Inductive patterning of the embryonic brain in Drosophila

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SUMMARY

In vertebrates (deuterostomes), brain patterning depends on signals from adjacent tissues. For example, holoprosencephaly, the most common brain anomaly in humans, results from defects in signaling between the embryonic prechordal plate (consisting of the dorsal foregut endoderm and mesoderm) and the brain. I have examined whether a similar mechanism of brain development occurs in the protostome Drosophila, and find that the foregut and mesoderm act to pattern the fly embryonic brain. When the foregut and mesoderm of Drosophila are ablated, brain patterning is disrupted. The loss of Hedgehog expressed in the foregut appears to mediate this effect, as it does in vertebrates. One mechanism whereby these defects occur is a disruption of normal apoptosis in the brain. These data argue that the last common ancestor of protostomes and deuterostomes had a prototype of the brains present in modern animals, and also suggest that the foregut and mesoderm contributed to the patterning of this ‘proto-brain’. They also argue that the foreguts of protostomes and deuterostomes, which have traditionally been assigned to different germ layers, are actually homologous.

Key words: Drosophila, Brain, Patterning, Foregut, Mesoderm

INTRODUCTION

Induction of neural tissue across tissue layers is a mechanism of brain development that has been extensively studied in deuterostomes (vertebrates). Tissues adjacent to the brain, such as the foregut endoderm and mesoderm (which constitute the prechordal plate) send developmental instructions to the brain (Ang and Rossant, 1993; Camus et al., 2000; Dale et al., 1997; Pera and Kessel, 1997; Shimamura and Rubenstein, 1997). Disruption of signaling between the prechordal plate and brain results in holoprosencephaly (HPE), a brain birth defect that includes a loss of ventral brain structures and incomplete separation of the forebrain into right and left hemispheres, and has a prevalence in humans of 1:250 in embryogenesis and 1:10,000 to 1:20,000 among live births (Chiang et al., 1996; Muenke and Beachy, 2000). Several of the molecular cues that are involved in the induction of pattern in the vertebrate brain have been identified and include members of the Hedgehog signaling pathway (Dale et al., 1997; Pera and Kessel, 1997; Shimamura and Rubenstein, 1997).

In contrast to the situation in deuterostomes, the induction of brain patterning in protostomes has been little studied. With the fruit fly Drosophila, there are a variety of genetic tools now available that allow us to address the question of whether the protostome brain is patterned by induction. By systematically removing tissues adjacent to the embryonic Drosophila brain, I have found that the foregut and the mesoderm are involved in establishing and refining brain pattern by influencing brain size and apoptosis. Furthermore, I have investigated the role of the Hedgehog signaling pathway in Drosophila brain patterning. The results argue that the protostome embryonic brain pattern is influenced by induction as it is in deuterostomes, and that Hedgehog signaling has a conserved role in this effect among these groups.

MATERIALS AND METHODS

Drosophila stocks

As a wild-type stock, Oregon-R flies were used. The following mutant strains were also used: forkhead, hedgehogE23, hedgehog-lacZ (hedgehogE230), hedgehog-GAL4 (unpublished line kindly provided by M. Calleja and G. Morata), Scer/GAL4,4.2hox-24B (mesodermal GAL4 driver), UAS-ricin (Rcomm3aScer/UAS.Cb), patched-GAL4 (Scer/GAL4 patched-558Δ), UAS-tau-GFP (Avic/GFP Scer/UAS.Btau.MAPT), 1407-GAL4 (Sweeney et al., 1995), UAS-patchedloop2 (Briscoe et al., 2001) and tinEC40. For analyzing gene expression, GAL4 lines were crossed with UAS-tau-GFP lines, thereby forming a microtubule-bound GFP reporter. For identifying mutant embryos, balancers expressing GFP or β-galactosidase were used.

Laser ablations

Dechorionated cellularized-blastula stage embryos were lined up on glass slides after staging on a dissecting microscope in Voltalef 3S oil. All staging of embryos was carried out using standard guidelines (Campos-Ortega and Hartenstein, 1997). The cells of the foregut anlage were ablated using a Laser Sciences VSL-337ND-S nitrogen laser. Cells were exposed to 1-second bursts at 10 Hz, and the area around where the laser was focused was checked for blebbing, thereby indicating cell death. I destroyed what appeared to be most of the cells around where the laser was focused using a Laser Sciences VSL-337ND-S nitrogen laser.
reached embryonic stage (ES) 15, as determined using a dissecting microscope. Approximately 60% of the laser-treated embryos clearly lacked a foregut; these were the embryos that were used for subsequent experiments.

**Immunohistochemistry and microscopy**

Embryos were collected, fixed and immunostained according to standard procedures (Patel, 1994). The following primary antibodies were used: BP102 [mouse, 1:200, Developmental Hybridoma Bank (DSHB)], anti-Fasciclin II (Bastiani et al., 1987) [mouse, 1:20], anti-Elav (rat, 1:100, DSHB), anti-Repo (Halter et al., 1995) [rabbit, 1:750], anti-GFP (rabbit, 1:1000, Molecular Probes), anti-β-galactosidase (rabbit, 1:1500, Cappel), anti-Hunchback (Struhl et al., 1989) [rat, 1:2000], anti-Prospero (mouse, 1:100, DSHB), anti-Fasciclin III (mouse, 1:100, DSHB) and anti-Hedgehog (Tabata and Kornberg, 1994) [rabbit, 1:1500]. For light microscopy, biotin-conjugated secondary antibodies were used (1:200, Jackson). Embryos were viewed and photographed on a Zeiss AxioPhot microscope. Secondary antibodies used for laser confocal microscopy were Texas Red-conjugated anti-mouse (1:200, Jackson), FITC-conjugated anti-rabbit (1:200, Jackson), and Cy5-conjugated anti-Rat (1:200, Jackson). Images were collected on BioRad MRC 1024 or Radiance confocal microscopes, and were processed using Adobe PhotoShop and Illustrator.

**Acridine Orange staining**

Staining for apoptosis using Acridine Orange was done according to standard procedure (Abrams et al., 1993). Briefly, embryos were dechorionated, then shaken for 5 minutes in a 1:1 mixture of 5 μg/ml Acridine Orange in PBS and heptane, and then mounted in Voltalev 105 oil. Embryos at late ES13 were examined on a BioRad Radiance confocal microscope for staining at the level of the pre-oral brain commissure at the dorsal midline of b1.

**Cell counting**

To quantify brain phenotypes, stained embryos were examined in the following ways:

1. To estimate the area occupied by neuronal nuclei and the number of glia in b1, confocal sections were collected from at the dorsal most region of the ES15 brain, progressing ventrally through the brain at 2.5 μm increments for 35 μm (which is a level slightly ventral to the preoral brain commissure in the wild-type brain), using a 60x oil immersion objective on a BioRad Radiance confocal microscope. These sections were then projected into one image using BioRad software. The number of glia present was counted directly and the area occupied by neurons was measured by tracing the outline of the anti-Elav marked brain using Adobe PhotoShop and obtaining the total pixel area within the outlined image. The pixel area was then multiplied by the conversion factor 0.154 μm²/pixel (this value was obtained by dividing the area of a scanning box provided by the BioRad confocal software by the number of pixels in such a box as measured in Adobe PhotoShop) to obtain an estimate of the area occupied by neuronal nuclei.

2. To estimate the number of glia and the area occupied by neurons in b2-S3, a procedure similar to that used in b1 was used except that optical sectioning began at the lateral edge of the foregut (or 5μm from the midline when the foregut was ablated) and progressed medially at 2.5 μm increments for 25 μm. Images were then projected and analyzed as described above. The boundaries of b2-S3 were estimated based on the positions of commissures.

3. For statistical analysis of the difference in cell counts between wild-type and mutant brains, Student’s t-test was used (Sokal and Rohlf, 1997).

It is important to note that the data provided are not intended to represent the absolute area or number of cells in the brains of embryos, but rather a relative comparison of brain size and glia number between groups of embryos as measured using consistent techniques.

**RESULTS**

**Ablation of the foregut results in brain patterning defects**

The embryonic brain of *Drosophila* develops in close association with the foregut ectoderm, as well as the visceral and procephalic mesoderm (Fig. 1A,B). Thus, these tissues are candidates for being involved in inductive patterning of the brain. The strategy employed was to remove these tissues systematically and see what effect this had on brain morphogenesis. The wild-type embryonic brain of *Drosophila* has a stereotyped arrangement of cells and axon tracts (Nassif et al., 1998a) (Fig. 1C,D), defects that can be readily detected using molecular markers. The structure consists of six segments: b1-b3 lie dorsal and lateral to the foregut and S1-S3 are ventrolateral and ventral to the foregut. All segments contain paired longitudinal connectives, which are called circumesophageal connectives lateral to the foregut. The preoral brain commissure is in b1, and connects the right and left hemispheres of the brain, which are otherwise separated at the midline, and the frontal commissure originates in b2 and extends into b1, where it joins with neuronal soma and glia to form the frontal ganglion. b3 and S1 have one commissure each, and S2 and S3 have two commissures each.

Does the removal of the foregut affect the development of the brain? To answer this question, I ablated the anlage of the embryonic *Drosophila* foregut using a laser. When the foregut did not invaginate, the following defects were seen in 90% of embryos (n=20): the pattern of neurons was disrupted so that the right and left hemispheres of the brain were either fused at the midline or separated by an abnormally small space (≤4μm), because of excess cells in this region; the area occupied by neuronal nuclei was decreased; the preoral brain commissure did not defasciculate normally, so that it appeared thickened; and the frontal commissure and ganglion were missing (Fig. 2A). In b2-S3, there was an apparent reduction in the area occupied by neuronal nuclei and a decrease in the number of glia (Fig. 2B). In addition, in 50% of these embryos, the longitudinal connectives in b2-S3 were disrupted.

Genetic ablation experiments agreed with these observations. Foregut formation is defective in embryos lacking function of the transcription factor Forkhead (Jürgens and Weigel, 1988). Seventy percent of Forkhead loss-of-function mutant embryos examined (n=20) exhibited defects in b1 that resembled the phenotype seen in embryos with laser ablated foreguts. This included an unusually small space between the brain hemispheres, abnormal defasciculation of the preoral brain commissure, and a decrease in the area occupied by neuronal nuclei in b1 and b2-S3, as well as a reduction in the number of glia in b2-S3 (Fig. 2C,D; Table 1). Examining the expression pattern of *forkhead* using an anti-Forkhead antibody showed that, while Forkhead is expressed in the central and lateral parts of the b1 hemispheres, it is not expressed at the dorsomedial edges of b1 up to ES13 (Fig. 2E). This suggested that the phenotype seen in the dorsal midline in Forkhead null embryos was not due to a loss of Forkhead function in this region. Interestingly, the vertebrate homolog of *forkhead*, Hnf3b, is expressed in the prechordal plate and brain, and embryos lacking function of this gene have severe defects in foregut morphogenesis and brain patterning (Ang and Rossant, 1994).
Disruption of mesoderm formation results in brain patterning defects

As the *Drosophila* foregut invaginates, it normally becomes sheathed by visceral mesoderm. Thus, when the foregut is ablated, visceral mesoderm is displaced from its normal position adjacent to the brain. How much does the loss of mesoderm contribute to the brain phenotype seen in foregut ablated animals? I examined embryos lacking function of the NK-2 class transcription factor Tinman, which have defects in forming mesoderm around the foregut, as examined using mesodermal markers for such as Fasciclin III expression, but do form foregut ectoderm (D. T. P., unpublished) (Bodmer et al., 1990; Azpiazu and Frasch, 1993). In 65% of Tinman loss-of-function embryos (*n*=20) there were excess cells at the dorsal midline of b1, the area occupied by neuronal nuclei was increased when compared with wild type in this region of the brain, and the preoral brain commissure was abnormally thin (Fig. 3A,B; Table 1).

To verify that the removal of the mesoderm had an effect on brain development, a pan-mesodermal driver (Brand and Perrimon, 1993) was used to express the toxin Ricin (Hidalgo et al., 1995), thereby specifically ablating mesodermal cells. The expression of this line in the anlage of the foregut mesoderm was verified using a GFP reporter (data not shown). In 45% of embryos expressing Ricin in the mesoderm (*n*=20), the brain phenotype resembled that of the *tinman* loss-of-function embryos in that there were excess cells at the dorsal midline and there was thinning of the preoral brain commissure (Fig. 3C,D); however, the frontal commissure and ganglion were also absent.

Ablation of the foregut and mesoderm results in changes in the pattern of brain apoptosis at the dorsal midline

Why were there excess cells at the dorsal midline in foregut- and mesoderm-ablated embryos? During normal brain development, more neurons are born than will be present in the adult brain and apoptosis eliminates the excess cells (for a review, see Hutchins and Barger, 1998). Defects in apoptosis could contribute to the observed defects in brain patterning by failing to remove excess cells. To see if apoptosis was perturbed when the foregut and mesoderm were ablated, Acridine Orange staining, which labels apoptotic cells (Abrams et al., 1993), was carried out. In *forkhead* loss-of-function embryos, the pattern of apoptosis in the brain at the level of the preoral brain commissure was clearly different from wild type at late ES13. In the wild-type b1 neuromere, there were groups of apoptotic cells at the dorsomedial edges of the hemispheres (Fig. 4A). This correlates with previous observations regarding the expression of the apoptosis regulatory protein Reaper (Nassif et al., 1998b). In *forkhead* loss-of-function embryos, there was a clear reduction in the number of these cells (Fig. 4B). Examination of *tinman* loss-of-function embryos showed that removal of mesoderm results in a similar reduction in the number of apoptotic cells at the dorsal midline (Fig. 4C), thus suggesting that the mesoderm and possibly the foregut have an influence on the normal pattern of apoptosis in brain development.
Loss of Hedgehog function results in brain patterning defects

The results of foregut and mesoderm ablation experiments strongly suggest that the brain is patterned by induction from these tissues. Did ablation of these tissues remove inductive signals required for normal brain development? What molecular signals could be mediating this effect? In vertebrates, Hedgehog signaling originating from the prechordal plate functions in forebrain patterning. Thus, the Hedgehog pathway in Drosophila seemed a good place to begin to look for inductive signals involved in brain development. Null mutations in Drosophila Hedgehog resulted in a phenotype that strongly resembled the one seen in the foregut ablation experiments in 70% of embryos (n=20). In b1, the right and left hemispheres of the brain were joined at the midline or separated by an abnormally small space because of excess cells in this region, and the preoral brain commissure showed abnormal defasciculation. In addition, in b1 the frontal commissure was missing, and there was a significant decrease in the area occupied by neuronal nuclei and the number of glia (Fig. 5A; Table 1). In b2-S3, the longitudinal connectives were disrupted, and the area occupied by neuronal nuclei and the number of glia was significantly reduced (Fig. 5B; Table 1), and the number of Fasciclin II-expressing neurons was reduced.

hedgehog and patched are expressed in the foregut and the brain

To see where the Hedgehog signal originated from, I analyzed hedgehog expression in the brain. Using a GFP reporter of hedgehog expression [hedgehog-GAL4 line kindly provided by M. Calleja and G. Morata, generated by the method outlined by Calleja et al. (Calleja et al., 1996)], I found that there was a source of Hedgehog in the foregut, i.e. immediately adjacent to the brain (Fig. 5C, D). This expression was present as the stomodeaum invaginated posterior through the region of brain neuroblasts, and continued through ES13, at which time the foregut expression

Table 1. Penetrance and expressivity of phenotypes

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Severity of defects (% penetrance)</th>
<th>Average cell counts in +++ embryos (s.d.)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>+</td>
<td>++</td>
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<tr>
<td>Wild type</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>forlheadg6</td>
<td>5</td>
<td>25</td>
</tr>
<tr>
<td>tinmanEc40</td>
<td>15</td>
<td>20</td>
</tr>
<tr>
<td>hedgehogE23</td>
<td>0</td>
<td>30</td>
</tr>
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</table>

*The value given for neurons is the average area (μm²) occupied by Elav-expressing nuclei. Statistically significant deviations from wild type (as determined using a t-test) are indicated by italicized numbers (n=20 in all cases). Severity of defects was rated as follows: +++, embryos showed the complete phenotype described for the mutation in the text and in Table 2; ++, embryos showed some of this phenotype; +, embryos did not show this phenotype.
Brain patterning was lost immediately adjacent to the brain. I also observed that hedgehog was expressed in a segmental pattern in the brain itself; this expression pattern is in agreement with previous reports (Lee et al., 1992; Taylor et al., 1993). A similar expression pattern was observed in a hedgehog-lacZ line and by staining with anti-Hedgehog antibody (data not shown). Importantly, patched, a gene encoding a putative receptor for Hedgehog (for a review, see Ingham, 1998), was found to be upregulated in brain cells surrounding the foregut Hedgehog source, including neurons, glia and precursors of these cells (Fig. 5E-G). In addition, these embryos lack the frontal commissure and ganglion.

Loss of Hedgehog function results in changes in apoptosis
To see if changes in apoptosis underlie the b1 dorsal midline defects seen in Hedgehog null embryos, Acridine Orange staining was carried out. This showed that there was a decrease in the number of apoptotic cells at the dorsal midline of the brain at late ES13 (Fig. 5H), suggesting that the defects in this region in Hedgehog null embryos were due to a disruption in the normal pattern of apoptosis.

Blocking Hedgehog signaling in neural cells influences brain size, but not apoptosis at the dorsal midline
To see if Hedgehog signaling is directly required in brain cells, I expressed a form of Patched that is insensitive to Hedgehog (Briscoe et al., 2001) specifically in neural tissues using the 1407-GAL4 line (Sweeney et al., 1995). In these embryos, the size of the brain was decreased; however, excess cells did not appear to be present at the dorsal midline (Fig. 5I). This suggests that Hedgehog signaling has a direct function in influencing brain size; however, its function in inducing apoptosis in the dorsal midline of b1 may be indirect.

Influence of the foregut and mesoderm on the formation of neural precursors
When do the inductive influences of the foregut and mesoderm exert their effect on brain development? To answer this question, I stained embryos lacking foregut (Forkhead null) or mesoderm (Tinman null) with anti-Hunchback antibody, which stains neuroblasts in the brain (Kambadur et al., 1998). At ES10, after the brain neuroblasts have started to delaminate, the pattern of Hunchback-expressing neuroblasts appeared to be the same as wild type in all these embryos (Fig. 6A-C). This suggested that the initial formation of brain neuroblasts was normal in the absence of a foregut or mesoderm. Next, I examined the pattern of ganglionic mother cells (GMCs) using the marker anti-Prospero (Doe et al., 1991). In this case, the number of brain GMCs in embryos lacking a foregut was clearly decreased at ES11 when compared with wild type (Fig. 6D,E). By contrast, in embryos lacking mesoderm, the pattern of GMCs did not appear to be affected (Fig. 6F). This suggested that there are two inductive events: an earlier event that is mediated by signals originating from the foregut, in which neural precursor cells of the brain receive a signal that influences the formation of GMCs. That Hedgehog may be involved in this signaling event is suggested by the fact that in a Hedgehog null background, the number of Prospero-expressing GMCs is reduced (Fig. 6G). The later event appears...
to be mediated by the visceral mesoderm and seems to influence the survival of late GMCs or postmitotic cells.

**DISCUSSION**

**Evidence that the foregut and mesoderm induce pattern in the brain**

When the foregut and mesoderm are ablated, specific sets of defects occur in the brain (summarized in Table 2), thus suggesting that these tissues induce patterning of the brain. Ablation of the foregut results in a smaller brain, and disruption of mesoderm formation enlarges b1. These results may be explained as follows: the foregut influences the size of the brain and the mesoderm influences apoptosis at the dorsal midline of b1. Thus, ablation of the foregut, which also removes the visceral mesoderm, results in a smaller brain size, in spite of excess cells resulting from apoptosis defects. By contrast, ablation of the mesoderm alone results in a slightly larger brain because of excess cells are present at the dorsal midline, but brain size is not reduced because the foregut is present.

Considering that the preoral brain commissure did not appear to defasciculate normally in foregut-ablated embryos, and appeared thinned in mesoderm-ablated embryos, the foregut and mesoderm may have additional distinct roles in influencing preoral brain commissure development. The cause for the differential effects of removing these tissues on the formation of this commissure is not entirely clear based on these experiments. However, these phenotypes may be related to the fact that removal of either tissue affects the arrangement of glia associated with this commissure; rearrangement of glia has been shown to influence commissure formation elsewhere in the *Drosophila* nervous system.

**Table 2. Effects on brain development of ablation of adjacent tissues**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Effects of ablation on gross brain morphology</th>
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<tr>
<td>Foregut</td>
<td>Fusion or abnormal closeness of brain hemispheres at midline in b1</td>
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<td></td>
<td>Decreased area occupied by neuronal nuclei in b1</td>
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<tr>
<td></td>
<td>Abnormal defasciculation of the pre-oral brain commissure</td>
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<td></td>
<td>Loss of frontal commissure</td>
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<tr>
<td></td>
<td>Decreased area occupied by neuronal nuclei in b2-S3</td>
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<tr>
<td></td>
<td>Reduction in number of glia in b2-S3</td>
</tr>
<tr>
<td>Mesoderm</td>
<td>Fusion or abnormal closeness of brain hemispheres at midline in b1</td>
</tr>
<tr>
<td></td>
<td>Increase in the area occupied by neuronal nuclei in b1</td>
</tr>
<tr>
<td></td>
<td>Thinning of pre-oral brain commissure</td>
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</tbody>
</table>
Involvement of Hedgehog in the induction of brain pattern

When function of Hedgehog is lost, brain patterning defects occur that resemble those seen in foregut ablated embryos, including a fusion of brain hemispheres in b1, a reduction in the size of the brain and abnormal defasciculation of the preoral brain commissure. Importantly, Hedgehog is expressed in the foregut adjacent to the brain, and patched, which encodes a putative receptor for Hedgehog, is expressed in brain cells surrounding the foregut. Loss of Hedgehog function causes a reduction in brain size that resembles what is seen when the mesoderm is removed; however, hedgehog does not appear to be expressed in the visceral mesoderm surrounding the foregut. This suggests that Hedgehog from the foregut may be received by the mesoderm (where patched is upregulated), which then responds by producing another signal that influences apoptosis at the dorsal midline. This hypothesis is supported by the observation that inhibiting Hedgehog signaling specifically in neural tissue using a mutant form of Patched results in a decrease in b1 size, but not in an excess of cells, at the dorsal midline.

Significance for understanding disease and evolution

Considering the reduction in the number of brain cells and the joining of the brain hemispheres, the foregut ablation brain phenotype resembled aspects of human HPE. Importantly, in the vertebrate nervous system, Sonic hedgehog is involved in the formation of oligodendrocytes and motoneurons, and in regulating apoptosis (Ericson et al., 1995; Nery et al., 2001). Disruption of Hedgehog signaling causes HPE; correspondingly, in Drosophila Hedgehog mutants, a HPE-like phenotype occurs, including a reduction in the number of glia and Fasciclin II expressing motoneurons. The recapitulation of aspects of the human HPE phenotype in Drosophila – i.e. the loss of brain cells and the defects in hemispheric separation – means that fly embryos might have use for understanding some mechanisms of this disease.

Furthermore, this work demonstrates that brain patterning via induction by the foregut and mesoderm appears to be a mechanism that is shared between protostomes and deuterostomes. This finding supports the hypothesis that the ground plan for the brain was established in the last common ancestor of bilaterally symmetric animals (Arendt and Nubler-Jung, 1996; Arendt and Nubler-Jung, 1999; Dohrn, 1875; Leydig, 1864). This also suggests how the brain of the much-debated last common ancestor of Bilateria may have developed. I hypothesize that this animal had a brain that formed in close association with the foregut, and molecules such as Hedgehog expressed in the foregut patterned this brain.

As regards the origins of the brain in evolution, if the foregut is assumed to be more ancient than the brain, then the possibility arises that the ventral neural mass of a bilaterian ancestor that lacked a brain could have expanded dorsally, using the foregut (which would probably have already been expressing patterning molecules such as Hedgehog) as a scaffolding, thus forming a brain.

The apparent homology between the foreguts of protostomes and deuterostomes raises a problem in nomenclature: the foregut of protostomes is considered ectodermal in origin, while the foregut of deuterostomes is considered endodermal. However, there appears to be homology in function, blastodermal fate map position (Arendt and Nubler-Jung, 1997), gene expression (Arendt et al., 2001) and induction between tissues derived from what have been traditionally regarded as distinct germ layers. Perhaps the assignment of the protostome foregut to the ectoderm and the deuterostome foregut to the endoderm should be reconsidered, as there are no absolute criteria for these assignments.

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