OTX1* and *OTX2* genes in flies defective in *otd* function. Nagao *et al.*[26] found that the human *OTX* genes could rescue the *ocelliless* phenotype (*oc* is a regulatory mutation causing defective *otd* expression in the fly pupa) just as well as *otd* could rescue. Not only the final phenotype, but also gene expression

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in the eye-antennal imaginal discs, is rescued. Leuzinger *et al.*[25] used a similar approach to drive transient ubiquitous expression of *otd*, *OTX1* or *OTX2*, to look at the better-studied embryonic function of *otd*. According to several criteria, *otd* expression can rescue the *otd* mutant phenotype, though in a variable proportion of embryos. *OTX2* also rescues, though at a lower frequency than *otd*, and *OTX1* rescues less efficiently still. The third paper[24] goes in the other direction: the mouse *Otx1* coding region was replaced by that of *Drosophila otd*. Many of the defects of *Otx1*$^{-/-}$ mice were rescued by *otd*, for example several brain regions previously missing are restored. Interestingly, the rescue was less efficient nearer to the MHB, for example the mesencephalon was never completely normal, and cerebellar foliation remained abnormal. In the sense organs, most defects were rescued, but the lateral semicircular canal in the inner ear was not.

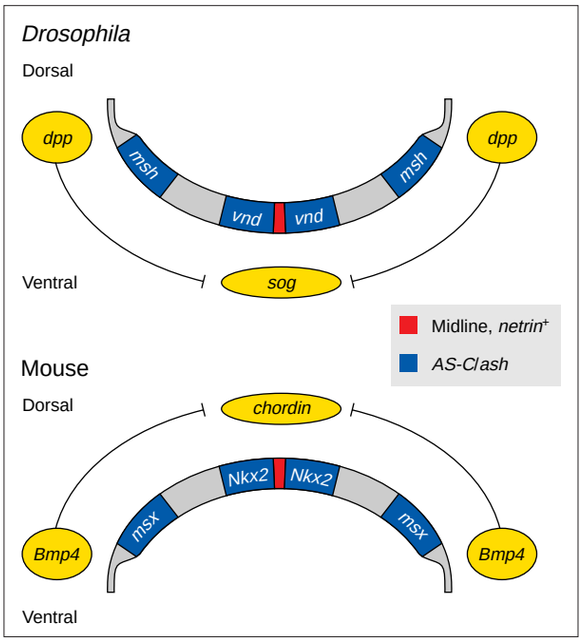

FIGURE 1. Transverse sections through the fly and vertebrate central nervous system primordia, showing similar dorsoventral regulation of pattern by the *sog* (*short gastrulation*)/*chordin*, *dpp* (*decapentaplegic*)/*BMP4*, *Msx*/*msh*, *Nkx2*/*vnd*, *AS-C* (*achaete-scute complex*)/*ash* (*AS-C homologues*) and *netrin* gene families. (Redrawn from Ref. 23, with extra information from Ref. 22.)

Hox genes in the fly nervous system

Hirth *et al.*[27] examine the expression and function of the *Drosophila* homeotic (*Hox*) genes in the CNS. In the mouse, the group 2 *Hox* genes have the most anterior expression boundaries, while group 1 *Hox* genes are expressed in restricted domains, one rhombomere posterior to the group 2 anterior boundary (Ref. 1 and Fig. 2). This lapse in the rule of colinearity has so far only been noted in vertebrates[28]; in the *Drosophila* ectoderm, strict colinearity is adhered to. Previous descriptions of homeotic gene expression in the fly nervous system put the anterior boundary of *labial* (*lab*; a group 1 *Hox* gene) and *proboscipedia* (*pb*; group 2) at the same level, within the deutocerebrum[29]. However, Hirth *et al.* have re-examined this expression using neural segmental markers, and now show that *lab* is actually expressed only in the posterior of brain segment b3 (the tritocerebrum), posterior to the anterior boundary of *pb* expression in segment b2 (the deutocerebrum) (Ref. 27; Fig. 2). The same exception to colinearity therefore occurs in the fly CNS as well as in vertebrates.

Homology of nervous systems

It is important to realize that the ability of homologous genes to replace each other functionally does not necessarily show that the regions in which they are expressed are homologous. The *Otx* and *otd* gene products, for example, are transcription factors. It is quite possible that the ability of OTX proteins to bind a particular DNA sequence has been conserved, while the downstream genes regulated by this binding have altered. This explanation would still allow the proteins to replace each other, but they would be regulating different downstream genes in each animal. The formal possibility has not been ruled out that expression of *otd* and *Otx* in the anterior brain could have arisen independently in insects and chordates. However, it is now clear that the similar expression of *Otx* genes in the two nervous systems has functional relevance; the similarity cannot be explained by the gain of a few enhancer elements, and thus is less likely to have arisen by convergent evolution.

These papers also show that, even though *Hox* and *Otx* genes are expressed in the early blastoderm in flies, this does not (as the auricularia hypothesis[20] suggests) mean that the ectoderm wall is homologous to the vertebrate neural tube, because the same genes are also expressed in the fly CNS, and in a very similar manner to vertebrates. Together with other evidence[17,22,30,31], these results might be the final nail in the coffin for theories like the auricularia hypothesis that support an independent origin for the insect and vertebrate nervous systems.

Significance of *otd/Otx* functional equivalence

The fly *otd* gene can replace mouse *Otx1* astoundingly well. We would have perhaps expected that *Otx1* would have evolved new functions to do with vertebrate-specific developmental programs, that *otd* could not perform. It appears that only the homeobox and the regulatory elements, plus perhaps some less well conserved regions like the acidic activation domains, are sufficient for most of the functions of *Otx1*. New expression domains acquired since the arthropod/vertebrate split might account for much of the differences between *otd* and *Otx1*; these differences can only be detected experimentally by examining and possibly replacing regulatory elements rather than just coding regions. Replacement of regions of the gene outside the homeobox would test whether these regions, or only the homeobox, is important.

Although *otd* can replace most functions of *Otx1*, some cannot be replaced. This could simply be a quantitative effect: Acampora *et al.*[9] have shown that in brain development *Otx* 'dosage' is more important than which individual *Otx* gene is present, although *Otx2* does appear to be more 'potent' than *Otx1*, since the *Otx2* mutant has a stronger phenotype. In the rescue experiments of Leuzinger *et al.*[25], *Otx1* is less effective at rescuing the *otd* phenotype than *Otx2*. *otd* could simply be less

'potent' in the mouse than either *Otx1* or *Otx2*, as is supported by the fact that mice with two copies of *Otx2* and no *Otx1* show a weaker phenotype than mice[24] with one copy of each gene (Ref. 32). Some of the lack of rescue cannot be explained in this way, however, and could reflect truly novel functions of *Otx1* that depend on its coding region. One example is the lateral semicircular canal phenotype of *Otx1*, which cannot be rescued by *otd*, suggesting that *Otx1* has taken on a role in development of this canal in the inner ear after duplication. Significantly, this canal evolved around the time when the *Otx* genes are thought to have duplicated, at the origin of jawed vertebrates[33] (agnathan fish have only two semicircular canals, and lack the lateral one[24]).

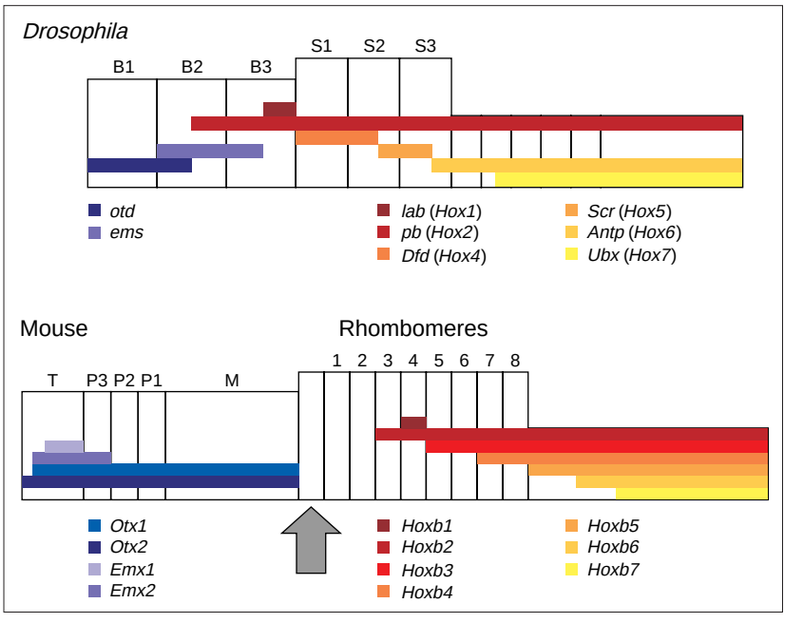

FIGURE 2. Anteroposterior gene expression in the fly and mouse central nervous systems showing *Hox*, *Otx/otd* and *Emx/ems* expression patterns. (Redrawn from Ref. 27, with extra information from Refs 6, 29, 38 and H. Reichert, pers. commun.) Arrow denotes the midbrain–hindbrain boundary; B1–B3, brain segments (proto-, deuto- and trito-cerebrum, respectively); S1–S3, mandibular, maxillary and labial segments, respectively; P1–P3, prosomeres in the diencephalon; T, telencephalon; M, mesencephalon.

The evolution of the midbrain–hindbrain boundary

A second way in which *otd* cannot replace *Otx1* is shown in the graded level of rescue in the brain: the midbrain–hindbrain boundary (MHB), midbrain and cerebellum, at the posterior of the *Otx* domain, are most sensitive to a low level of *otd/Otx* function and thus can be less easily rescued, while the telencephalon (at the anterior of the *Otx* domain) is least sensitive[24]. The vertebrate MHB is known to act as an organizer, producing signals that pattern the midbrain and anterior hindbrain[35,36], but no such organizer has been described in the brain of *Drosophila*. Like the lateral semicircular canal, the organizer may have arisen early in vertebrate evolution, since lampreys and all jawed vertebrates, but not hagfish or cephalochordates, have an undeniable midbrain and cerebellum[34]. Thus the inability of *Drosophila otd* to rescue the midbrain–hindbrain boundary phenotype of mouse *Otx1* may be because the MHB is a new structure that evolved, or was at least greatly elaborated, early in the vertebrate lineage. The *Otx* genes themselves may have helped in the evolution of the MHB organizer; changing *Otx* expression patterns could have produced a gap between *Otx* and *Hox* expression domains (*otd* and *Hox* are adjacent in the fly; Fig. 2), which was then filled by MHB-specific transcription factors and signalling molecules such as EN, PAX2, PAX5, PAX8, WNT1 and FGF8 (Ref. 36). A thorough analysis of the development of this region in a range of vertebrates is clearly needed.

Perspectives

The papers reviewed here answer some questions while raising others. The list of known similarities between the vertebrate and arthropod nervous systems has been expanded significantly, suggesting that they are probably homologous structures. If this is so, has the auricularia hypothesis been disproved? Enteropneusts, a group of invertebrate chordates, have both a dorsal neural tube and a ventral nerve plexus, and it has been argued[37] that this shows that the vertebrate dorsal neural tube cannot be homologous to the invertebrate ventral nervous system. However, it is possible to argue that they are, if one of the enteropneust nervous systems is an independently-derived novelty, or if the two enteropneust nervous systems arose from a duplication of the single ancestral one. Investigation of expression patterns of developmental genes in enteropneusts may resolve the discrepancy.

Additionally, it is still not resolved whether the functional equivalence of genes, as shown by their ability to replace each other, really demonstrates that the genes are doing the same thing in the two animals concerned. It would be interesting, for example, to test whether mammalian *Hoxb2* can rescue the phenotype of *Drosophila lab* mutants (*lab* being a group 1 *Hox* gene, *Hoxb2* a group 2). So far, genes in the same subfamily have mostly been tested for rescuing ability [*otd* and *Otx1* (Refs 24–26), *hh* and *shh* (Ref. 30) or *Hoxb1* and *labial* (Ref. 17)], but this ability may simply show that the important coding-region functions of a whole gene family have remained constant, rather than that two genes are the closest homologues within the family. The ability to rescue should be tested for a range of related genes, not just the ones suspected to be most closely related to each other. Certainly, these studies emphasize the need to compare regulatory elements as much as coding regions between species, since it appears that evolution has relied predominantly on regulatory changes.

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Update

CBP/p300 transgenic mice

Last month's issue of *Trends in Genetics* featured a review of the disease-related potential of the transcriptional cofactors CREB-binding protein (CBP) and the adenovirus E1A-associated protein, p300 (Ref. 1). Shortly after the publication of this review, the combined efforts of David Livingston's laboratory (Harvard Medical School, Boston, MA, USA) and Richard Eckner's laboratory (Univ. of Zurich, Switzerland) appeared in *Cell*, describing the deleterious effects of inactivating one or both murine *CBP* and/or *p300* alleles[2].

The results-at-a-glance are presented in Table 1. When a single *p300* allele is inactivated, the resultant embryos suffer a significantly reduced viability (up to 55% died *in utero*, depending on genetic background), although heterozygotes that do survive do not suffer from further p300-insufficiency after birth. Mice homozygous for p300 mutations always die in *utero*, between days 9 and 11.5 of gestation. These nullizygous embryos are much smaller than their littermates and exhibit severe open neural tube and heart defects. Interestingly, cells removed from the p300 homozygous mutants displayed poor proliferation properties, implying that p300 is required for growth stimulation, an idea that is contrary to the general opinion that CBP and p300 are tumor suppressor proteins. Unlike p300, CBP heterozygous mutant mice, described earlier[3], manifest skeletal abnormalities consistent with the human congenital Rubinstein–Taybi syndrome, in which one *CBP* allele is inactivated[4]. CBP homozygous mutant mice, however, strongly resemble the p300 mutants, and also die *in utero*, between days 9 and 11.5 of gestation.

TABLE 1. Mouse models for CBP and p300 mutations

CBP	p300	Phenotype	Refs
++	++	Normal	–
+–	++	Skeletal abnormalities	3
––	++	Embryonic lethal	2, 3
++	+–	Reduced viability	2
++	––	Embryonic lethal	2
+–	+–	Embryonic lethal	2

Abbreviations: +, normal allele; –, inactive allele.

Crossing the p300 and CBP heterozygous mutants produced double heterozygous CBP/p300 mutant embryos, which died *in utero* but otherwise shared phenotypic similarities to both CBP and p300 homozygous mutants. This remarkable result suggests that the two proteins exert certain common embryonic survival functions and that the combined dose of CBP and p300 is critical for mouse embryonic development. Although CBP and p300 are not completely redundant physiologically, these results suggest that a 25% drop in combined CBP/p300 levels (through the loss of one *CBP* or *p300* allele) is enough to interfere seriously with embryonic development, while a 50% drop results invariably in embryonic death.

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