Embryonic development of the Drosophila brain: formation of commissural and descending pathways

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SUMMARY

The establishment of initial axonal pathways in the embryonic brain of Drosophila melanogaster was investigated at the cellular and molecular level using antibody probes, enhancer detector strains and axonal pathfinding mutants. During embryogenesis, two bilaterally symmetric cephalic neurogenic regions form, which are initially separated from each other and from the ventral nerve cord. The brain commissure that interconnects the two brain hemispheres is pioneered by axons that project towards the midline in close association with an interhemispheric cellular bridge. The descending longitudinal pathways that interconnect the brain to the ventral nerve cord are prefigured by a chain of longitudinal glial cells and a cellular bridge between brain and subesophageal ganglion; pioneering descending and ascending neurons grow in close association with these structures. The formation of the embryonic commissural and longitudinal pathways is dependent on cells of the CNS midline. Mutations in the commissureless gene, which affects growth cone guidance towards the midline, result in a marked reduction of the brain commissure. Mutations in the single-minded gene and in other spitz group genes, which affect the differentiation of CNS midline cells, result in the absence or aberrant projection of longitudinal pathways. The analysis of axon pathway formation presented here reveals remarkable similarities as well as distinct differences in the embryonic development of the brain and the segmental ganglia, and forms the basis for a comprehensive genetic and molecular genetic dissection of axonal pathfinding processes in the developing brain.

Key words: axonal pathfinding, CNS, glia, embryo, Drosophila, commissureless, single-minded

INTRODUCTION

The embryonic CNS of Drosophila has become an important model system for investigations of neuronal development. Insight into the mechanisms that control neurogenesis, axonal pathfinding and target recognition has been gained by exploiting the powerful genetic and molecular genetic techniques available in Drosophila (for reviews see Campos-Ortega and Knust, 1990; Grenningloh et al., 1990; Goodman and Doe, 1993). However, most of the investigations on development in the embryonic CNS of Drosophila have been carried out on the ventral ganglia. Thus, little is known about the development of the Drosophila brain (see Campos-Ortega and Hartenstein, 1985).

Insight into neurogenesis and axogenesis in an insect brain has been attained recently in the grasshopper. Embryonic neuroblasts in the grasshopper brain have been identified and some of their molecular expression patterns characterized (Zacharias et al., 1993). Moreover, the role of glial cell-bound proliferative clusters in prefiguring brain pathways and the processes that generate the axon tracts in the brain have been studied (Boyan et al., 1995a,b,c). However, it is unclear if the results of these investigations can be applied to Drosophila. In the hemimetabolous grasshopper, embryogenesis gives rise to adult-like structures (Bate, 1976; Chapman, 1982). In contrast, in the holometabolous Drosophila, embryonic development produces a highly derived larval stage, which is refigured postembryonically (Campos-Ortega and Hartenstein, 1985; Truman and Bate, 1988). The degree to which the embryonic brain of Drosophila is restructured postembryonically has not yet been resolved (see Truman et al., 1993). (For a review of older literature on insect brain development see Malzacher, 1968.)

In this report, we analyse the cellular and molecular processes that give rise to an initial set of axonal projections in the embryonic brain of Drosophila. We first use histology, immunocytochemistry and enhancer detection in combination with light microscopy, laser confocal microscopy and electron microscopy to determine how the commissural and descending pathways are established. We find that commissural interconnections are formed by axons that project along an interhemispheric cell bridge and that descending connections are prefigured by a chain of glial cells along which pioneering axons extend. We then use specific mutants to show that CNS midline cells are involved in the formation of both commissural and descending brain pathways. We find that mutations in the commissureless gene, which is involved in growth cone
guidance towards the midline, result in a marked reduction of the commissure, and that mutations in the single-minded gene and in other spitz group genes that are involved in the differentiation of midline cells, result in the absence or ectopic projection of descending pathways.

These findings demonstrate that the embryonic brain of Drosophila can be characterized at a level of resolution that is comparable to that attainable in the more simple ventral ganglia. Moreover, they reveal both distinct differences and remarkable similarities in the cellular processes and molecular mechanisms that are involved in early embryonic development in the brain as compared to the ventral nerve cord. Finally, these results show that many features of brain development in the holometabolous Drosophila are strikingly similar to those that occur in the hemimetabolous grasshopper, thus suggesting that similar fundamental processes might underlie embryonic brain development in all insects.

MATERIALS AND METHODS

Fly strains

The wild-type strain was Oregon-R. The following alleles were used: comm1, logo1 and lola1 (Seeger et al., 1993); simβ21.2 (Hilliker et al., 1980); slitβ107 and spiiβ14 (Nüsslein-Volhard et al., 1984); S1 (Bridges and Morgan, 1919); rhoβ644 and pntβ874 (Jürgens et al., 1984); faslab1 and faslab2 (Elkins et al., 1990); fasTTE (Zinn et al., 1988). Enhancer detector strains, 2138 P[repo4/ lacZ] (Xiong et al., 1994) and 2035 P[3.7 sim/ lacZ] (Nambu et al., 1990) were used. Embryos were staged according to Campos-Ortega and Hartenstein (1985) and Klämbt et al. (1991).

Immunocytochemistry

Embryos were dechorionated, fixed and labelled according to Patel (1994). Primary antibodies were mouse BP102 diluted 1:20; rabbit anti-HRP diluted 1:250 (Jackson Immunoresearch); mouse anti-Fasciclin I diluted 1:5; mouse anti-Fasciclin II diluted 1:5; rat RK2 diluted 1:1250 (Campbell et al., 1994); rabbit anti-prospero diluted 1:1500 (Vaessen et al., 1991). Secondary antibodies were HRP-conjugated goat anti-mouse, HRP-conjugated goat anti-rabbit, DTAF-conjugated goat anti-mouse, DTAF-conjugated goat anti-rabbit, DTAF-conjugated goat anti-rabbit, Cy3-conjugated goat anti-mouse and Cy3-conjugated goat anti-rabbit (Jackson Immunoresearch) all diluted 1:250, usually after preabsorption against fixed embryos. Embryos containing a P[lacZ] element were incubated with a rabbit antibody against β-galactosidase (Aurion) which was detected according to Patel (1994). Fluorescence-labelled embryos were mounted in Vectashield H-1000 (Vector).

Histology and histochemistry

Stained embryos were imbedded in Spurr’s (EMS), oriented according to neuraxis and cut into 4 μm sections. Sections were mounted on coated glass slides. Histochemical detection of β-galactosidase was in whole mounts according to Jacobs et al. (1989).

Light and laser confocal microscopy

For light microscopy, a Zeiss compound microscope (Axioskop) equipped with fluorescence and Nomarski optics was used. For laser confocal microscopy, a Leica TCS 4D or a Noran video speed confocal system were used. Optical sections ranged from 0.8 μm to 3 μm.

Electron microscopy

Embryos were dechorionated, devitellinized and fixed with 6% glutaraldehyde in phosphate buffer (pH 7.2) for 1 hour at room temperature. Six 10-minute washes with phosphate buffer were followed by postfixation in 1% OsO4 in the same buffer for 90 minutes at 4°C. Dehydration was in a graded ethanol series and embedding was in Spurr’s. Ultrathin sections were mounted on Formvar-coated single slot grids and viewed on a Zeiss EM 10 electron microscope.

RESULTS

The embryonic brain hemispheres derive from the procephalic neurogenic regions

Development of the Drosophila brain begins with the segregation of neuroblasts from the procephalic neurogenic ectoderm (see Campos-Ortega and Hartenstein, 1985). Following segregation, these brain neuroblasts start generating their neuronal progeny and, during this time, the prospero gene product (Doe et al., 1991; Vaessen et al., 1991) is expressed in the brain (Fig. 1A–C). In the early neurogenesis phase, the cells that express prospero most intensely are these progeny located interior to superficial layer of brain neuroblasts (Fig. 1D,I). Longitudinal stripes of these prospero-expressing cells are observed in both brain hemispheres (Fig. 1G).

The two procephalic neurogenic regions that give rise to the brain hemispheres are initially separated from each other (Hartenstein and Campos-Ortega, 1984). This is seen in Fig. 1A where the two regions of prospero-expressing cells in the two developing brain hemispheres are widely separated from each other. At the 12/0 stage, the brain hemispheres become linked to each other at the midline by a bridge-like structure of prospero-expressing cells (Fig. 1B,C). This is where the brain commissure will form.

A gap also initially separates the procephalic and the ventral neurogenic regions (Hartenstein and Campos-Ortega, 1984). This is seen in Fig. 1D,E,H where the prospero-expressing regions of the brain hemispheres are clearly separated from the prospero-expressing ventral neurogenic regions. As neurogenesis proceeds, prospero-expressing cells extend from the brain to the ventral nerve cord, thus establishing cellular contiguity around the ingrowing foregut (Fig. 1F,I). This is where the circumesophageal connectives will form.

Formation of the embryonic brain commissure

In the adult insect brain, all of the commissures located within the brain are preoral structures (see Strausfeld, 1976). Developmental studies in the locust indicate that all of the 73 identified preoral commissural bundles of the adult brain differentiate from a single preoral ‘primary brain commissure’ in the embryo (Boyan et al., 1995a,b). In Drosophila, a single embryonic brain commissure is also formed during embryonic development. We refer to this structure as the (preoral) embryonic brain commissure. The postoral tritocerebral commissure and the frontal commissure run outside of the brain (Boyan et al., 1993); in this report we will not refer to these two structures as brain commissures.

One of the first signs of brain commissure formation is the generation of two cellular column-like protrusions that extend from the medial edges of the brain hemispheres towards the midline. Immunocytochemical analysis with the neuron-specific anti-HRP antibody (Jan and Jan, 1982; Snow et al., 1987), the neuronal-cell-body-specific anti-elav antibody
Brain development in *Drosophila* (Campos et al., 1987) and the glia-cell-specific anti-repo (anti-RK-2) antibody (Campbell et al., 1994; Xiong et al., 1994; Halter et al., 1995) indicate that these protrusions are made up of axons, neuronal cell bodies and a small number of glial cells (Fig. 2A-C). These protrusions, once they reach the midline, establish an interhemispheric cell bridge. Both neurons and glial cells contribute to the completed cell bridge; however, neuronal and glial cell bodies are in spatially separate layers. A small group of neurons and glial cells is found at the brain midline and may also be involved in the formation of this interhemispheric bridge (Fig. 2A-C).

The first axonal projection across the midline in the brain is established by neurons that differentiate near the medial edge of each hemisphere and send their axons along the nascent cellular protrusions towards the midline (Figs 2A-C). As they do so, these axons express the cell adhesion molecule Fasciclin I (Bastiani et al., 1987; Zinn et al., 1988). At the midline, the axons make contact and fasciculate with their homologs and, subsequently, grow along their homologs towards the opposite hemisphere (Figs 2D, 4D). Throughout its period of formation, this nascent initial brain commissural fascicle is very closely associated with the interhemispheric cell bridge (Fig. 2E,F). Indeed, both the developing cellular bridge and the extending pioneering commissural axons reach the midline at the same time. As soon as the first commissural axonal pathway in the brain across the midline is established, it is followed by other fasciculating commissural axons and, during subsequent embryogenesis, this commissural fascicle differentiates further to become the massive preoral commissure of the embryonic brain (Fig. 2G). Throughout embryogenesis, a row of glial cells that extends across the brain midline continues to be associated with this preoral brain commissure (Fig. 2H,I).

Two minor commissural structures are formed anterior to the subesophageal ganglion but outside of the embryonic brain; they will be mentioned briefly here. The postoral tritocerebral commissure is pioneered before the preoral brain commissure. This commissure runs outside of the brain and connects the circusesophageal connectives and the brain hemispheres at the level of the developing tritocerebrum. The frontal commissure is pioneered by two symmetrical neuronal cell bridges that run from the developing frontal ganglia to each of the brain hemi-

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**Fig. 1.** Early embryonic brain development. prospero immunoreactivity in cephalic neurogenic regions. (A-C) Two initially separated prospero-expressing regions (A, arrow) become linked by cellular bridge at midline (C, arrow); dorsal view of wholemounts. (D-F) Initially separated prospero-expressing regions in hemispheres (E, arrow) and ventral nerve cord are linked by cellular bridges (F, arrow); lateral section (D). Lateral views of wholemounts (E,F). (G-I) Location of prospero-expressing cells in the hemispheres; dorsal section (D), lateral section (I). Stages 11 (A,D,E,G,H), 12/5 (F,I), 12/0 (B,C). Scale bar, 50 μm.
spheres. It is established at approximately the same time as the preoral brain commissure.

**Development of circumesophageal axon pathways**

Before the first descending longitudinal axonal pathways are established, two bilaterally symmetrical longitudinal arrays of repo-immunoreactive glial cells are seen extending from the brain hemispheres to the subesophageal neuromeres (Fig. 3B,C). In the ventral nerve cord, these longitudinal glial cells have been described in detail (Jacobs and Goodman, 1989a; Jacobs et al., 1989). In the head, the longitudinal glial cells are found at the medial edges of the proscephalic and subesophageal neurogenic regions and also extend around the gap between the brain and ventral ganglia caused by the ingrowing foregut. The location of these longitudinal glial cells prefigures the path of the circumesophageal connectives.

The first axonal pathways that interconnect brain and ventral nerve cord are formed once the longitudinal glial cells are in place. Initially, a small gap separates the neurons of each cephalic hemisphere from the neurons of the subesophageal ganglion (Fig. 3D-F). Subsequently, a cellular bridge composed of anti-HRP immunoreactive neuronal cell bodies is formed across this gap anterior to the row of longitudinal glial cells (Fig. 3G-I). Then the first longitudinal axon pathways are pioneered by axons that project across the neuronal cell bridge in close association with the row of longitudinal glial cells (Fig. 3A, arrows); they meet and fasciculate approximately half-way around the ingrowing esophagus. This initial axonal pathway is followed by other fasciculating descending and ascending axons. Longitudinal glia cells continue to be closely associated with this longitudinal fascicle as it differentiates further into the circumesophageal connective.

In contrast to the ventral ganglia, in the brain the first longitudinal fascicle is established before the first brain commissural fascicle. Indeed, the first circumesophageal interconnection appears to be formed as the foregut grows through the embryonic CNS (Fig. 3A). This relatively early time of formation is advantageous, since the foregut grows and

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**Fig. 2.** Commissure formation. Frontal views through the brain hemispheres (laser confocal microscopy). Neuron-specific anti-HRP immunoreactivity (A,D,E,F,G), glial cell-specific repo immunoreactivity (H), combined anti-HRP immunoreactivity (red) and repo immunoreactivity (green/yellow) (B,C,I). (A-C) Cellular protrusions (A, arrows) composed of neurons and glial cells extend from medial edges of hemispheres towards midline. Small group of neurons and glial cells at midline (open arrow). (A) Reconstruction of brain hemispheres, (B,C) different optical sections. (D-F) Establishment of initial commissural axonal projection (D,F, arrows) in close association with interhemispheric cell bridge (E, arrowhead). (D) Reconstruction of brain hemispheres, (E,F) different optical sections. (G-I) Differentiation of commissural pathway leads to formation of preoral brain commissure (G, arrow); a row of glial cells extending across midline (H, arrowhead) is closely associated with commissure. Stages 12/0 (A-C), 13 (D-F), 16 (G-I). Scale bar, 10 μm.
expands rapidly causing a substantial increase in the distance along which pioneering circumesophageal axons would later have to project.

**A primary axon scaffold in the embryonic brain**

After the establishment of the initial brain commissural and circumesophageal fascicles, these structures grow rapidly in size due to the addition of numerous axons. Longitudinal axonal pathways extend from the most anterior (according to the neuraxis) parts of the hemispheres, past the brain commissure and around the site of foregut ingrowth into the first subesophageal neuromere. (Due to head involution, the brain longitudinal tracts that lie most posteriorly in the embryo correspond to the most anterior part of the brain according to the neuraxis; see Boyan et al., 1995c.) The preoral embryonic brain commissure and the postoral embryonic tritocerebral

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**Fig. 3.** Formation of circumesophageal axon pathways. (A–C) Sections, (D–L) lateral optical sections in plane of a circumesophageal connective (laser confocal microscopy). Neuron-specific anti-HRP immunoreactivity (A, D, G, J), P[repo4lacZ] revealed with anti-β-galactosidase (B, C), glial cell-specific repo immunoreactivity (E, H, K), combined anti-HRP immunoreactivity (red) and repo immunoreactivity (green/yellow) (F, I, L). (A) Ascending and descending axonal processes (arrows) growing around ingrowing foregut (arrowhead); lateral section. (B–C) Rows of longitudinal glial cells (arrowheads) extend from brain hemispheres around foregut to ventral nerve cord; frontal sections. (D–F) Neurons of brain and subesophageal ganglion are initially separated by distinct gap (D, arrow); this gap is bridged by row of glial cells (E, arrowhead). (G–I) Subsequently, a neuronal cell bridge (G, arrow) is formed across gap in close association with row of longitudinal glial cells (H, arrowhead). (J–L) The first axonal pathways (J, arrow) are established between brain and subesophageal ganglion across neuronal cell bridge and in close association with longitudinal glial cells (L, arrowhead). Stages 11 (D–F), 12/5 (G–I), 12/3 (A), 12/0 (J–L) and 13 (B, C). (Note that stage 12/5 precedes stage 12/3 which precedes stage 12/0; see Klämbt et al., 1991.) Scale bar, 10 μm.
commissure form parallel axonal pathways around the esophagus. Thus, a quasi-orthogonal primary axon scaffold is established in the brain at the end of embryogenesis.

Although this axonal scaffold is discernible in whole mount (Fig. 4B), it is more clearly evident in histological sections (Fig. 4A), which show that the preoral brain commissure, the circumesophageal connectives, the tritocerebral commissure and the commissures of the three subesophageal neuromeres are all in the same plane at the end of embryogenesis. The salient features of the postoral axonal structures (tritocerebral commissure, circumesophageal commissures and connectives) are comparable to those found in the adult. In contrast, the primary preoral brain commissure in the embryo is still relatively undifferentiated; the numerous individual commissures characteristic of the mature brain are formed postembryonically. Ultrastructural analysis of the developing brain commissure at the end of embryogenesis confirms this apparently unitary, compact arrangement of the many commissural axons (Fig. 5A). It also shows that the brain commissure is intimately surrounded by a ring-like array of cells, which may be glial (Fig. 5D). In contrast to the compact nature of the embryonic brain commissure, there are discrete spaces in the hemispheres (Fig. 5B,C) some of which are associated with ultrastructural indications of cell death (Abrams et al., 1993).

A number of cell adhesion molecules are dynamically expressed in the ventral nerve cord; their expression patterns have led to the labelled pathway hypothesis for axonal guidance (Raper et al., 1984; Goodman et al., 1984). Many of these molecules are also found in the developing brain indicating that the labelled pathway hypothesis might also apply to the formation of axon tracts in the brain. For example, many brain axons express the Fasciclin I cell adhesion molecule (Bastiani et al., 1987; Zinn et al., 1988). Fig. 4D shows the expression of Fasciclin I on the axons of the neurons that pioneer the brain commissure. Fig. 4E shows the intense Fasciclin I-immunoreactivity in the differentiated brain commissure at a later stage. A more restricted expression pattern is seen for Fasciclin II (Snow et al., 1988; Harrelson and...
Brain development in *Drosophila*

**Fig. 5.** Ultrastructure of embryonic brain hemisphere and preoral commissure. Stage 15. Sections perpendicular to orientation of commissural axons near site at which commissure leaves brain hemisphere. (A) Overview of brain hemisphere showing peripheral cell body layer (arrows) and central neuropil (asterisk). (B) Higher magnification showing individual brain neurons and their axons (arrow), and remnants of apoptotic cells (stars). (C) Higher magnification showing individual cortex glial cells and apoptotic cell (star). (D) Cross-section through brain commissure. Ring of putative glial cells (asterisks) surrounds axons of commissure C. Axon fascicle (arrowhead) projects into commissure. Scale bar, A, 10 μm; D, 3 μm.

**Fig. 6.** Commissure differentiation is perturbed in *commissureless* mutants. (A-E) Frontal views through brain hemispheres (laser confocal microscopy). (F-G) Sections. Neuron-specific anti-HRP immunoreactivity (A), combined anti-HRP immunoreactivity (red) and glial cell-specific repo immunoreactivity (green/yellow) (B,C), combined axon-specific BP102 immunoreactivity (red) and repo immunoreactivity (green/yellow) (D), BP102 immunoreactivity (E,G), Fasciclin II immunoreactivity (F). (A-C) An initial commissural axon pathway (A, arrow) is established along midline cellular bridge (B, arrowheads) composed of neurons and small number of glial cells. (A) reconstruction of hemispheres, (B,C) different optical sections. (D,E) Subsequent differentiation of commissural pathway is perturbed and only single fascicle of commissural axons (E, arrows) is formed. Associated glia cells (D, arrowheads) spanning midline appear normal. (F) Commissural fascicle contains Fasciclin II-expressing cells. Frontal section. (G) Mutant preoral commissure (filled arrow) is highly reduced and all postoral commissures are missing (open arrow); descending longitudinal tracts (arrow heads) appear normal. Frontal section in plane of both circumsophageal connectives. Stages 13 (A,B,C), 14 (D,E), 16 (F,G). Scale bar, 10 μm.
Goodman, 1988; Grenningloh et al., 1991). Three major Fasciclin II-expressing fascicles are found in the circumesophageal connectives and in the preoral brain commissure; one Fasciclin II-expressing fascicle is found in the tritocerebral commissure (Fig. 4C,F). Fasciclin III (Patel et al., 1987; Snow et al., 1989) expression was not found.

The embryonic brain commissure is reduced in commissureless mutants

The characterization of the commissural and longitudinal pathways represents a first step in the analysis of brain axogenesis. An important second step is the investigation of the molecular and genetic mechanisms that underlie the formation of these axon pathways. One way to elucidate these mechanisms is by using mutants to analyze the role of specific genes in building the axon pathways of the brain.

In the ventral ganglia, mutations in the gene commissureless have a dramatic and specific phenotype; all commissural pathways are lacking while other axon pathways appear normal (Seeger et al., 1993). In the brain, the cellular events involved in the initial formation of a first brain commissural fascicle appear normal in commissureless mutants. Thus, cellular protrusions extend from each hemisphere towards the midline, a cellular bridge spans the gap between the two hemispheres and pioneering axons establish an interconnection across the midline (Fig. 6A-C). Subsequently several other commissural axons project along the pioneered midline pathway, thus, giving rise to a first brain commissural fascicle (Fig. 6E). This brain commissural fascicle develops in close association with a row of repo-expressing glial cells that extends across the midline (Fig. 6D), and many of the axons in this fascicle express Fasciclin II as in the wild type (Fig. 6F).

However, in striking contrast to the wild type, subsequent differentiation of the brain commissure involving the projection of many more axons across the midline and the formation of additional commissural fascicles does not take place in the commissureless mutants. For example, the two more anterior Fasciclin II-expressing fascicles are not formed. Thus, despite the fact that a cellular bridge has been established across the midline and that both glial cells and pioneering commissural axons are in place, many of the follower axons cannot project across the midline in the absence of the commissureless gene product. As a result, the brain commissure that is formed during embryogenesis is markedly reduced; considerably less than half of the normal commissural axons are formed (Fig. 6G). Thus, although the commissureless gene product is not necessary for the initial pioneering of a brain commissural pathway, it is essential for further differentiation of the brain commissure through the additional projection across the midline of numerous follower axons.

In commissureless mutants, the tritocerebral commissure and the commissures of the ventral ganglia do not form. In contrast, the longitudinal descending connectives appear normal and the frontal commissure forms in a normal manner.

The circumesophageal connectives fail to form in single-minded mutants

The single-minded gene encodes an HLH transcription factor that acts as a master regulator of midline cell development including the differentiation of midline cells into mature neurons and glial cells (Crews et al., 1988; Nambu et al., 1990, 1991). Mutations in the gene single-minded have a striking phenotype in the ventral ganglia; commissures fail to form and the longitudinal tracts collapse into a fused tract at the midline (Thomas et al., 1988; Klämbt et al., 1991). In the developing embryonic brain, mutations in the single-minded gene have an equally striking yet morphologically completely different phenotype. In most cases, the longitudinal circumesophageal connectives fail to form (Fig. 7A,K).

The absence of connectives in single-minded mutants may be related to a gap in the longitudinal glial cell array. A subset of the longitudinal glial cells that normally form around the ingrowing foregut are missing in the single-minded mutants (Fig. 7B,C). Tenuous neuronal bridges across this gap are sometimes observed; however, they appear to be unstable and subsequently disappear. Mutations in the single-minded gene do not cause a collapse of the longitudinal tracts at the brain midline, as is the case for the longitudinal tracts in the ventral nerve cord. A preoral brain commissure is formed in single-minded mutants, and the brain tracts and associated glial cells that develop anterior to the esophagus appear to differentiate normally (Fig. 7D-F).

In approximately 20% of the single-minded mutants, ectopic descending pathways from the brain to the ventral ganglia are observed. In these cases, the two main longitudinal tracts in the two brain hemispheres do not project separately around the esophagus. Rather they fuse medially at the level of the dorsal esophagus and then project posteriorly and ventrally along the gut towards the fused segmental ganglia (Fig. 7G,J). Repo-immunoreactive glial cells are not associated with this ectopic pathway (Fig. 7H,I).

The brain phenotypes observed in single-minded mutants correlate with the spatial expression of the single-minded gene in the wild type as assayed by a P[spitz/lacZ] transformant line (Nambu et al., 1991). The P[spitz/lacZ] marker indicates that single-minded is expressed in cells located in the lower part of the brain (just anterior-dorsal to the subesophageal ganglion), and some of these cells appear to be glial (Fig. 7L). In the wild type, the circumesophageal connectives develop in close proximity to these cells; normal differentiation of these cells may, therefore, be important for the formation of the paired circumesophageal connectives.

Based on the P[spitz/lacZ] marker, the single-minded gene does not appear to be expressed in more anterior parts of the brain. Specifically, there is no evidence for single-minded expression among the midline neuronal and glial cells at the site of brain commissure formation. This implies that the functional role of single-minded as a master regulator of midline cell development does not apply to the major anterior regions of the embryonic brain.

Other mutations that affect brain pathway formation

A common feature of mutants with defects in either the commissureless gene or the single-minded gene is that the mutant phenotypes seen in the embryonic brain are strikingly different from those observed in the embryonic ventral nerve cord. A difference in mutant phenotype in the brain versus the ventral ganglia is apparent for several other genes that are involved in axonal pathway formation.

The single-minded gene is a member of the spitz group (Mayer and Nüsslein-Volhard, 1988) that also includes the genes spitz, Star, pointed and rhomboid. Mutations in these
four genes have comparable phenotypes in the ventral nerve cord, and these phenotypes resemble those seen in single-minded mutants (Klämbt et al. 1991) as well as in slit mutants (Rothberg et al., 1988, 1990). Given this similarity, we wondered whether mutations in all of these genes might have similar effects on the formation brain axon pathways. Our analysis shows that this is indeed the case, but these defects are very different from those observed in the ventral nerve cord. Mutations in the genes spitz and Star typically result in the absence of circumesophageal connectives. Mutations in rhomboid and pointed result in the absence or in the formation of abnormally shortened circumesophageal connectives. Mutations in slit result in the formation of aberrant ectopic connections between the brain and the ventral ganglia. In all of these cases, the absence or abnormal formation of connective pathways correlates with gaps in the set of longitudinal glia that normally link the brain to the ventral ganglia (Hirth et al., unpublished data).

Mutations in the genes longitudinals lacking and longitudinals gone are known to affect the guidance and extension of longitudinal axons in the ventral ganglia (Seeger et al., 1993). This does not appear to be the case in the developing brain. No obvious defects in either longitudinal or commissural axon pathway formation are observed in the developing brain of longitudinals gone mutants. Mutations in the longitudinals lacking gene result in an approximately 50% reduction in the length and thickness of the embryonic brain commissure. However, the longitudinal circumesophageal pathways seem completely normal in longitudinals lacking mutants.

**DISCUSSION**

Studies of nervous system development in insects have been significant for understanding the cellular and molecular mechanisms involved in axon outgrowth and pathway formation (for reviews see Goodman and Doe, 1993; Reichert, 1993). Most of this work has, however, focused on the segmental ganglia or the peripheral nervous system; little is known about insect brain development. In this report, we investigate the processes that lead to the formation of the major axonal pathways in the embryonic brain of *Drosophila*, and we use mutants to analyze the role of specific genes in building these brain pathways.

The principle problem addressed in this paper stems from the fact that the insect brain originates as two neural masses that are separate from each other as well as from the chain of segmental ganglia, raising the question of how these separate entities become connected. The manner in which this problem is solved during embryogenesis reveals both differences and similarities in the embryonic development of the brain as compared to the ventral nerve cord. In the following, these differences and similarities are discussed.

**Linking the brain hemispheres**

In the brains of higher animals, the two hemispheres are connected by numerous commissural axons. During commissure formation, axonal pathways must be established across the midline, which provides a natural boundary in pattern formation. In insects and in vertebrates, specific cells at the CNS midline play a prominent role in commissure formation (for reviews see Dodd and Jessel, 1988; Goodman and Shatz, 1993).

In *Drosophila*, a small set of pioneering axons establishes the first brain commissural pathway. These pioneering axons extend in close association with bridge-like aggregates of neuronal and glial somata that extend across the brain midline. Such aggregates of neurons and glial cells extending across the midline are not observed during commissure formation in the segmental ganglia. However, surprisingly similar features are seen in the developing corpus callosum and anterior commissure of the mammalian brain (Silver et al., 1982). There, glia cells from each hemisphere form an interhemispheric bridge (‘glial sling’) that is used by the growth cones of the first commissural axons. This suggests that the formation of cellular bridges across the midline may be a general feature of commissure formation in complex brains, related to the problem of interconnecting separate brain hemispheres. The mechanisms that lead to the formation of interhemispheric cellular bridges in *Drosophila* are not yet known. Cell migration, local division or delayed differentiation of neurons and glial cells may be involved.

Molecules involved in commissure formation in vertebrate spinal cord have been identified recently (Tessier-Lavigne et al., 1988; Kennedy et al., 1994; Serafini et al., 1994), but little is currently known about their involvement in brain development. In *Drosophila*, molecules involved in brain commissure development are now known. One of these is the commissureless gene product, which is thought to be secreted from specific midline cells (Seeger et al., 1993). In commissureless mutants, the initial commissural fascicles in the brain develop normally, yet most of the subsequently formed commissural fascicles are missing. This suggests that molecular signals sufficient for the initial formation of a brain commissural fascicle are still present in the commissureless mutant, but that these signals are not sufficient to allow the majority of commissural axons to form. The longitudinals lacking gene (Seeger et al., 1993) may also be involved in differentiation of the embryonic brain commissure. Brain commissural fascicles are established in longitudinals lacking mutations; however, both length and thickness of these embryonic commissures are markedly reduced.

The specific molecular roles of the genes commissureless, longitudinals lacking and longitudinals gone in brain commissure and connective formation remain to be determined. However, in all three cases, our genetic analysis demonstrates the existence of clear differences in the mechanisms of commissure and connective formation in the brain as compared to the ventral nerve cord. We, thus, posit that brain-specific mechanisms exist that allow the formation of an initial commissural fascicle in the absence of the commissureless gene product, and that brain-specific mechanisms exist that allow the formation of longitudinal pathways even in the absence of the genes longitudinals lacking or longitudinals gone.

**Connecting the brain to the ventral nerve cord**

The formation of longitudinal pathways that interconnect brain and ventral nerve cord must deal with two developmental constraints: the initial separation of two neurogenic regions (see Campos-Ortega and Hartenstein, 1985) and the ingrowing foregut. The solution to these constraints seems to involve the early generation of neuronal cell bridges and rows of longitudinal glial cells, which extend from the brain hemispheres around the ingrowing foregut towards the ventral ganglia and, thus, link the cephalic and ventral neurogenic
Fig. 7. Paired circumesophageal connectives fail to form in *single-minded* mutants. (A-C) Lateral optical sections in plane in which circumesophageal connective would normally develop (laser confocal microscopy). (D-I) Frontal optical sections in plane in which both circumesophageal connectives would normally develop (laser confocal microscopy). (J) Midsaggital section. (K) Frontal section. (L) Lateral view through brain and subesophageal ganglion of P[**sim**/lacZ] transformed wildtype (laser confocal microscopy). Neuron-specific anti-HRP immunoreactivity (A,D,G), glial cell-specific repo immunoreactivity (B,E,H), combined anti-HRP immunoreactivity (red) and repo immunoreactivity (green/yellow) (C,F,I), axon-specific BP102 immunoreactivity (J,K), combined anti-HRP immunoreactivity (red) and anti-β-galactosidase immunoreactivity (green/yellow) (L). (A-C) Longitudinal axon pathways across a gap (A-C, arrows) between brain hemispheres and subesophageal ganglion do not form and longitudinal glial cells do not bridge gap. (D-F) Commissural fascicles, preoral brain tracts and associated glial structures are established between brain hemispheres but paired circumesophageal connectives (D, asterisks) do not form (compare Fig. 2G-I). (G-I) Ectopic midline descending pathways (G, arrow) seen in 1/5 of the mutants. Longitudinal axons fuse medially and project aberrantly from brain to the ventral ganglia (compare Fig. 2G-I). (J) Aberrant ectopic midline pathway (arrows) projecting along foregut-associated structures. (K) Preoral commissure (filled arrow) and preoral brain tracts (arrowheads) are present despite absence of paired circumesophageal connectives (asterisks). (L) P[**sim**/lacZ] enhancer detector staining indicates that *single-minded* is expressed in the lower part of the brain (arrow) located just anterior and dorsal to subesophageal ganglion. Some of the stained cells in this region are also labeled by the glial cell-specific anti-repo antibody and may, thus, be longitudinal glial cells. Stages 12/3 (A,B,C), 14 (L), 16 (D-K). Scale bar, 10 μm.
regions. As in the ventral nerve cord (Jacobs and Goodman, 1989a), the longitudinal glial cells may constitute a preformed pathway for pioneering axons.

The formation of the longitudinal glial cells linking the cephalic and ventral CNS depends on the single-minded gene. In the absence of this gene, a gap in the longitudinal array of glia appears between the brain and the ventral nerve cord. This gap is not bridged by longitudinal pioneers and, in consequence, the circumesophageal connectives fail to form correctly. Since similar events occur in other spitz group genes, it seems likely that complex molecular genetic interactions among all of these regulatory genes are involved in controlling the development of the longitudinal connective pathways.

The single-minded gene product, a transcription factor, is thought to be a master developmental regulator of CNS midline lineage (Crews et al., 1988; Thomas et al., 1988; Nambu et al., 1990, 1991). In the ventral nerve cord of single-minded mutants, specific midline cells do not differentiate properly. In consequence, the commissures are lacking and the longitudinal tracts collapse at the midline (Thomas et al., 1988; Klämbt et al., 1991). The phenotype of single-minded mutants in the brain is strikingly different. In single-minded mutants, a brain commissure is established and the longitudinal tracts within the brain do not collapse. The expression pattern of single-minded in the brain is also completely different compared to the ventral nerve cord. We have no evidence for the expression of single-minded anywhere in the brain midline anterior to circumesophageal connectives. These findings suggest that the role of single-minded as a master developmental regulator may be limited to the midline of the ventral CNS and to those midline structures that are involved in circumesophageal connective formation. Other parts of the brain, including the anterior midline at the site of brain commissure formation, do not appear to be under the control of the single-minded gene. The developmental regulator genes that do control midline differentiation in these major parts of the brain remain to be identified.

Similar features of neuronal development in the brain and in the segmental ganglia

Although the Drosophila brain derives from a separate neurogenic region, differentiates into an enormously complex structure and, in doing so, manifests a series of brain-specific developmental processes, several features of its embryonic development are reminiscent of the development of the more simple segmental ganglia. Thus, in both cases: (i) glial cells are involved in axogenesis; longitudinal glial cells prefigure descending pathways and glial cells are involved in commissure formation (Jacobs and Goodman, 1989a; Klämbt and Goodman, 1991); (ii) pioneer neurons establish a single axon scaffolding that is used by subsequently outgrowing neuronal processes for pathfinding (Klämbt et al., 1991; Jacobs and Goodman, 1989b; Grenningloh et al., 1991); (iii) molecularly labelled axon pathways are formed during differentiation of the axon scaffolding (e.g. Raper et al., 1984; Goodman et al., 1984); Fasciclin II, for example, is expressed in a similar regionalized manner by specific longitudinal pathways in brain and ventral ganglia (Grenningloh et al., 1991).

Interestingly, all of the above mentioned features of embryonic brain development in Drosophila are also found in grasshopper brain development (Boyan et al., 1995a,b,c), suggesting the generality of these developmental processes in all advanced insects. Furthermore, many of the developmental processes that are involved in generating the fly brain are involved in vertebrate brain development. For example, the roles of glial cells in prefuriging axon pathways, the function of pioneer neurons in establishing axon pathways and the formation of a primary axon scaffolding are also features of embryonic brain development in vertebrates, including mammals (Steindler, 1993; Silver, 1993; Chitnis and Kuwada, 1990; Stainier and Gilbert, 1990; Wilson et al., 1990; Easter et al., 1993).

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