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An urbilaterian origin of the tripartite brain: developmental genetic insights from *Drosophila*

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SUMMARY

Studies on expression and function of key developmental control genes suggest that the embryonic vertebrate brain has a tripartite ground plan that consists of a forebrain/midbrain, a hindbrain and an intervening midbrain/hindbrain boundary region, which are characterized by the specific expression of the Otx, Hox and *Pax2/5/8* genes, respectively. We show that the embryonic brain of the fruitfly *Drosophila melanogaster* expresses all three sets of homologous genes in a similar tripartite pattern. Thus, a *Pax2/5/8* expression domain is located at the interface of brain-specific *otd/Otx2* and *unpg/Gbx2* expression domains anterior to Hox expression regions. We identify this territory as the deutocerebral/tritocerebral boundary region in the embryonic *Drosophila* brain. Mutational inactivation of *otd/Otx2* and *unpg/Gbx2* result in the loss or misplacement of the brain-specific expression domains of *Pax2/5/8* and Hox genes. In addition, *otd/Otx2* and *unpg/Gbx2* appear to negatively regulate each other at the interface of their brain-specific expression domains. Our studies demonstrate that the deutocerebral/tritocerebral boundary region in the embryonic *Drosophila* brain displays developmental genetic features similar to those observed for the midbrain/hindbrain boundary region in vertebrate brain development. This suggests that a tripartite organization of the embryonic brain was already established in the last common urbilaterian ancestor of protostomes and deuterostomes.

Key words: Brain development, Brain evolution, Midbrain/hindbrain boundary, Otx, Hox, Pax, *Drosophila melanogaster*

INTRODUCTION

Classical phylogenetic, neuroanatomical and embryological studies have led to the view of an independent evolutionary origin of protostome and deuterostome brains. Accordingly, bilaterians have been divided into two major groups with different CNS morphologies: the gastroneuralia, which are characterized by a ventral nerve cord, include protostomes such as arthropods, annelids and molluscs; and the notoneuralia, which are characterized by a dorsal nerve cord, include all chordates (Siewing, 1985; Brusca and Brusca, 1990; Nielsen, 2001). By contrast, more recent studies examining the expression patterns and functions of orthologous genes in embryonic nervous systems have revealed similarities in the embryonic brains of protostomes, such as annelids and arthropods, and deuterostomes like tunicates and mammals. These results suggest that lophotrochozoan and ecdysozoan invertebrate brains and chordate brains of vertebrates, acraniates and urochordates are all characterized by a rostral region specified by genes of the *otd* (*oc* – FlyBase)/Otx family and a caudal region specified by genes of the Hox family (reviewed by Bruce and Shankland, 1998; Arendt and Nübler-Jung, 1999; Hirth and Reichert, 1999; Holland and Holland, 1999; Reichert and Simeone, 1999; Wada and Satoh, 2001; Reichert, 2002).

In ascidian and vertebrate chordates, a *Pax2/5/8* expression domain is located between the anterior Otx and the posterior Hox expression regions of the embryonic brain (reviewed by Holland and Holland, 1999; Wada and Satoh, 2001). In vertebrate brain development, this *Pax2/5/8* expression domain is positioned at the interface of the brain-specific *Otx2* and *Gbx2* expression domains; it is an early marker for the isthmic organizer at the midbrain-hindbrain boundary (MHB), which controls the development of the midbrain and the anterior hindbrain (reviewed by Liu and Joyner, 2001; Rhinn and Brand, 2001; Wurst and Bally-Cuif, 2001). The central role of this MHB region in brain development together with the conserved expression patterns of *Pax2/5/8* genes in this region have led to the proposal that a fundamental characteristic of the ancestral chordate brain was its tripartite organization characterized by Otx, *Pax2/5/8* and Hox gene expressing regions (Wada et al., 1998). To investigate whether protostomes also possess a tripartite organization of the brain, and to gain insight into the evolution of the bilaterian brain, we carried out a comparative analysis of expression and function in the ecdysozoan

Drosophila melanogaster of the orthologues that pattern the vertebrate MHB region.

Our study reveals striking similarities in the expression and function of genes that pattern the embryonic brains of *Drosophila* and vertebrates. We find that a *Pax2/5/8* expressing domain is located between an anterior *otd*-expressing region and a posterior Hox-expressing region in the embryonic brain of *Drosophila*. Moreover, in *Drosophila*, as in vertebrates, we find that this *Pax2/5/8* expressing domain is positioned at the interface between the *otd/Otx2* expression domain and a posteriorly abutting *unplugged*/*Gbx2* expression domain. Finally, we demonstrate that inactivation of *otd/Otx2* or of *unplugged/Gbx2* results in comparable effects on mispositioning or loss of brain-specific expression domains of orthologous genes in both embryonic brain types. These developmental genetic similarities indicate that the tripartite ground plan, which characterizes the developing chordate brain, is also at the basis of the developing insect brain, conferring on all bilaterians a deep similarity in brain development. This suggests that a corresponding tripartite organization already existed in the brain of the last common urbilaterian ancestor of insects and chordates.

MATERIALS AND METHODS

Fly culture

Wild-type flies are of the strain *Oregon-R*. All mutant stocks are from the Bloomington Stock Center unless indicated otherwise. The following reporter *lacZ* lines and mutant alleles were used: *P{lacZ}unpg*^{f85} (an *unpg-lacZ* reporter gene that expresses cytoplasmic β-galactosidase in the same pattern as endogenous *unpg*) (Chiang et al., 1995), *P{lacZ}Pax2*$^{\Delta 122}$ (a *Pax2-lacZ* reporter gene that expresses β-galactosidase in the same pattern as endogenous *Pax2*; E.F., M. Daube, J. Kronhamn, A. Rasmuson-Lestander and M.N., unpublished), *otd*^{1A101} (Hirth et al., 1995), *P{3′lacZ}unpg*^{r37} (*unpg* null allele with a *unpg-lacZ* reporter gene that expresses nuclear β-galactosidase in the same pattern as endogenous *unpg*) (Chiang et al., 1995).

In situ hybridization and immunocytochemistry

For in situ hybridization experiments, digoxigenin-labelled sense and antisense RNA probes of the *spa/Pax2* cDNA clone cpx1 (Fu and Noll, 1997) were generated in vitro with a DIG-labelling kit (Roche diagnostics) and hybridized to *Drosophila* whole-mount embryos by following standard procedures (Tautz and Pfeifle, 1989). Whole-mount immunocytochemistry was performed as previously described (Hirth et al., 1998). Rabbit anti-PAX2 antiserum (Fu and Noll, 1997) and monoclonal anti-POXN antibodies (Hassanzadeh et al., 1998) were used as primary antibodies at 1:50 and 1:20 dilutions, respectively. To generate an anti-Otd antiserum, a 835 bp fragment from an *otd* cDNA (position 1708-2542) (Finkelstein et al., 1990) was amplified by PCR and cloned into the pCR2.1-TOPO vector (Invitrogen), from which it was removed by digestion at flanking *Eco*RI sites and cloned in frame into the pGEX-2T expression vector (Smith and Johnson, 1988). The glutathione-S-transferase/Otd fusion protein was purified according to Smith and Johnson (Smith and Johnson, 1988), except that induction occurred at 18°C overnight. Immunization of rabbits was carried out by Pab Productions (Hebertshausen). For whole-mount immunocytochemistry, anti-Otd antiserum was used as primary antiserum at a 1:100 dilution.

Embryos were staged according to Campos-Ortega and Hartenstein (Campos-Ortega and Hartenstein, 1997).

Laser confocal microscopy

For laser confocal microscopy, a Leica TCS SP microscope was used. Optical sections ranged from 0.4 to 2 μm, recorded in line average mode with picture size of 512×512 pixels. Captured images from optical sections were arranged and processed by the use of IMARIS (Bitplane). Figures were arranged and labelled by the use of Adobe Photoshop.

RESULTS

Pax2 and *Poxn* expression in the embryonic brain

In the embryonic CNS of vertebrates, the *Pax2*, *Pax5* and *Pax8* genes are expressed in specific domains that overlap in the presumptive MHB region (reviewed by Liu and Joyner, 2001; Rhinn and Brand, 2001; Wurst and Bally-Cuif, 2001). *Drosophila* has two *Pax2/5/8* orthologues, *Pox neuro* (*Poxn*) and *Pax2* (Noll, 1993; Fu and Noll, 1997).

The embryonic brain of *Drosophila* can be subdivided into the protocerebrum (PC or b1), deutocerebrum (DC or b2) and tritocerebrum (TC or b3) of the supra-oesophageal ganglion and the mandibular (S1), maxillary (S2) and labial (S3) neuromeres of the sub-oesophageal ganglion. Expression of *engrailed* (*en*) delimits these subdivisions by marking their most posterior neurones (reviewed by Hartmann and Reichert, 1998) (Fig. 1). Because of morphogenetic processes, such as the beginning of head involution, the neuraxis of the embryonic brain curves dorsoposteriorly within the embryo. Accordingly, henceforth anteroposterior coordinates refer to the neuraxis rather than the embryonic body axis.

We first determined whether *Pax2* is expressed in specific domains of the *Drosophila* brain, by analysing its expression pattern using in situ hybridization, immunolabelling and *lacZ* reporter gene expression. *Pax2* transcripts initially appear during gastrulation and at stage 9/10 are observed in a segmentally reiterated pattern of the developing procephalic and ventral neuroectoderm, with its anteriormost expression domain located at the future deutocerebral-tritocerebral boundary (Fig. 2A, arrow). Expression of *Pax2* transcripts in the developing brain begins at stage 10/11 and is most prominent in a longitudinal stripe at the medial part of the protocerebrum and in a transversal stripe at the posterior border of the deutocerebrum (Fig. 2B-D, arrowheads). Immunolabelling with a Pax2-specific polyclonal antibody (Fu and Noll, 1997) revealed that Pax2 protein distribution resembles that of *Pax2* transcripts, as does a *Pax2-lacZ* reporter gene expressing β-galactosidase (Fig. 3A-C). In addition to its expression in the developing anterior brain, *Pax2* expression is also seen in six to eight cells located at the lateral margin of each hemisegment throughout the more posterior CNS regions of the sub-oesophageal ganglion and ventral nerve cord (Fig. 3C).

To determine the expression of the second *Drosophila Pax2/5/8* orthologue, we characterized *Poxn* expression using immunolabelling and *lacZ* reporter genes. POXN protein is first detected in the developing brain at the end of germband extension (stage 10/11) in two stripes of the procephalic neuroectoderm, which subsequently become restricted to the posterior protocerebrum and the posterior deutocerebrum (Fig. 3D,E). *Poxn* expression in more posterior regions of the CNS also occurs in segmentally reiterated patterns (Fig. 3F) (Bopp

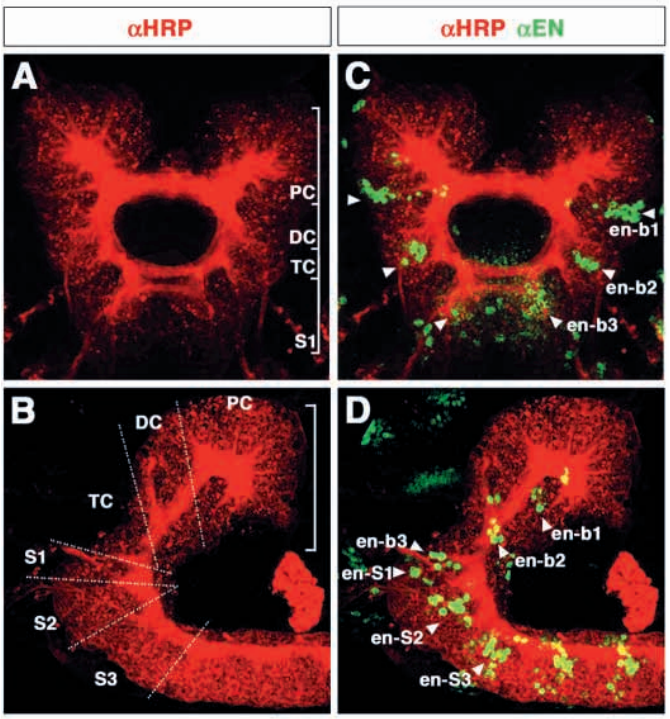


Fig. 1. Expression of *engrailed* (*en*) delimits the neuromeric subdivisions of the embryonic brain and ventral nerve cord. Laser confocal microscopy of stage 13/14 wild-type embryos, reconstructions of optical sections. (A,C) Frontal views; (B,D) lateral views. The bracket in B indicates the extend of optical sections used to reconstruct the frontal views of A,C. (A,B) Immunolabelling with a neurone-specific anti-HRP antibody (red). (C,D) Double-immunolabelling with anti-HRP (red) and anti-EN (green, yellow). The embryonic brain can be subdivided into the protocerebrum (PC or b1), deutocerebrum (DC or b2) and tritocerebrum (TC or b3) of the supraesophageal ganglion and the mandibular (S1), maxillary (S2) and labial (S3) neuromeres of the sub-oesophageal ganglion. *en*-expressing cells delimit the posterior boundaries of the PC (en-b1), DC (en-b2) and TC (en-b3), and of the mandibular (en-S1), maxillary (en-S2) and labial (en-S3) neuromeres.

et al., 1989). A comparison between *Pax2* and *Poxn* expression domains reveals, that *Pax2* and *Poxn* are never co-expressed in the same cells of the CNS. Moreover, expression of *Pax2* and *Poxn* does not occur at a comparable anteroposterior position along the neuraxis (Fig. 3I), with one exception. This exception is in the posterior deutocerebrum where adjacent *Pax2* and *Poxn* expression domains define a transversal domain immediately anterior to the tritocerebral brain neuromere (arrows in Fig. 3G,H). This transversal domain of adjacent *Pax2* and *Poxn* expression is distinguishable from segmentally reiterated expression in more posterior regions by the fact that it is the only position along the neuraxis where expression of both genes coincides with a neuromere boundary (compare Fig. 3H with Fig. 1D). We refer to this transversal domain of adjacent *Pax2/5/8* orthologue expression as the deutocerebral-tritocerebral boundary (DTB) region.

It is important to note that the DTB is located anterior to the expression domain of the *Drosophila Hox1* orthologue *labial* (*lab*), which is expressed in the posterior tritocerebrum (Hirth et al., 1998). Moreover, the DTB is located posterior to the expression domain of the *Drosophila Otx* orthologue *otd* in the protocerebrum and anterior deutocerebrum (Hirth et al., 1995). Thus, in *Drosophila* as in vertebrates, a *Pax2/Poxn* (*Pax2/5/8*) expression domain is located between the anterior *otd/Otx2* and the posterior Hox-expressing regions. This raises the question of whether the DTB in the embryonic *Drosophila* brain might have developmental genetic features similar to those observed for the MHB in vertebrate brain development.

Gene expression patterns at the deutocerebral-tritocerebral boundary region

In the embryonic vertebrate brain, *Otx2* is expressed anterior to and abutting *Gbx2*. The future MHB as well as the overlapping domains of *Pax2*, *Pax5* and *Pax8* expression are positioned at this *Otx2-Gbx2* interface (Liu and Joyner, 2001; Rhinn and Brand, 2001; Wurst and Bally-Cuif, 2001). To investigate if comparable expression patterns are found in the embryonic fly brain, we determined the brain-specific expression of the *Drosophila Gbx2* orthologue *unplugged*

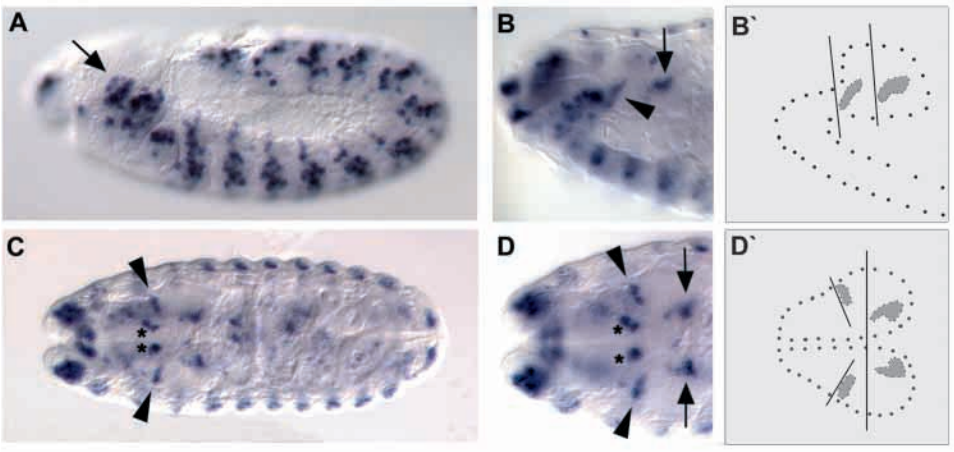


Fig. 2. Whole-mount in situ hybridization of *Pax2* transcripts in wild-type embryos. (A,B,B′) Lateral views, (C,D,D′) dorsal views; anterior is towards the left. (A) By stage 9/10, *Pax2* expression is detectable in a segmentally reiterated pattern of the developing procephalic and ventral neuroectoderm, with its anteriormost expression domain located at the future deutocerebral-tritocerebral boundary (arrow). (B-D) At stage 14, *Pax2* transcripts are most prominent in a longitudinal stripe at the medial part of the protocerebrum (arrows) and in a transversal stripe (arrowheads) at the posterior border of the deutocerebrum. Asterisks in C,D indicate *Pax2* expression in cells associated with the developing hypopharyngeal organ outside the CNS (see also Fig. 3A). (B′) Simplified summary scheme of B with brain-specific *Pax2* expression domains (grey) shown in relation to protocerebral and deutocerebral neuromere boundaries (unbroken lines). (D′) Simplified summary scheme of D with brain-specific *Pax2* expression domains (grey) shown in relation to protocerebral and deutocerebral neuromere boundaries (unbroken lines).

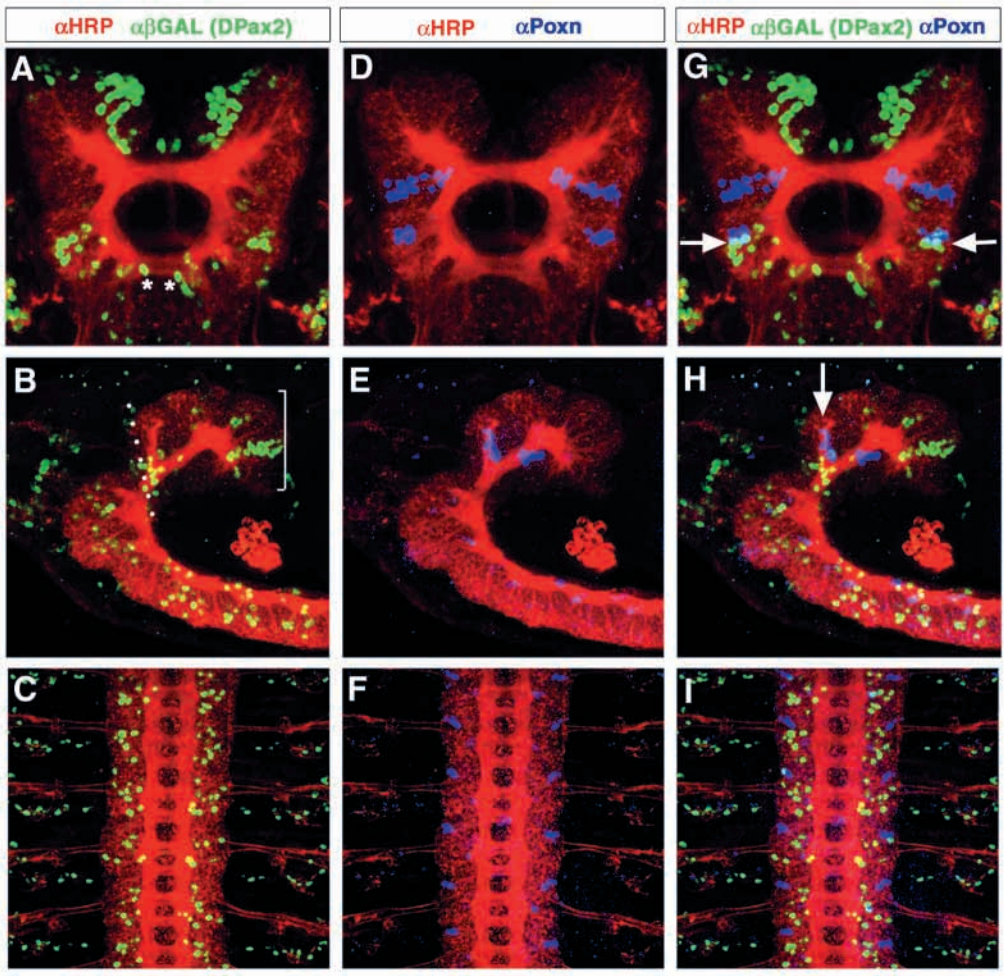


Fig. 3. *Pox neuro* (*Poxn*) and *Pax2* are expressed in distinct domains in the embryonic brain and ventral nerve cord. Laser confocal microscopy of stage 13/14 embryos, reconstructions of optical sections. (A-I) *P{lacZ}Pax2* $^{\Delta 122}$/+. (A,D,G) Frontal views, (B,E,H) lateral views, (C,F,I) ventral views. The bracket in B indicates the extend of optical sections used to reconstruct the frontal views of A,D,G, the dotted line demarcates the deutocerebral/tritocerebral neuromere boundary. (A-C) Double-immunolabelling with anti-HRP (red) and anti-β-gal (green, yellow). (D-F) Double-immunolabelling with anti-HRP (red) and anti-POXN (blue). (G-I) Triple-immunolabelling with anti-HRP (red), anti-β-gal (green, yellow) and anti-POXN (blue, white); arrows in G,H indicate *Pax2* and *Poxn* expression at the same anteroposterior position along the neuraxis in the posterior deutocerebrum where they define a transversal domain. This transversal domain of adjacent *Pax2/5/8* orthologue expression is located anterior to the deutocerebral/tritocerebral neuromere boundary. Note that expression of *Pax2* and *Poxn* are never observed in the same cells. The apparent co-expression of *Pax2* and *Poxn* shown in G (white) is an artefact caused by superimposed optical sections. Asterisks in A indicate *Pax2* expression in cells associated with the developing hypopharyngeal organ outside the CNS.

(*unpg*) in relation to that of *otd*, using immunolabelling and an *unpg-lacZ* reporter gene that expresses β-galactosidase like endogenous *unpg* (Chiang et al., 1995). The *otd* gene is expressed in the protocerebrum and anterior deutocerebrum of the embryonic brain (Fig. 4A,B) (Hirth et al., 1995), as well as in midline cells in more posterior regions of the CNS (Fig. 4C) (Finkelstein et al., 1990; Wieschaus et al., 1992). Expression of *unpg-lacZ* in the embryonic CNS is first detected at stage 8 in neuroectodermal and mesectodermal cells at the ventral midline, with an anterior limit of expression at the cephalic furrow (Chiang et al., 1995). Subsequently, the *unpg* expression domains in the CNS widen and have their most anterior border in the posterior deutocerebrum (Fig. 4D-F). Double immunolabelling of OTD and β-galactosidase revealed that the posterior border of the brain-specific *otd* expression domain coincides with the anteriormost border of the *unpg* expression domains along the anteroposterior neuraxis (Fig. 4G,H, arrows). There is no overlap of *otd* and *unpg* expression in the brain or in more posterior regions of the CNS (Fig. 4G-I).

These findings indicate that the *otd-unpg* interface is positioned at the anterior border of the DTB. This was confirmed by additional immunolabelling studies examining *unpg-lacZ*, *otd*, *Poxn* and *en* expression in the protocerebral/deutocerebral region of the embryonic brain. Thus, double immunolabelling of OTD and EN confirms that the posterior border of *otd* expression extends beyond the protocerebral en-b1 stripe into the anterior deutocerebral domain (Fig. 5A). Labelling OTD and POXN confirms that the *Poxn* expression domain of the DTB is posterior to this deutocerebral *otd* expression boundary (Fig. 5B). Labelling EN and β-galactosidase, which is indicative of *unpg* expression, confirms that the anteriormost *unpg* expression domain overlaps with the en-b2 stripe (Fig. 5C). Finally, labelling β-galactosidase and POXN confirms that this anteriormost *unpg* expression domain overlaps with the *Poxn* expression domain of the DTB (Fig. 5D). Therefore, in terms of overall gene expression patterns, we find that a transversal domain of adjacent *Pax2/Poxn* expression defines the DTB region of the embryonic *Drosophila* brain. Furthermore, this region is located between an anterior *otd* expression domain and a posterior *Hox* expression domain. Moreover, it is located abutting and posterior to the interface of *otd* and *unpg* expression along the anteroposterior neuraxis. A schematic summary of these expression patterns in the embryonic fly brain is illustrated in Fig. 8A.

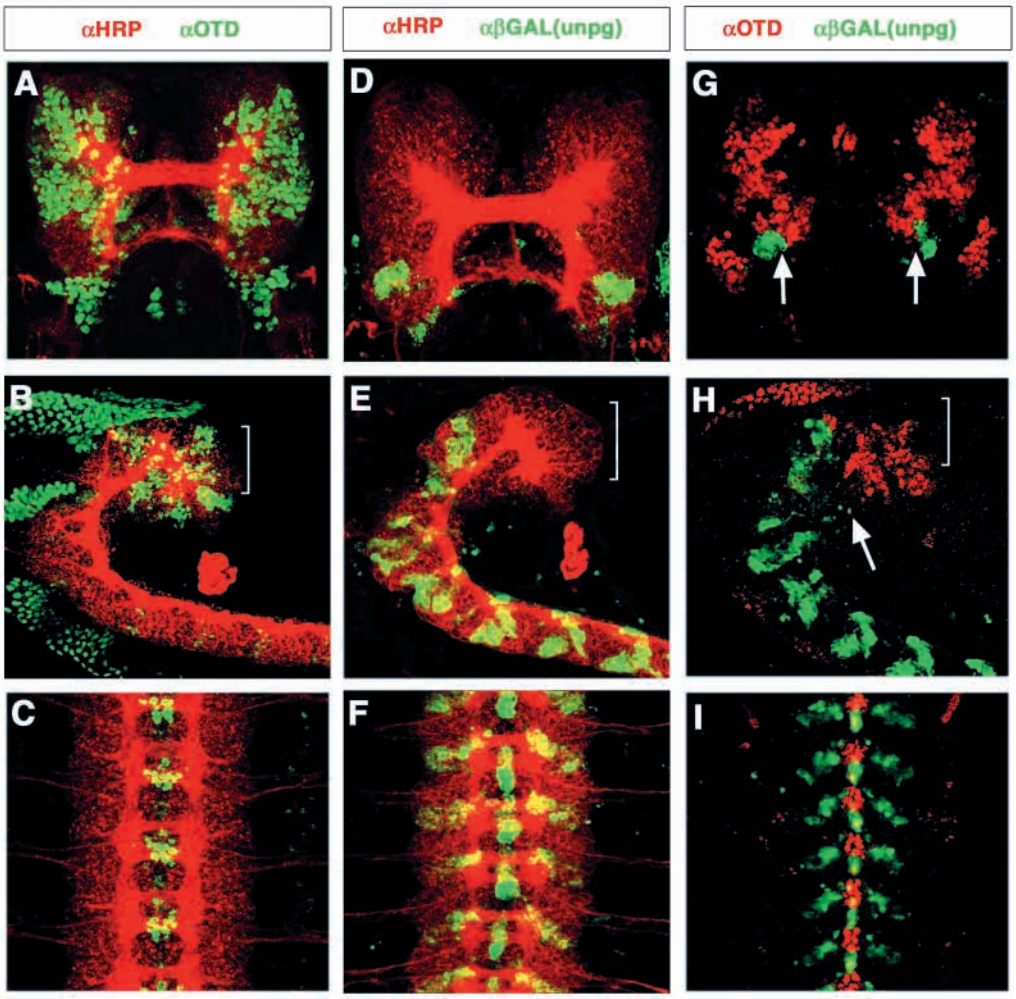


Fig. 4. *otd* and *unpg* expression domains in the embryonic brain and ventral nerve cord. Laser confocal microscopy of stage 13/14 embryos, reconstructions of optical sections. (A-C) Wild type, (D-I) *P{lacZ}unpg*^{f85}/+. (A,D,G) Frontal views, (B,E,H) lateral views, (C,F,I) dorsal views. The brackets in B,E,H indicate the extend of optical sections used to reconstruct the frontal views of A,D,G, respectively. (A-C) Double-immunolabelling with anti-HRP (red) and anti-OTD (green, yellow). (D-F) Double-immunolabelling with anti-HRP (red) and anti-β-gal (green, yellow). (G-I) Double-immunolabelling with anti-OTD (red) and anti-β-gal (green, yellow); arrows indicate interface of *otd* and *unpg* expression at the anterior border of the deutocerebral-tritocerebral boundary (DTB) region. Note that a direct interface of the *otd* and *unpg* expression domains only occurs on the ventral (according to neuraxis) side of the brain; on the opposing side, a small gap between the two expression domains is seen (H, arrow).

Mutation of *otd* and of *unpg* result in loss or misplacement of brain-specific gene expression domains

In mammalian brain development, homozygous *Otx2*-null mutant embryos lack the rostral brain, including the MHB-specific *Pax2/5/8* expression domain (Acampora et al., 1995; Matsuo et al., 1995; Ang et al., 1996), whereas *Gbx2* null mutants misexpress *Otx2* and *Hoxb1* in the brain (Wassarman et al., 1997). Moreover, *Otx2* and *Gbx2* negatively regulate each other at the interface of their expression domains (Li and Joyner, 2001; Liu and Joyner, 2001; Martinez-Barbera et al., 2001; Rhinn and Brand, 2001; Wurst and Bally-Cuif, 2001; Ye et al., 2001). To test if similar regulatory interactions occur in the embryonic brain of *Drosophila*, we analysed the expression of the corresponding orthologues in *otd* and *unpg* mutant embryos.

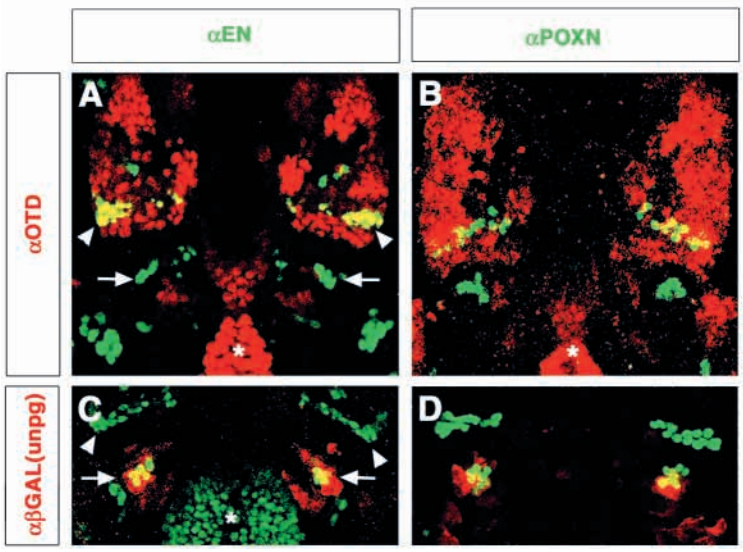


Fig. 5. Brain-specific expression of *otd*, *unpg*, *en* and *Poxn*. Laser confocal microscopy of stage 13/14 embryos, reconstructions of optical sections, frontal views. The extend of optical sections used to reconstruct the frontal views is more dorsal (according to neuraxis) than for Fig. 4A,D,G. (A,B) Wild type, (C,D) *P{lacZ}unpg*^{f85}/+. (A) Double-immunolabelling with anti-OTD (red) and anti-EN (green, yellow). (B) Double-immunolabelling with anti-OTD (red) and anti-POXN (green, yellow). (C) Double-immunolabelling with anti-β-gal (red) and anti-EN (green, yellow). (D) Double-immunolabelling with anti-β-gal (red) and anti-POXN (green, yellow). Arrowheads indicate en-b1 stripes, arrows indicate en-b2 stripes. Asterisks in A-C indicate non-neuronal expression domains of OTD and EN in the foregut.

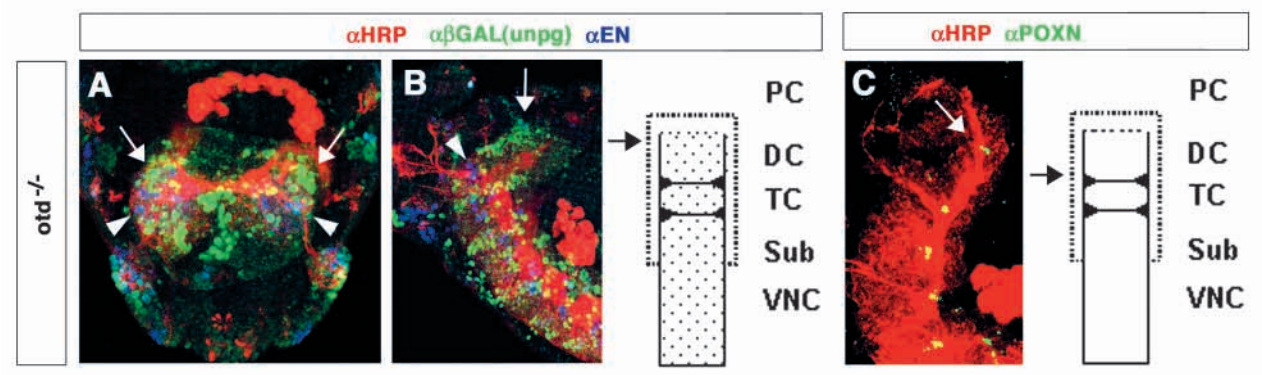
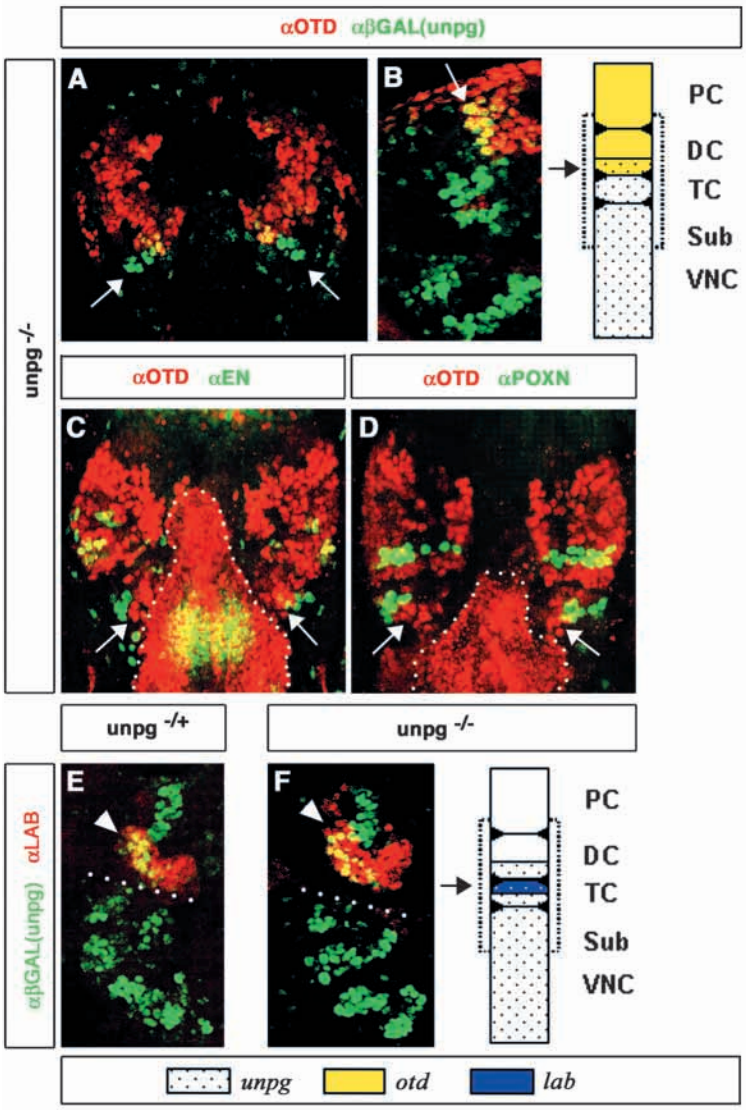


Fig. 6. Altered expression patterns of *unpg*, *en* and *Poxn* in brains of $otd^{-/-}$ embryos. Laser confocal microscopy of stage 13/14 embryos, reconstructions of optical sections. (A) Frontal view, (B,C) lateral views. (A,B) otd^{JA101}; $P\{3'lacZ\}unpg^{r37}$/+, (C) otd^{JA101}. The extend of optical sections used to reconstruct the frontal view in A is the same as in Fig. 3B. Broken rectangles in the summary schemes indicate the region of the embryonic brain shown at higher magnification in B,C. (A,B) Triple-immunolabelling with anti-HRP (red), anti-β-gal (green, yellow) and anti-EN (blue, white). Arrows indicate the anterior shift of brain-specific *unpg* expression into the anterior deutocerebrum (compare with Fig. 4D,E). Note that the protocerebrum is missing in *otd* mutants (Hirth et al., 1995), resulting in the absence of en-b1-expressing cells, whereas en-b2-expressing cells delimiting the deuto-tritocerebral boundary are present (A,B, arrowheads). (C) Double-immunolabelling with anti-HRP (red) and anti-POXN (green, yellow). Arrow indicates the normal position of the posterior POXN stripe in the posterior deutocerebrum, which is absent in the *otd*-null mutant embryo. PC, protocerebrum; DC, deutocerebrum; TC, tritocerebrum; Sub, sub-oesophageal ganglion; VNC, ventral nerve cord.



In *otd*-null mutant embryos, the protocerebrum is absent because protocerebral neuroblasts are not specified (Hirth et al., 1995; Younossi-Hartenstein et al., 1997). Analysis of *unpg*, *en* and *Poxn* expression in *otd*-null mutant embryos revealed that the anteriormost border of *unpg* expression shifts anteriorly into the anterior deutocerebrum (Fig. 6A,B), while *Poxn* fails to be expressed in the deutocerebrum (Fig. 6C). In contrast to

Fig. 7. Altered expression patterns of *otd* and *lab* in brains of $unpg^{-/-}$ embryos. Laser confocal microscopy of stage 13/14 embryos, reconstructions of optical sections. (A,C,D) Frontal views, (B,E,F) lateral views. (A-D,F) $P\{3'lacZ\}unpg^{r37}$, (E) $P\{3'lacZ\}unpg^{r37}$/+. The extend of optical sections used to reconstruct the frontal views in A,C,D is the same as in Fig. 3B; this results in increased visualization of gut-specific *otd* and *en* expression. Broken rectangles in the summary schemes indicate the region of the embryonic brain shown as magnified view in B,F. (A,B) Double-immunolabelling with anti-OTD (red) and anti-β-gal (green, yellow). Arrows indicate the posterior shift of brain-specific *otd* expression into the anteriormost *unpg* expression domain. (C) Double-immunolabelling with anti-OTD (red) and anti-EN (green, yellow). Arrows indicate the posterior shift of brain-specific *otd* expression up to the deutocerebral en-b2 stripe into the posterior deutocerebrum (compare with Fig. 5A). Broken line indicates non-neuronal expression domains of OTD and EN in the foregut. (D) Double-immunolabelling with anti-OTD (red) and anti-POXN (green, yellow). Arrows indicate that the posterior border of brain-specific *otd* expression extends posteriorly up to the *Poxn* expression domain of the DTB (compare with Fig. 5B). Broken line indicates non-neuronal expression domains of OTD and POXN in the foregut. (E,F) Double-immunolabelling with anti-LAB (red) and anti-β-gal (green, yellow) in $unpg^{+/-}$ versus $unpg^{-/-}$ embryos; the broken line demarcates the tritocerebral/mandibular neuromere boundary. Arrowheads indicate the anterior shift of *lab* expression from the posterior tritocerebrum (E) (Hirth et al., 1998) into the anterior tritocerebrum (F). PC, protocerebrum; DC, deutocerebrum; TC, tritocerebrum; Sub, sub-oesophageal ganglion; VNC, ventral nerve cord.

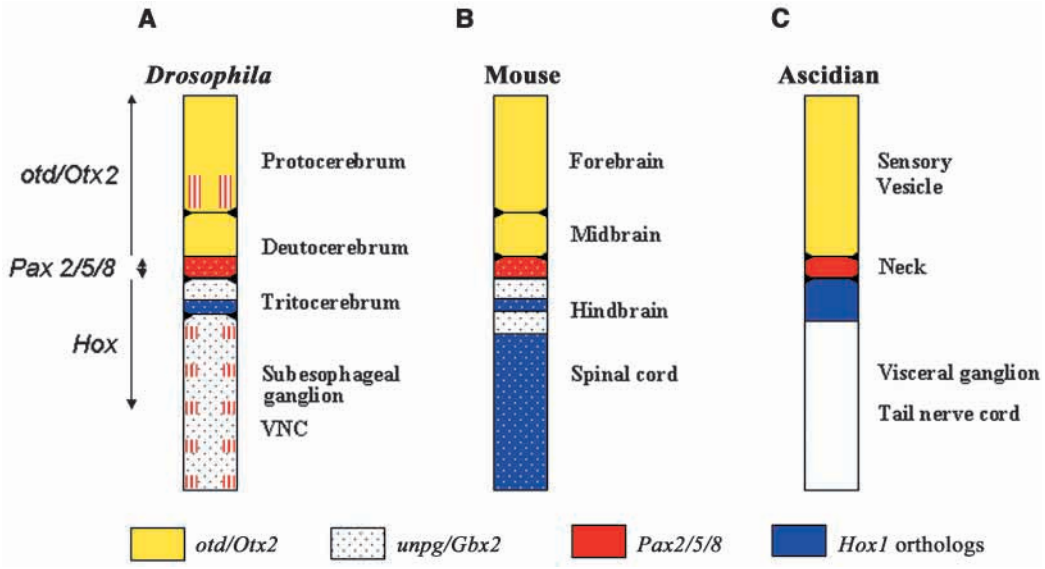


Fig. 8. Tripartite organization of the *Drosophila*, mouse and ascidian brain, based on expression patterns of orthologous genes. The expression of *otd/Otx2*, *unpg/Gbx2*, *Pax2/5/8* and *Hox1* gene orthologues in the developing CNS of (A) stage 13/14 *Drosophila* embryo, (B) stage E10 mouse embryo (Wurst and Bally-Cuif, 2001) and (C) neurula ascidian embryo (Wada et al., 1998). In all cases, a *Pax2/5/8*-expressing domain is located between an anterior *otd/Otx2*-expressing region and a posterior *Hox*-expressing region in the embryonic brain. (Note that in *Drosophila*, the *Pax2/5/8* orthologues *Pax2* and *Poxn* also show a segmentally reiterated expression pattern as outlined with striped red boxes in A.) Moreover, in *Drosophila*, as in mouse, a *Pax2/5/8*-expressing domain is positioned at the interface between the *otd/Otx2* expression domain and a posteriorly abutting *unplugged*/*Gbx2* expression domain. This *otd/Otx2*-*unpg/Gbx2* interface displays similar developmental genetic features in both *Drosophila* and mouse (see text for details).

inactivation of *otd*, inactivation of *unpg* does not result in a loss of cells in the mutant domain of the embryonic brain, as is evident from the expression of an *unpg-lacZ* reporter construct in *unpg*-null mutant embryos (Chiang et al., 1995). Analysis of *otd* expression in *unpg*-null mutants shows that the posterior limit of brain-specific *otd* expression shifts posteriorly into the posterior deutocerebrum, thus extending into the DTB (Fig. 7A,B). This was confirmed by additional immunolabelling studies examining *otd*, *Poxn* and *en* expression in the protocerebral/deutocerebral region of the embryonic brain in *unpg*-null mutants. Double immunolabelling of OTD and EN in *unpg*-null mutants confirms that the posterior border of brain-specific *otd* expression extends posteriorly to the deutocerebral en-b2 stripe into the posterior deutocerebrum (Fig. 7C, compare with Fig. 5A). In addition, double immunolabelling of OTD and POXN in *unpg*-null mutants confirms that the posterior border of brain-specific *otd* expression extends posteriorly into the *Poxn* expression domain of the DTB (Fig. 7D, compare with Fig. 5B). Moreover, analysis of *lab* expression in *unpg*-null mutants shows that brain-specific *lab* expression shifts anteriorly into the anterior tritocerebrum (Fig. 7E,F). Thus, in both *Drosophila* and mammals, mutational inactivation of *otd/Otx2* and *unpg/Gbx2* result in the loss or misplacement of the brain-specific expression domains of orthologous Pax and Hox genes. Moreover, *otd* and *unpg* appear to negatively regulate each other at the interface of their expression domains.

In vertebrate brain development, the *Pax2* gene, and subsequently the *Pax5* and *Pax8* genes, are among the first genes expressed at the *Otx2/Gbx2* interface, followed by the overlapping expression of *En1* and *Fgf8* genes (Ye et al., 2001). Inactivation of *Pax2*, *Pax5*, *En1* or *Fgf8* results in the loss of the midbrain and cerebellum because of a failure to maintain development of this brain region (reviewed by Joyner, 1996; Wassef and Joyner, 1997; Acampora and Simeone, 1999; Martinez et al., 1999; Liu and Joyner, 2001; Rhinn and Brand, 2001; Wurst and Bally-Cuif, 2001). In *Drosophila*, no obvious brain phenotypes were seen after mutational inactivation of *Pax2*, *Poxn*, *en/inv* or the *Drosophila* Fgf homologue *branchless* (*bnl*) (data not shown). The absence of brain phenotypes in these mutants contrasts with those observed in the vertebrate brain following mutational inactivation of the orthologous *Pax2*, *Pax5*, *En1* and *Fgf8* genes (Liu and Joyner, 2001; Rhinn and Brand, 2001; Wurst and Bally-Cuif, 2001).

DISCUSSION

Comparative developmental studies in urochordates and vertebrates have led to the notion that the basic ground plan for the chordate brain consists of a forebrain/midbrain region characterized by Otx gene expression, a hindbrain region characterized by Hox gene expression, and an intervening boundary region characterized by expression of *Pax2/5/8* genes (Wada et al., 1998; Holland and Holland, 1999; Wada and Satoh, 2001). Our data show that this tripartite ground plan is also found in the insect brain (Fig. 8). This suggests that a corresponding, evolutionarily conserved, tripartite organization also characterized the brain of the last common ancestor of insects and chordates.

A comparison of the brain-specific topology of gene expression patterns that define this tripartite organization in *Drosophila* and in mouse suggests that the vertebrate midbrain/hindbrain boundary (MHB) region corresponds to the insect deutocerebral-tritocerebral boundary (DTB) region. If this is the case, one might expect that other patterning genes that characterize the MHB region are also expressed at the insect DTB. Although this expectation is fulfilled for the segment-polarity genes *en* and *wingless* (*wg*) in *Drosophila*, these two genes are expressed at the borders of all CNS neuromeres, as well as at parasegmental boundaries in the

epidermis; hence, their expression may not be indicative of brain-specific requirements.

In addition to remarkable similarities in orthologous gene expression between insects and chordates, our study also shows that several functional interactions among key developmental control genes involved in establishing the *Pax2/5/8*-expressing MHB region of the vertebrate brain are also conserved in insects. Thus, in the embryonic brains of both fly and mouse, the intermediate boundary regions, DTB and MHB, are positioned at the interface of *otd/Otx2* and *unpg/Gbx2* expression domains (Fig. 8A,B). These boundary regions are deleted in *otd/Otx2*-null mutants and mispositioned in *unpg/Gbx2*-null mutants. Moreover, *otd/Otx2* and *unpg/Gbx2* genes engage in crossregulatory interactions, and appear to act as mutual repressors at the interface of their brain-specific expression domains. However, not all of the functional interactions among genes involved in MHB formation in the mouse appear to be conserved at the *Drosophila* DTB. Thus, in the embryonic *Drosophila* brain, no patterning defects are observed in null mutants of *Pax2*, *Poxn*, *en* or *bnl*. It remains to be seen if these genes play a role in the postembryonic development of the *Drosophila* brain.

It is conceivable that the similarities of orthologous gene expression patterns and functional interactions in brain development evolved independently in insects and vertebrates. However, a more reasonable explanation is that an evolutionary conserved genetic program underlies brain development in all bilaterians. This would imply that the generation of structural diversity in the embryonic brain is based on positional information that has been invented only once during evolution and is provided by genes such as *otd/Otx2*, *unpg/Gbx2*, *Pax2/5/8* and Hox, conferring on all bilaterians a common basic principle of brain development. If this is the case, comparable orthologous gene expression and function should also characterize embryonic brain development in other invertebrate lineages such as the lophotrochozoans. This prediction can now be tested in lophotrochozoan model systems such as *Platynereis* (Arendt et al., 2001), *Helobdella* (Kourakis et al., 1997) or *Dugesia* (Pineda et al., 2002).

Taken together, our results indicate that the tripartite ground plan that characterizes the developing chordate brain is also present in the developing insect brain. This implies that a corresponding tripartite organization already existed in the brain of the last common urbilaterian ancestor of insects and chordates. Therefore, we propose an urbilaterian origin of the tripartite brain.

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