Cell fate determination at the insect CNS midline

Thesis by

Te-Yi Kung

In Partial Fulfillment of the Requirements

for the degree of

Doctor of Philosophy

California Institute of Technology

Pasadena, California

1998

(Submitted March 19, 1998)

Dedicated to my parents

Acknowledgments

First of all, I would like to thank Dr. Kai Zinn, my research and academic adviser. I thank him for all the inspirations, encouragements, and understanding that he has unselfishly given to me. His passions for science and wildness are always an amusement to observe and a pleasure to follow. His honest heart makes him not only a fair and excellent scientific mentor, but also a trustworthy adviser for things beyond science and the academic.

Thank you, Kai: without you, this thesis would not have existed.

I would also like to express my gratefulness to other members on my committee: Dr. David Anderson, Dr. Mary Kennedy, Dr. Howard Lipshitz, and Dr. Paul Sternberg. Their useful advises helped me to go through the most critical parts in my years at Caltech as a Ph.D. student.

I would also like to thank Dr. Hsin Tsai, my college adviser. He has broadened my life, opened my eyes, and prepared my mind for the wonderful world a scientist can see.

I can not say enough "thanks" to my colleagues in Kai's lab, past and current. They are wonderful people with humanity, humor, and wisdom. They are my endless source of ideas, discussions, and laughers. Thanks to Shin-Shay Tian, Sarah Fashena, Barry Condron, Jonathan Bradley, Bruce Burkamper, Chand Desai, Yinong Zhang, Ania, Lakshimi, Doris, Ina, and Evgenya. They have made my life different.

A special thank goes to Caltech, especially the Biology Department, which has provided me with a warm, stable, and inspiring environment to work in.

Finally, I would like to thank all the friends that I made at Caltech. They have given me such a collection of wealth to treasure throughout my life time. Thanks to

Kaushiki Menon for her amusing complaints and for her patience to listen to my stories. Thanks to Susan Ou for making great antibodies and for giving me heartfelt supports. Thanks to Qi Sun as an invaluable source for advises in fly genetics, computer operations, and daily life. Thanks to Yuri Levin for those nice tea times and walks that refreshed my mind and stabilized my mood. I would also like to thank Sergey Cherkis as the same reasons I am grateful to Yuri, although their mind clarifying capabilities function with subtle differences.

And at last, I have my great thanks to Wenying Shou, for the way she is and the way she helps - I simply can not ask anymore from a friend as what she has done for me.

Since there are still so many people to thank, and I could not possibly list every one here, let me say my last thanks to the God. After all, He is the one who has brought me here.

Abstract

The central nervous system (CNS) of insect embryos develops from a neuroepithelial sheet that is patterned by the activities of segmentation genes. Neuroblasts delaminate from this sheet and divide once to produce two ganglion mother cells (GMCs). There are ~ 30 lateral neuroblasts arranged in a regular array in each thoracic and abdominal segment. The GMC progeny differentiate and give rise to neurons and glial cells. These are born in specific positions, and the neurons extend axons along stereotyped trajectories.

Our group has always been interested in the cellular and molecular mechanisms involved in the formation and maintenance of the CNS cell fate map.

In the second chapter of this thesis, I report the isolation and characterization of a cDNA clone encoding a novel *Drosophila* nonreceptor protein tyrosine phosphatase (PTP), denoted DPTP61F. DPTP61F is closely related to the mammalian PTP1B and the T cell PTP. We show that DPTP61F mRNA is selectively expressed in a pattern of 15 segmental stripes in germband extended embryos. This expression pattern is characteristic of zygotic segment polarity genes, and suggests that DPTP61F activity may be involved in the process of segmental determination. Later in development, DPTP61F is expressed in the CNS.

In Chapters III and IV, I focus on the embryonic development of the CNS midline. In Chapter III, I describe the cloning of the grasshopper *single-minded* (*Gsim*) gene, and the characterization of its expression patterns as well as its putative functions as the master regulator of midline genes.

In Chapter IV, I describe the roles of the cAMP-dependent protein kinase (PKA) in the development of the *Drosophila* CNS midline. We find that ectopic expression of PKA in the midline increases the number of *BarH1*-expressing midline neurons. We also

show that local inhibition of PKA in the developing midline results in the death of midline glia during late embryogenesis. The loss of the glia apparently cause CNS axons to follow abnormal pathways.

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Chapter I

Introduction

Early cell fate decisions in insect CNS midline development

Before reviewing cell fate decisions during insect CNS midline development, I would like to go over some basic knowledge about morphogenesis at early stages of insect embryonic development. Since my thesis work has been conducted in the fruit fly *Drosophila* and the grasshopper *Schistocerca*, two insect species that have adopted different developmental programs, I shall concentrate this review on contrasting the two. Intense studies on *Drosophila* have accumulated an enormous amount of information with respect to the genetic and molecular aspects, whereas the grasshopper system can provide better morphological information due to the large size of neuronal cells and of the overall nervous system. Our studies are intended to provide links between the two systems.

Section I: Early embryonic morphogenesis in *Drosophila*, from the fertilized egg to gastrulation

a) The initial specification of the ventral nerve cord midline - gastrulation

Ventral furrow formation marks the beginning of mesoderm development, and is the first morphogenic event of *Drosophila* gastrulation. It is a particularly clear example of epithelial folding. Several morphogenetic processes occur in parallel during *Drosophila* gastrulation. Three invaginations create the germ layers. First, the presumptive mesoderm begins to invaginate as a broad band of cells on the ventral side of the embryo. Then, while mesoderm invagination continues, the endoderm is made from two invaginations, one at the posterior pole (the posterior midgut invagination), and one on the ventral side of the head region (the anterior midgut invagination). During this time, a process called germ band extension moves the posterior end of the embryo onto the dorsal side.

Before gastrulation begins, all 5000~6000 cells in the blastoderm epithelium look morphologically identical, except that cellularization is not completed on the dorsal side.

However, cells in different regions can already be distinguished by their gene expression patterns. The genes *twist* and *snail* are expressed in ventral cells, including all future mesodermal cells, and some endodermal and ectodermal cells (Thisse et al., 1988; Leptin and Grunewald, 1990). The mesoderm is made from *snail*-expressing cells that are situated between ~10% and ~70% egg length from the posterior end of the embryo. All of these cells also express *twist*, but unlike *snail*, *twist* expression extends slightly beyond the lateral edge of the prospective mesoderm (Kosman et al., 1991; Leptin, 1991).

As soon as the cells on the ventral side of the embryo have formed, they begin to invaginate. The apical sides (facing the outside of the embryo) of the prospective mesodermal cells first flatten, and then a subpopulation, a central band of cells approximately 8-10 cells wide, constrict apically and their nuclei migrate towards their basal ends. The cells are thereby turned from a nearly columnar to a wedge shape. The band of cells that undergo these changes will be called the "central population." A 4- to 5-cell-wide band of prospective mesoderm cells on each side of the central population, called the peripheral population, does not constrict apically. Within about ten minutes, the changes in the central population result in the appearance of an indentation in the ventral surface of the embryo (the ventral furrow), which deepens and invaginates into the interior of the embryo, followed by the peripheral cells on each side which have not constricted apically. This first phase of mesoderm invagination, which lasts about 15 minutes, is characterized by the absence of individual cell movements either within the plane of the epithelium or out of the epithelium, and of cell division or growth. The epithelium remains intact as it invaginates (Leptin et al., 1992).

The cell shape change is a prominent phenomenon of this stage, and the actin cytoskeleton probably plays a major role in apical constriction. This is supported by the finding that myosin, concentrated at the base of the cell before ventral furrow formation,

becomes localized to the apical side as constriction occurs (Young et al., 1991). However, apical constriction can not alone be responsible for the movement of nuclei and shape changes. In *twist* mutant embryos, no apical constriction occurs, but nuclei nevertheless move away from the apical end of the cells and the cells change their shape sufficiently to make a small transient furrow (Leptin and Grunewald, 1990; Sweeton et al., 1991). Surprisingly, apical flattening and nuclear movement occur even in mutants in which the blastoderm is not properly cellularized (Leptin et al., 1992). All of these findings are consistent with the notion that the movement of nuclei is passive, that is to say, that nuclei are merely being released from the apical end of the cell. This would mean that the role of nuclear movements is to permit the cell shape changes rather than to cause them.

The actual mesodermal germ layer is formed in the next phase of mesoderm invagination. *snail* expression is now lost from the mesoderm, but *twist* continues to be expressed (Thisse et al., 1988; Alberga et al., 1991; Leptin 1991; Kosman et al., 1991). The tube of prospective mesoderm created by the invagination of the ventral furrow loses its epithelial structure and disperses into single cell. These cells divide, migrate out on the ectoderm to form a single cell layer and then divide again. The cell divisions during this phase have no morphogenetic role, as shown by the finding that the mesoderm disperses and spreads out normally in mutant embryos in which these divisions fail to occur (Leptin and Grunewald, 1990).

Germ band extension begins as soon as the ventrolateral furrow has begun to invaginate and the posterior midgut cells are beginning to change their shapes. The germ band consists of the invaginated mesoderm and the overlying ectoderm, situated between the head fold and the hindgut primodium. This region begins to lengthen, but since the embryo is enclosed in membranes and can not change its overall dimension, this results in the posterior midgut primodium being displaced dorsally. The dorsal epithelium of the

embryo, the future amnioserosa, does not lengthen but becomes thin and folds up as the advancing posterior midgut is pushed dorsally. Germband extension is later reversed by germ band retraction so that the original anterior-posterior order is re-established (reviewed in Leptin et al., 1992).

b) Molecular mechanisms of gastrulation in *Drosophila*

Mutations that affect different parts of the embryo were used to assess the roles of different cell populations in gastrulation. Mutations in both maternal-effect and zygotic genes have been useful for this purpose. Most of these are mutations that delete or change certain cell fates in the developing embryo either along the anterior-posterior axis, or along the dorsoventral axis.

Mutations in maternal-effect genes that pattern the dorsoventral axis affect the fates of most cells along the dorsoventral axis (Ray et al., 1991). Ventralizing mutations lead to the expression of ventral-specific genes in all cells of the embryo, while dorsalizing mutations produce embryos in which no ventral genes are expressed. In contrast, mutations in zygotic genes have more restricted effects. Usually only the fates of the cells that normally express the gene are changed, while other cells in the embryo develop according to their normal program. Mutations in the genes *twist* and *snail* abolish or change the fates of the cells that normally give rise to the mesoderm, and mutant embryos fail to make mesoderm (Simpson, 1983).

Maternally dorsalized embryos do not form a ventral furrow, nor do any of their cells undergo the changes usually seen in ventral cells. Their apical surfaces remain round and the nuclei stay in the apical parts of the cells (Leptin and Grunewald, 1990). In contrast, in completely ventralized embryos, the apical surfaces of all cells flatten, and nuclei at all positions along the dorsoventral axis migrate basally, a behavior characteristic

of most ventral cells in wildtype embryos. This indicates that this behavior is part of an autonomous morphogenetic program of cells expressing ventral genes, and not simply a mechanical response to activities from neighboring non-ventral cells.

The timing of the ventral furrow formation seems much more likely to be the effect of accumulation of one or more crucial zygotic gene products above a critical level. This notion is based on the observation that embryos that have only one functional copy of the zygotically active gene *twist* (and therefore only one half of the normal amount of this transcription factor) begin ventral furrow formation several minutes later than embryos with two copies (Leptin et al., 1992). Thus, the product of one or more genes transcribed under the control of *twist* must be limiting for furrow formation.

According to this presumption, the cells nearest the midline have a higher chance of constricting early compared to more lateral cells because the early *twist* (and *snail*) expression is restricted to a narrow ventral band corresponding in width to the central population of prospective mesoderm cells (Leptin, 1991) and, within this band, *twist* levels are highest near the midline. And this is in fact observed.

Drosophila belongs to one of the most phylogenetically derived insect orders (Diptera). Thus, although it is the most well-studied insect, it may not be representative of other insect groups, especially those of more primitive orders. Grasshopper, on the other hand, represents the primitive end of the phylogenetic tree. Interestingly, germband length is indicative of how advanced an insect species is: the germband lengthens toward the more advanced end of the phylogenetic tree, a point that will be elaborated in the next section.

Section II: Long and short germband types of body plan

a) Definitions of long germband and short germband types

All arthropod embryos, including those of insects, undergo a highly conserved, "phylotypic" point in development called the germband stage. This stage occurs after the embryo has completed gastrulation, when it assumes an elongated shape in which the body is overtly segmented. At this stage, in *Drosophila,* the earlier expression of the maternal gap and pair-rule genes has decayed. Morphological segmentation and the regional specifications of the body plan are clearly visible. Segment polarity gene expression is maintained, and the cellular signaling systems mediated by these genes continue to refine the patterns within each segment. Homeotic genes are expressed in their characteristic domains, but their expression is also constantly refined and they continue to influence the development of region- and segment-specific structures.

It is this germband stage that represents the phylotypical point for arthropods. All arthropod embryos look very similar at the morphological level during this stage, and a comparison of insects reveals even more extensive similarities. At the germband stage, all insect embryos possess a head composed of a procephalic region plus three gnathal segments that form the mouth parts, three thoracic segments, and eight to eleven abdominal segments (Patel, 1994). Various insect embryos reach this stage through drastically different routes. These differences can be illustrated by comparing the development of *Schistocerca* (grasshopper) and *Drosophila* (fruit fly), representatives of the two extremities of insect developmental strategies.

Drosophila oogenesis occurs in a meroistic type of ovary, in which nurse cells are connected by a cytoplasmic bridge to the anterior end of the egg. The nurse cells provide the developing egg with large quantities of the maternal RNA. In contrast, *Schistocerca* oogenesis takes place within a panoistic ovary, where there are no nurse cells. The RNA

inside the oocyte comes from the oocyte nucleus itself (for review, French, 1988). These differences in ovary types may account for the differences in the relative rates of development (eggs from panoistic ovaries tend to develop much slower than those from meroistic ovaries), and in some of the earliest steps in anterior-posterior pattern formation.

After the initial events of fertilization, the *Drosophila* embryo undergoes a highly synchronous series of nuclear divisions in the absence of cell divisions, resulting in a syncytium. The nuclei begin migrating toward the periphery of the egg, and shortly thereafter become separated by cell membranes, resulting in the formation of a cellular blastoderm. The embryo at this stage appears to comprise a homogeneous field of cells spread across the entire egg, but a multitude of manipulations such as ligation, fragmentation, centrifugation, and localized irradiation of the blastoderm embryo indicate that the cells are anything but homogeneous. The cells of the *Drosophila* blastoderm are already developmentally committed to contribute to specific regions of the final body plan, and thus, the *Drosophila* blastoderm embryo contains a precise representation of the complete larval animal. The same types of manipulation also indicate that positional values are specified in *Drosophila* eggs even before the blastoderm stage, leading Sander (1976) to suggest that gradients of positional information are established in the oocyte and that interactions between these gradients are able to establish the entire body plan by the blastoderm stage.

This highly determined blastoderm stage is not characteristic of grasshopper development. After fertilization, like *Drosophila* embryos, the grasshopper embryo develops syncytially as the nuclei divide and begin to migrate toward the periphery of the egg, although the migrating nuclei and their surrounding islands of cytoplasm were separated by yolk granules. After the nuclei reach the periphery, cellularization takes

place. Unlike in *Drosophila*, it is only those cells at the posterior pole of the egg that give rise to the embryo itself. The remaining cells give rise to extraembryonic membranes (Moloo, 1971). The cells at the posterior end form a small disc of about 300 μm in diameter that is located within the 7-mm long egg. This disc of cells, which represent the *Schistocerca* blastoderm, differs from the highly determined *Drosophila* blastoderm in that it does not appear to contain a complete projection of the final body plan. Various observations and manipulations of the *Schistocerca* embryo similar to those carried out in *Drosophila* indicate that although the *Schistocerca* blastoderm embryo contains cells determined to give rise to the head, a posterior growth zone gives rise to the remaining segmented cellular proliferation (Mee and French, 1986). The more posterior regions are specified one segment at a time during this growth phase. Manipulations before the blastoderm stage indicate that the more posterior regions of the egg contains information specifying the location for the initial blastoderm disc, and that the egg does not contain information for the specification of the different regions of the embryo (Moloo, 1971).

Embryos, such as those of *Drosophila,* that contain a complete representation of the body plan at the blastoderm stage, are classified as long germband embryos by G. Krause, whereas embryos, such as those of *Schistocerca*, that develop segments sequentially during a growth phase after the blastoderm stage are said to have intermediate, or "semi-long" germband embryos. Hemipterans are an example of the intermediate germband type. The blastoderm contains information for the specification of head, gnathal, and thoracic segments, but the entire abdomen arises from a growth zone (Sander, 1976). The distribution of germband types is interesting from an evolutionary standpoint because it raises the possibility that representatives of intermediates in the revolution of short to long germband development can be found extensively among

insects. However, the precise nature of the relationship of intermediate-germband development to long and short germband development is unclear.

The initial specification of an anterior domain with progressive addition of posterior segments is a common characteristic of the development of other arthropods, such as crustaceans, and is even comparable to the early development of annelids. Additionally, panoistic ovaries appear to be ancestral to meroistic ovaries. Thus, the production of eggs in a panoistic ovary and the short germband mode of embryonic development may be most similar to the development strategy utilized by the common ancestor of all insects and, most likely, all arthropods (Tear et al., 1988). Our understanding of the molecular and genetic basis of segmentation, however, is mostly confined to the development of the more highly evolved long germband embryo of *Drosophila.*

b) Segmentation in long germ- and short germband embryos

In *Drosophila*, maternal contributions to the oocyte, supplied by the nurse cells, are essential to the initial specification of the long germband embryo. These maternal influences establish the initial gradients of information, such as the *bicoid* protein gradient, within the egg. This initial information is utilized to sequentially subdivide the embryos into smaller and smaller units, which is reflected in the subsequent expression of gap and pair-rule genes. The expression patterns of the segment polarity and homeotic genes indicate that the entire body plan has been established by the end of the blastoderm stage. Simultaneously, rapidly, and without growth, the blastoderm is simply subdivided into smaller and smaller units by the sequential expression of the gap, pair-rule, and segment polarity genes.

Short germband embryos, by contrast, may be less dependent on maternal contributions. The egg appears to contain information only for the location of the developing blastoderm disc and for the initial growth polarity. The generation of segments is tied to the subsequent expansion of the proliferative zone of the initial blastoderm disc. The segments appear one at a time as the embryo elongates, rather than simultaneously. Thus, metameric patterning in short germband embryos is associated with a proliferative growth zone that generates segments one at a time in a rostrocaudal gradient after the formation of the blastoderm.

The existence of these two modes of segmentation - progressive subdivision of a sheet of cells versus addition of segments one at a time - raises several questions. What mechanisms are conserved between long and short germband development, and what mechanisms are unique to each mode? How did simultaneous segmentation evolve from sequential segmentation?

Comparison between homologous segmentation genes of *Drosophila* and *schistocerca* seems to be the best approach for addressing these questions. So far the *Schistocerca* homologues of the *Drosophila* homeotic genes *abdominal-A* and *Antennapedia*, the segment polarity gene *engrailed*, and the pair-rule gene *even-skipped* have been isolated and their protein distribution patterns studied in detail (Patel et al., 1989a,b,c; Tear et al., 1990; Patel, 1995). These studies reveal that certain aspects of *Drosophila* pattern formation are shared by short germband insects, whereas other aspects may be unique to long germband development.

Take ***engrailed*** expression for example: *Drosophila engrailed* is a segment polarity gene expressed in the posterior portion of each segment throughout development, and thus can be considered a molecular marker defining *Drosophila* segments (Kornberg et al., 1975; Fjose et al., 1985; Dinardo et al., 1985). At the germband stage (stages 10-13 in

Drosophila, 30% to 50% in grasshopper), the expression patterns of *engrailed* in both organisms are very similar. However, long and short germband embryos establish this expression pattern very differently. In *Drosophila*, the *engrailed* stripes begin to form during the blastoderm stage. They appear in a rapid anterior to posterior progression across the length of the embryo, subdividing the existing sheet of cells at regular space intervals (Patel, 1989a). At this time the expression of *engrailed* in *Drosophila* shows a pair-rule pattern of initiation. This is thought to reflect the fact that *engrailed* expression is controlled by the pair-rule genes (reviewed by Ingham, 1991). This data is also consistent with the embryological data indicating that the *Drosophila* blastoderm contains a complete representation of the final larval body plan. The same types of embryological studies in grasshopper suggest, however, that segments arise one at a time during development. This is confirmed at the molecular level by the observation that *engrailed* stripes in *Schistocerca* appear one at a time during a growth phase, rather than simultaneously at the blastoderm stage. Stripe formation begins in the region of the first and second thoracic segments during gastrulation and spreads both anteriorly and posteriorly. The sequential formation of stripes is most striking in the abdominal segments, where stripe formation follows well behind the advancement of the growth zone and about two segments ahead of the first morphologically visible signs of segmentation. At no point does the *engrailed* pattern show the existence of a compressed striped pattern that simply expands with growth, and there is no indication of a "pair-rule" pattern to the stripe formation. Thus, the analysis of *engrailed* expression provides the conclusion that *Schistocerca* segments are generated one at a time after the blastoderm stage, and might not be under the control of pair-rule genes.

Similar studies with ***even-skipped***, one of the three master pair-rule genes in *Drosophila* (Small and Levine, 1991), provides more insights into this issue: In

Drosophila, there are two stages of *even-skipped* expression. The earlier expression starts during the blastoderm stage in a pattern of 7 stripes, and this is required for the later expression of *engrailed* in 14 stripes, with the anterior margin of each *even-skipped* stripe coinciding with an odd-numbered *engrailed* stripe (Lawrence et al., 1987). At the on set of gastrulation, *even-skipped* stripes have sharply defined anterior borders and *engrailed* stripes show a pair-rule pattern of intensity reflecting their orders of initiation. Later in its second phase of expression, *even-skipped* has an expression pattern detected in the developing CNS, in dorsal mesoderm and in the ectodermal cells of the anal pad. *Even-skipped* is required for development of specific neurons, including the anterior corcer cell (aCC).

In grasshopper however, prior to (12%-16% of development) and during (17%-30% of development) the initiation of *engrailed* stripe expression, there is no corresponding expression of *even-skipped.* At this stage, *eve* does not show any pair-ruled pattern of expression, nor does its expression overlap with that of *engrailed.* Later in development, when the *engrailed* stripes appear in the abdominal region, there is also no detectable pair-rule pattern of *even-skipped* expression. To conclude, according to Patel's (Patel, N.H. 1992) observation, at no time in grasshopper development is there any expression pattern that could suggest *even-skipped* is involved in the initiation of *engrailed* stripe expression. However, despite the lack of pair-rule expression, *even-skipped* is expressed in the posterior mesoderm in early grasshopper embryo, which is reminiscent of the early expression patterns of *Xhox3* and *Evx-1, even-skipped* homologues in *Xenopus* and mouse respectively (Ruizi Altaba and Melton, 1989a; Bastian and Gruss, 1990; Dush and Martin, 1992). The conserved expression pattern of *even-skipped* is postulated to be involved in early anterior-posterior axial patterning.

On the other hand, midway through the grasshopper embryogenesis, *eve* is expressed in a segmentally repeated pattern of neurons, in an ectodermal ring at the anal pad, and in a segmentally repeated pattern of dorsal mesoderm, a pattern identical to that seen in *Drosophila* at an equivalent stage of development. In both insects, the dorsal mesodermal cells that express *even-skipped* go on to form part of the dorsal vessel (heart) and the dorsal-most muscle fibers of the body wall. Moreover, careful examination shows the neural expression of *even-skipped* in two insects can be traced to individual homologous neurons. The vertebrate *even-skipped* homologues are also expressed within the CNS in a subset of spinal cord neurons.

More recent comparison of ***Antennapedia*** expression patterns in *Drosophila* and grasshopper yielded similar results to those obtained for *even-skipped. Antennapedia* is an ANT-C homeotic gene. It has divergent early expression patterns in these two insects, reflecting different modes of early development. However, by the germband stage, its expression patterns become similar: both insects show relatively uniform epithelial expression throughout the thoracic and abdominal segments which is subsequently restricted to the thorax. Later, expression is also observed in subsets of PNS and CNS neurons in both insects (Patel, 1995).

All these observations support the notion that while certain aspects in *Drosophila* and grasshopper development are conserved throughout the evolution, some aspects of *Drosophila* pattern formation may instead represent recent evolutionary advances and may differ from the mode of pattern formation used in the ancestors of present-day arthropods and vertebrates (Patel, 1993).

Section III CNS and midline morphogenesis in *Drosophila* and *Schistocerca*

a) The developmental commitment of grasshopper CNS midline cells

In grasshopper embryos, neuroblasts undergo repeated asymmetrical divisions to produce from a few to more than 100 neural progeny (Bate, 1976). A neuroblast divides to produce a neuroblast and a smaller ganglion mother cell. The ganglion mother cell undergoes a terminal symmetrical division to produce two ganglion cells, which differentiate to become neurons. The segmental ganglia of the ventral nerve cord are thus composed of groups or families of related neurons, each resulting from divisions of a single neuroblast.

Studies on these cell lineages suggest that most of the midline cells come from a single precursor cell: the unpaired median neuroblast (MNB) (in thoracic segment 3, the MNB lineage in grasshopper produces more than 90 progeny.). The MNB gives rise to the vast majority of midline neurons, including the efferent dorsal unpaired median (DUM) neurons and the local and segmental interneurons (Thompson and Siegler, 1991). The numbers of MNB progeny in each of these neuronal classes differ between thoracic and abdominal segments (Thompson and Siegler, 1993), suggesting that the lineage may be regulated by homeotic gene function. Direct tracing of the MNB lineage in embryo cultures has demonstrated that the midline glia are also derived from the MNB. The first 3-4 divisions of the MNB generate neuronal GMCs that divide to produce neurons. The next 8-10 MNB divisions (31-40% of the development) generate glial precursors, which divide to produce approximately 31 midline glia per segment. At about 40%, the MNB switches back to generating neuronal GMCs (Condron and Zinn, 1994a; for a review on cell fate decisions in grasshopper, see Condron and Zinn, 1994b). The first cell fate transition in the MNB lineage requires the activity of *engrailed* gene (Condron and Zinn,

1994c), whereas the second cell fate switch requires the activation of PKA (Condron and Zinn, 1995).

b) Determination of cell identities in the *Drosophila* CNS midline

Each segment of the fly CNS (also called neuromere) originates after gastrulation from the ventral neuroectoderm (or ventral neuroepithelium) and is composed of three regions: the left and right neurogenic regions which generate the bulk of neurons, and a midline region which generates a small number of special midline neurons and glial cells and separates the left and right neurogenic regions (Crews et al., 1988; Thomas et al., 1988; Klambt et al., 1991). In both vertebrates and insects, special midline cells appear to play a key role in the formation of the axonal commissures which connect both sides of the developing CNS. In *Drosophila,* genetic analyses have further shown that midline cells perform important functions during the establishment of the CNS axonal pattern (Klambt et al., 1991; Seeger et al., 1993).

The CNS midline precursors become molecularly distinct from the neighboring ventral (presumptive mesoderm) and dorsal (neurogenic region) cells as early as the cellular blastoderm stage, when they first express *single-minded*, the master regulatory gene of midline development (Crews et al., 1988; Thomas et al., 1988). At this stage, the midline precursors are arranged in two bilaterally symmetric columns of cells along the anterior-posterior axis; each column is a single cell wide and about four cells long per segment. After the invagination of the mesoderm during gastrulation, the two columns of midline precursors are brought together at the ventral midline to form a single column of eight cells. All eight cells delaminate into the same layer as neuroblasts, leaving a transient gap in the surface epidermis that is later filled by the rearrangement of the epidermal cells (figure 1, adopted from Goodman and Doe, 1993). After one round of division, each

segment has 16 midline cells. The anterior five pairs of cells are post-mitotic and differentiate into identified glia (from pairs 1-3) and neurons (from pairs 4-5), whereas the posterior-most two (possibly three) pairs continue dividing to generate a final complement of about 25-30 cells per segment along the ventral midline (figure 2, adopted from Goodman and Doe, 1993). From anterior, the first three pairs of precursor cells give rise to the three pairs of identified midline glia (MGP, MGM, and MGA, respectively), whereas the fourth and fifth pairs of midline cells produce neurons (MP1 and V, respectively). The pair of V neurons is the anterior-most pair of ventral unpaired median (VUM) neurons (Goodman et al., 1984). The lineage of the sixth through the eighth pair of midline cells has not yet been completely determined, but their progenies include an additional four VUM neurons, the median neuroblast (MNB), and a small number of glial cells associated with both the VUM and the MNB (Klambt et al., 1991).

However, more recent observation by Bossing and Technau (1994) argues the number of midline cells per segment is variable (on average 7.5 per segment). Moreover, their data suggest that there is only one midline progenitor per segment that gives rise to all three pairs of midline glia.

The MNB lineage in fly is not yet well classified as in grasshopper. Using the DiI labeling method (Udolph et al., 1993), however, it appears that this midline progenitor gives rise to 5-8 neurons and most of them are identified as interneurons. In late embryos, one of the fibers from MNB clones on either side enters the intersegmental nerve and, thus, may represent a motorneuronal projection (Bossing and Technau, 1994) These MNB progeny and the MNB itself are confirmed to express *engrailed,* as in the case of grasshopper.

It is apparent that there are some similarities in the projection patterns between cells of the putative MNB clone in the late *Drosophila* embryo and those in the

grasshopper embryo. However, these similarities are even more obvious in the fly VUM neurons (possibly homologous to grasshopper DUM neurons) (Goodman et al., 1984), although VUM neurons do not derive from the putative MNB (Klambt et al., 1991).

c) The midline genes in *Drosophila*

In *Drosophila*, the nervous system can be subdivided into two lateral halves connected by a special set of cells found at the CNS midline. These midline cells or mesectodermal cells are the first ones to be specified in *Drosophila* neurogenesis (Crews et al., 1988; Thomas et al., 1988). The early development of the CNS midline might be controlled by inductive signals originating from the mesoderm, since there are no midline cells in mutants lacking the mesoderm (Kosman et al., 1991; Leptin et al., 1991). However, the earliest indication of midline differentiation is the expression of the ***single-minded*** (*sim*) gene in the midline progenitors.

A master regulator of CNS midline development (Nambu et al., 1991), the *sim* gene encodes a nuclear protein expressed in the midline cells throughout embryogenesis (Crews et al., 1988). Mutations in *sim* are embryonic lethals characterized by fusion of the longitudinal axon bundles and collapse of the axon scaffold (Thomas et al., 1988). Genetic and cell biological studies have indicated that *sim* is required for proper midline cell differentiation and gene expression (Nambu et al., 1990). In the *sim* null mutant background, the midline cells fail to undergo synchronous postgastrulation cell division, exhibit defects in the formation of nerve cell precursors, and do not properly autoregulate *sim* gene expression. Functions of the *sim* gene are necessary for all aspects of midline cell development, including early events in the formation of midline nerve cell precursors as well as later events in their differentiation into mature neurons and glia. Induction of ectopic *sim* during early embryogenesis results in the expression of midline cell

properties, including cellular morphology and patterns of gene expression, in most or all cells of the lateral CNS. The ectopic expression of *sim* is thus sufficient to cause other cell types to take on midline identities (Nambu et al., 1991).

As a transcription factor belonging to the basic helix-loop-helix (bHLH) family, the sim protein is thought to function by transcriptionally regulating other genes important for development of midline cells. Consistent with this notion is the finding that the midline expressions of a number of genes, such as *slit*, *Toll*, *rhomboid*, *engrailed* and *center divider* (*cdi*) are dependent upon *sim* function (Numbu et al., 1990; Muralidhar et al., 1993). In the *sim* mutant, the expressions of these genes are either eliminated completely, or reduced significantly.

Other midline genes identified as mutants that alter cell lineages at the ventral midline have also been reported. This group of midline genes including *spitz*, *orthodenticle, Star* (reviewed in Klambt et al., 1991), and *pointed* (reviewed in Scholz et al., 1997). Although these genes show unchanged expression patterns in *sim* mutant, all of their normal functions still require normal *sim* expression in the midline. Studies of these genes suggest that they regulate midline morphogenesis by participating in the signaling pathways involving the above described *sim*-dependent genes.

Phenotypic classifications on these midline genes subdivide them into several groups (Klambt et al., 1991). The first phenotypic class, including *sim* and *slit*, shows a complete collapse of CNS axon pathways toward the midline, the absence of commissures, and thus the fusion of longitudinal axon bundles into a single tract. However, in the *sim* mutant, all midline processor cells divide at least once but then die during germband retraction, whereas in *slit* embryos no cell death is observed at the

midline. Rather, the midline cells are displaced ventrally and do not appear to differentiate properly.

The second class of midline genes is represented by *orthodenticle* (*otd*). In *otd* mutant, the posterior commissure is missing in every segment and the overall nervous system becomes narrower. Studies using enhancer trap reporters in an *otd* background reveal that VUM and probably the MNB neurons are strongly affected at an early stage. Considering the fact that VUM neurons are the set of midline cells that locate just bellow the posterior commissures, it is reasonable that their absence disrupts the formation of posterior but not anterior commissures. Midline glia appear to be normal in *otd* mutants, though the MGMs fail to migrate. This glial defect might only be a secondary effect caused by the death of VUM neurons, because the MGMs follow the growth cones of the VUM neurons during normal migration.

The third class of midline genes includes *spitz*, *Star*, and *rhomboid*. Mutations in any of these genes lead to a fusion of the two commissures. However, in *rho* mutant embryos, the midline glial cells appear to be missing from early stages, suggesting a function for this gene in the determination or differentiation of midline glial cells. In *S* mutant embryos, the midline glial cells initially develop normally, but then the MGM fail to migrate, and during stage 14, all midline glia eventually die. In contrast, in *spitz* mutant embryos, the MGM also fail to migrate, but all midline glia come to lie abnormally on the dorsal surface of the CNS, where they persist until the end of embryogenesis (Klambt et al., 1991). It is interesting that more recent studies have shown that all three of these genes are involved in the DER/*spitz* signal transduction pathway, which functions with Argos to determine whether midline glia survive or die during late embryogenesis. The DER/Argos signaling pathway will be discussed in detail in the Discussion sections of chapters III and IV (or see the review by Scholz et al., 1997).

There is another class of midline genes whose function is to provide growth cone guidance across the midline during the formation of the axon commissures. This group of genes includes *Netrins A* and *B* (Mitchell et al., 1996), *commissureless* and *roundabout* (Seeger et al., 1993; Tear et al., 1996; Kidd et al., 1998 a and b). Both *comm* and *Robo* encode novel membrane proteins. Comm proteins are expressed in the midline glia, while *Robo* is expressed on neuronal growth cones. *Robo* functions as a gatekeeper to repel axons from the midline. *Comm* acts to neutralize *Robo* function, aloowing commissural axons to cross the midline. These two genes function together to control midline crossing. Although these mutations alter the CNS scaffold, they leave midline cell lineages unchanged.

Conclusions

At first glance, the overall structure of the embryonic CNS in the grasshopper is quite similar to that in the fruit fly. This similarity extends to axonal projection patterns, as in the case of motorneuron/interneuron sibling pair aCC/pCC (figure 3a), and to gene expression patterns of individual identified neurons as in the case of *single-minded* expression in the midline neurons (figure 3b). Many identified neurons in the fly CNS were first characterized as homologues of previously identified grasshopper neurons.

However, the midline cell lineages in these two insects are rather different, just as the mechanisms leading to germband formation are divergent between these two insects. Therefore, there are processes for which we can assume that fly and grasshopper use the same mechanisms, and other processes for which we cannot make these assumptions. Although we know some basic rules for a few developmental processes in *Drosophila* and

Schistocerca, we know very little about the mechanisms involved in these morphogenetic events. How do cells, after their fates assigned and positions determined, build ordered structures and organs? Morphogenetic mechanisms include cell proliferation and growth, cell migration, shape changes, programmed cell death of individual cells and of groups of cells - but what are the genetic and molecular bases behind them? Our work addresses some of these questions.

This chapter draws its main sources from several references, and is updated with the most recent data to meet the purpose for the introduction section. These major references are listed in the following Reference section in bold font to emphasize their contributions to this review.

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Figure 1. Development of the neurogenic region and the ventral midline

The development of the midline is depicted as a series of four schematic cross sections of embryos from four successive developmental stages (dorsal is up). During the blastoderm stage, the mesectodermal or midline precursor cells, indicated in black, are located on either side of the mesectodermal anlage; they separate the presumptive mesoderm from the presumptive neurogenic ectoderm. As gastrulation proceeds, mesodermal cells invaginate, and the two rows of four mesectodermal cells per segment become juxtaposed at the ventral midline. During germband elongation, these cells intermingle to form a row of eight midline precursor cells per segment. Before these precursor cells start to divide to give rise to the neurons and glial cells of the ventral midline, they all delaminate from the ventral ectoderm into the same layer as the delaminating neuroblasts (NBs), leaving behind a transient gap that is later filled in by the rearrangement of epidermal cells. (Adopted from Klambt et al., 1991.)

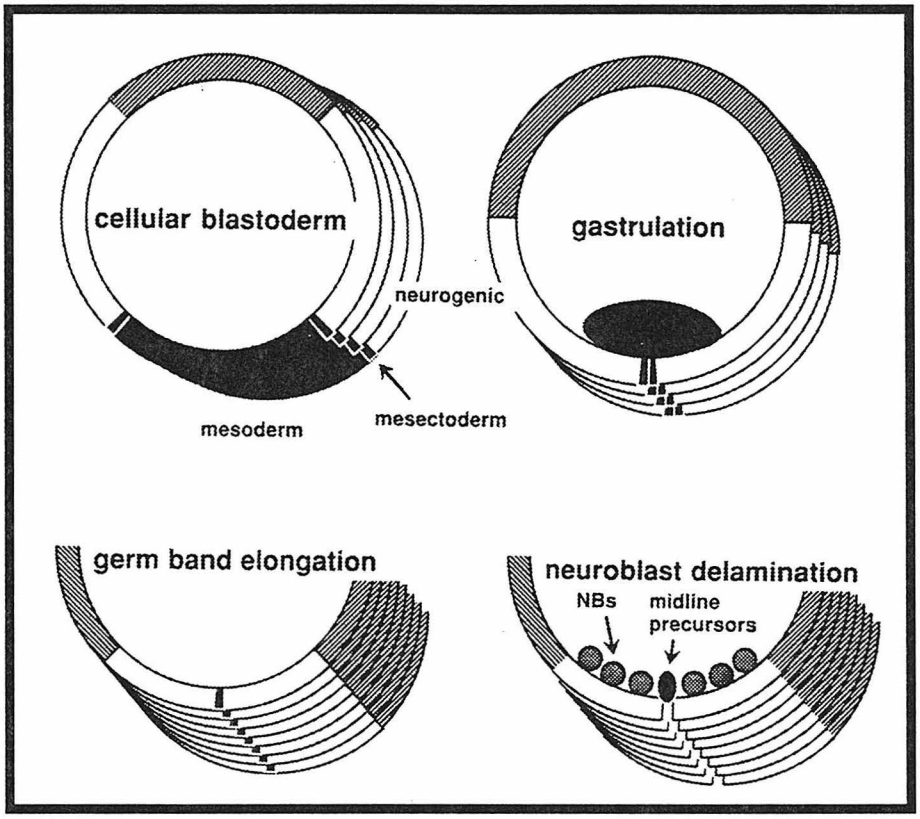

cellular blastoderm
neurogenic
mesoderm
mesectoderm
gastrulation
germ band elongation
neuroblast delamination
NBs
midline
precursors

Figure 2. Development of the midline and axon commissures (from Klambt et al., 1991)

Development of the midline and axon commissures. (*A*) During gastrulation, the two rows of mesectodermal cells (each row is four cells per segment) initially come together at the midline (*B*) and then intermingle during germ-band elongation to form the row of eight midline precursor cells per segment (C). As revealed using enhancer-trap lines (described in Klämbt et al. 1991), the eight midline precursors become determined to the following fates (from anterior to posterior) (*D*). The anterior-most three precursors give rise to the midline glia: (MGP) posterior; (MGM) middle; (MGA) anterior. The relative order of these cells changes during development as a result of cell migrations.

The fourth precursor is MP1 (midline precursor 1) that gives rise to the two MP1 neurons. These anterior four midline precursors divide only once (*E*). The posterior four midline precursors give rise to the VUM (ventral unpaired median) neurons, the median neuroblast, and their support cells. Two to three precursors give rise to the VUMs (and possibly their support cells), and the fourth precursor gives rise to the MNB (and possibly its support cells). The midline precursors marked by the asterisk are likely to divide more than once. (*F-I*) Development of the two axon commissures takes place in a series of discrete steps: (*F*) Pioneering of the posterior commissure (PC); (*G*) pioneering of the anterior commissure (AC); (*H*) migration of the midline glia; (*I*) the resulting separation of the anterior and posterior commissures. (*F*) Growth cones that pioneer the posterior commissure extending directly toward the anterior-most VUM neurons, called the V cells. Once these growth cones meet the V cells, they extend around the anterior end of these cells and cross the midline in the position where the MP1 neurons had just been, as shown in panel *G*. The growth cones that pioneer the anterior commissure extend directly toward the posterior edge of the MGA glia. (*H*) Subsequent separation of the two commissures results from the migration of the MGM glia. (*J-L*) The migration of the midline glia with respect to the VUM neurons and the formation of the two commissures is shown in schematic sagittal views. (*J*) Posterior commissure (PC) forms just anterior to the VUM cell bodies on their anterior extensions that are the beginning of their growth cones. The midline glial cells extend endfeet posteriorly which contact the VUM growth cones. (*K*) Anterior commissure forms in contact with the MGA and MGM glia on the anterior side of the VUM growth cones (in contrast to the posterior commissure which is now on the posterior side of the VUM growth cones). During this period, the VUM cell bodies begin to drop away from the inner surface and the MGM cells migrate over the top of the MGA cells. (*L*) Commissures are separated by the migration of the MGM glia that contact the VUM axons. The VUM growth cones now extend along the MGM glia to the inner surface of the anterior commissure where they bifurcate , as their cell bodies continue to drop away from the inner surface.

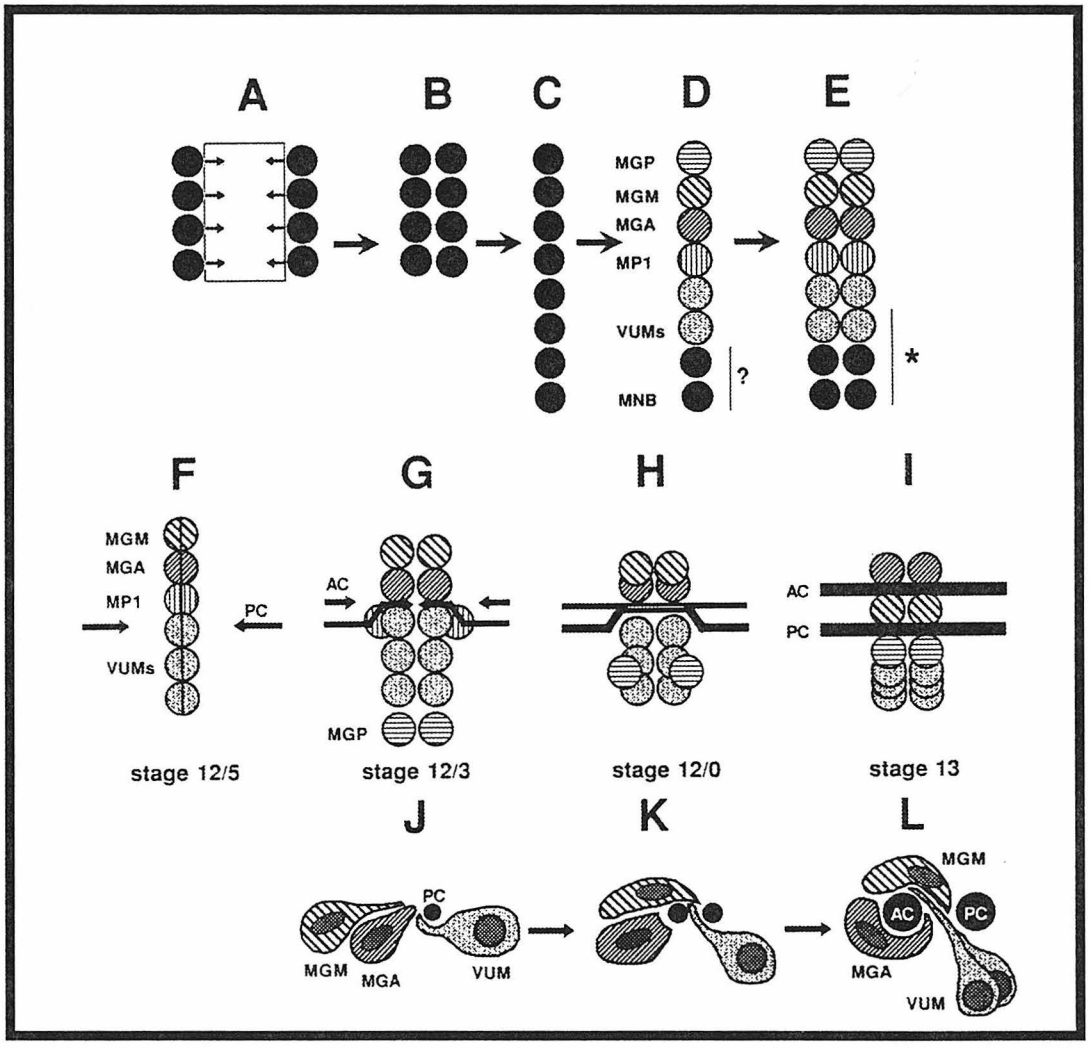
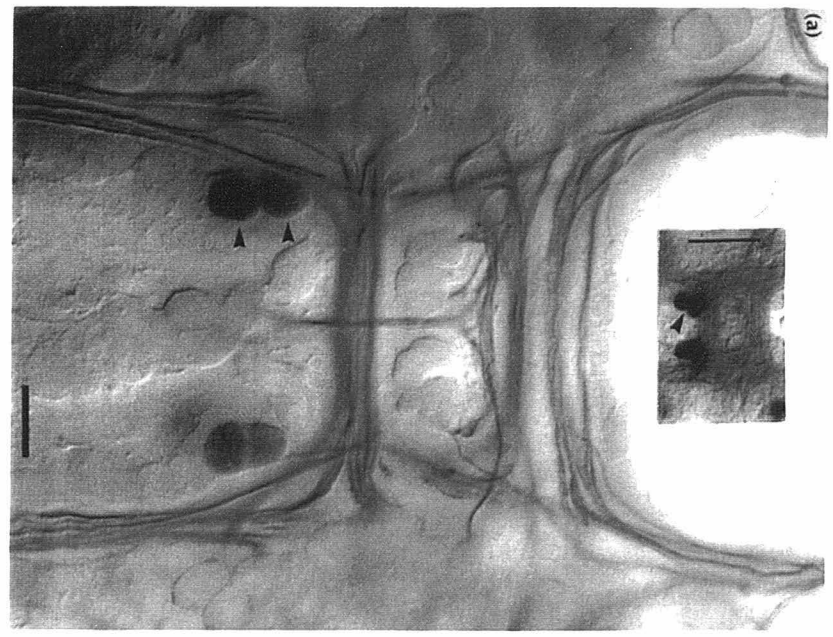
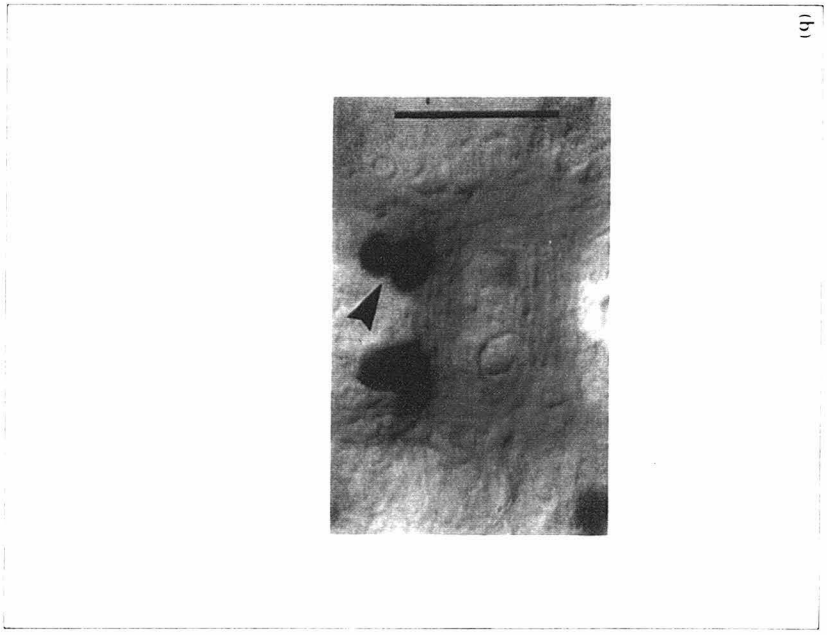

A
B
C
D
E
MGP
MGM
MGA
MP1
VUMs
MNB
?
*
F
G
H
I
MGM
MGA
MP1
VUMs
PC
AC
MGP
AC
PC
stage 12/5
stage 12/3
stage 12/0
stage 13
J
K
L
PC
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MGA
VUM
MGM
AC
PC
MGA
VUM

Figure 3. Development of the grasshopper CNS in vivo and in culture

a) Single segments of the grasshopper and fly CNS, double stained with antibodies against *even-skipped* (black) and axonal antigen (brown). The segments are at the same magnification. The motorneuron/interneuron sibling pair aCC and pCC are indicated by arrowheads in the main photograph and inset. Note the larger size and clearer visualization of the cell bodies and axon bundles in the grasshopper embryo.

b) Single segment of the fly CNS (enlargement of the inset in a).


(a)


(b)

Chapter II

Identification of a Drosophila protein tyrosine phosphatase expressed in a pattern of segmental stripes in the germ band extended embryo

Te-Yi Kung, Shin-Shay Tian, and Kai Zinn

Abstract

Tyrosine kinases are essential elements of developmental signaling mechanisms. Protein tyrosine phosphatases (PTPs) are also likely to be involved in these signaling pathways, but their precise roles are more poorly understood. Here we describe the cloning of cDNAs encoding a novel nonreceptor PTP, DPTP61F, which is expressed in early Drosophila embryos. DPTP61F is most closely related to the mammalian PTP1B and T cell PTPs, and, like them, contains a hydrophobic C terminal tail. DPTP61F mRNAs are expressed throughout embryonic development, but are most abundant during the first 12 hrs. Whole-mount embryo in situ hybridization experiments show that DPTP61F mRNA is expressed in a pattern of 15 segmental stripes in the germ-band extended embryo. These stripes correspond approximately to the most anterior five cells of an eight cell long segment. There is also expression in a patch of cells in the head. Later in development, DPTP61F mRNA is enriched in the central nervous system and in the dorsal margin of the pharynx. The segmentally striped expression pattern of DPTP61F suggests that it may be involved in the process of segmentation. We propose a model in which DPTP61F regulates the activity of the zw3 serine-threonine kinase, which has been shown to control expression of the segment polarity gene engrailed.

Introduction

Signal transduction via control of tyrosine phosphorylation is essential for many developmental processes in both vertebrates and invertebrates. In Drosophila, three receptor tyrosine kinases have been shown to control specific cell determination events. The torso tyrosine kinase is uniformly distributed in the egg, and is locally activated by a ligand expressed on the follicle cells that abut the ends of the embryo. Localized activation of torso is required for the correct fate specification of the cells comprising the termini of the embryo. The Drosophila EGF receptor homologue (DER) is defined by several classes of mutations (flb, torpedo, Ellipse) that cause aberrant cell determination at different developmental stages. DER is involved in the maternal specification of dorsoventral fates in the embryo, in the zygotic determination of the midline of the embryonic nervous system, and in photoreceptor development in the eye. The sevenless tyrosine kinase controls the determination of R7 photoreceptor identity.

In contrast to these well-defined roles for tyrosine kinases, the functions of protein tyrosine phosphatases (PTPs) during Drosophila development are not well understood. PTPs are a distinct class of molecules that are not homologous to serine/threonine phosphatases. Many PTPs have been recently identified, and these can be grouped into receptor-linked and nonreceptor classes. Receptor PTPs (R-PTPs) are transmembrane glycoproteins, some of which have adhesion molecule-like extracellular regions consisting of immunoglobulin-like domains and/or 'fibronectin type III' repeats. Nonreceptor PTPs often contain other domains in addition to the PTP homology unit. These include phosphotyrosine-binding SH2 domains and domains related to cytoskeletal proteins (for reviews see CHarbonneau and Tonks, 1992; Pot and Dixon, 1992).

cDNA clones encoding four Drosophila receptor PTPs have been sequenced. All of these PTPs are expressed on central nervous system axons in the embryo (Hariharan et al., 1991; Yang et al., 1991; Tian et al., 1991; Streuli et al., 1989; Desai et al., 1994). A null mutation in the DPTP99A gene has been isolated, but has not been shown to cause any specific developmental defect (Hamilton et al., 1995). The only Drosophila nonreceptor PTP that has been identified thus far is the product of the corkscrew (csw) gene. This is an SH2 domain-containing PTP that is maternally deposited in the embryo. Embryos derived from mothers lacking csw function have defective terminal structures due to insufficient torso PTK activity (7). These data suggest that the csw PTP may dephosphorylate a tyrosine residue on torso itself or on an associated molecule, and that this dephosphorylation stimulates torso PTK activity. Binding of the csw SH2 domain to phosphotyrosine also activates its phosphotase activity.

In this chapter, we describe the isolation and characterization of a cDNA clone encoding a novel Drosophila nonreceptor PTP, denoted DPTP61F, that is closely relatedto the mammalian PTP1B and T cell PTP sequences. We show that DPTP61F is selectively expressed in a pattern of 15 segmental stripes in germ band extended embryos. This expression pattern is characteristic of zygotic segment polarity genes, and suggests that DPTP61F activity may be involved in the process of segmental determination.

MATERIALS AND METHODS

Isolation of a full-length DPTP61F cDNA clone. One of the subcloned 300 bp PTP PCR fragments, known as 153, identified in the study of Tian et al. (1991), was used to screen a λgt11 library prepared from 9-12 hr. old embryos (Zinn et al., 1988). The resulting clone was used for in situ hybridization experiments, and also used to screen a plasmid library from 4-8 hr. old embryos (Brown and Kafatos, 1988). Several clones were obtained in this screen that had inserts of about 3.4 kbp. One of these was selected and sequenced, using unidirectional exonuclease III digestion to generate subclones (Henikoff, 1984). Molecular biology techniques were performed using standard methods (Sambrook et al., 1989).

Analysis of DPTP61F expression. The subcloned λgt11 insert was used for Northern blot experiments on staged RNA samples. Total RNA was isolated from fly embryos and Northern analyses performed as described in (Tian et al., 1991). In situ hybridization to fly embryos was done essentially as described (Tautz and Pfeifle, 1989), using the Genius Kit (Boehringer Mannheim) to generate digoxigenin-labeled probe, and detecting hybridization using the Vector ABC substrate kit II. Embryos were dehydrated and cleared in methyl salicylate.

RESULTS AND DISCUSSION

Molecular characterization of a cDNA encoding DPTP61F. Tian et al. (1991) used polymerase chain reaction (PCR) techniques to identify cDNA clones encoding seven different PTPs that are expressed in 9-12 hour old Drosophila embryos. Three of these sequences were initially chosen for further study because they are selectively expressed in the central nervous system. These encode the R-PTPs DLAR, DPTP10D, and DPTP99A (Hariharan et al., 1991; Yang et al., 1991; Tian et al., 1991; Streuli et al., 1989). We also identified cDNAs encoding the csw PTP (Perkins et al., 1992), DPTP (Streuli et al., 1989); we have denoted this sequence DPTP69D based on its cytological location to distinguish it from the other Drosophila PTPs), and a widely expressed R-PTP, DPTP4E, that is very similar in sequence to DPTP10D. The final cDNA identified in this study encodes DPTP61F, and the corresponding gene is located at 61F/62A on the polytene chromosomes.

We identified a full-length DPTP61F cDNA clone and determined its sequence. This cDNA encodes a protein of 548 amino acids, having a molecular weight of 62 kilodaltons (figure 1). DPTP61F lacks an N-terminal signal sequence, but a Kyte-Doolittle hydropathy plot shows that it contains a 23 residue hydrophobic region at its extreme C terminus (data not shown).

The N-terminal 300 residues of DPTP61F are highly homologous to other PTP sequences. DPTP61F contains the characteristic VHCSAG sequence found in almost all PTPs. The cysteine residue within this sequence has been found to be essential for catalytic activity of a number of PTPs (for reviews see Charbonneau and Tonks, 1992; Pot and Dixon, 1992). The two PTP sequences that are most similar to DPTP61F are those of PTP1B (known as PTP1 in rat) and T cell PTP (TC-PTP) (Guan et al., 1990;

Charbonneau et al., 1989; Chernoff et al., 1990; Brown-Shimer et al., 1990; Cool et al., 1989; Mosinger et al., 1992; Swarup et al., 1991). Residues 64-298 of DPTP61F are 58.1% identical to the human PTP1B sequence (residues 44-279), and 56% identical to the human T cell PTP sequence (residues 46-277; figure 2). Like DPTP61F, PTP1B and TC-PTP have hydrophobic C-terminal sequences about 20 residues in length. The remainder of the C-terminal half of DPTP61F, however, shows no significant similarity to other sequences in the current databases.

Expression of DPTP61F mRNA during embryonic development. We examined the temporal expression pattern of DPTP61F during embryonic development by Northern blot analysis (figure 3). We made RNA from staged embryo collections, each representing a 3 hr. time period. A 3 hr. AEL (after egg laying) embryo corresponds to the cellular blastoderm stage. This stage is followed by gastrulation and germ band extension. The embryo remains in the germ band extended stage for several hours, and the germ band begins to retract at about 7.5 hr. Axonogenesis in the central and peripheral nervous systems occurs predominantly during the 9-12 hr. period.

A major transcript migrating at a position corresponding to a 3.3 kb marker RNA is observed at the beginning of embryogenesis (0-3 hr. AEL; figure 3). The level of this transcript decreases slightly in 3-6 hr. AEL embryos, then increases again in 6-9 and 9-12 hr. embryos. The decrease in expression between the 0-3 hr. and the 3-6 hr. periods suggests that all or part of the DPTP61F RNA present at the beginning of embryogenesis may be maternally contributed. After 12 hr., expression diminishes drastically, and much lower levels of transcript are detected in late embryos (18-24 hr). In addition to the major transcript, a smaller transcript of about 2.8 kb is also observed between 0 and 12 hrs. Finally, a weak band at about 3.5 kb is seen in 9-12 hr. embryos.

To examine the distribution of DPTP61F mRNA in the embryo, we used in situ hybridization to whole-mount embryos (Tautz and Pfeifle, 1989). A DPTP61F cDNA clone was labeled with digoxigenin and hybridized to whole-mount embryo collections of various ages. DPTP61F mRNA expression was detected using alkaline phosphatase-conjugated anti-digoxigenin antibodies. No localized expression of DPTP61F mRNA is observed in syncitial or cellular blastoderm embryos. However, at the cellular blastoderm stage, darker alkaline phosphatase staining is observed than with control probes, suggesting that DPTP61F is ubiquitously expressed at a low level (data not shown).

After the cellular blastoderm stage, gastrulation in Drosophila begins with the formation of the ventral furrow. This is followed by germ band extension, in which the segmented region of the embryo (three gnathal, three thoracic, and nine abdominal segments) elongates and folds over the dorsal side of the embryo. The first localized expression of DPTP61F mRNA is observed in early germ-band extended embryos (stage 8-9 of ref. 20). At this stage, a patch of cells expressing DPTP61F mRNA at very high levels is observed in the head region, just anterior to the invagination of the anterior midgut primordium (figure 4A). There is also lower-level expression in stripes within the segmented region. In the photograph of figure 4A, three clear stripes are observed. In other embryos, weak stripes at other positions are also seen. These data indicate that the segmental striping seen at later stages (see below) does not develop uniformly, but that some stripes appear earlier than others. There is some suggestion of a pair-rule (7-stripe) pattern in certain embryos, but we can not state definitively that expression is initiated in alternating segments.

The germ band extended phase of Drosophila embryogenesis lasts for about 3.5 hours (stages 8-11), and during this period there are no dramatic morphological changes in the embryo. In stage 10 and 11 embryos DPTP61F mRNA is expressed at highest levels

in a pattern of 15 stripes. Figure 4B is a lateral view of a stage 10 embryo. One stripe can be seen anterior to the cephalic furrow; this corresponds to the first gnathal segment. The second gnathal stripe is superimposed on the cephalic furrow, and there are 13 stripes posterior to the furrow. It should be noted that DPTP61F mRNA is not exclusively expressed in the stripes. The staining between the stripes is darker than the background observed with control probes (data not shown), indicating that the DPTP61F gene is widely expressed, but that transcripts are present at higher levels within the segmental stripes. Figure 4C is a ventral view of a stage 10 embryo, showing several clear stripes.

Alignment of the stripes of DPTP61F mRNA expression in the germ band extended embryo with the forming segmental furrows indicates that the PTP transcript is present at highest levels posterior to the furrow, and at low levels anterior to it. The region within the stripes of expression corresponds approximately to the anterior half of each segment (figure 4B, 4C). In segments that are about eight cells in length, DPTP61F mRNA is expressed at elevated levels in the anterior four or five cells. The most anterior two or three cells of the segment sometimes appear to contain a particularly high level of DPTP61F transcript.

In addition to the segmental stripes, there are two other patterns of high-level DPTP61F expression observed in stage 10-11 embryos. A stripe or patch of hybridization in the head (figure 4B) may correspond to the same cells observed in stage 9 embryos (figure 4A). A longitudinal stripe of expression is also observed in the neuronal layer of stage 10-11 embryos. This extends the entire length of the germ band, and shows no segmental modulation (data not shown; this stripe is out of focus in figure 4B).

As germ-band retraction begins, the segmental stripes fade. During much of stage 12, no clearly localized DPTP61F expression is evident. However, by the beginning of

stage 13, higher levels of transcript are observed in the ventral nerve cord and brain (figure 4D). A large fraction of the cell bodies in the nerve cord appear to contain DPTP61F mRNA. There is also localized expression in a row of cells along the dorsal margin of the pharynx. This is interesting, because these cells may be derived from the region adjacent to the anterior midgut primordium in which the first localized DPTP61F expression is observed. It is thus possible that DPTP61F is involved in determining the identity of these pharyngeal cells.

Implications of the DPTP61F expression pattern. The segment polarity genes control cell identity and patterning within each segment of the Drosophila embryo. Mutations in segment polarity genes cause alterations in the fate of cells within a particular region of each segment. Many of the zygotic segment polarity genes that have been molecularly cloned are expressed in a characteristic segmentally reiterated pattern of stripes in the germ band extended embryo. These genes include *engrailed* (*en*), *gooseberry* (*gsb*), *cubitus interruptus Dominant* (*ciD*), *wingless* (*wg*), *hedgehog* (*hh*), and *patched* (*ptc*) (for reviews see Nusse and Varmus, 1992; Ingham and Martinez-Arias, 1992; Peifer and Bejsovec, 1992). The first three of these genes encode transcription factors. *wg* and i encode secreted proteins (Lee et al., 1992; Mohler and Vani, 1992), and *ptc* encodes a surface protein. The segment polarity genes are expressed in the following patterns within an eight cell long segment: *ciD* and *ptc* in the first six cells (numbering from the anterior end of the segment), *wg* in the fifth cell, *gsb* in the last three cells, and *en* and *hh* in the last two cells. The expression pattern of DPTP61F is thus most similar to that of *ciD* and *ptc*.

Patterning within the segment occurs by a complex series of cell-cell interactions, most of which are not well understood. The control of *en* expression by interaction between the adjacent *en*- and *wg*- expressing cells has been most intensively studied. The

pattern of *en* expression is initially established by the action of pair-rule genes. Subsequently, maintenance of *en* expression involves positive autoregulation by en protein, and this autoactivation requires signaling by the adjacent *wg*-expressing cells. *en* autoactivation is blocked by the activity of a ubiquitously expressed serine-threonine kinase, *zw3*. In the posterior part of the segment, *wg* protein secreted by the adjacent cell causes inactivation of *zw3*, thus permitting continued *en* expression (figure 5; Seigfried et al., 1992). There is also a reciprocal signal from the *en*-expressing cells to the *wg*-expressing cells that is required to maintain *wg* expression. This signal involves the products of the *hh* and *ptc* genes (Nesse and Varmus, 1992; Ingham and Martinez-Arias, 1992; Pheifer and Bejsovec, 1992).

Although none of the segment-polarity genes thus far identified encode proteins that are obviously involved in control of tyrosine phosphorylation, it has been shown that *zw3* and its mammalian homologue, glycogen synthase kinase (GSK-3), are phosphorylated on tyrosine in vivo. This tyrosine phosphorylation is essential for biological activity of GSK-3 (Hughes et al., 1993). It is thus likely that a tyrosine kinase exists which is responsible for *zw3* phosphorylation and activation in the embryo.

These results suggest that DPTP61F may be involved in the control of *zw3* activity by regulating its phosphorylation on tyrosine. It could do this by dephosphorylating *zw3* directly and thereby inactivating it. However, given the expression pattern of DPTP61F, such a model would not correlate well with our current knowledge of the mechanisms of signaling within the segment. DPTP61F is expressed in the first five cells of an eight cell long segment (figure 5A). In the posterior three of these cells (which are part of the *wg* equivalence group), there is no requirement for *zw3* function, as removal of *zw3* has no apparent effect in these cells. The anterior two cells expressing DPTP61F are part of the en equivalence group, but they do not normally

express *en* because, being >2 cell diameters away from the *wg* cells, they receive an insufficient amount of *wg* signal to cause inactivation of *zw3*. When *zw3* activity is eliminated by mutation, or when *wg* is ubiquitously expressed, these cells express *en* (Siegfried et al., 1992; Noordermeer et al., 1992). Thus, under normal conditions, *zw3* is active in the first two cells of the segment (which express DPTP61F), but in the last two its activity is blocked by the *wg* signal. This is the reverse of what would be expected if DPTP61F dephosphorylated *zw3*.

Another possibility is that DPTP61F positively regulates the activity of the tyrosine kinase that phosphorylates *zw3*. Such a mechanism would be more consistent with our current knowledge about PTP function in signaling pathways. The only PTPs for which there is information about their roles in signaling are the CD45 PTP in mammalian hematopoietic cells and the csw PTP in flies. In both cases, PTP activity causes an increase in activity of a tyrosine kinase. In the case of CD45, this is thought to occur via dephosphorylation of an autophosphorylated tyrosine residue on the *lck* tyrosine kinase. This dephosphorylation stimulates *lck* activity (reviewed in Cantley et al., 1991). For the *csw* PTP, genetic data indicate that a decrease in *csw* activity causes a decrease in the activity of the *torso* tyrosine kinase (Perkins et al, 1992). It is not known whether this effect is direct, however.

Dephosphorylation and activation of a tyrosine kinase by DPTP61F could increase tyrosine phosphorylation of *zw3* kinase in the anterior portion of the segment where DPTP61F is maximally expressed. This would facilitate the maintenance of high *zw3* activity in those cells in which *zw3* function is required to block autoactivation of *en* expression. This hypothetical model is depicted in figure 5B. In such a model, DPTP61F would be part of the mechanism for repressing ectopic *en* expression in the cells immediately posterior to the normal en stripe (figure 5A). DPTP61F is likely to be

involved in the maintenance rather than in the initiation of repression, because it is not expressed in a clear striped pattern until stage 10. The zygotic segment polarity genes described above are all expressed in stripes by stage 9, at the beginning of the germ band extended phase.

The above model suggests that a null mutation in the gene encoding DPTP61F might have a segment polarity phenotype similar to that of embryos lacking *zw3*. In these embryos, the domain often expression is expanded toward the posterior, resulting in the replacement of denticle bands with naked cuticle (Siegfried et al., 1992). There are no candidate segment polarity genes in the vicinity of 61F. The DPTP61F gene may have been missed in previous screens for zygotic segment polarity mutations. It is also possible, however, that a segment polarity phenotype would only be observed in embryos lacking maternally contributed as well as zygotic DPTP61F activity, because the generalized early expression of DPTP61F mRNA suggests that it may be deposited in the egg. A number of other genes, including *zw3*, *disheveled*, and *porcupine* (Siegfried et al, 1992; Perrimon et al, 1989) are in this category of maternal-effect segment polarity genes. Mutations in such genes would not be recovered in standard screens for zygotic embryonic lethal mutations.

While this manuscript was in preparation, McLaughlin and Dixon published the sequence of DPTP61F, and showed that the protein exists in two forms. One of them (DPTP61Fm) is located on internal membranes, while the other (DPTP61Fn) is located in the nucleus. These two forms are encoded by alternatively spliced mRNAs. Our sequence corresponds to DPTP61Fm. Our work first showed that DPTP61Fm is expressed in a striped pattern. Subsequently, Ursuliok et al. (1995) published this striped expression pattern, showed that DPTP61Fn is not expressed in stripes, and

analyzed the control of the DPTP61Fm mRNA stripes by pair-ruled and segment polarity genes.

We thank Pantelis Tsoulfas for performing the Northern blot of figure 3, Sharon McLaughlin and Jack Dixon for communicating data before publication, Nicholas Brown for cDNA libraries, and Shane Sauby for technical assistance. This work was supported by National Institutes of Health grant #NS28182 to K.Z., as well as by Basil O'Connor Starter Scholar Research Award #5-816 from the March of Dimes Birth Defects Foundation, a Pew Scholars Award, and a McKnight Scholars Award.

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Figure 1. Nucleotide and predicted amino acid sequence of a cDNA clone encoding DPTP61F. The DNA sequence is numbered at the right. The amino acid sequence is indicated in one-letter code under the DNA sequence.

```
CCTCACTAAA GGGAACAAAA GCTGGTACCG CGCCCCCCCT CGAGGTCGAC      50
GGTATCGATA AGCTTGCTTG TTCTTTTTGC AGAAGCTCAG AATAAACGCT     100
CAACTTTGGG ACCTGCACCC CCCCCGGTCC AGCTCTAGTT CTCCTCTGAA     150
ATTAACCAGC CGCCTGATAA CCAAATTAAA AGTTATTAAA TCGGAACAAA     200
TCGCAGCAAA GTGTGCACAA TGAGCGAGCA GAAAACGAGT GGGAGTGGGT     250
                M  S  E  Q  K  T  S  G  S  G  S
CGGCGGCCGC TGCCCGTCTG CAAATCGAGG CGGAGTACAA GGACAAGGGG     300
 A  A  A  A  R  L  Q  I  E  A  E  Y  K  D  K  G
CCGCAATGGC ATCGCTTCTA CAAGGAAATT TGTGAGACCT GCGACCGGGA     350
P  Q  W  H  R  F  Y  K  E  I  C  E  T  C  D  R  E
GGCGAAAGAG AAGCAGTTCA GCACCTCGGA ATCGGAACGC CACACGAACC     400
 A  K  E  K  Q  F  S  T  S  E  S  E  R  H  T  N  R
GCGGCCTGAA TCGCTATCGG GATGTGAATC CGTACGATCA TTCCCGCATT     450
 G  L  N  R  Y  R  D  V  N  P  Y  D  H  S  R  I
GTTTTGAAGC GCGGCAGCGT AGACTACATC AATGCGAACC TGGTTCAGCT     500
V  L  K  R  G  S  V  D  Y  I  N  A  N  L  V  Q  L
GGAGCGCGCC GAGCGCCAGT ACATCCTGAC CCAAGGACGG CTGGTGGACA     550
E  R  A  E  R  Q  Y  I  L  T  Q  G  P  L  V  D  T
CAGTGGGCCA CTTCTGGCTG ATGGTATGGG AGCAGAAGTC CAGGGCTGTT     600
 V  G  H  F  W  L  M  V  W  E  Q  K  S  R  A  V
CTCATGCTCA ACAAGCTGAT GGAGAAGAAA CAGATCAAGT GCCACCTCTA     650
L  M  L  N  K  L  M  E  K  K  Q  I  K  C  H  L  Y
CTGGCCCAAC GAGATGGGAG CCGACAAGGC CCTGAAACTC CCCCACGTCA     700
 W  P  N  E  M  G  A  D  K  A  L  K  L  P  H  V  K
AACTCACCGT GGAGCTCGTC CGTCTCGAGA CCTACCAGAA CTTTGTCCGG     750
 L  T  V  E  L  V  R  L  E  T  Y  Q  N  F  V  R
CGATGGTTCA AACTAACCGA TCTCGAAACG CAGCAGAGTC GTGAAGTGAT     800
R  W  F  K  L  T  D  L  E  T  Q  Q  S  R  E  V  M
GCAGTTCCAC TACACCACAT GGCCGGACTT TGGCATTCCC AGCTCGCCGA     850
 Q  F  H  Y  T  T  W  P  D  F  G  I  P  S  S  P  N
ACGCATTCCT TAAGTTCTTG CAGCAGGTTC GCGACTCCGG CTGCCTCAGC     900
 A  F  L  K  F  L  Q  Q  V  R  D  S  G  C  L  S
CGCGACGTGG GTCCAGCCGT AGTCGACTGC AGTCCCGGAA TCGGCCGTTC     950
R  D  V  G  P  A  V  V  D  C  S  A  G  I  G  R  S
GGGCACCTTC TGCCTGGTGG ACTGTTGCCT GGTACTGATC GACAAGTACG    1000
 G  T  F  C  L  V  D  C  C  L  V  L  I  D  K  Y  G
GAGAGTGCAA TGTGTCCAAG GTGCTCTGCG AACTGCGCAC GTACCGCATG    1050
 E  C  N  V  S  K  V  L  C  E  L  R  T  Y  R  M
GGCCTCATAC AGACCGCCGA CCAGCTGGAC TTCTCCTACC AGGCGATCAT    1100
G  L  I  Q  T  A  D  Q  L  D  F  S  Y  Q  A  I  I
CGAGGGCATT AAAAAGCTGC ACGATCCCAC CTTTCTTGAC GCTGAACAAC    1150
E  G  I  K  K  L  H  D  P  T  F  L  D  A  E  Q  P
CCCTCATTAG CAACGACACA GAGACTCACA CACTGGATGA GCTGCCGCCG    1200
 L  I  S  N  D  T  E  T  H  T  L  D  E  L  P  P

CCATTGCCCC CACGCGTCCA ATCGCTTAAT CTGCCACTGG CCCCCAATTC    1250
P  L  P  P  R  V  Q  S  L  N  L  P  L  A  P  N  S
CGCCGGTATA CTTTCGCTCA ATATGCCGCC GGCCCAGGCT AATGGAGCTG    1300
 G  G  I  L  S  L  N  M  R  A  A  Q  A  N  G  A  E
AAAGTATCGG CAAAGAGCTG AGCAACCACG CCCTGAACAA CTTCATTAAT    1350
 S  I  G  K  E  L  S  N  H  A  L  N  N  F  I  N
CAGCACGACA TGATCCACGA TGCCGAGGTG GCCGACAGTC GTCCCTTGCC    1400
Q  H  D  M  I  H  D  A  E  V  A  D  S  R  P  L  P
GCCATTACCC GTAAGGGCTT TCAACGATTC GGACAGCGAT GAGGATTACT    1450
P  L  P  V  R  A  F  N  D  S  D  S  D  E  D  Y  L
TGCTGGATGA CGATGACGAG GACGACACCG ATGAGGATGA GGAGTACGAG    1500
 L  D  D  D  D  E  D  D  T  D  E  D  E  E  Y  E
ACCATTAACG AGCACGATGC CGATCCAGTT AATGGACACG TGCCCCGCAC    1550
T  I  N  E  H  D  A  D  P  V  N  G  H  V  P  A  T
AACACAGCCA CATGCCGACG ATGTTAATGC CAACAACGAG AAGCCAGCCG    1600
 T  Q  P  H  A  D  D  V  N  A  N  N  E  K  P  A  V
TGCCAGTCGA CGAGCAGCAC AAGGCGAACG GCATCGATCC AATTCCAGGC    1650
 P  V  D  E  Q  H  K  A  N  G  I  D  P  I  P  G
CAGCTCCCAG CCAGTCCCGA GAACGAGCTG AAGCGGCGAA AACGCAACGA    1700
Q  L  P  A  S  P  E  N  E  L  K  R  R  K  R  N  E
ATACCAGGCC AGCCTGGAGC AGAAGGTCAA CGACATGAAG CGGAAGCAGC    1750
 Y  Q  A  S  L  E  Q  K  V  N  D  M  K  R  K  Q  R
GGGAGAACGA GGACAAACAG CTGGCGGCAA AGAAACGAAG GTCATTACTC    1800
 E  N  E  D  K  Q  L  A  A  K  K  R  R  S  L  L
ACATATATTG CGCCTGGTGT TGTGGTGGGC GTGATCTGCG CCTACGCATA    1850
T  Y  I  A  A  G  V  V  V  G  V  I  C  A  Y  A  Y
CACGAAGCTA GGTTAAGTGC CCCGTAGCCA CTAAGTAGTC GTAGATCAGT    1900
 T  K  L  G
AGTAGCAATA GCTGTATGAC GTATTTAGTT GCCGTAGGAG ACCGATTCCT    1950
CCCCTCATAC CGATTTCCGA CTTCCGATCC CCACCTGCTC GTCCGCCTGA    2000
CCCGGCATCT AATGCGGGCA ACGAATCAAG GACGACGTGC AACGGATCGG    2050
AAAGGATCAG AGATCGGACG GAATCGCATC GCAAACGCCA TGCAATCGCA    2100
CACGAATTGC GGATGGCGGA TTGATTGAGC TGAACGAACG ATACAAGATG    2150
CATAAATATT AGGGAATTTT TTATATACGA AATCAAGAAG GAAAACAAAA    2200
GAACAAATG AAAGAAAAGA AGCAACTAAA CTAAACTAAC GAAAATCGG     2249
```

Figure 2. Alignment of the N-terminal DPTP61F amino acid sequence (indicated as 61F) with the homologous N-termini of the human PTP1B (indicated as pt1b) and human T cell PTP (indicated as tcpt) sequences. Each sequence is numbered at the right. Residues conserved in all three sequences are shaded in gray. Dashes indicate gaps introduced during alignment of the sequences.

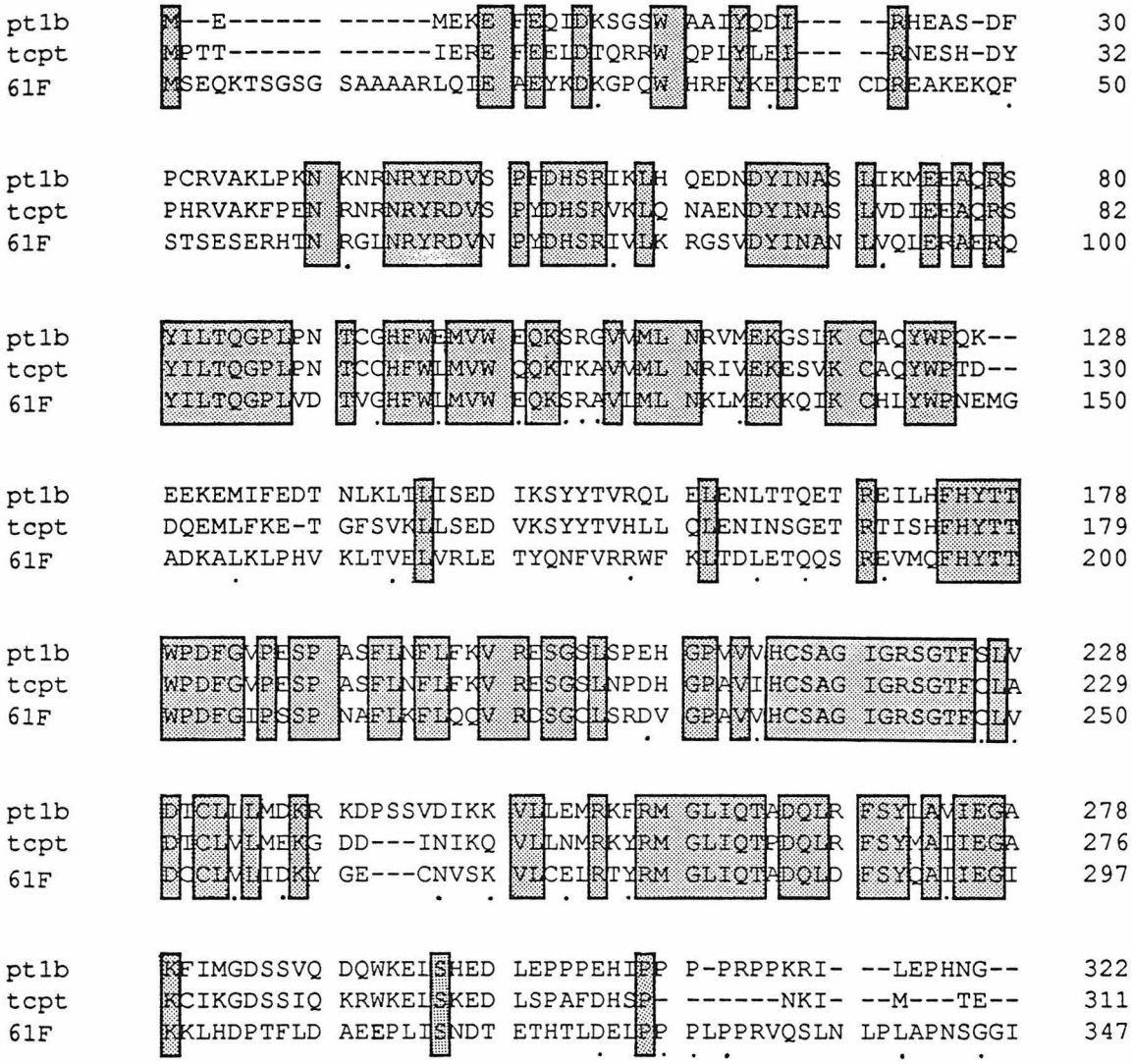

pt1b M--E------ ------MEKE FEQIDKSGSW AAIYQDI--- --RHEAS-DF 30
tcpt MPTT------ ------IERE FEEIDTQRRW QPLYLEI--- --RNESH-DY 32
61F MSEQKTSGSG SAAAARLQIE AEYKDKGPQW HRFYKEICET CDREAKEKQF 50
pt1b PCRVAKLPKN KNRNRYRDVS PFDHSRIKLH QEDNDYINAS LIKMEEAQRS 80
tcpt PHRVAKFPEN RNRNRYRDVS PYDHSRVKLQ NAENDYINAS LVDIEEAQRS 82
61F STSESERHTN RGLNRYRDVN PYDHSRIVLK RGSVDYINAN LVQLERAERQ 100
pt1b YILTQGPLPN TCGHFWEMVW EQKSRGVVML NRVMEKGSLK CAQYWPQK-- 128
tcpt YILTQGPLPN TCCHFWLMVW QQKTKAVVML NRIVEKESVK CAQYWPTD-- 130
61F YILTQGPLVD TVGHFWLMVW EQKSRAVLML NKLMEKKQIK CHLYWPNEMG 150
pt1b EEKEMIFEDT NLKLTLISED IKSYYTVRQL ELENLTTQET REILHFHYTT 178
tcpt DQEMLFKE-T GFSVKLLSED VKSYYTVHLL QLENINSGET RTISHFHYTT 179
61F ADKALKLPHV KLTVELVRLE TYQNFVRRWF KLTDLETQQS REVMQFHYTT 200
pt1b WPDFGVPESP ASFLNFLFKV RESGSLSPEH GPMVVHCSAG IGRSGTFSLV 228
tcpt WPDFGVPESP ASFLNFLFKV RESGSLNPDH GPAVIHCSAG IGRSGTFCLA 229
61F WPDFGIPSSP NAFLKFLQCV RQSGCLSRDV GPAVVHCSAG IGRSGTFCLV 250
pt1b DICLLLMDKR KDPSSVDIKK VLLEMRKFRM GLIQTADQLR FSYLAVIEGA 278
tcpt DICLVLMEKG DD---INIKQ VLLNMRKYRM GLIQTPDQLR FSYMAIIEGA 276
61F DCCLMHDKY GE---CNVSK VLCEMRTYRM GLIQTADQLD FSYQAIIEGI 297
pt1b KFIMGDSSVQ DQWKELSHED LEPPPEHIPP P-PRPPKRI- --LEPHNG-- 322
tcpt KCIKGDSSIQ KRWKELSKED LSPAFDHSPF- ------NKI- --M---TE-- 311
61F KKLHDPTFLD AEEPLISNDT ETHTLDELPP PLPPRVQSLN LPLAPNSGGI 347

Figure 3. Northern blot analysis of the temporal pattern of DPTP61F mRNA expression during embryonic development. The labels at the top of each lane indicate the ages of the embryo collections (in hrs. AEL) from which the RNA samples were isolated. 15 mg of total RNA were loaded in each lane of the gel. Standardization with control probes and staining of filters with methylene blue (data not shown) indicated that equal amounts of RNA were in fact present in each lane. The numbers at the left side of the figure indicate the sizes of the various mRNAs observed, as determined by comparison with RNA size markers.

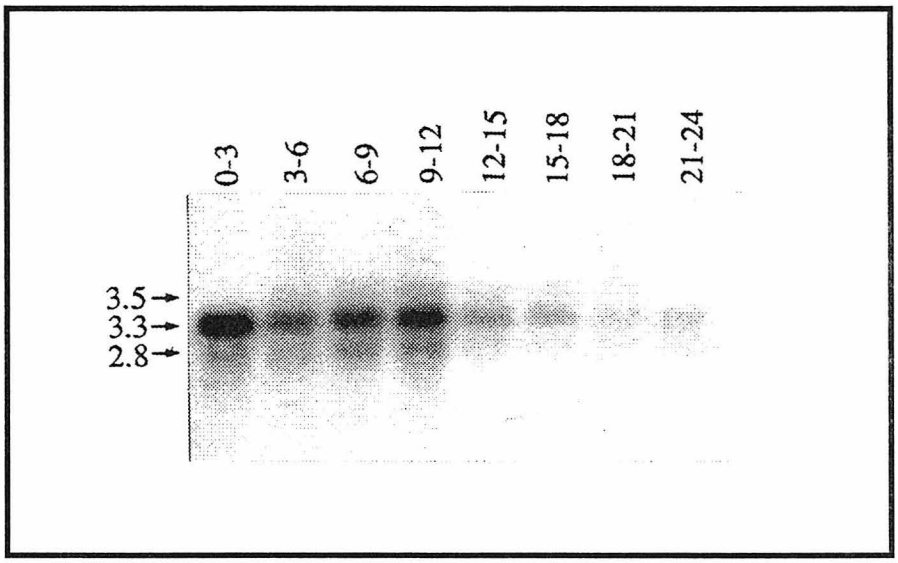

0-3
3-6
6-9
9-12
12-15
15-18
18-21
21-24
3.5
3.3
2.8

Figure 4. Spatial expression patterns of DPTP61F mRNA in the Drosophila embryo. Whole-mount embryos were hybridized to digoxigenin-labeled probes, and hybridization was detected using alkaline phosphatase immunocytochemistry. This generates a purplish reaction product, which corresponds to the dark areas seen in these photographs. All embryos are oriented with anterior to the left; A, B, and D are lateral views, with dorsal up. C is a ventral view. A. Stage 9 embryo, showing the patch of DPTP61F expression in the head (large arrowhead), and the forming segmental stripes (small arrowhead). B. Stage 10 embryo, showing the segmentally striped expression pattern. Two segmental grooves are indicated by small arrowheads. The borders of one stripe of DPTP61F expression are indicated by dots. The patch of expressing cells in the head is also indicated by a large arrowhead. C. Ventral view of stage 10 embryo. Two stripes are indicated by arrowheads. D. Stage 13 embryo. The brain, which is darkly stained, is indicated by a large arrowhead. The ventral nerve cord runs along the bottom of the embryo. There is also dark staining in the gut and yolk granules. The ventral ectoderm does not express DPTP61F. The small arrowhead points toward the strip of cells on the dorsal side of the pharynx which express DPTP61F mRNA at high levels. Note the sharp dorsal border of this zone of expression. (The arrowhead is at the dorsal edge of the embryo, so there is a row of nonexpressing cells between the point of the arrowhead and the expressing pharyngeal cells. These are difficult to see in the photograph.)

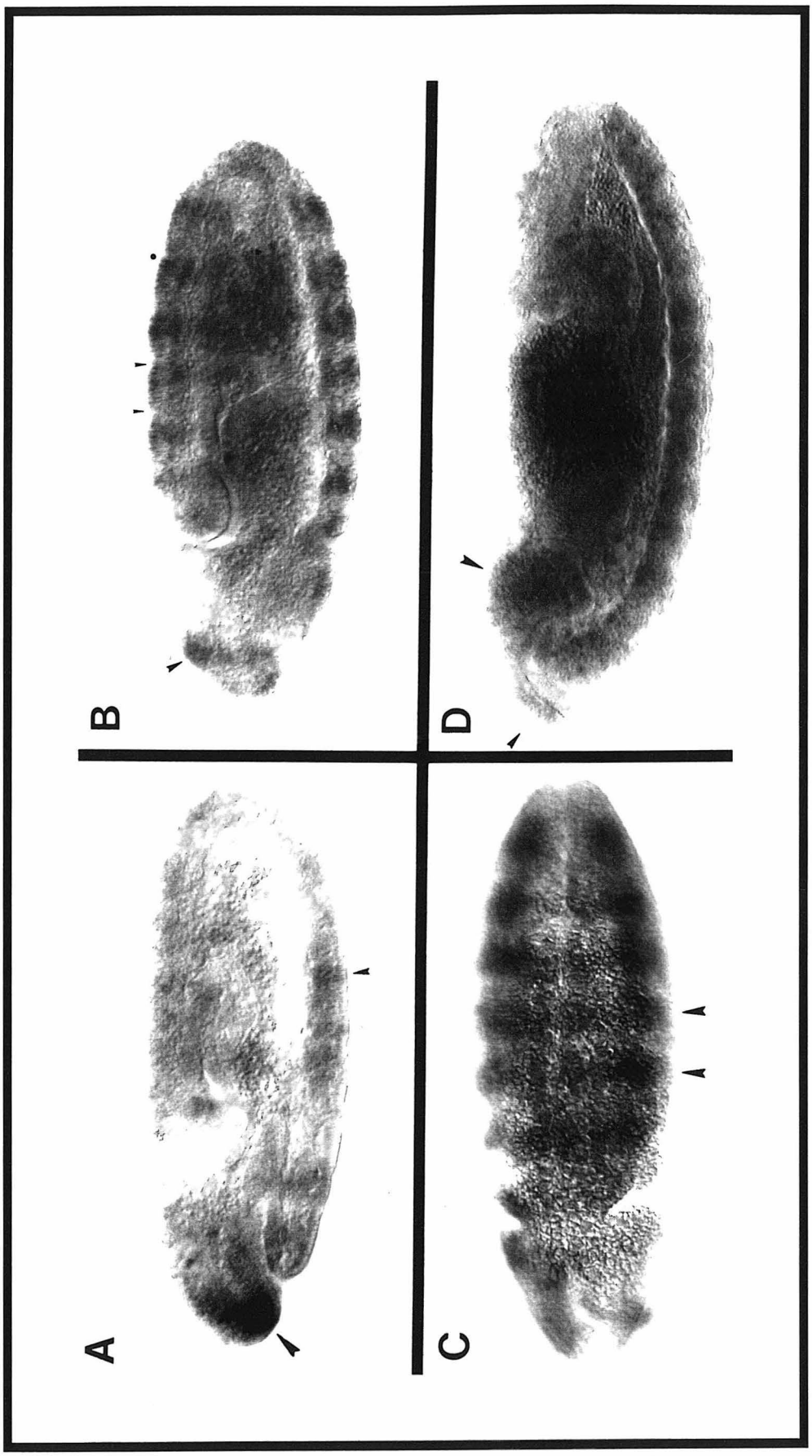
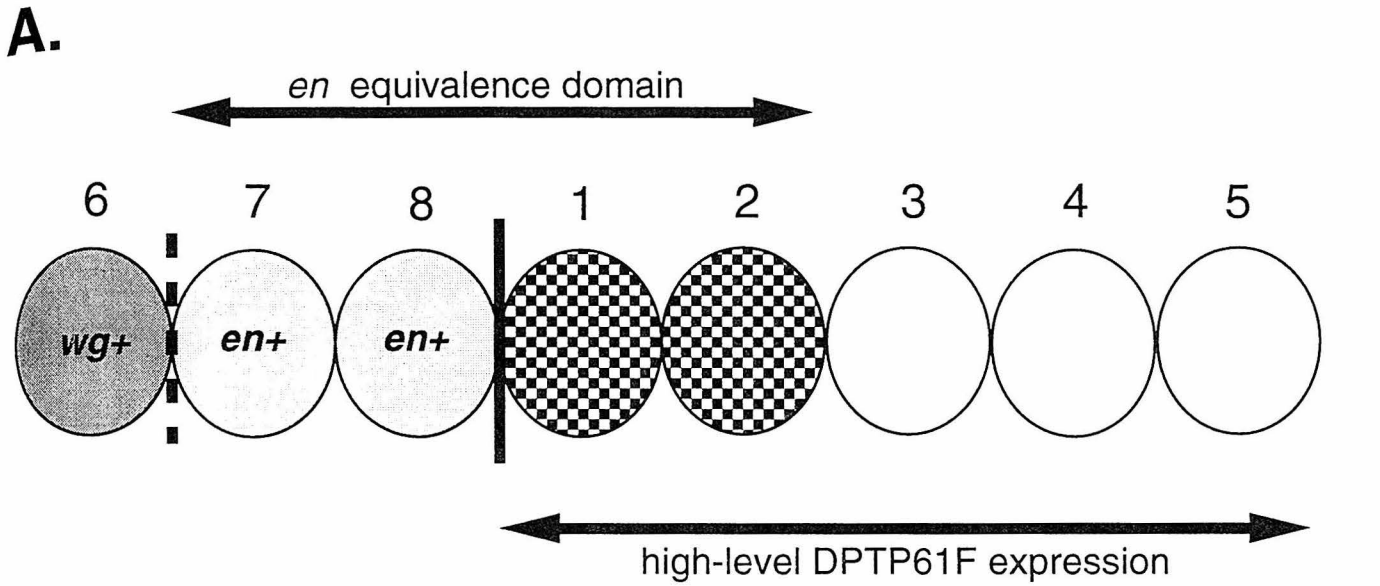
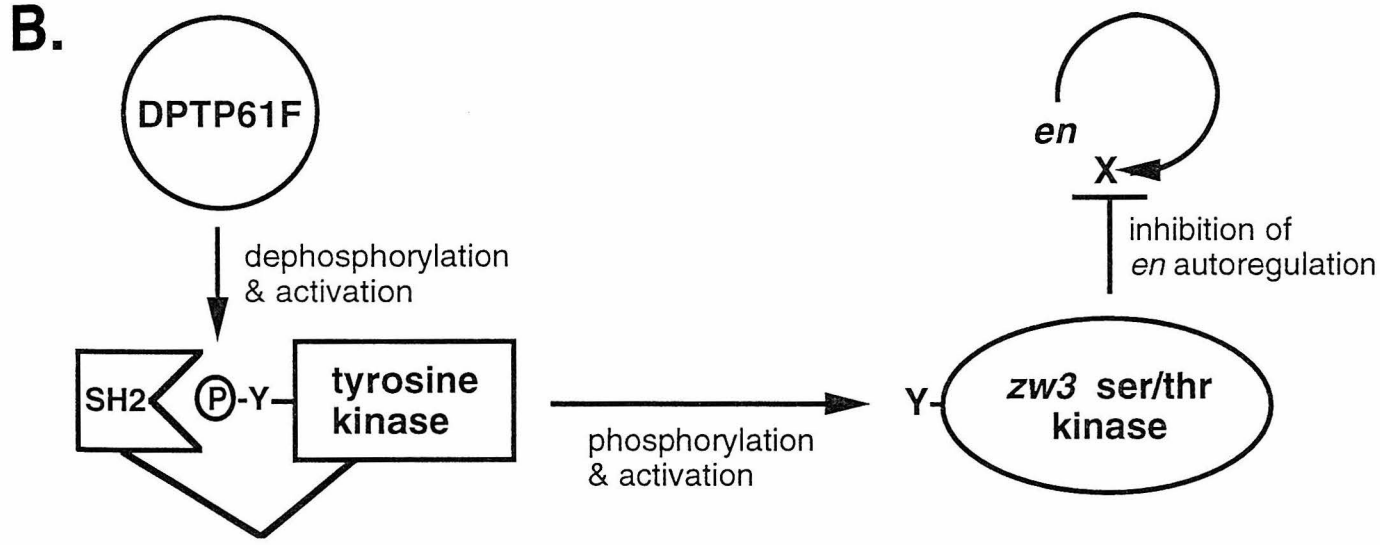

A
B
C
D

Figure 5. A. Schematic diagram of the expression pattern of DPTP61F within an eight cell long segment of the germ band extended embryo (after Peifer and Bejsovec, 1992; Siegfried et al., 1992. Noordermeer et al, 1992 depicts segments at this stage as 12 cells in length). The essential elements of the expression patterns shown are not the exact numbers of cells in each domain, but the borders of these domains relative to the segmental (dark vertical line) and parasegmental (dotted vertical line) boundaries. Eight cells are shown in two adjacent segments, and cells are indicated by the numbered circular outlines. Cell 6 of the anterior segment is on the left, and cell 5 of the posterior segment at the far right. The region of high-level DPTP61F expression is indicated by the dark line below the cell outlines. The line above the cell outlines indicates the region within which cells are competent to express *engrailed* (the *en* equivalence domain). In these cells (#s 7,8,1,2) *zw3* represses en autoactivation unless it is inactivated by the *wg* signal emanating from the *wg*-expressing cell 6 (indicated as *wg*+ and shaded in dark gray). Cells 7 and 8 (*en*+-light gray) normally receive enough *wg* signal to inactivate *zw3*, so *en* is expressed in these cells. Cells 1 and 2 (checkerboard pattern) are too far from the *wg*-expressing cell, and so do not normally express *en*. However, in a *zw3*-null embryo these cells ectopically express *en* (Siegfried et al., 1992). The expression of DPTP61F in cells 1 and 2 suggests that it may be involved in keeping *zw3* active, and thus in maintaining repression of *en*. B. A hypothetical model, as discussed in the text, for the role of DPTP61F in maintaining *en* repression. DPTP61F is depicted as dephosphorylating an SH2-domain containing tyrosine kinase, by analogy with CD45 dephosphorylation of *lck*. In this case, dephosphorylation is thought to cause dissociation of the SH2 domain, which occludes the active site when bound to the autophosphorylated tyrosine (reviewed in Cantley et al., 1991).


A.
en equivalence domain
6
7
8
1
2
3
4
5
wg+
en+
en+
high-level DPTP61F expression


B.
DPTP61F
dephosphorylation
& activation
SH2
P-Y
tyrosine
kinase
phosphorylation
& activation
Y
zw3 ser/thr
kinase
en
X
inhibition of
en autoregulation

Chapter III

Cloning of a grasshopper homologue of *Drosophila single-minded* gene, and analysis of its midline-specific expression patterns during grasshopper embryogenesis

Te-Yi, Kung; Kaushiki, Menon, Kai, Zinn

Summary

The overall structure of the embryonic CNS in the grasshopper (*Schistocerca americana*) and fruit fly (*Drosophila melanogaster*) is very similar. However, in grasshopper the cell bodies of neurons are much larger, and individual axon tracts are also much more easily visualized. The similarity of CNS structures in these two organisms extends to the axon trajectories and gene expression patterns of individual identified neurons. Recent technical advances have allowed the molecular mechanisms involved in grasshopper embryonic development to be determined (Condron and Zinn, 1994 a and b, 1995), we decided to clone the grasshopper homologue of *Drosophila single-minded* (*sim*) gene. We hope to characterize the functions of this so called "master regulator of CNS midline development" gene by taking advantage of the grasshopper system, in which a much better microscopic resolution can be achieved. With this in mind, we would like to elucidate how individual identities of cells are established through *sim* regulated embryonic morphogenesis.

Mutations in the *Drosophila sim* gene result in the loss of precursor cells which give rise to the midline cells in fly embryonic CNS. Sim protein belongs to the bHLH-PAS transcription factor family, and either activates or represses transcription of different midline cell-fate-determining genes, such *as rhomboid, Toll, engrailed, slit, spitz, Star, orthodenticle*, etc. *sim* directs the establishment of most, if not entire, *Drosophila* CNS midline cell identities. Using a 660 b.p. probe from the amino-terminal sequence of *Drosophila sim*, we screened a grasshopper embryonic cDNA library under low stringency, and obtained two clones one of which harbored a full-length DNA sequence homologous to *Drosophila sim*. We named this *sim* homolog *Gsim* to indicate its grasshopper origin. Like all other *sim*s identified so far, *Gsim* encodes a protein

containing the highly conserved bHLH-PAS domains in the N-terminal half to serve its role as a gene-specific transcription factor. However, the carboxy-terminal halves of *sim* proteins share no similarities among *Drosophila*, grasshopper, and mammals, though they all contain peptide motifs consistent with their roles as transcription regulators. The sequence comparison among *Gsim*, *Drosophila sim* and mammalian *sim*s reveals that *Gsim* is more similar to the human *SIM2* gene than to its *Drosophila* homologue. This similarity can be of special interest, because human *SIM2* has been recently identified as one of the genes located within the Down syndrome critical region. Murine *Sim1* and *Sim2* both have stereotypical expression patterns in the embryonic and adult mouse brain. In this chapter we describe the cloning of *Gsim* and its RNA expression patterns throughout grasshopper embryogenesis. We will also compare these expression patterns to those of the fly, murine and human *sim* homologues; finally, we will discuss the possible functions of this highly conserved gene using protein sequence comparison analysis based on the genebank database.

Introduction

single-minded (*sim*) was first identified through analysis of a deletion mutation which drastically altered the pattern of *Drosophila* embryonic neuronal precursor cells (Crews et al. 1988; Thomas et al. 1988), *sim* is now denoted as the "master regulatory gene" of *Drosophila* midline development, based on the observations that *sim* is required for midline expression of a group of genes including *slit, Spitz, Star, Toll, rhomboid, cdi, engrailed,* etc. (Nambu et al. 1990; Nambu et al. 1991; Muralidhar et al. 1993; Kim, S.H. and Crews S.T. 1993; Golembo et al. 1996). Loss of *sim* function leads to the absence or mislocalization of several classes of midline neurons and glia, and results in the fusion of the two longitudinal tracts of *Drosophila* CNS. It is from this phenotype that *single-minded* was given its name.

In 1996, two murine *Sim*s were also cloned (Ema et al. 1996; Fan et al. 1996; Moffett et al. 1996; Yamaki et al. 1996), followed by the identification of two human *SIM*s in 1997(Chrast et al. 1997). Now we report the successful cloning of *Gsim,* another *single-minded* homologue, from the grasshopper *Schistocerca americana.*

Grasshopper and *Drosophila* represent two extremes in insect developmental patterns. Grasshopper is a hemimetabolous insect with an extreme "short-germ band" mode of development, while *Drosophila* is an advanced, long-germ band, holometabolous insect. We wished to clone *Gsim* to provide a valuable evolutionary comparison of midline development in these two species. In addition, we hoped to visualize *sim* expression at a higher resolution, due to the larger size of grasshopper cells. Finally, these studies set the stage for experiments to manipulate *sim* expression in single lineages and observe effects on midline development.

The *sim* genes have attracted interest because of their unique role in embryonic CNS development. Now they have become even more interesting, as one of the two human *sim* genes is potentially involved in the pathogenesis of Down syndrome (DS)(Dahmane et al. 1995).

Consistent with its multi-gene regulatory function, *sim* belongs to the basic helix-loop-helix (bHLH) family of transcription factors, which are known to regulate the differentiation of various cell types in fly neurogenesis, as well as in vertebrate myogenesis (reviewed by Jan and Jan 1993). The bHLH domain in *sim* is a 49 amino acid section at the N-terminus, which is followed by another highly conserved domain, termed PAS. PAS is a ~250 amino acid sequence originally found in Period, ARNT, and *sim* proteins (Nambu et al. 1991; Hoffman et al. 1991). Many other bHLH-PAS proteins have been identified since then, and four molecular functions of bHLH-PAS proteins have been inferred (reviewed by Hankinson 1995; Schmidt and Bradfield 1996; Zelzer et al. 1997): (1) The basic domain contributes to sequence-specific DNA binding; (2) the HLH and the PAS domains together form a dimerization interface; (3) ARNT seems to be the universal dimerization partner for other bHLH-PAS proteins; and (4) amino acid sequences carboxy-terminal to each bHLH-PAS domain appear to participate in transcriptional activation or repression of the target genes.

Murine and fly *sims* can dimerize with ARNT or *tango* (*Drosophila* ARNT homolog)(Swanson et al. 1995; Ema et al. 1996; Probst et al. 1997) and bind to their cognitive DNA sequence, called the CME (CNS midline element; Wharton et al. 1994). However, while the fly *sim* can both activate and repress transcription through its carboxyl terminus (Franks and Crews 1994; Xiao et al. 1996), the murine *sim*s seem to function only as repressors in transient transfection assays (Ema et al. 1996; Probst et al.

1997). The carboxyl termini of fly and murine *sim*s are not conserved, which is consistent with their observed divergent activities.

So far, few approaches could be taken to study the functions of human *SIM* genes. However, due to the high similarity to their mouse counterparts throughout the entire coding region, human *SIM*s are suggested to also dimerize with ARNT, and work through a repressive regulation mechanism. Nevertheless, it remains to be determined whether the *SIM* proteins function exclusively as repressors in mammals.

In *Drosophila*, *sim* is expressed in the CNS midline cells. Cell fate experiments have established that *sim* is required for early events in midline cell development, including synchronized cell division, proper formation of nerve cell precursors, and positive autoregulation of its own midline expression. Induction of ectopic *sim* protein under the control of the hsp 70 promoter shows that *sim* can direct cells of the lateral CNS to exhibit midline cell morphology and gene expression patterns. These observations have led Steve Crews and his colleagues to conclude that *sim* functions as a "master regulatory gene" for *Drosophila* CNS midline development (Nambu et al. 1990; Nambu et al. 1991).

In humans, Down syndrome (DS)/trisomy 21 is the most frequent genetic cause of mental retardation. Although most cases of DS can be explained by the trisomy of chromosome 21 (existence of three full copies of chromosome 21), there are also rare patients who carry DS by chromosomal rearrangements resulting in triplication of partial chromosome 21. Molecular characterization of these "partial trisomy" cases has allowed the delineation of a DS critical region (DSCR) located at the sub-band 21q22.2, and the correlation of this region with many DS abnormalities (Delabar et al. 1993). Using the exon-trapping technique to isolate potential coding sequences within this region, two

groups have identified exons that predict an open reading frame highly homologous to the *Drosophila sim* gene product (Chen et al. 1995; Dahmane et al. 1995).

Mouse models mimicking the chromosome 21 trisomy reveal neurological abnormalities identical to human DS symptoms, including abnormal behavior and age-related degeneration of some cholinergic neurons.

Here we report the cloning of a grasshopper homologue of *Drosophila sim* gene, named *Gsim* to describe its origin from grasshopper. Although *Gsim* has an overall length similar to that of fly *sim* (674 in grasshopper, 673 in fly), its amino terminal half which contains the highly conserved bHLH-PAS domains is 23 amino acids shorter than that of fly *sim*, but only two amino acids longer than those of mammalian *sim*s (*Sim*1/2 in mouse, *SIM*1/2 in human). In fact, *Gsim* possesses a higher percentage of identical amino acids to the mammalian *sim*s than to the *Drosophila sim*. This homology is especially pronounced when it is compared to the human *SIM2* (the gene located within the DSCR) in the view of overall similarity in the conserved N-terminal halves, or in any of the bHLH, PAS1, PAS2 or HTS domains (the latter three are all contained in the PAS domain).

Whole mount in situ hybridizations of *Gsim* RNA probe to grasshopper embryos display expression patterns which initiate at a stage of 33% embryonic development, continue on until stage of 45%, and become undetectable by 50%. *sim* mRNA expression patterns are detected in one to two rows of cells along the midline of the ventral nerve cord, including the median neuroblast (MNB), which is the precursor cell giving rise to most midline neurons and glia in grasshopper.

Results

A 3.8 kb cDNA fragment encodes a 674 amino acid bHLH-PAS protein

Considering that the bHLH domain in *Drosophila sim* is also conserved in many non-*sim* transcription factors of the HLH family, when we decided to clone the grasshopper homologue of *Drosophila single-minded*, we chose a 660 base pair stretch devoid of the N-terminal bHLH region as the probe, in order to minimize background that is often observed under low stringency hybridization conditions. From $5x10^5$ viral plaques transfected from a λ ZapII grasshopper 40-45% embryonic cDNA library kindly provided by M.J., Bastiani, we obtained two positive clones which consistently showed intense signals after three rounds of sub-screening. We sequenced one clones which contained an insert of 3.8 kb. The amino-terminal half of this clone is homologous to *Drosophila single-minded.* We thus identified this clone as a cDNA encoding the grasshopper *sim* homologue, referred to hereafter as *Gsim.*

Gsim encodes a protein of 674 amino acids, as shown in figure 1. Immediately downstream of the starting methionine is a 49 amino acid stretch which represents the basic helix-loop-helix (bHLH) motif, first characterized by Murre et al., in 1989 (for reviews, see Murre et al. 1989; Benezra et al. 1990). This motif is composed of two amphipathic helices separated by a variable loop. Found in a wide variety of transcription factors such as the mammalian proteins *Myc, Max, MyoD, and E2A*, as well in *Drosophila* proteins *Achaete-scute, Daughterless*, *Trachealess* (*trh*), etc., the bHLH structure has been demonstrated to harbor subdomains that play roles in both DNA binding (basic region) and protein dimerization (HLH). The other important motif found in *Gsim* is the PAS domain of about 250 amino acids which extends from amino acid residues 98 to 335 in *Gsim*. First characterized by Crews et al. (1988), PAS was described as a region that

contains two direct repeats of ~50 amino acids, named PAS1 and PAS2 respectively, that function to confer target gene specificity (Zelzer et al., 1997).

As illustrated in figure 1, the bHLH and PAS domains together represent almost the entire N-terminal half of the *Gsim* protein, which is also the highly conserved region among different *sim*s identified so far (see next section for comparisons). However, the *Gsim* C-terminal half starting around amino acid residue 350, like those of other *sim*s, shares no significant similarity with any other protein in the database. The serine, threonine, glutamine and proline rich regions in *Dsim* identified as transcriptional activation domains (Franks and Crews,1994) are not conserved in *Gsim* in an alignable fashion. However, the c-terminal half of *Gsim* protein is also serine / threonine / proline rich. As sequences rich in these amino acids have been shown to function as activation domains in other transcription factors (Mitchell and Tijan, 1989), this part of *Gsim* might function like the corresponding regions of *Dsim*.

Similarities among *Gsim* and other related proteins

As a member of the *sim* gene family, *Gsim* shares overall identity of > 76% at the amino acid level in the amino terminal half when compared with other *sim*s. However, it is unexpected that *Gsim* bears a stronger resemblance to its mammalian homologues than to *Drosophila*'s. In figure 2a and 2b we represent alignments and trees comparing *sim*s and other bHLH-PAS proteins in individual homologous domains. It is worth mentioning that between the two repeats of PAS1 and PAS2 in the PAS domain, there is a stretch of 39 amino acid residues (S140 to I178) in *Drosophila sim* which feature the most divergent region in all the homologous N-terminal halves of *sim*s. However, in grasshopper, this divergent region was reduced to 16 amino acid residues (S141 to F156), closer to the

length of 14 residues in other mammalian sims (T141 to Y154 in *hSIM2*). In fact, as shown in all the comparisons in different domains, *Gsim* shares a higher homology with mammalian *sim*s than with the *Dsim* (see introduction).

The *Gsim* RNA expression patterns in grasshopper midline

Gsim mRNA expression is first observed at the midline in 33% grasshopper embryos (figure 3a). Later, in 40% embryos, *Gsim* expression is restricted to median neuroblast (MNB) and its neuronal progeny, the H cell (MP3 progeny), and anterior midline glia. Figure 3b shows an overall view of the CNS midline in a 40% grasshopper embryo, while figure 3c is a close-up view which allows the identification of individual neural cells, including the MNB, the anterior commissure (AC) and the posterior commissure (PC)(indicated by arrowheads). *Gsim* expression disappears by 50% of grasshopper embryonic development (result not shown).

Discussion

Sims as the master regulator of CNS midline development

Within the embryonic CNS of *Drosophila*, there exist specialized groups of cells that lie along the midline of the ventral nerve cord and are characterized by a number of unique anatomical, developmental, and morphological properties (Klambt et al., 1991). These midline cells consist of both neurons and glia, and define a discrete cellular substructure that plays an important role in the proper elaboration of the axon scaffold during CNS formation. In the blastoderm, the midline cells arise as two single-cell-wide stripes that occupy a characteristic position between mesoderm precursors and lateral neuroectoderm precursors. As gastulation proceeds, the two stripes of midline cells are brought together along the ventral furrow to form a single column. The midline cells constitute a discrete mitotic domain and undergo a synchronous postgastrulation cell division. During neurogenesis, the midline cells extend their nuclei into the nerve cell precursor layer, yet characteristically retain cytoplasmic connections with the underlying epidermis. They later differentiate into 25-30 neurons and glia per segment, which constitute the axis of symmetry for the ventral nerve cord.

First identified as one of the mutations that disrupt specific features of embryonic neuronal development, the *single-minded* gene is suggested to play an important role in the development of midline cells and the formation of the fly CNS. Mutations in *sim* are embryonic lethals, characterized by fusion of the longitudinal axon bundles and collapse of the axon scaffold (Thomas et al., 1988).

The *sim* genes themselves encode nuclear proteins which define a new subclass of the bHLH-PAS family of transcription factors, in which the bHLH domains are believed to be involved in DNA binding and protein dimerization, while PAS domains are

concerned with specific gene targeting. Thus, the *sim* proteins are thought to function by transcriptionally regulating other genes important for development of the midline cells, and are considered the master regulators of the CNS midline development.

a) The information revealed by the midline expression patterns of *sims*

Consistent with its predicted role as a master regulatory gene of CNS midline development, the *sim* transcripts are first observed in the midline progenitors of the *Drosophila* blastoderm embryo at cellularization (figure 4a), while the protein is detected within nuclei following gastrulation along the ventral midline of the ectoderm at stage 6-7 (Thomas et al., 1988; Crews et al., 1988). *Sim* continues to be expressed in all of the midline progenitors until they differentiate into neurons, glia, and other non-neuronal cells. At this point, detectable expression becomes restricted to the midline glia.

In the fly *sim* null mutants, the expression of *slit*, *Toll*, *rhomboid*, *engrailed*, and *spitz* all become undetectable or significantly reduced (Nambu et al., 1990). In fly embryos with ectopic *sim* expression, not only do all the CNS cells express midline genes, but they also possess extended nuclei and long cytoplasmic projections (figure 4b). Furthermore, there is a lack of ventral epidermis in these embryos in which *sim* has been ectopic expressed, suggesting there is a transformation of ventral ectodermal cells into CNS midline cells which results in an overproduction of the CNS midline cells at the expense of both lateral CNS and ventral epidermis (Nambu et al., 1991). Based on these results, Nambu et al. proposed that *sim* might regulate the expression of a battery of genes that act in the development of fly CNS midline cells.

In the grasshopper, we performed whole mount *in situ* hybridization using a probe against *Gsim*. The expression of *Gsim* is first observed in the CNS midline of 33% grasshopper embryos, which is about the stage when the first pioneer neurons of CNS are

extending their growth cones. Later in 40% embryos, *Gsim* expression is restricted to the MNB and its neuronal progeny, H cell (MP3 progeny) and in anterior midline glia. At this stage, the longitudinal axon bundles of CNS have extended along the entire length of the grasshopper embryo, and commissural axon tracts can be seen in all segments as well (Bentley et al., 1979. These longitudinal and commissural axon bundles establish the major scaffold of the CNS. *Gsim* midline expression continues until stage 47% of grasshopper embryonic development, a stage when the entire CNS ladder scaffold has been completed, then disappears by the stage of 50% (result not shown).

Although exactly parallel staging of *Drosophila* and grasshopper embryos is not possible given their different modes of development, the expression patterns of *Dsim* and *Gsim* apparently start and terminate in a similar fashion, judging from the very similar expression patterns of CNS cell markers such as engrailed and evenskipped in *Drosophila* and grasshopper (data not shown), we thus propose that *Gsim* might play a similar role in the grasshopper midline cell fate determination to *Dsim* in *Drosophila.*

Considering the highly conserved protein sequences, it is not surprising that the expression patterns of murine *Sim* genes during development are reminiscent of those of the *Drosophila* and Grasshopper *sim*s. For example, figure 4d shows that the mouse *Sim2* is expressed in the developing ventral diencephalon, including its midline, while *Sim1* is expressed in regions immediately adjacent to the ventral midline of the diencephalon and of the spinal cord (figure 4e).

b) bHLH, PAS and the C-terminal half of the *sim* proteins in transcriptional regulation

All the *sim* proteins identified so far share a very similar structure: the amino-terminal half consists of a bHLH domain followed by the PAS domain, both highly

conserved from insects to mammals. However, the carboxyl-terminal halves of *sim* proteins are highly divergent among fly, grasshopper and mammals, although they all contain different types of peptide motifs that could potentially serve a role in transcriptional regulation.

By expressing *Dsim* protein in fly salivary gland nuclei using a heat-shock inducible *Hsp70* gene promoter, Muralidhar et al. (1993) has demonstrated that *sim* protein associates with the *sim* late promoter on polytene chromosomes. In addition, *sim* protein also directly binds to a small number of additional chromosomal sites, among which are the promoter regions of the *47F* gene, *Toll, and cdi.* Since the expression of all these four gene is *sim*-dependent, *sim* is likely to be a transcription factor.

In fact, the transcriptional enhancers of *sim, Toll,* and *slit* genes all containing the CNS midline element (CME), a conserved 5-base pair sequence (ACGTG), which is absolutely required for the transcription of genes expressed in fly CNS midline. CME has been proposed to be the target sequence of the *sim* bHLH domain (Wharton et al. 1994).

However, if the CME represents the DNA sequence *sim* protein recognizes and binds to, there must be another mechanism which provides the target gene specificity.

One of the best characterized bHLH-PAS proteins is the aryl hydrocarbon receptor (AHR), which is an inducible transcription activator when bound to its ligand dioxin. Upon ligand binding, the receptor-ligand pair translocates into the nucleus, forms a heterodimer with another bHLH-PAS protein — the AHR nuclear translocator protein, Arnt -- and activates a specific set of target genes (reviews by Hankinson 1995; Schmidt and Bradfield 1996).

When *tango* (or dArnt), a *Drosophila* homologue of ARNT was isolated, it was shown to dimerize with *Dsim* and another *Drosophila* bHLH-PAS protein *trachealess* which shares very similar DNA binding sites with *Dsim*. The interesting experiment,

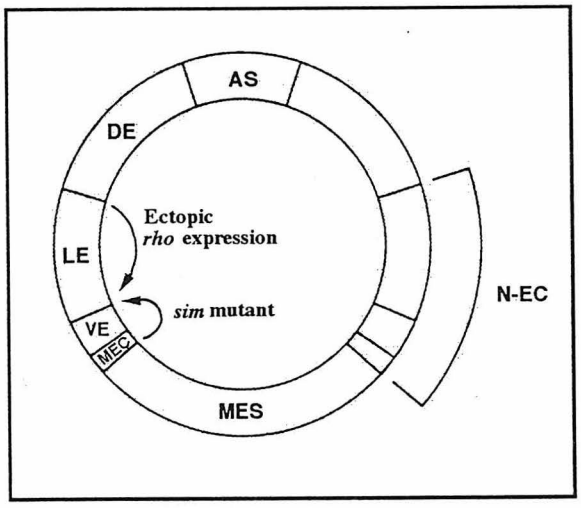
however, was done by making *Trh-Sim* chimera proteins, in which when the *Trh* PAS domain was replaced by the analogous region of *sim*. The resultant chimera protein could replace function of *sim* protein (Zelzer et al., 1997). This observation suggested that the ability of bHLH-PAS heterodimers to associate, through the PAS domain, with additional distinct proteins that bind target-gene DNA, is essential to confer specificity.

In mouse, two *Sim*s were also shown to dimerize with mArnt in the yeast two hybrid analysis and coimmunoprecipitation experiments (Ema et al., 1996; Probst et al., 1997). However, when the carboxy termini of *mSim*s were constructed into the BOS expression vector, and cotransfected with GAL DBD-Arnt into the COS cells along with the pG5EC reporter gene, CAT expression was markedly reduced in a dose-dependent manner, while the cotransfection of a control *Dsim* plasmid with GAL DBD-Arnt and the reporter pG5EC resulted in a maximal threefold increase over the basal CAT activity (Ema et al., 1996). Since in fly germ line transformation experiments three individual transcription activating regions within the carboxy terminus of *Dsim* have been identified (Franks, R.G. and Crews, S.T.) suggesting *Dsim* functions as a positive regulater, the *mSims* might be the first example of bHLH-PAS proteins with transcriptional repressing activity through their carboxy-terminal sequences.

However, as studied in many other transcription factors (Reviewed by Mitchell, P.J. and Tjian, R.; 1989), protein sequences rich in serine, threonine, glutamine and proline often serve as transcriptional activation domains, which are exactly what we find in the *Dsim* and *Gsim*, but not in the analogous regions of mammalian *sim*s. This difference might explain why insect *sim*s and mammalian *sim*s regulate transcription in opposite manners.

c) A possible mechanism of how *sim* plays its master regulatory role in the CNS midline development, and beyond

To explain the mechanism , we start with the blastoderm fate map. Shown in figure 6a and here are schematic cross sections representing the cellular blastoderm with the dorsal part of the embryo at the top of the drawing (adopted from fig1 by Kim and Crews; 1993). AS, aminioserosa; DE, dorsal epidermis; LE, lateral epidermis; VE, ventral epidermis; MEC, mesectoderm; MES, mesoderm. The neuroectoderm (N-ECT) gives rise to both the ventral-lateral epidermis and the CNS. It includes the blastoderm precursors that give rise to the LE, VE and MEC. The MEC gives rise to the midline cells of the CNS.



In the cellular blastoderm stage, the neurogenic ectoderm specifies the region of blastoderm precursors that will give rise to lateral epidermis (LE), ventral epidermis (VE), and the mesectoderm (MEC) where the midline cells of the CNS arise. It is worth mentioning at this point that almost the entire neuroectoderm can potentially respond to *spitz* signaling and activate the ventral epidermal development pathway. This hypothesis comes from the observation that in the *sim* mutant, the mesectodermal cells which normally constitute the midline adopt the fate of neighboring ventral ectodermal cells (Xiao et al.; 1996). Ectopic expression of *rhomboid*, a key component in the *spitz* signaling pathway required for the normal VE fate, can result in a lateral-to-ventral neuroectodermal cell fate transformation (Xiao et al., 1996). Thus, coth LE and MEC can take on the VE identity

At this stage, the initial specification of the mesectoderm stripes requires 1) the dorsal/ventral patterning genes such as *snail* to be expressed in mesoderm to repress *sim* expression (Nambu et al., 1990), and 2) cell/cell interactions with adjacent mesodermal cells mediated by the *Notch* signaling pathway, which, together with many other neurogenic genes such as *Delta, Enhancer of split*, *neuralized*, *big brain,* and *mastermind*, was also shown to repress *sim* expression in the mesodermal muscle precursor cells (Lewis and Crews, 1994).

In summary, the repression of *sim* expression at regions other than mesectoderm is required for the mesectodermal fate specification. Expression of *sim* and hence the activation of *sim*-dependent midline genes, including *slit*, *Toll*, *rhomboid*, *engrailed*, and *center divider*, in the mesectodermal cells (Nambu et al., 1990) mark this region to take on the midline MEC fate.

In the ventral ectoderm located adjacent to the mesectoderm, activation of *spitz* signaling is responsible for ventral cell fates. *rhomboid* and *Star* are thought to be required

for processing of the membrane bound Spitz precursor to release soluble Spitz, a ligand of DER, in order to activate the signaling for the ventral cell fates (Kolodkin et al., 1994). Sereted Spitz was identified as the only *sim*-dependent element contributed by the midline to ectoderm patterning, so it was concluded that the midline is the site of *spitz* processing (Golembo et al., 1996a). In other words, the proper expression of *sim* promotes the correct expression patterns of *rhomboid* and *Star*, which then process the ubiquitously expressed membrane Spitz precursor, to release the functional soluble Spitz into the intercellular matrix, and thereby to activate the ventral cell fate in the adjacent ventral ectoderm (figure 6b).

The contribution of *sim* patterning to the lateral ectoderm comes from the activation of *argos*, the repressor of Spitz signaling pathway, being induced by activation of Spitz/DER signaling in the ventral ectoderm (Galembo et al. 1996b). The diffusion of Argos to the neighboring lateral ectoderm then inhibits or reduces Spitz signaling there, thus determining lateral epidermal cell fates(figure 6b).

In summary, the expression of *sim* gene exclusively in the mesectoderm at the blastoderm stage, is determined through the activation and repression by dorsal/ventral patterning genes such as *dorsal, twist,* and *snail* in the mesoderm. The proper expression of *sim* in midline then activates a group of *sim*-dependent midline genes, including *rhomboid*, which is involved in the processing of the Spitz precursor. The soluble Spitz diffuses to the neighboring ventral ectoderm, activates the DER/Spitz signaling pathway which is required for the patterning of ventral epidermal cells. The DER/Spitz signaling, however, does not contributes to ventral cell fates in the *sim*-expressing midline. DER/Spitz signaling triggers expression of the DER antagonist *argos*, which then diffuses to the lateral ectoderm, to block the DER/Spitz signaling so that these cells can adopt the lateral cell fates.

d) *SIM*s and the Down syndrome

The fact that *SIM2* gene maps to the 21q22.2 Down syndrome critical region (DSCR) raises the possibility that *SIM* contributes to the DS phenotype when triplicated.

Mouse models have been generated by several groups to gain insight into the molecular basis of DS. One such model in which the Ts65Dn mouse carries a reciprocal translocation resulting in the triplication of a segment of chromosome 16 (the homologue of chromosome 21 in human) syntactic to the DSCR and containing mouse *Sim2* (Reeves et al. 1995) is of particular interest. Neurological abnormalities have been reported in this mouse, including abnormal behavior (Reeves et al. 1995), and age-related degeneration of some cholinergic neurons, a phenotype also observed in DS brains (Holtzman et al. 1996).

SIM2, however, is not the only gene identified in the 21q22.2 band on human chromosome 21. In fact, other interesting genes such as *Drosophila minibrain* (*mnb*) homolog, *Drosophila enhancer-of-zeste* (*EZH2*) homolog, rat pep-19 gene homolog, and human chromatin assembly factor-I (*CAF1P60*) gene are also mapped to the DSCR. Their putative functions also make them good candidates for DS-responsible genes.

The use of YACs (yeast artificial chromosomes) in mouse transgenesis will be an elegant approach to test whether dosage imbalance of *Sim2* contributes to the Ts65Dn mice phenotype.

Loss and gain of function experiments will be required to address whether the murine *Sim* homologues also direct the development of cells located adjacent to or within the CNS midline. In fact, preliminary results indicate that *Sim1* mutant mice generated by homologous recombination display neurological defects. Mice carrying *Sim2* mutant

alleles have also been generated (Michaud, J. and Fan, C.-M., 1997; personal communication with Rosenquist et al. and Shamblott et al.). It will be of great interest to determine their phenotypes in the near future, to bring more insight into the understanding of the roles that the *sim* gene family plays in CNS development.

Materials and Methods

Cloning of *Gsim*

A 660 b.p. fragment (a.a. residues 212-870) from *Drosophila sim* was labeled with P^{32} dATP to serve as a probe under low stringent hybridization conditions. $5x10^5$ phage plaques transfected from a ZapII phage library (from Bastiani, M.J.) containing cDNA transcripts from 40-45% grasshopper embryos were screened. In one of the two positive clones, a 3.8 kb insert was isolated by digestion with EcoRI and XhoI.

Whole mount in situ hybridization of embryos

Devitellinized, fixed embryos were processed for hybridization by a modified procedure of Wiemers et al. (1983), digoxigenin-UTP labeled probes were generated using the Ambion Megascript in vivo transcription kit as per instructions. Probes were subsequently hydrolyzed to 100-400 b.p. fragments, heated at 80 degree for 5-10 minutes, and chilled on ice. Hybridization was performed overnight at 72 degree with ~200ng probe per ml of hybridization solution.

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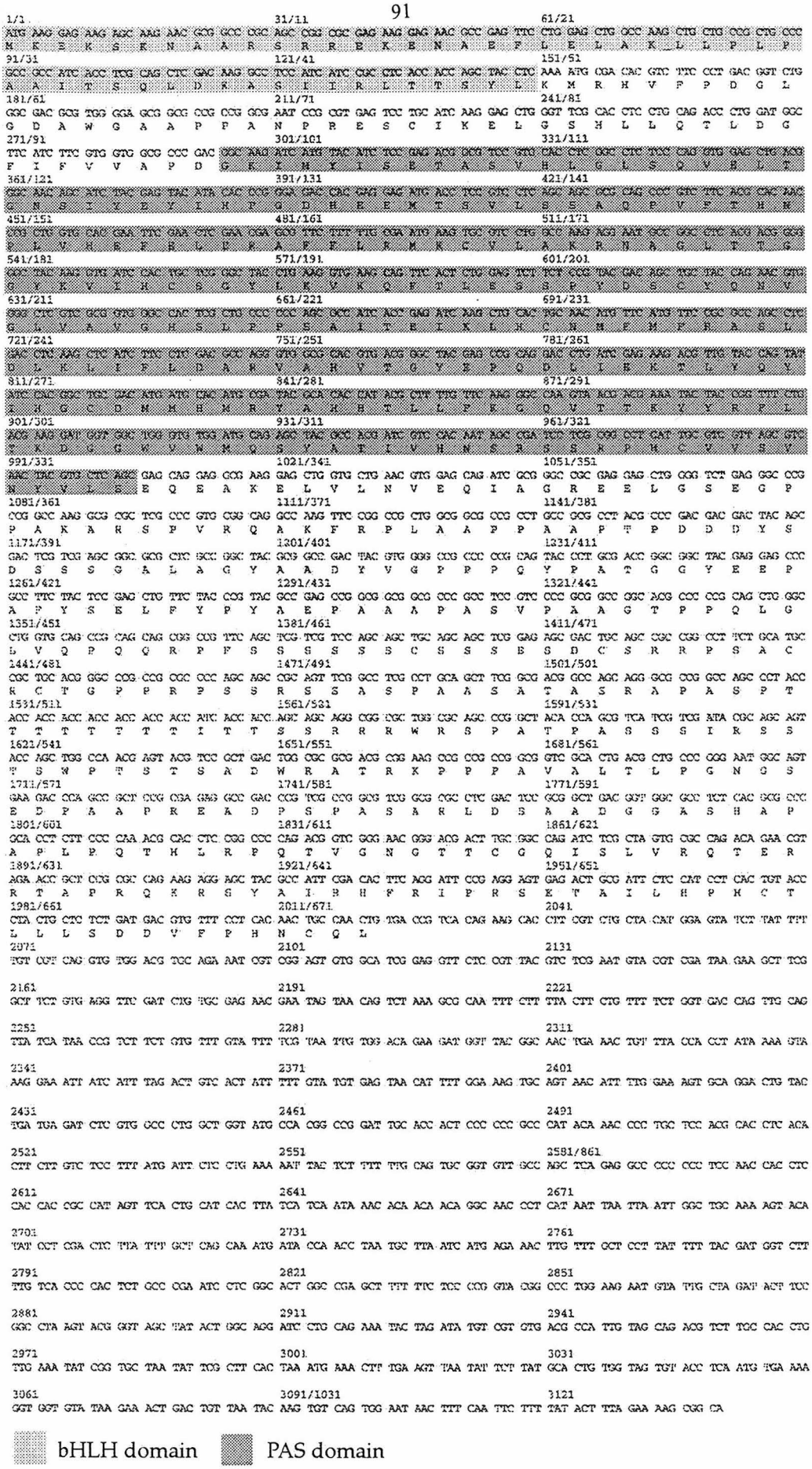
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Figure 1. The Nucleotide and Deduced Amino Acid Sequences of the *Gsim*

The nucleotide and the deduced amino acid sequences of the Gsim cDNA coding strand are both numbered from the first residue. The bHLH and PAS domains are shown as shadowed boxes, with individual subdomains indicated by underlining.



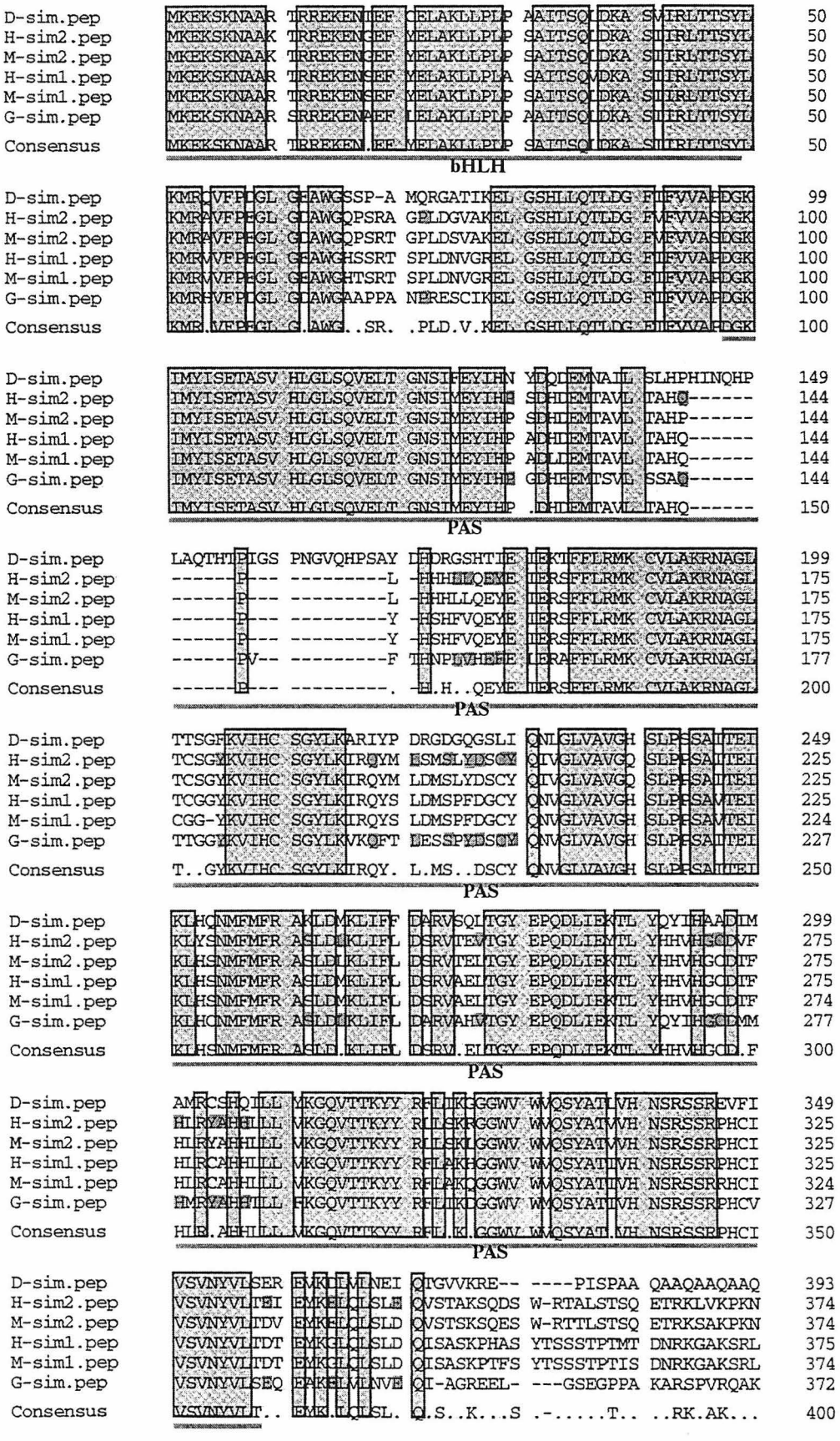
bHLH domain PAS domain

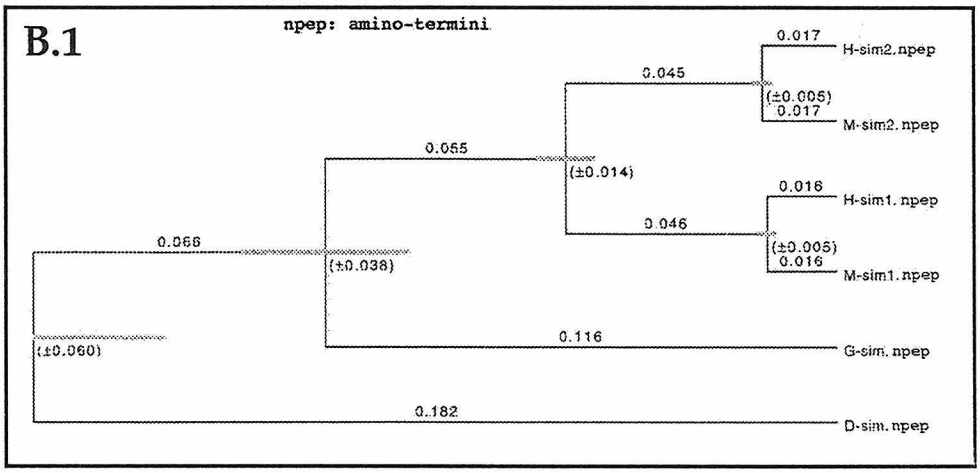
Figure 2. Sequence comparisons among Gsim and other related proteins

a) Sequence alignments of Gsim, Dsim, two murine sims Sim1 and Sim2, and two human sims SIM1 and SIM2. Different subdomains are underlined and individually labeled.

b) Sequence comparison trees of conserved domains in *sim* related genes (bHLH-PAS family of genes). Each protein name is prefixed by an abbreviation of the organism where the gene was isolated from: D, *Drosophila melanogaster*; H, human; M, mouse.

A


D-sim.pep
H-sim2.pep
M-sim2.pep
H-sim1.pep
M-sim1.pep
G-sim.pep
Consensus
bHLH
PAS
PAS
PAS
PAS


B.1
npep: amino-termini
0.017
H-sim2.npep
0.045
(±0.005)
0.017
M-sim2.npep
0.055
(±0.014)
0.016
H-sim1.npep
0.046
0.066
(±0.005)
0.016
M-sim1.npep
(±0.038)
0.116
(±0.060)
G-sim.npep
0.182
D-sim.npep

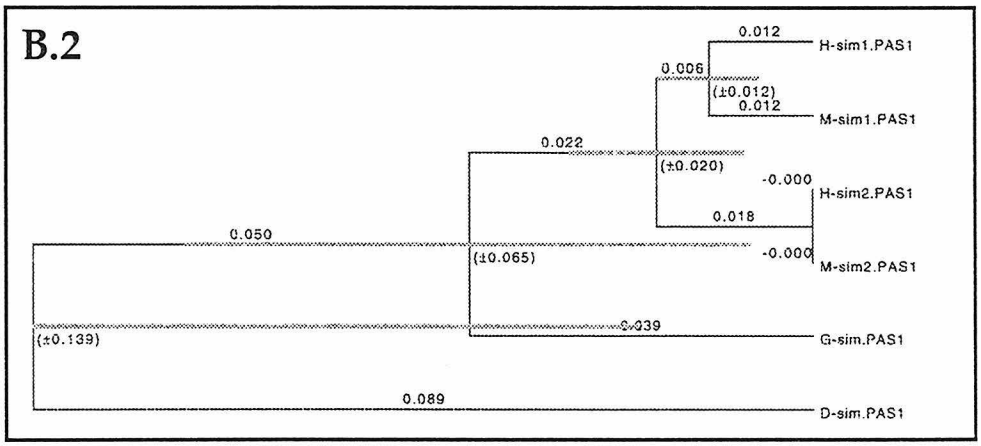

B.2
0.012
H-sim1.PAS1
0.006
(±0.012)
0.012
M-sim1.PAS1
0.022
(±0.020)
-0.000
H-sim2.PAS1
0.018
0.050
-0.000
(±0.065)
M-sim2.PAS1
0.039
(±0.139)
G-sim.PAS1
0.089
D-sim.PAS1

Figure 3. The *Gsim* mRNA Expression Patterns

a) A dosal view of 33% grasshopper embryonic CNS where *Gsim* transcripts are distributed as a continuous strip of cells along the entire midline.

b) , c) At 40% of grasshopper embryonic development, the *Gsim* expression is restricted to the median neuroblast (MNB) and its neuronal progeny, the H cell (MP3 progeny), and the anterior midline glia. Figure 3b is an overall view of the CNS midline in 40% grasshopper embryo, while figure 3c is a close-up view which allows the identification of individual neural cells, including the MNB. The anterior commissure (AC) and posterior commissure (PC) are indicated by arrowheads.

In all panels, *Gsim* mRNA is shown in dark blue, and monoclonal antibody 8B7 as a neuronal marker is shown in brown color.

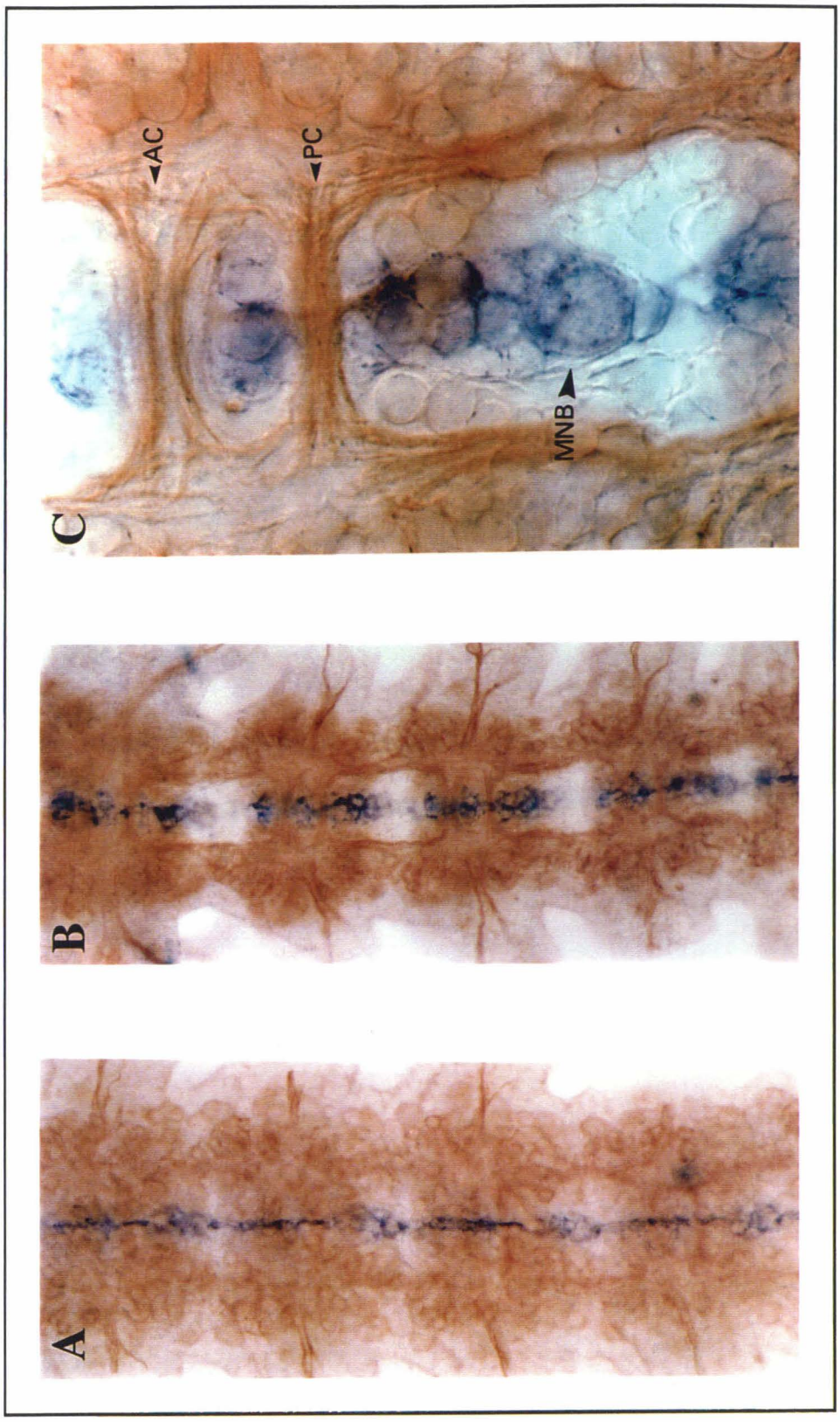

A
B
C
AC
PC
MNB

Figure 4. A comparison of the expression patterns of the *Dsim, mSim1* and *mSim2* genes

a) *Drosophila sim* protein is expressed in the cells at the ventral midline (indicated by the arrow).

b,c) Bright-field and dark-field views, respectively, of a transverse section of a *Drosophila* embryo of the same stage as that in a. The *sim* transcripts are found in the midline cells (white arrows) at both sides of the ventral furrow (vf).

d,e) Transverse sections of an embryonic day E9.5 mouse brain at the level of the diencephalon (di). The mouse *mSim2* is expressed in the ventral diencephalon including its midline(de), whereas *mSim1* is expressed in cells adjacent to the midline (e).

f) A transverse section of an E10.5 mouse spinal cord shows that *Sim1* is expressed in cells at both sides of the floor plate (fp), the ventral midline of the spinal cord.

Figure 4 is adopted from Michaud, J. and Fan, C.-M. (1997).

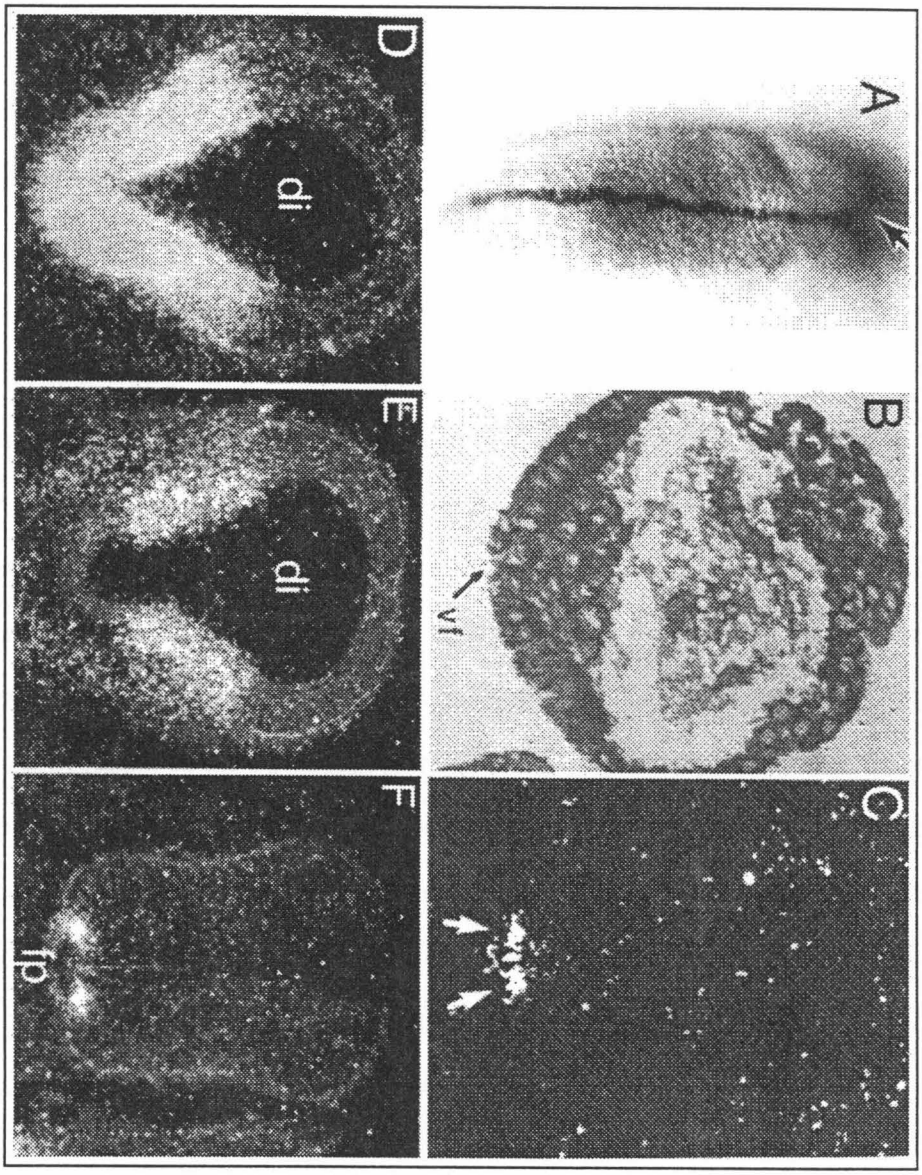
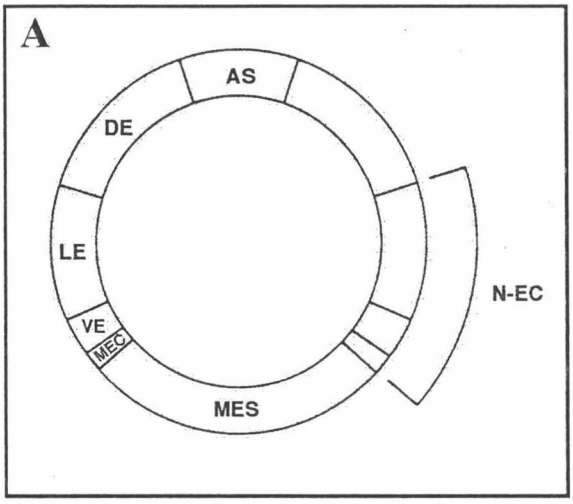
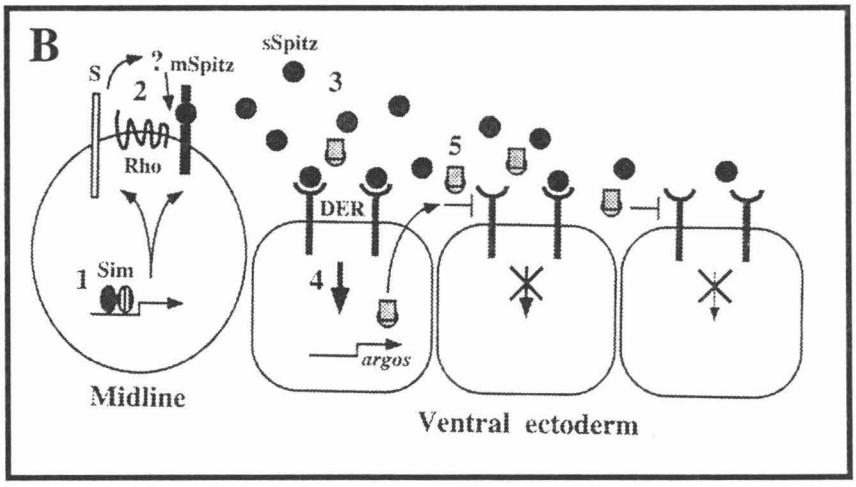

A
B
C
D
E
F
vf
di
di
fp

Figure 5. A possible mechanism for *sim* plays a master regulatory role in the development of the CNS midline and the ventral and lateral neuroectoderm

a) A schematic cross section representation of the cellular blastoderm with the dorsal part of an embryo at the top of the drawing (adapted from Kim and Crews, 1993, figure1a). AS, aminioserosa; DE, dorsal epidermis; LE, lateral epidermis; VE, ventral epidermis; MEC, mesectoderm; MES, mesoderm. The neurogenic ectoderm (N-ECT) gives rise to both the ventral-lateral epidermis and the CNS. It includes the blastoderm precursors that give rise to the LE, VE and MEC. The MEC gives rise to the midline cells of the CNS.
b) A model for establishment and maintenance of graded DER activation in the ectoderm (adopted from Golembo et al. 1996a; figure 6). In this model, secreted Spitz is required to activate DER and to pattern the ventral ectoderm. However, the major, if not the exclusive, source of secreted Spitz is the midline. In these cells, the expression of *spitz, rhomboid* and *Star* depends directly or indirectly on Sim (1). Expression of *rho* and *Star* allows processing of the Spitz precursor (2). (3) Secreted Spitz diffuses from the midline, and forms a gradient that is responsible for graded activation of DER in the ectoderm. (4) In the cells closest to the midline, the highest level of DER activation will take place. Consequently, expression of *argos* will be induced. (5) Diffusion of Argos to the neighboring ectodermal cells will terminate or reduce DER signaling, thereby promoting them to adopt the lateral epidermis fate.


A
AS
DE
LE
VE
MEC
MES
N-EC


B
S
?
mSpitz
sSpitz
2
3
Rho
5
DER
1
Sim
4
argos
Midline
Ventral ectoderm

Chapter IV

Protein Kinase A activation and inhibition can alter cell fates at the *Drosophila* CNS midline

Kung, Te-Yi; Zinn, Kai

Summary

The development of the *Drosophila* central nervous system (CNS) begins shortly after gastrulation, when CNS progenitor cells delaminate from the neuroectoderm in a stereotypical spatio-temporal pattern. These cells form a distinct mitotic domain (Foe, 1989) that gives rise to a variety of neuronal and glial cells, and it is these descendant neuronal and glial cells that in turn build up the CNS structure, with the commissural axon tracts interconnecting the longitudinal halves of the nerve cord in a ladder-like pattern.

Mutations in a number of genes have been shown to selectively affect the differentiation of midline cells (Nambu et al., 1991; Klambt et al., 1991. For review, see Chapters I and III). A detailed map of molecular markers for individual neuroblasts and ganglion mother cells has been established (Doe, 1992). The complete lineages of specific neuroblasts in the map also became available several years ago (Udolph, et al., 1993; Bossing and Technau, 1994). However, the mechanisms that lead to the specification of cell fates in the CNS midline still remain unclear, because most regulatory components underlying these mechanisms are shared among many common signal transduction pathways. One approach to the study of mechanisms involved in the development of CNS midline is to perturb these common components selectively at the midline, so as to minimize the alterations of normal development in other parts of the organism.

A successful example of such experimental design comes from the Grasshopper *Schistocerca*. Grasshopper and *Drosophila* share an overall similarity in their CNS structures, including the axon trajectories and gene expression patterns of individual identified neurons. Most identified neurons in the fly CNS were first characterized as homologs of grasshopper neurons, which can be studied easily due to their large size as well as the overall large size of the CNS in this organism. One very well characterized

lineage in grasshopper is the lineage to the median neuroblast (MNB), which is a multipotent progenitor cell that produces most neurons and glia in the grasshopper CNS midline. The MNB generates pioneer neurons during its first few divisions, and then switches to the production of midline glial precursors. After the glia have been produced, the MNB reverts to generating neurons.

In an attempt to identify signal transduction pathways that affect these temporally regulated cell-fate switches, microinjections of different perturbing agents to the MNB were performed at specific temporal stages in the cultured grasshopper embryos, and the results suggested that when cAMP-dependent-protein-kinase (PKA) is prematurely activated in the MNB, the glial-to-neuronal cell-fate switch takes place prematurely. Conversely, inhibition of PKA activation in the MNB blocks the switch.

In this chapter we report another piece of evidence on PKA regulation of the midline cell fates in the insect CNS. When we specifically expressed in the *Drosophila* midline a mutant mouse PKA catalytic subunit that is resistant to inhibition by regulatory subunits, we observed an increased number of *BarH1*-expressing midline neurons. We also report that when we expressed a mutant regulatory subunit of PKA, which behaves as a PKA inhibitor, midline glia died or disappeared during late embryogenesis. The absence of midline glia apparently produces a *roundabout-like* phenotype, in which longitudinal axons now cross the midline. Loss of midline glia also causes narrowing of the nerve cord, bringing the longitudinal tracts closer together.

Introduction

In eukaryotes, the cAMP-dependent protein kinase (PKA) has been known to mediate the majority of cellular responses to the intracellular second messenger, cAMP. The inactive PKA holoenzyme is a tetramer (R_2C_2) consisting of regulatory subunits (R) and catalytic subunits (C) (Toda et al., 1987). The rate of cAMP synthesis, catalyzed by adenylyl cyclase, is modulated via a stimulatory (Gs) or inhibitory (Gi) G protein that itself responds to an activated transmembrane receptor. In response to extracellular stimuli, such as hormones, neurotransmitters, and local chemical mediators that activate adenylyl cyclase-coupled receptors, cAMP is produced and binds to the regulatory subunits which thus dissociate from the PKA complex. The catalytic subunits are then freed to enter the nucleus and to phosphorylate a spectrum of protein substrates, such as the transcription factor cAMP-response-element-binding protein (CREB)(reviewed by Lee, 1991), thereby leading to altered patterns of gene transcription. A simple diagram illustrating this signal transduction pathway is shown in figure 1.

PKA has been shown to control several developmental switches in simple organisms such as Dictyostelium (reviewed by Devreotes, 1994). However, its roles in fly development could not be easily defined by analysis of zygotic PKA mutations, due to maternal rescue. Loss of maternal PKA in ovaries causes oogenesis defects, and PKA-negative eggs are not laid. Eggs lacking zygotic PKA expression, however, display no morphological abnormalities, due to perdurance of maternal PKA protein (Lane and Kalderon, 1993).

More recently, with the enhancer trap technique which allows the spatial and temporal-specific expression, and with the use of dominant mutants of either catalytic or

regulatory subunits, the roles of PKA have been identified during the development of *Drosophila* imaginal discs (Pan and Rubin, 1995; Willis et al., 1995; Jiang and Struhl, 1995; Strutt et al., 1995; Lepage et al., 1995). In these cases, PKA acts antagonistically to the anterior-posterior compartment defining gene *hedgehog* to determine cell fates in the eye, wing, and limb imaginal discs.

PKA also controls early developmental switches during embryogenesis. THis was demonstrated by studies in the Grasshopper *Schistocerca*, using embryo cultures and drug perturbation techniques (Condron and Zinn, 1995). In grasshopper, the MNB is a multipotent progenitor cell that produces neurons and midline glia in distinct temporal phases. Like other neuroblasts, the MNB divides regularly every 1% of grasshopper development (~5 hours), and generates three to four ganglion mother cells and their neuronal progenies (six to eight neurons) in its first few divisions. The NMB then produces glial precursors for approximately eight divisions, and these precursor cells migrate anteriorly and divide to form groups of commissural and sheath glia. At 39-40 % of development, the NMB switches back to produce ganglion mother cells (Condron and Zinn, 1994). The second transition in this MNB lineage is triggered by the activation of PKA. PKA-C translocates into the nucleus at this time during development in vivo, indicating that the emzyme is activated. When PKA is prematurely activated in the MNB by microinjection of purified PKA-C, or by pharmacological agents that elevate intracellular cAMP levels, the glial-to-neuronal cell fate switch occurs prematurely. Inhibition of PKA activity by microinjection of the Walsh inhibitor or by a non-hydrolyzable cAMP analog blocks the glial-to-neuronal switch (figure 2, adopted from Condron and Zinn, 1995).

Encouraged by these results, we decided to examine the roles of PKA in *Drosophila* midline development, by using two fly lines where the expression of mutant

PKA is driven by Gal4, the availability of which can be temporally and spatially controlled by crosses to different enhancer trap Gal4 lines (see figure 3 for details of the enhancer trap technique). In this chapter, we report that a mutant mouse PKA catalytic subunit, resistant to the inhibition by regulatory subunits in *Drosophila* (referred to as UIC in this chapter) and therefore considered constitutively active PKA, increases the number of *BarH1*-expressing midline neurons. We also report here that in late embryogenesis, the expression of mutant regulatory subunits which dominantly inhibit PKA functions (referred to as BGDr in this chapter) in the CNS midline cause death of midline glia. Loss of the glia causes narrowing of the nerve cord and abnormal midline crossing by longitudinal axons.

Results

Ectopic PKA activation results in an increased number of BarH1 expressing midline neurons

To examine the effect of increased PKA activity on midline development in *Drosophila*, we took advantage of an available PKA mutant transgenic fly line named UIC. In this UIC line, mutant mouse PKA catalytic subunit resistant to the inhibition by regulatory subunit (Orellana and McKnight, 1992) was expressed from a GAL4-responsive promoter (Li et al., 1995). To drive the GAL4-responsive promoter in a spatio-temporal specific manner, we crossed the UIC line to different GAL4 source lines which express GAL4 in distinct subsets of midline cells with different temporal specificities.

Among six GAL4 lines we tested, *Rhomboid*-GAL4 and *Slit*-GLA4 produced the strongest effects on midline development. In normal embryos of stage 15 or later, BarH1 expressing CNS neurons in each abdominal segment adopt a very stereotypical pattern. For convenience, we divide these BarH1 positive neurons into subgroups: one unpaired neuron located at the midline, then several bilaterally symmetric groups, one central most (CM) neuron, two anterior median (AM) neurons, one median median (MM) neuron, one median posterior (MP) neuron, and three lateral most (LM) neurons per hemisegment (See Figure 4, column 2). Given the spatially restricted expression of GAL4 from *Rhomboid*-GAL4 or *Slit*-GAL4, only the central unpaired neuron and the two CM neurons have ectopic expression of the mutant PKA. This spatial restriction of PKA availability corresponds to the results we observed where the midline unpaired neuron and the central most neurons are the most seriously affected groups of cells (figure 4). In Rhomboid-GAL4 driven UIC embryos, 27-37% (varies with different genetic

backgrounds) of the hemisegments (n>700) display an increase in the number of CM neurons from one to two (compared with 0.7% in wildtype, n=280). The phenotype is consistently observed in different genetic backgrounds. There are also cases in which the number of the CM neuron number increases from one to three, though this situation is rather rare (4 out of 700 hemisegments). In 10 out of 350 segments, we observed an increase in the number of the midline unpaired neuron from one to two or even three. Although this is a relatively rare event, we have to point out that this change has never been observed in wild type or in any other combination of mutant PKA transgenic background. Thus we consider this phenotype meaningful.

The number of AM, MM, PM, and LM groups of neurons also changed at a penetration of 24%, 5%, 4%, and 16%, respectively (n=112), with the wildtype showing 1-2% abnormalities on these groups of neurons.

We observed similar phenotypes in *Slit*-GAL4 driven UIC embryos, though the numbers are not as statistically significant.

Localized PKA inactivation at the midline leads midline glia to enter abnormal apoptosis at late embryogenesis

The PKA inactivation described in this section was performed with a transgenic fly line called BGDr, where a construct encoding a mutated regulatory subunit driven by a GAL4-responsive promoter is located on the second chromosome. Again, we crossed BGDr to different midline-GAL4 lines for spatio-temporal specific inhibition of PKA activity. Embryos from stages 8-17 were collected, fixed with standard procedures, and hybridized with anti-β-galactosidase or/and anti-fasciclin II monoclonal antibodies to observe the resultant phenotype.

There are no apparent defects in midline cells or in the CNS scaffold in fly embryos younger than stage 15). However, starting from late stage 15 (figure 5) to early stage 16, some segments in *Rhomboid*-GAL4 driven BGDr embryos showed a narrowed-in phenomenon at the junction points where two traverse commissures connect to the longitudinal axon tracts. As embryos aging to late stage 16 and stage 17, this phenotype (figure 5) becomes even more pronounced that the distance between two longitudinal tracts is one half as much as that in the wildtype. Thick, irregular multiple connections are formed at the locations of two commissural tracts, on which the expression of fasciclin II was detected, although in wildtype at this stage of embryogenesis the fasciclin II expression should no longer be present on traverse axonal tracts.

We further explored the mechanism underlying this phenotype, and found that corresponding to the onset of CNS scaffold defects, enhancer trap AA41 and *single-minded* expressing glia in the midline displayed additional cell death other than the usual apoptosis which reduces midline glia from 6 to an average of 3.2 per segment in late fly embryogenesis. In the embryos collected from crosses of BGDr/+; AA41-lacZ/+ to Rhomboid-GAL4 homozygotes or crosses of BGDr/+; sim-3.7-lacZ/+ to Rhomboid-GAL4 homozygotes, half of the embryos show midline β-galactosidase positive glia which were induced to express β-galactosidase under the AA41 or *sim*-3.7 lacZ enhancer trap promoter. Half of these β-galactosidase positive embryos should carry BGDr, a mutant regulatory subunit that inhibits local PKA activation. Indeed, we observed that about half of these embryos exhibit disturbed CNS scaffold (n=84). It is in these and only these embryos that glial cells were absent. We would like to note that it is only in those segments where AA41 or *sim* glia are completely eliminated that there is the *roundabout*-like astray axon phenomena occur. In segments where as long as one single glial cell is still left, even if it is mislocated, the CNS can still maintain its normal scaffold. As a result, no

fasciclin II expressing axons traverse the midline, and thus the distance between two longitudinal tracts remains normal, unless affected by neighboring abnormal segments.

Other minor abnormalities induced by mutant PKA expression in the midline during fly embryogenesis

Other minor abnormalities outside the midline are observed in our crosses of UIC and BGDr to six different midline GAL4 lines, including *single-minded*-GAL4, *Rhomboid*-GAL4, 52-GAL4, X11-GAL4, *Slit*-GAL4, and *Scabrous*-GAL4.

At late stage embryos of the *Rhomboid*-GAL4/+; BGDr/+ genotype, we observed minor motorneuronal axon projection defects, such as by-pass and stall (see figure 7). These abnormalities, however, are with very low penetrance.

In embryos collected from crosses of UIC to *Scabrous*-GAL4, we observed another phenotype that at late stage 17 to first instar larvae, UIC causes hypertrophy of the CNS tail or complete disruption of the CNS formation. The hypertrophy is accompanied with prolonged fasciclin II expression (usually no staining is detectable by anti-fasciclin II MAb at late stage 17), which often protrudes from the dorsal epidermis (see figure 8). This specific phenotype occurs with a frequency of 8 out of 67 from the UIC/*Sca*-GAL4 eggs aged to late embryonic stage 17 or beyond. It was never observed in any other PKA mutant crosses we have performed so far, and has never been reported either. Complete disruption of the CNS formation occurred 2 out of 67 cases.

Discussion

Ectopic activation of PKA in Drosophila CNS midline during embryogenesis

Midline PKA activation increases BarH1 expressing neurons

BarH1, identified at the *Bar* locus on the X chromosome, is a fly homeobox gene specifically expressed in the nervous system during embryogenesis (Higashijima et al., 1992 a and b). In the CNS, however, *BarH1* is only expressed in a subset of neurons. In this chapter we report that when the constitutively active PKA catalytic subunit (UIC) is expressed in the midline by the *Rhomboid*-GAL4 induction, we observed an increasing number on *BarH1* positive cells. This phenotype may reflect a late PKA influence on midline development, because the increased neuron number can only be observed at late embryogenesis approximately starting from stage 15. It is also at this stage that *BarH1*-expressing midline neurons have fully acquired their late expression patterns.

We examined the existence of other defects in UIC/*Rhomboid*-GAL4 embryos by using different midline cell or CNS fascicle specific antibodies (monoclonal antibodies to *engrailed, eveskipped, gliolectin, fasciclin II, BP102,* and polyclonal antibodies to *netrin A , B)*. However, we did not identify any other major morphological changes at the current resolution (results not shown).

We also varied the GAL4 source with different spatio-temporal distributions in midline by crossing UIC to *Sim*-GAL4, *Slit*-GAL4, *X11*-GAL4, 52-GAL4, and *Scabrous*-GAL4. However, only the *Slit*-GAL4 line displayed similar phenotypes. In fly embryogenesis, *Rhomboid* and *single-minded* do indeed have at least partially overlapping expression patterns, since the expression of Rhomboid in the midline is induced by single-minded (Nambu et al., 1990, Golembo et al., 1996). The fact that these two spatially overlapping GAL4 lines could have brought completely different results may be explained

readily by their different temporal phases. *Rhomboid* has a sustained expression in the midline throughout the entire neurogenesis, while *single-minded* transcription is controlled under two promoters, one early and one late. The expression *of single-minded* from the late promoter does not start until stage 9, and that is the promoter in the *sim*-GAL4 line we used. The late induction of PKA activity in this case may miss the specific temporal phase that is required to commit the cell fate to *BarH1*-expressing neurons. We also can not exclude the possibility that the promoter of *sim*-GAL4 line, as those of X11-GAL4, 52-GAL4 and *Scabrous*-GAL4, are not strong enough to produce the phenotype.

Comparison of the mechanism between fly and grasshopper

In Grasshopper, the elevation of intracellular cAMP level and therefore the activation of PKA in the midline progenitor cell MNB is shown to be the key event for the second cell fate transition (see figure 2), in which MNB ceases to generate glial precursors and resumes the production of neuronal ganglion mother cells. When PKA activity is prematurely elevated in the MNB (at 30-35% of development), the second neuronal production phase also occurs prematurely. The total number of neurons in MNB clusters where PKA activity is elevated at the beginning of the glial production period is consistent with the uninterrupted production of neuronal ganglion mother cells during the entire culture period (Condron and Zinn, 1995).

However, at the corresponding stage (stage 13) in *Drosophila*, we failed to see such a distinct shift of cell fate from glia to neuron when we misexpressed PKA catalytic subunit in midline. This could be because the PKA-C gene used in UIC is encoded by *DC0*, which is responsible for most PKA activity in adult flies (Lane and Kalderon, 1993). It is unclear, however, that how much DC0 contributes to the CNS development in embryogenesis. There are two other genes (*DC1* and *DC2*) that could also encode PKA-

like catalytic subunits (Kalderon and Rubin, 1988). Further experiments using promoters of these genes can be performed.

In addition, the perdurance of the maternally encoded *DC0* protein, which can last at least until 12 hours post-fertilization, makes the study of mutant PKA phenotype very difficult, since the entire embryogenesis is almost finished at this stage. Successes of using UIC and BGDr in the study of PKA function in the anterior imaginal discs were actually achieved in a much later stage of larval development, when the maternal supply of PKA has been depleted.

Alternatively, it is also possible that the controlling mechanism for the MNB lineage in grasshopper, as if the lineage itself, is simply not conserved in fly, although the resultant CNSs from different neuroblast lineages of these two organisms still share amazing overall similarities.

Local PKA inhibition in the CNS midline during *Drosophila* development

From the studies of grasshopper MNB lineage, we were expecting to see an increased number of midline glia as a result of failed glia-to-neuron switch (Condron and Zinn, 1995) when PKA activity is inhibited in *Drosophila* midline. Using six different GAL4-enhance trap lines described above, we tested this hypothesis by expressing BGDr, a mutant regulatory subunit that functions as a PKA inhibitor, to locally suppress PKA activation during midline morphogenesis. The phenotypes, again, differed from our expectation, but as well brought some new insights into how PKA is involved in the midline morphogenesis. We report here that when driven by *Rhomboid*-GAL4 in a subset of midline cells, inhibiting of PKA activation by BGDr resulted in distortions in the CNS scaffold correlating to the death of midline glia (see figure 8). This event, again, has a late onset that doesn't start until stage 15 of *Drosophila* development.

In *Drosophila*, programmed cell death in midline cells starts at as early as stage 11 (~7 hours), peaking at stages 12 and 16 (Abrams et al., 1993). In the *Drosophila* ventral nerve cord, midline glia (MG) exist transiently to establish the position and morphology of the commissural axon tracts of the nervous system in midline embryogenesis. They express surface proteins such as *D-netrin* (Mitchell et al., 1996) and *Commissureless* (Tear et al., 1996), both of which are implicated in growth cone guidance. During commissural tract morphogenesis, a subset of the MG migrate and interpose themselves between axon fascicles traversing the midline to separate the anterior and posterior commissures (Klambt et al., 1991). Subsequent to the establishment of commissure morphology, between stage 13 and 16 of embryogenesis, programmed cell death takes place which in turn reduces the number of MG from 6 to 3.2 per segment at hatching (Sonnenfeld and Jacob, 1995).

The genetic basis of determination and early differentiation of the MG is not clear. The expression of genes in the MG lineage is regulated by *single-minded* and segment polarity genes (Nambu, 1991) which are required by all mesectodermal cells. In early stage 12, up to twelve mesectodermal cells express genes encoding structural proteins characteristic of differentiated MG (Dong and Jacobs, 1997). However, only six of these mesectodermal cells participate in commissure establishment, which occurs during stages 12 and 13 (Sonnenfeld and Jacob 1995).

The process of eliminating extra glia to 3.2 per segment after the commissure establishment is regulated by Argos and Spitz group genes (Stemerdink and Jacob, 1997; Scholz et al., 1997; Galembo et al., 1996). Argos is a secreted molecule with an atypical EGF motif that functions as an inhibitor of *Drosophila* EGF receptor (DER) signaling. DER signaling is required for the survival of glia, while Argos blocks this signaling and leads cells to apoptosis. A model for this process is that *rhomboid*, DER and components

of the membrane skeleton associate on the membrane between migrating MGs function in a juxtacrine fashion: Within the junction, Rhomboid facilitates trans-membrane Spitz signaling, or the release of secreted Spitz (sSpitz) from membrane bound Spitz (mSpitz), which then activates DER that also localizes within the junction. Sufficient DER signaling triggers Argos production, which is then secreted into the environment. However, released Argos has a minimal effect upon signaling within junctions because of restricted inward diffusion, while it effectively reduces DER signaling outside the junctions, such as distant MG with reduced junction formation (see figure 9 adopted from Stemerdink and Jacob, 1997).

It is likely that during the MG eliminating process from stage 12/5 to stage 15, a subset of the equivalent glial cells, usually those that have close contact with commissural axons, receive sufficient DER signaling, thus initiate *argos* expression to work as a long-range inhibitor of DER activation, and thus restrict the number of equivalent cells acquiring MG fate at hatching.

To summarize, misexpression of Argos reduces midline MG number and disturbs the CNS regularity (see figure 10 adopted from Stemerdink and Jacob, 1997).

A close comparison of our results and the defects induced by Argos in the CNS scaffold as well as its effect on the survival of MG, shows at least an amazing similarity at morphological level. It is tempting to postulate that the PKA regulates of midline glia number through Argos signaling. In fact, the linkage of PKA signaling pathway to Ras signaling pathway has already been provided in the cultured PC12 cell system, in which elevation of cAMP level triggers the activation of mitogen-activated protein kinase (Frodin et al., 1994; Young et al., 1994). The Ras signaling often transduces signals from the EGF-receptor, and is shown to control the number of midline glial cells as well (Scholz et al., 1997).

We feel that it is too early to draw a conclusion at this point. However, considering the timing of onset of the phenotypes, and the morphological similarities in the defects, it will certainly be of interest to examine the association of PKA signaling to the Argos signaling in the regulation of midline glia survival.

Conclusions

In this chapter, we presented the first clear example on the regulation of *Drosophila* embryonic neurogenesis by PKA activity. The midline specific activation of PKA increases the number of a certain set of neurons, while midline specific inhibition of PKA activity reduces the number of midline glia, which results in altered CNS scaffold. There are also minor defects in the projection of motorneuronal axons when PKA activity is blocked, though we can not determine if it is due to the missing of midline glia which normally provides certain membrane bound guidances for the motorneurons, or to the altered CNS structure. The observation of CNS tail hypertrophy when PKA is misexpressed in midline is another interesting phenotype that we have no explanation of. However, since PKA activation in PC12 cells can affect cell growth and differentiation, we do not regard this phenotype to be an extreme case.

Our observation on the PKA function during fly embryogenesis, however, is restricted to very late stages of embryonic development (stages 15-17), when the maternal protein can no longer be detected by standard immunohistochemistry methods.

Materials and Methods

UIC, abbreviation of yw; UIC; is the GAL4-UAS-MC* described in Li et al., 1995. It is located on 2nd chromosome, homozygous viable, and is marked with mini-w+.

BGDr, abbreviation of yw; BGDr15/p[y+]cyo; hsGAL4. BGD is the shortest GAL4-UAS-R* as described in Li et al., 1997. Not homozygous viable, recombinated with Df(22)r15 that lacks DC0. hsGAL4 is hsp70 GAL4 homozygous on 3rd (also marked with mini w+).

Both UIC and BGDr are kindly provided by D. Kalderon.

We performed the experiments described in this chapter by crossing these two PKA mutant lines to different enhancer trap GAL4 lines to drive the mutant PKA expression in specific subsets of cell in the midline. We also used enhancer trap-lacZ reporters to observe the morphological changes associated with different midline cells.

Embryo fixation and immunohistochemistry were performed with standard methods. In double staining experiments, Vectastain Elite ABC kit was used to improve the signal.

Immunohistochemistry

Affinity purified rabbit anti-BarH1Ab S12 antibodies were kindly provided by T. Kojima.

Antibody signals were enhanced by biotinylated anti-mouse-goat IgG secondary anitibodies, followed by color reactions with avidin-peroxidase, using the Elite ABC Vectastain kit (Vector labs, Burlingame, CA).

β-gal lines

AA41, an enhancer trap line that carries a P element insertion at 95A. It expresses β-gal in the midline glia.

p[3.7sim-lacZ], on the X chromosome, with the early onset expression persisting through the rest of embryogenesis.

GAL4 lines

Sim-GAL4, viable insertion, homozygous on the second chromosome.

p[rhomboid-GAL4], homozygous on the second chromosome.

p[52-GAL4], on 2nd, midline GAL4 enhancer trap strain.

p[X11-GAL4], on 2nd, midline GAL4 enhancer trap strain.

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Figure 1. Schematic diagram of PKA signaling

The rate of cAMP synthesis, catalyzed by adenylyl cyclase, is modulated via a stimulatory (Gs) or inhibitory (Gi) G protein that itself responds to an activated transmembrane receptor. The inactive PKA holoenzyme is a tetramer (R_2C_2) consisting of regulatory subunits (R) and catalytic subunits (C). In response to extracellular stimuli, such as hormones, neurotransmitters, and local chemical mediators that activate adenylyl cyclase, the second messenger cAMP is produced. cAMP binds to the regulatory subunits of PKA, and cause them to dissociate from the complex. Activated catalytic subunits enter the nucleus to phosphorylate a spectrum of proteins including the transcription factor cAMP-response-element-binding-protein (CREB), which then leads to altered patterns of gene transcriptions.

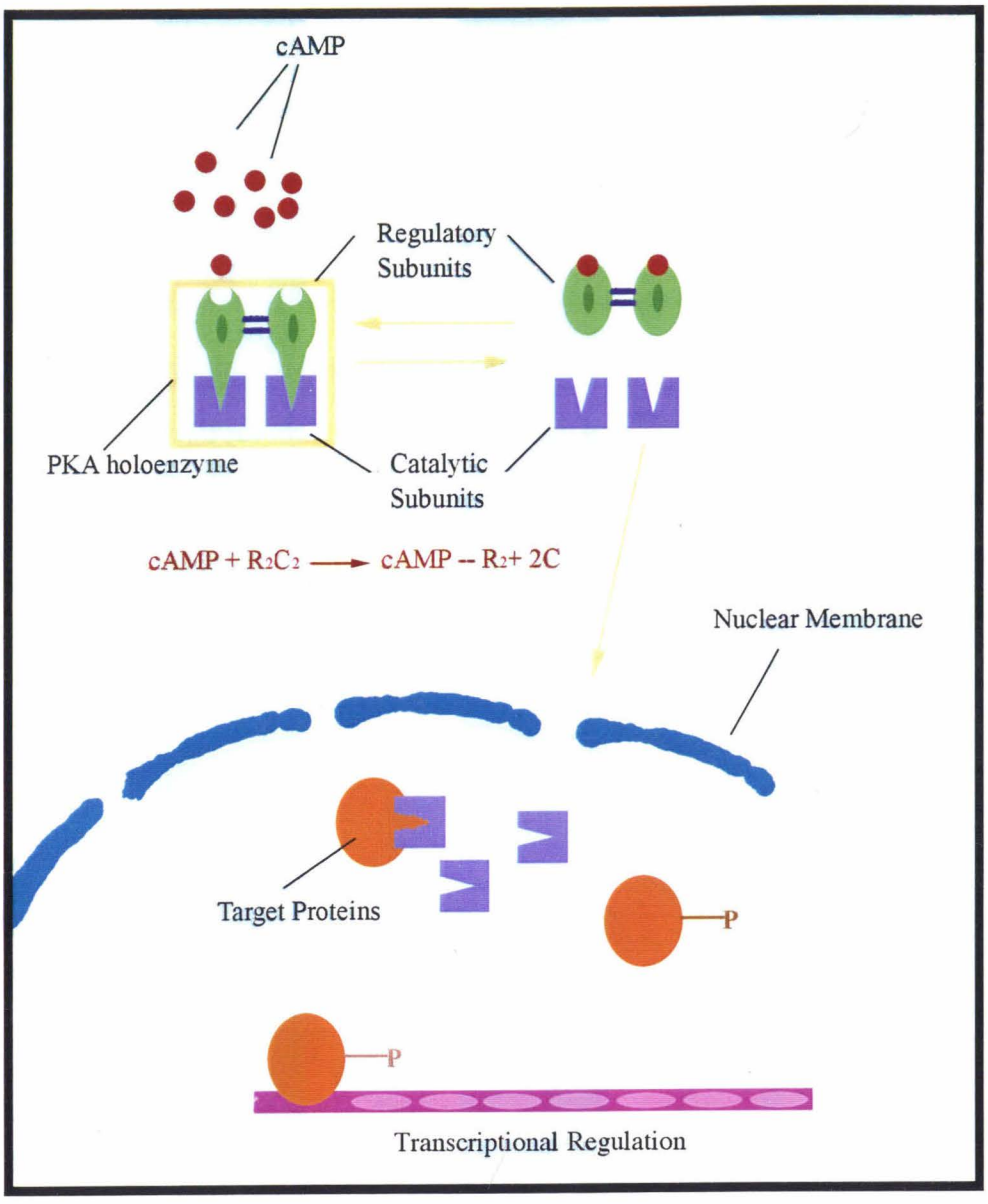

cAMP
Regulatory Subunits
PKA holoenzyme
Catalytic Subunits
cAMP + R2C2 → cAMP -- R2+ 2C
Nuclear Membrane
Target Proteins
P
P
Transcriptional Regulation

Figure 2. PKA mediates the neuronal-glial cell fate decisions in the grasshopper CNS midline

This diagram summarizes the cell-fate decisions in the normal and perturbed MNB lineages. Developmental stages (normalized to the T3 segment) and the period of culturing and PKA activation are indicated in the top panel. In normal development, the MNB generates neurons (red), then glia (green), and finally neurons again. When the *engrailed* expression is inhibited, no glial cells are generated, and extra neurons are produced that pile up in a disorganized manner. PKA-C translocation may be impaired in *engrailed*-inhibited segments (indicated by the hatched MNB nucleus). The same effect is produced by premature activation of PKA. When PKA is inhibited, glia are generated, but the second phase of neuronal production does not occur. Extra glia may be produced during this phase. (Adopted from Condron and Zinn, 1995).

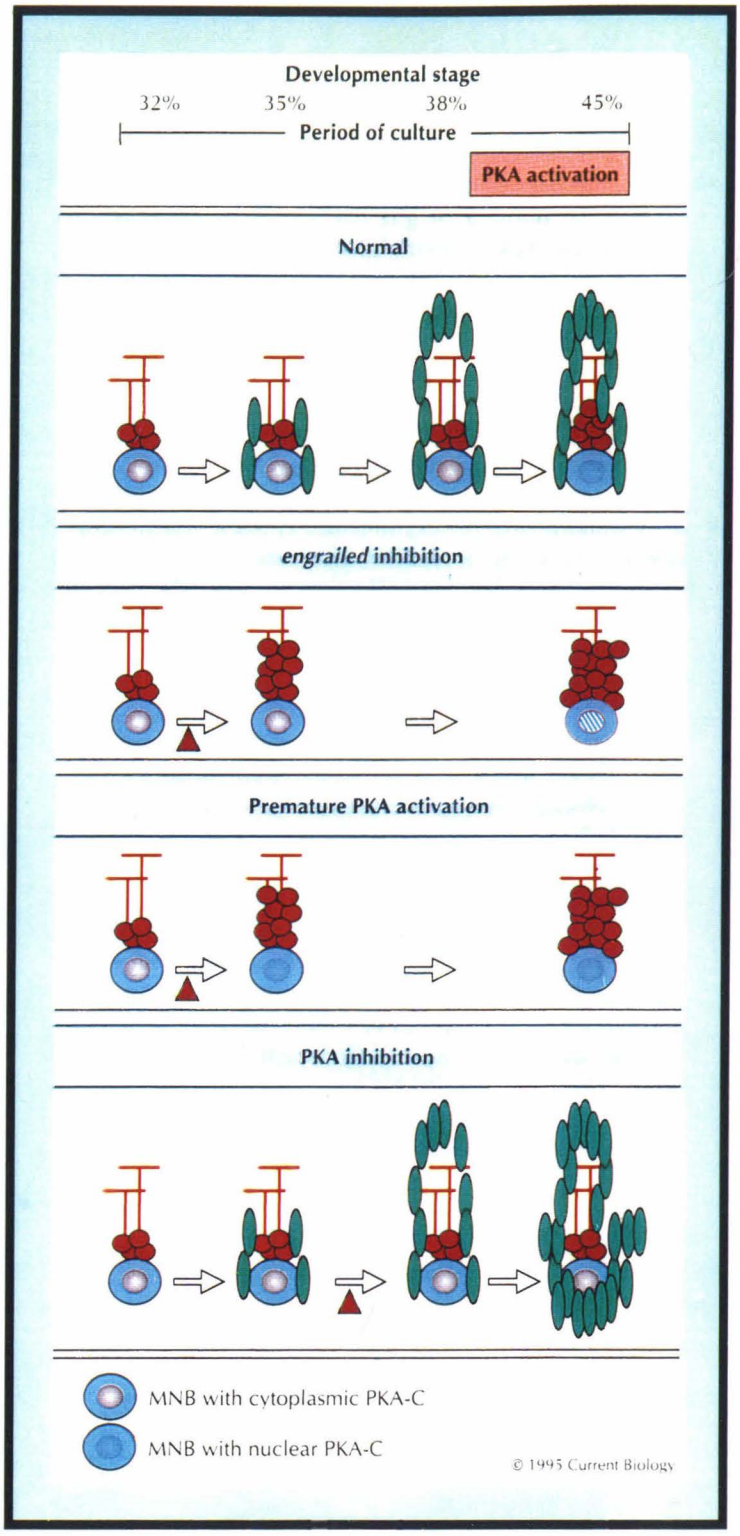
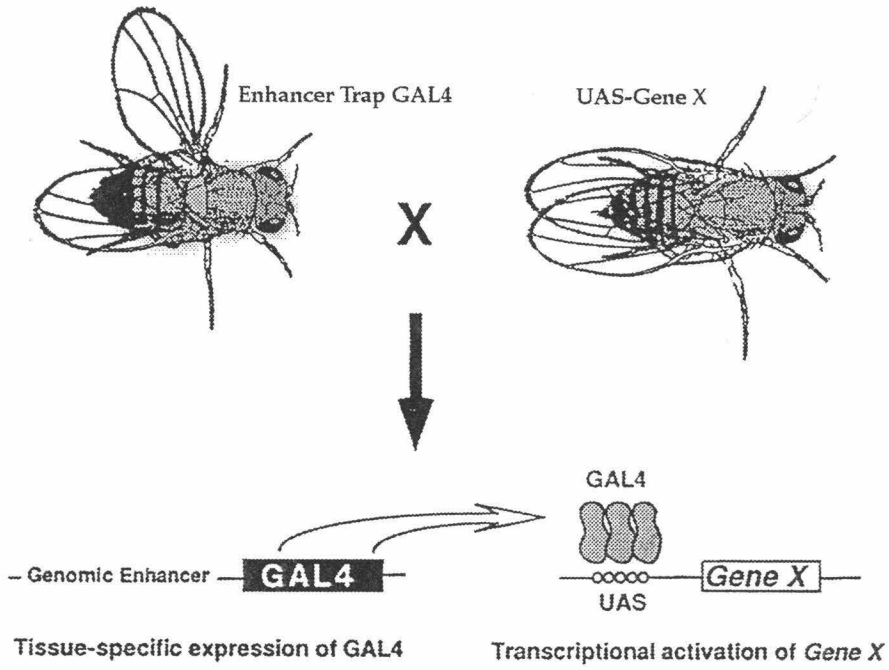

Developmental stage
32%
35%
38%
45%
Period of culture
PKA activation
Normal
engrailed inhibition
Premature PKA activation
PKA inhibition
MNB with cytoplasmic PKA-C
MNB with nuclear PKA-C
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Figure 3. A schematic diagram on enhancer trap strategy used in this chapter



To generate transgenic lines expressing GAL4 in cell- and tissue- specific patterns, the GAL4 gene is inserted randomly into the genome, driving GAL4 expression from numerous different genomic enhancers. A GAL4-dependent tartget gene can then be constructed by subcloning any sequence behind GAL4 binding sites. The target gene is silent in the absence of GAL4. To activate the tartget gene in a cell- or tissure- specific pattern, flies carrying the target (UAS-gene X) are cross to flies expressing GAL4 (Enhancer Trap GAL4). In the progeny of this cross, it is possible to activate UAS-Gene X in cells where GAL4 is expressed and to observe the effect of this directed misexpression on development.

GAL4 source lines:

rhomboid-GAL4 **scabrous-GAL4**
sim-GAL4 **slit-GAL4**
X11-GAL4 **52-GAL4**

Target gene lines

UAS-BGDr **UAS-UIC**
AA41-lacZ **sim-lacZ**

Figure 4. Ectopic PKA activation results in an increase in BarH1 expressing neurons

UIC expression was driven by *rhomboid*-GAL4 in stage 17 fly embryos. Embryos were immunolabeled with anti BarH1 antibodies. In panels 1a/1b/1c, white arrows indicate ectopic BarH1-expressing neurons. Panel 2, a wildtype embryo. All the embryos are photographed with the anterior side up.

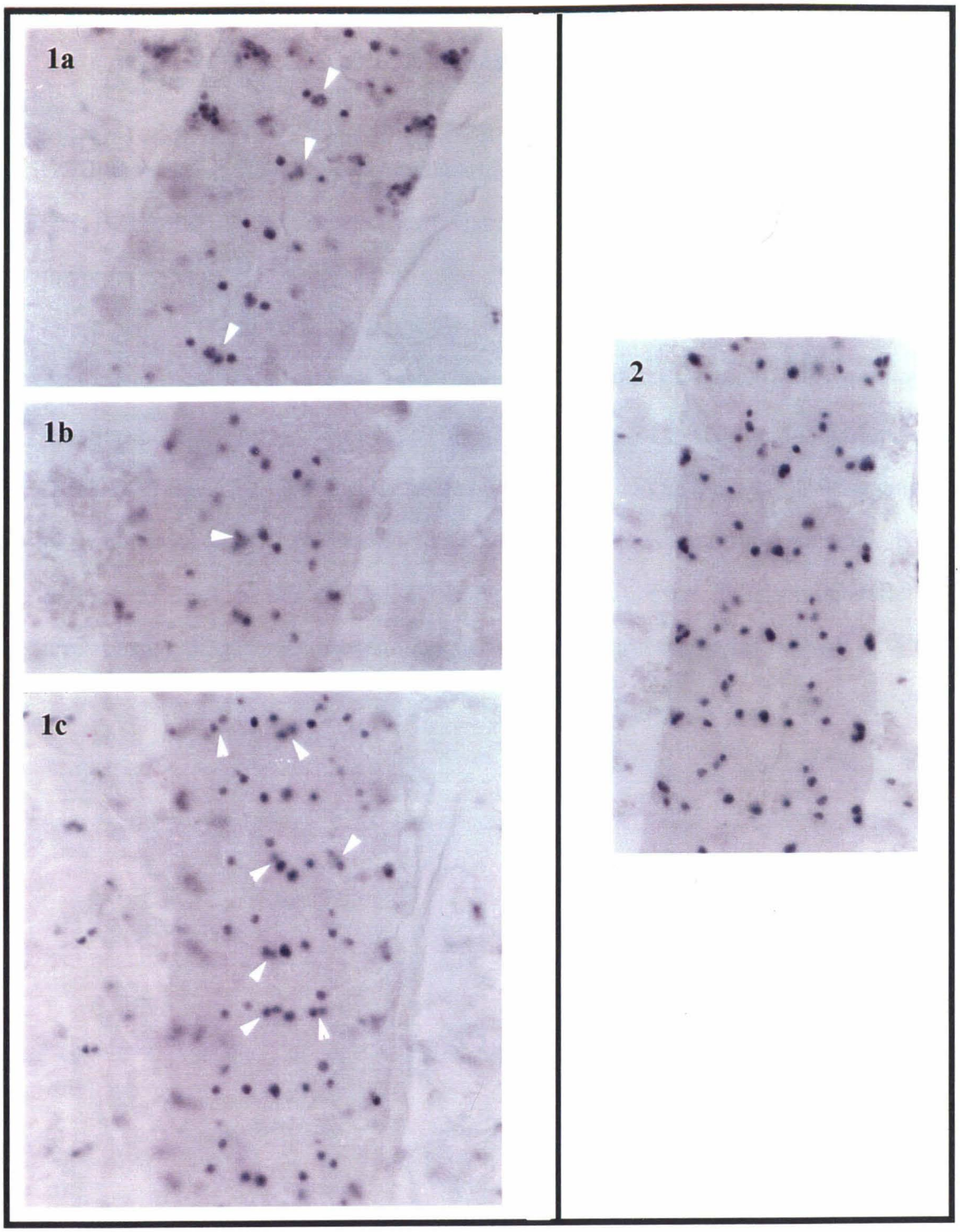

1a
1b
1c
2

Figure 5. Localized PKA inhibition in the midline leads to glia apoptosis and CNS structural defects

BGDr expression was driven by *rhomboid*-GAL4 in stage 16 (A1, A2, A3) and stage 17 (B1, B2) embryos. Midline glia were visualized with MAbs to β-galactosidase (which is the reporter in the AA41 enhancer-trap line). The CNS scaffold was probed with MAbs raised against Fasciclin II. In the wildtype, there are 2-4 midline glia per segment (panel A1), whereas in the mutant, some segments show a narrowed-in phenotype at the junction points where two traverse commissures connect to the longitudinal axon tracts (panels A2 and A3). Note that there are no midline glia in all defective CNS segments. In older embryos (stage 17, panel B), the CNS defects become more pronounced. Note that as long as there is one single MG left per segment, even if it is mislocated (white arrowheads), the distance between longitudinal axonal tracts remains normal.

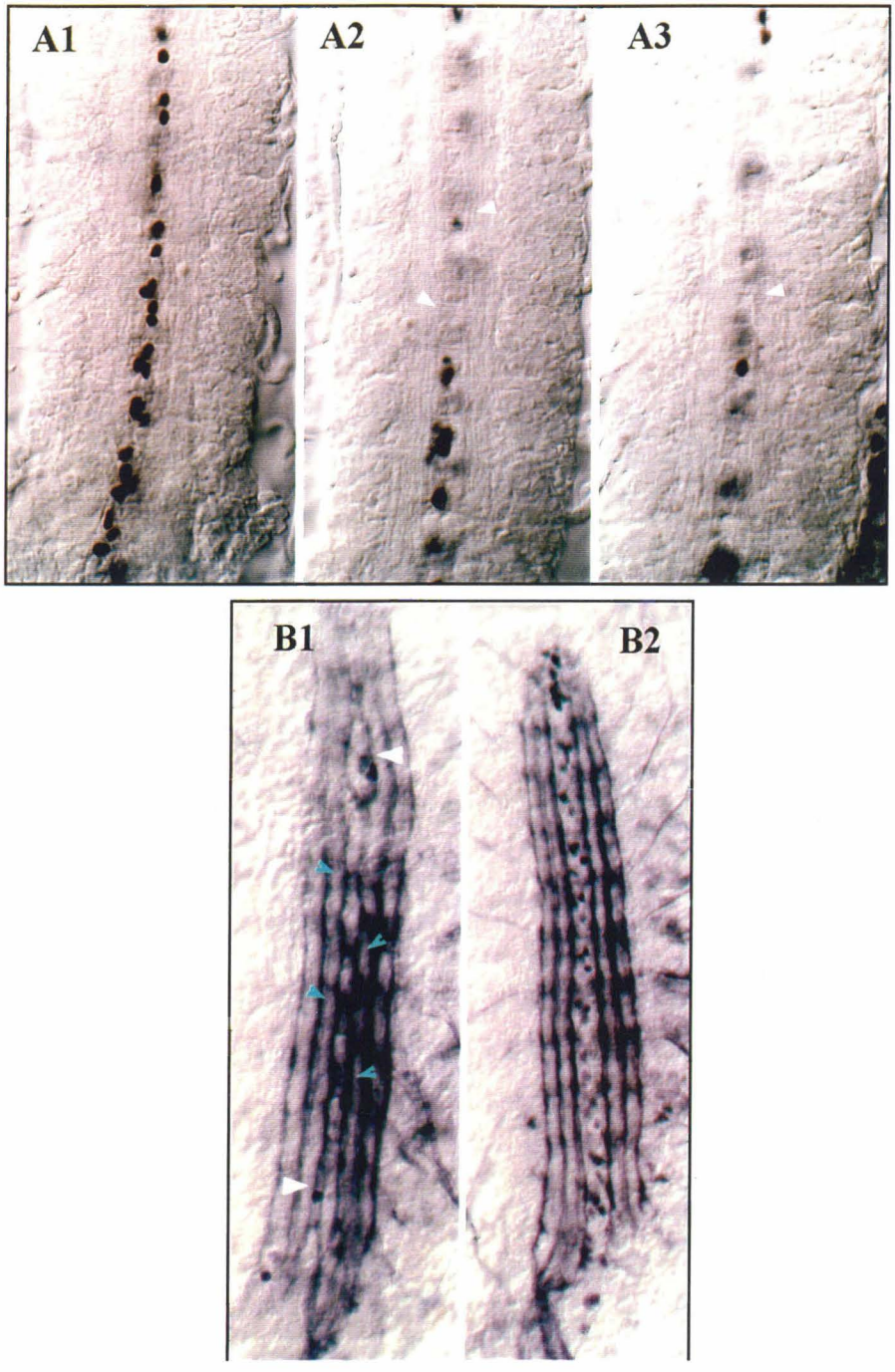

A1
A2
A3
B1
B2

Figure 6. Localized PKA inhibition in the midline leads to glia apoptosis and CNS structural defects - II

BGDr expression was driven by *rhomboid*-GAL4 in late stage 16 embryos. Midline glia were visualized with MAbs to β-galactosidase (which is the reporter in the AA41 enhancer-trap line). The CNS scaffold was probed with MAbs raised against Fasciclin II.

A) wildtype, with 2-4 midline glia per segment.

B) significantly reduced glia number (0 to 2 per segment) in BGDr-expressing embryos.

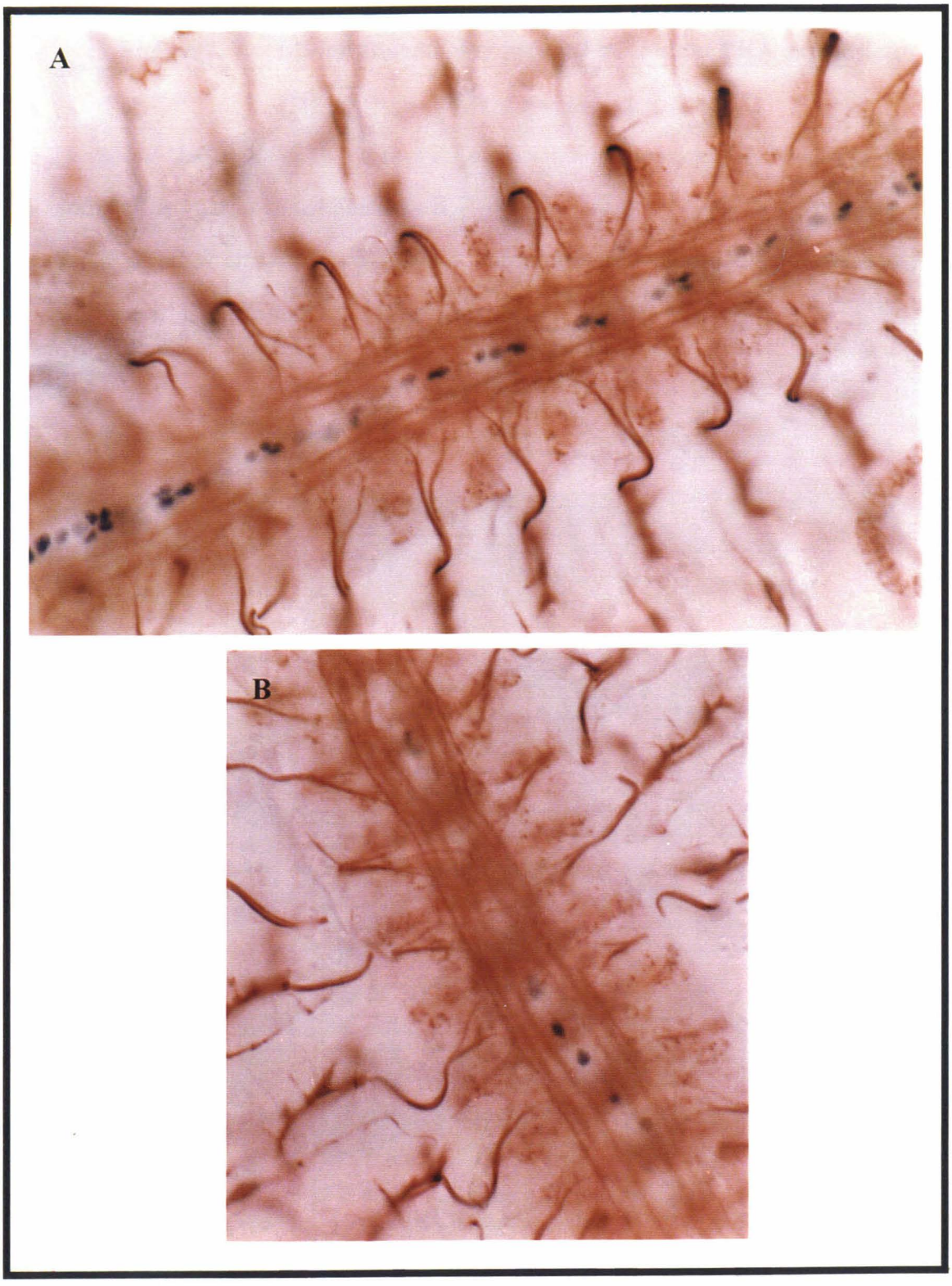

A
B

Figure 7. Minor abnormality induced by mutant PKA expression - I

rhomboid-GAL4 drove BGDr expression in stage 17 embryos. CNS scaffold was probed with MAbs raised against Fasciclin II.

White or black arrowheads indicate minor motorneuronal projectory defects as A) stall, B) bypass, C) SNb stall.

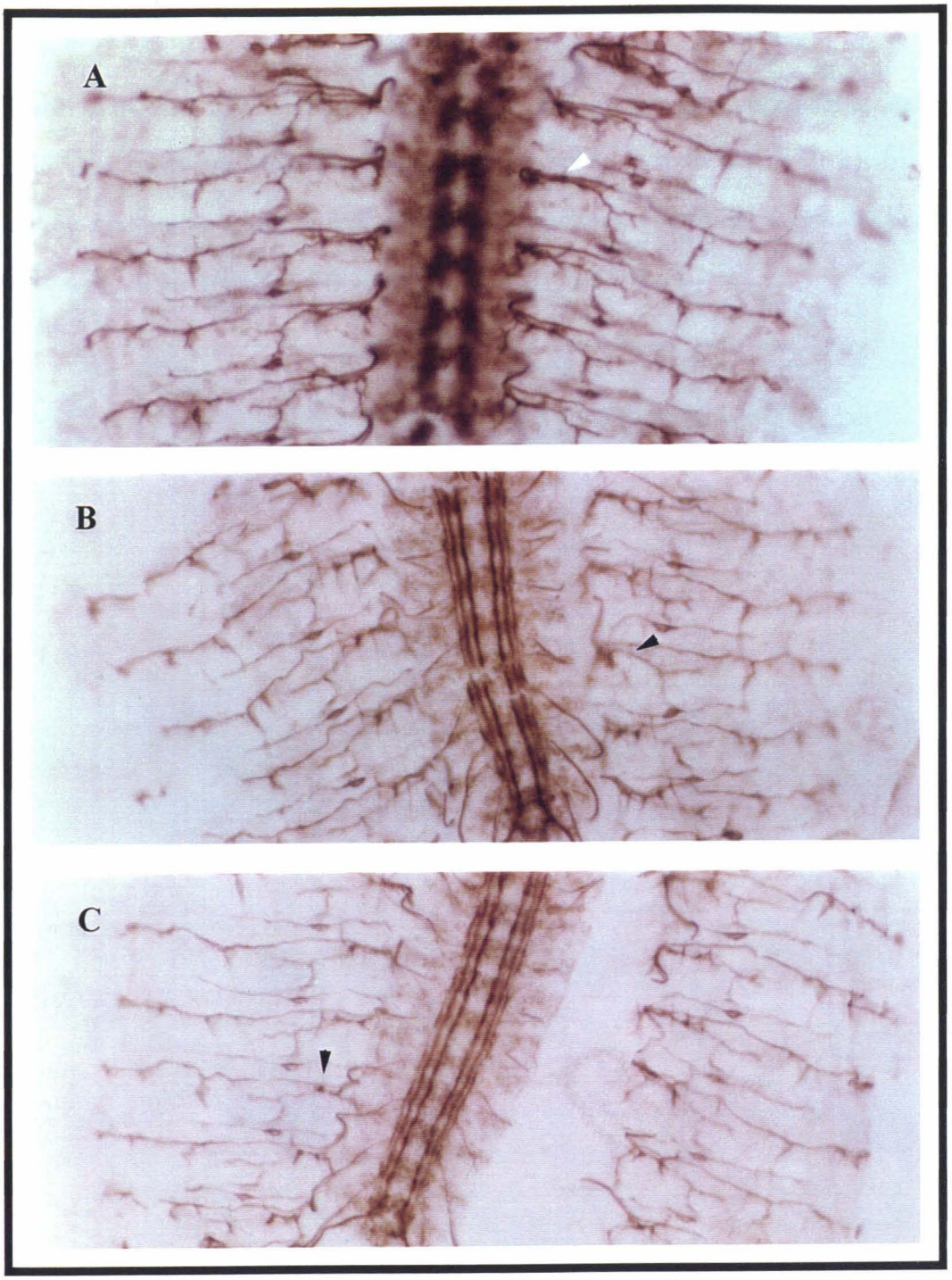

A
B
C

Figure 8. Minor abnormality induced by mutant PKA expression - II

Scabrous-GAL4 driven UIC expression in late stage 17 embryos and first instar larvae. CNS structures are visualized by MAbs raised against fasciclin II. a), b), c), show embryos and larvae with prolonged Fasciclin II expressing on the CNS and hypertrophic CNS tails; d) shows an embryo with completely disrupted CNS.

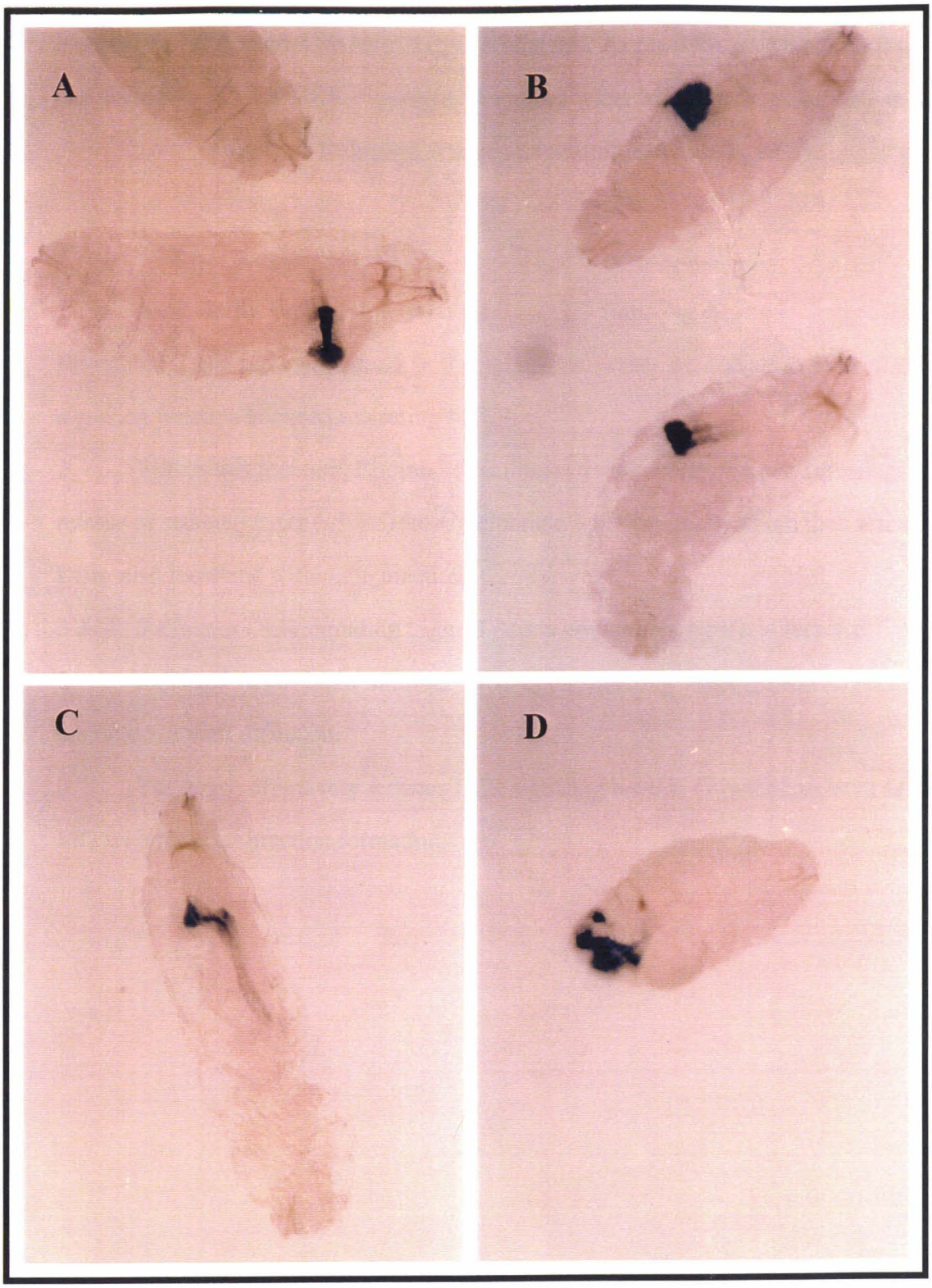

A
B
C
D

Figure. 9 A model on how Argos might act: Argos diffuses and functions as a juxtacrine to inhibit DER signaling in neighboring MG, leading the MG to enter apoptosis (adopted from Stemerdink and Jacob, 1997).

1. A modle for suppression of autocrine Argos signaling during MG signaling. Rhomboid, DER and components of the membrane skeleton associate in juxtacrine signaling junctins between migrating MG.

2. Within this junction, Rhomboid facilitates transmembrane Spitz signaling, or the release of secreted Spitz (sSpitz) from membrane Spitz (mSpitz), which then activates DER, also localized within the junction.

3 & 4. Sufficient DER signaling triggers Argos production, which is secreted.

5. Released Argos has a minimal effect upon signaling within junctions because of restricted inward diffusion.

6. Yet Argos effectively reduces DER signaling outside of junctions, such as distant MG with reduced junction formation.

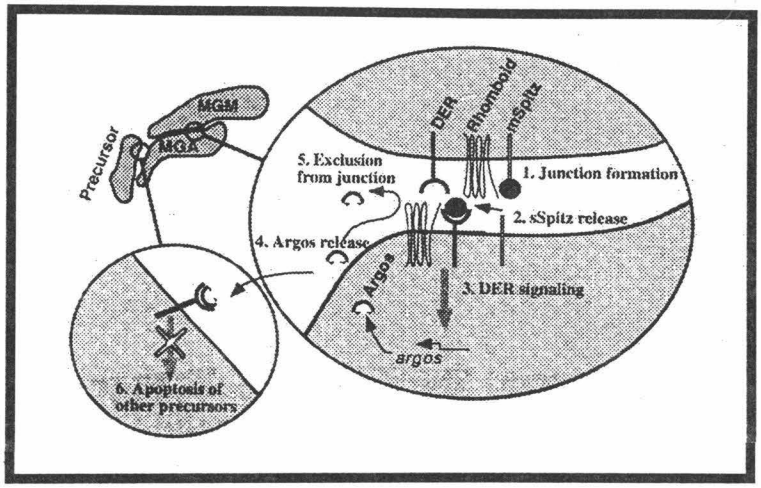

MGM
MGA
Precursor
DER
Rhomboid
mSpitz
5. Exclusion
from junction
1. Junction formation
2. sSpitz release
4. Argos release
Argos
3. DER signaling
argos
6. Apoptosis of
other precursors

Figure 10. Argos misexpression alters commissure regularity

The CNS axon tracts are visualized by Fasciclin II immunolabeling followed by HRP histochemistry. Stage 12/3 embryos of A) wild-type, and B) *hs-argos* were subjected to three 45-minute heat shocks and aged to stage 16 before fixation and immunolabeling. Arrows in B) indicate fasciclin II misexpression on commissural axons.

