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EGFR signaling is required for the differentiation and maintenance of neural progenitors along the dorsal midline of the *Drosophila* embryonic head

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SUMMARY

EGFR signaling has been shown in recent years to be involved in the determination, differentiation and maintenance of neural and epidermal cells of the ventral midline (mesectoderm and ventromedial ectoderm). Localized activation of the TGFα homolog Spitz (Spi) in the mesectoderm is achieved by the products of the genes *rhomboid* and *Star*. Spi binds to its receptor, the *Drosophila* epidermal growth factor receptor homolog (*Egfr*), and triggers the Ras pathway which is needed for the survival and differentiation of ventral midline cells. The results reported here indicate that EGFR signaling is also required in a narrow medial domain of the head ectoderm (called 'head midline' in the following) that includes the anlagen of the medial brain, the visual system (optic lobe, larval eye) and the stomatogastric nervous system (SNS). We document that genes involved in EGFR signaling are expressed in the head midline. Loss of EGFR signaling results in an almost total absence of optic lobe and larval eye, as well as severe reduction of SNS and medial brain. The cellular mechanism by which this phenotype arises is a failure of neurectodermal cells to differentiate combined with apoptotic cell death. Overactivity of EGFR signaling, as achieved by heat-shock-driven activation of a wild-type *rhomboid* (*rho*) construct, or by loss of function of *argos* (*aos*) or *yan*, results in an hyperplasia and deformity of the head midline structures. We show that, beside their requirement for EGFR signaling, head and ventral midline structures share several morphogenetic and molecular properties.

Key words: EGFR signaling, brain, visual system, stomatogastric nervous system, differentiation, apoptosis

INTRODUCTION

During early neurogenesis parts of the ectoderm transform into neural progenitor cells which segregate from the surface of the embryo, proliferate inside the embryo and give rise to a multitude of different neuronal and glial cell types. In vertebrates, a large coherent domain of the ectoderm, called the neurectoderm, invaginates and forms the neural tube; proliferation of neural progenitors and subsequent differentiation takes place in the wall of this tube (for review, see Jacobson, 1991). In most invertebrates, including insects, most of the neurectoderm stays at the surface and delivers individual neural progenitor cells, called neuroblasts, into the embryo (for review, see Campos-Ortega, 1993; Goodman and Doe, 1993). However, there are some neurectodermal domains, such as the mesectoderm and parts of the neurectoderm of the head which are the object of this study, that behave more similar to the vertebrate neurectoderm in that they invaginate from the surface as coherent groups of cells.

Early neurogenesis is a period of intense 'crosstalk' between cells of the neurectoderm. Many decisions have to be made and carried out in regard to the morphogenetic behaviors of neurectoderm cells. For example, the boundary between neurectoderm and epidermal ectoderm has to be delineated. Neurectoderm cells have to constrict apically in a well-coordinated manner to invaginate (in vertebrates) or delaminate (in insects). Cells along the boundary of the vertebrate neurectoderm have to break lose from their epidermal neighbors in order for the neural tube to close. The pattern of proliferation of neural progenitors has to be coordinated with neural differentiation. A large number of secreted and membrane-bound molecules have been identified that play a role in these different morphogenetic processes. Adhesion molecules, such as N-cadherin in vertebrates and E-cadherin in *Drosophila*, are essential for early neurogenesis (Detrick et al., 1990; Yan et al., 1991; Kintner, 1992; Levine et al., 1994; Tepass et al., 1996). Secreted and membrane-bound signal molecules, their receptors and intracellular signal transduction systems are expressed in the neurectoderm. The epidermal growth factor (EGF) receptor and its ligands, EGF and TGFα, form one system that has been implicated in different events of early neurogenesis (for review, see Plata-Salaman, 1991; Ferrer et al., 1996). In a variety of different cultured vertebrate cell lines, EGF has an effect on cell motility, proliferation, differentiation and cell survival. EGFR and its ligands are widely expressed in the developing vertebrate nervous system (Hall and Ekanayake, 1991; Ferrer et al., 1996). Experimental in vivo studies suggest a function of EGFR signaling in the

control of cell proliferation and cell fate (e.g., Lillien, 1995; Craig et al., 1996).

In *Drosophila*, EGFR signaling has been investigated in detail in several systems, in particular the compound eye, embryonic mesectoderm and ventral ectoderm, and oogenesis. In the compound eye, EGFR signaling is required to promote the differentiative fate of all ommatidial cells, including photoreceptor, cone cells and pigment cells (for recent review, see Freeman, 1997; Schweitzer and Shilo, 1997). It has been shown that the *Drosophila* TGFα homolog, Spi, is secreted by photoreceptor R8 and other photoreceptors; this ligand activates in the cells surrounding R8 the *Drosophila* EGF receptor, encoded by the *Egfr* (or *DER*) gene, which in turn triggers the Ras pathway. Ras signaling regulates the activity of several transcription factors, among them those encoded by the *yan* and *pointed* (*pnt*) genes, which modulate an (unknown) program of genes required for ommatidial cell differentiation.

In the embryo, Egfr and its ligand Spi are expressed widely (Zak et al., 1990; Rutledge et al., 1992). However, Spi is activated at specific locations, notably the mesectoderm, by *rho* and *Star* (*S*) (Schweitzer et al., 1995a,b; Golembo et al., 1996a). Spi acts upon the mesectoderm cells themselves, as well as the directly adjacent ventral ectoderm cells. Cells receiving the Spi signal activate the Ras pathway and express *argos* (*aos*), which encodes a secreted protein inhibiting further EGFR activation by Spi (Schweitzer et al., 1995a,b; Golembo et al., 1996b). The Ras pathway activates *pnt* and inhibits *yan*, the same transcriptional modulators identified in the eye (Klaes et al., 1994; Gabay et al., 1996). Loss of EGFR signaling results in failure of the mesectoderm cells to differentiate, as well as changes in differentiative fate of the ventral ectoderm cells (Raz and Shilo, 1992, 1993; Sonnenfeld and Jacobs, 1994; Klaes et al., 1994; Dong and Jacobs, 1997).

In this study, we have investigated the role of EGFR signaling in the neurectoderm of the embryonic head (procephalon). Most of the procephalic neurectoderm produces neuroblasts in a mode that is similar to what is observed in the ventral ectoderm of trunk segments (Younossi-Hartenstein et al., 1996). However, there are several populations of neural progenitors located along the medial fringes of the procephalic neurectoderm that differ profoundly from their lateral neighbors in the way that they develop. These cells, which will be called head midline cells in the following, give rise to the visual system (optic lobe, larval eye), ventromedial and dorsomedial parts of the brain, and the stomatogastric nervous system. Like their counterparts in the mesectoderm, the head midline cells do not give rise to typical neuroblasts by delamination, but stay integrated in the surface ectoderm for an extended period of time. The proneural gene *l'sc*, which transiently (for approximately 30 minutes) comes on in all parts of the procephalic neurectoderm while neuroblasts delaminate (Younossi-Hartenstein et al., 1996), is expressed continuously in the head midline cells for several hours. Finally, head midline cells, similar to ventral midline cells of the trunk, require the EGFR pathway. In embryos carrying loss-of-function mutations in *Egfr*, *spi*, *rho*, *S* and *pnt*, most of the optic lobe, larval eye, SNS and dorsomedial brain are absent. This phenotype arises by a failure of many neurectodermal cells to segregate (i.e., invaginate) from the ectoderm; in addition, around the time when segregation should take place, there is an increased amount of apoptotic cell death which removes many head midline cells. In embryos where EGFR signaling is activated ectopically by inducing *rho*, head midline structures are variably enlarged. A typical phenotype resulting from the overactivity of EGFR signaling is a 'cyclops' like malformation of the visual system, in which the primordia of the visual system stay fused in the dorsal midline.

MATERIALS AND METHODS

Fly stocks and egg collections

As wild-type stock, we used Oregon R. The following mutations, which are described in Lindsley and Zimm (1992) if not otherwise indicated, were used in this study: $Egfr^{f5}$ (flb^{2G31}; kindly provided by Dr U. Banerjee), spi^{1} (spi^{IIA}; kindly provided by Dr U. Banerjee), pnt^{1} (pnt^{8B74}; kindly provided by Dr C. Klämbt), yan^{e2d} (Rogge et al., 1995), aos^{d7} (Freeman et al., 1992; kindly provided by Dr C. Klämbt), rho^{M3} (kindly provided by Dr E. Bier), *hs-rho*^{IC} (kindly provided by Dr E. Bier) and *Df(3R) H99*. (kindly provided by Dr J. Lengyel). Egg collections were done on yeasted apple juice agar plates. Embryonic stages are given according to Campos-Ortega and Hartenstein (1985). For heat-shock-induced *rho* expression, agar plates with staged collections of embryos carrying the *hs-rho* construct were raised at 25°C for various time intervals in the period between 3 and 8 hours after egg laying, placed in a 37°C incubator for 1 hour and allowed to complete development up to stage 16 (approx. 13 hours at 25°C) before fixing them.

Markers

To visualize the pattern of expression of *rho*, *aos*, *pnt* and *yan* the following markers were used: a full-length cDNA for *aos* (Freeman et al., 1992; kindly provided by Dr M. Freeman) and *S* (Kolodkin et al., 1994; kindly provided by Dr U. Banerjee), P*lacZ* insertion in *rho* (Bier et al., 1990; kindly provided by Dr E. Bier), P*lacZ* insertion in *aos* (Freeman et al., 1992; kindly provided by Dr C. Klämbt), P*lacZ* insertion in *pnt* (Klaes et al., 1994; kindly provided by Dr C. Klämbt) and monoclonal antibody against Yan (Lai and Rubin, 1992; kindly provided by Dr Z. Lai). A commercial monoclonal antibody against activated MAPK (dp-ERK) (available through Sigma) was used to visualize the embryo domains in which the Ras signaling pathway was activated (Gabay et al., 1997a,b). To characterize the phenotype arising from loss or overactivity of EGFR signaling, the following markers were used: monoclonal antibody against the Fasciclin II protein (Grenningloh et al., 1991; kindly provided by Dr C. Goodman), which labels subsets of neuronal precursors, among them part of the optic lobe, larval eye and dorsomedial brain; mAb22C10, which labels sensory neurons (Zipursky et al., 1984; kindly provided by Dr S. Benzer); a monoclonal antibody against the Crumbs protein, which labels apical membrane of ectodermal tissues (Tepass et al., 1990; kindly provided by Dr E. Knust) and P*lacZ* insertion in *sine oculis* (*so*; Cheyette et al., 1994) to label optic lobe.

Immunohistochemistry and histology

Expression of β-galactosidase (β-Gal) in enhancer-trap lines and promoter constructs was detected with a polyclonal anti-β-galactosidase antibody (Cappel; dilution 1:2000). The anti-Fas II antibody was diluted 40-fold, anti-Crumbs 1:20, mAb22C10 1:50, anti-dp-ERK 1:1000. For staining, embryos were collected, dechorionated and fixed for 30 minutes in a mixture of 4% formaldehyde in PEMS (0.1 M Pipes, 2 mM $MgSO_4$, 1 mM EGTA, pH 7.0) with heptane. They were devitellinized in methanol and further prepared for antibody labelling following the standard procedure (e.g., Ashburner, 1989). Sectioning of embryos was done on an LKB Ultrotome V. Serial sections of 3-5 μm thickness were mounted on slides, weakly counterstained with methylene

blue/toluidine blue (Ashburner, 1989) and covered with Permount (Sigma).

In situ hybridization

Digoxigenin-labeled DNA probes were prepared following manufacturers' instructions (Genius kit; Boehringer) using full-length cDNAs of the AS-C gene *l`sc* (Cabrera et al., 1987; kindly provided by Dr S. Campuzano), the E(SPL)-C genes *m5* and *m7* (Klämbt et al., 1989; Knust et al., 1992; kindly provided by Dr E. Knust), *aos* and *S*. In situ hybridizations to whole-mount embryos were prepared according to the protocol of Tautz and Pfeifle (1989). Embryos were dehydrated and embedded in Epon.

RESULTS

Neurectodermal cells of the mesectoderm and head midline differ from lateral neurectoderm cells in their pattern of early neurogenesis

The development of the mesectoderm has been followed in detail in several previous studies (Klämbt et al., 1991; Sonnenfeld and Jacobs, 1994; Bossing and Technau, 1994). Mesectoderm cells give rise to neurons as well as glia cells; they differ from the adjacent 'lateral' neurectoderm in two important aspects (Figs 1, 2A,B). First, mesectoderm cells never form typical neuroblasts. Instead, they remain integrated as epithelial cells in the surface ectoderm until quite late in development (stage 12; 8-9 hours), as opposed to neuroblasts, which segregate from the ectoderm during stages 9 to 11 (before 6 hours; Tepass and Hartenstein, 1994). Mesectoderm cells undergo a single division in the horizontal plane (i.e., parallel to the surface; Hartenstein and Campos-Ortega, 1985), whereas neuroblasts divide in a stem cell mode with a perpendicularly oriented mitotic spindle. Secondly, whereas neuroblasts forming in the lateral neurectoderm are surrounded by cells that stay at the surface and later form the epidermis, mesectoderm cells invaginate from the surface ectoderm as a coherent group and uniformly give rise to neurons or glia cells, but not epidermis.

'Atypical' neurectodermal cells with the same properties as those described above for mesectoderm cells can also be found in the head of the embryo. These cells, which we propose to call 'head midline cells' in the following, are located in four domains: the anlage of the stomatogastric nervous system (SNS), dorsomedial and ventromedial domain of the brain (DMD, VMD), and optic lobe/larval eye (OL; Fig. 1). The anlage of the SNS forms a placode that moves inside the embryo along with the stomodeum (for a detailed description, see Hartenstein et al., 1994). Only a few individual SNS progenitors delaminate from this placode; the majority invaginate as a coherent group and form three epithelial vesicles (Fig. 2D,E). The DMD, VMD and OL are not located directly in the midline; instead, they form bilateral symmetric domains, which are separated by a median strip of flat epithelial cells (DMD and OL) or the clypeolabrum (VMD). Cells of the DMD and VMD invaginate as multiple small clusters around stage 13 (9 hours); shortly after this stage, they can still be recognized on the dorsomedian and basal brain surface as multiple minute vesicles with an internal lumen (Fig. 2D; see also Younossi-Hartenstein et al., 1996). Later, the vesicles dissociate and become incorporated into the brain, or undergo programmed cell death. The OL forms a placode that invaginates around the same stage as the SNS and DMD (Fig. 2G,H; for detailed description, see Green et al., 1993). The invaginated optic lobe cells attach themselves to the lateral brain surface as an epithelial vesicle; they remain quiescent during the remainder of embryonic development and start to proliferate during the larval period. A small contingent of the optic lobe placode forms the larval eye (Bolwig's organ).

Neurectodermal cells of the mesectoderm and head midline are characterized by an extended period of proneural and neurogenic gene expression

Proneural genes, which encode transcription factors of the bHLH family, are expressed in small clusters ('proneural clusters') of cells in the ventral and procephalic neurectoderm (Cabrera et al., 1987; Skeath and Carroll, 1992; Younossi-

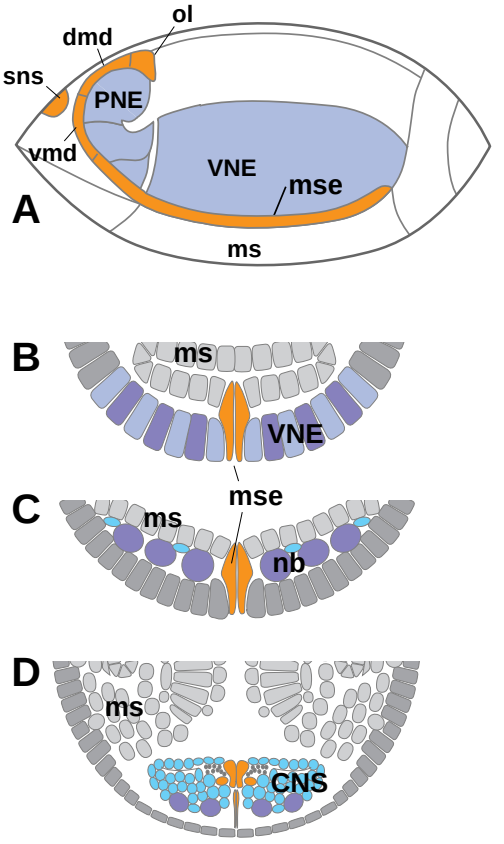


Fig. 1. Topology and properties of midline neurectoderm. (A) Schematic representation of blastoderm fate map, indicating location of lateral neurectoderm (purple; PNE procephalic neurectoderm; VNE ventral neurectoderm) and midline neurectoderm (orange; mse mesectoderm; ol optic lobe; dmd dorsomedial brain; vmd ventromedial brain; sns stomatogastric nervous system). (B-D) Schematic representations of cross sections of embryos of increasing age (B, stage 8; C, stage 10; D, stage 14). In lateral neurectoderm, individual neuroblasts (dark purple) delaminate and divide in a stem cell mode, producing ganglion mother cells and neurons (blue). By contrast, mesectodermal cells remain epithelial for a long period and do not divide in a stem cell mode. Between stages 12 and 14, they invaginate and form the midline structures of the CNS, including glia cells and pioneer neurons of the connectives. Other abbreviations: fg, foregut; ms, mesoderm; nb, neuroblast.

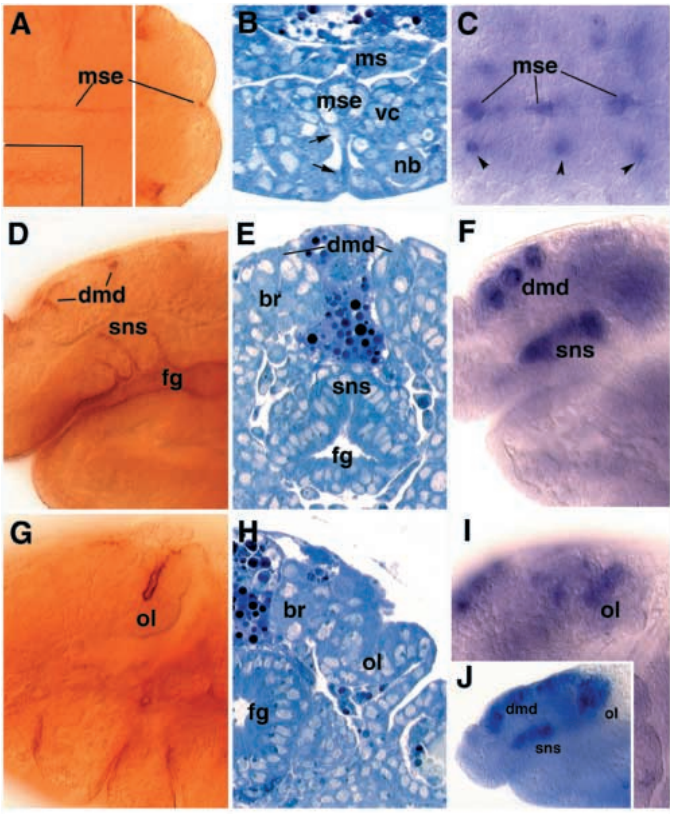


Fig. 2. Early neurogenesis of midline neurectoderm. (A,D,G) Whole mounts of stage 12 embryos labeled with anti-Crb antibody (A, ventral view; D,G, lateral view). (B,E,H) Cross sections of stage 12 embryos stained with methylene blue/toluidine blue/borax. (C,F,I,J) Whole mounts of stage 12 embryos probed with a *l'sc* cDNA (C, ventral view; F,I, lateral view) and a *E(spl) m5* cDNA (J, lateral view). These and pictures presented in the following figures were prepared as 'digital montages' by superimposing 2-3 digitized images to show structures at different focal planes. (A-C) Upper row documents epithelial character of mesectoderm cells (mse). Note Crb expression on narrow apical surface of mesectoderm cells (inset shows these cells at higher magnification; in right part of panel, curvature of germband at posterior pole of embryo is in focus). Cross section in B depicts long processes of mesectodem cells (arrows) whose cell bodies (mse) have shifted interiorly and lie between neurons of ventral nerve cord (vc; nb labels lateral neuroblast). C shows l'sc expression in mesectoderm(mse); lateral staining (arrowheads) corresponds to clusters of mesodermal cells. (D-F) The second row shows representative views of segregating progenitors of the stomatogastric nervous system (sns) and dorsomedial brain (dmd). The former invaginate as three pouches from the foregut epithelium (fg in D); the latter form a series of small, linearly arranged invaginations which become attached to the dorsomedial surface of the brain (br). *l'sc* (F) and *E(spl) m5* (J) are continuously expressed in the SNS and DMD. (G-I) The lower row focuses on optic lobe invagination (ol), which appears at the dorsoposterior surface of brain (br). (I,J) Expression of *l'sc* and *E(spl) m5* in optic lobe.

Hartenstein et al., 1996). Expression of the proneural genes renders cells competent to form neuroblasts. By means of inhibitory cell-cell interaction within each proneural cluster, proneural gene expression becomes restricted to one single cell, which subsequently segregates as a neuroblast (Artavanis-Tsakonas et al., 1991; Campos-Ortega, 1993); shortly after delamination, proneural gene expression in the neuroblast ceases. The time period from the first appearance to the decline of proneural gene expression in a given neuroblast is only about 30 minutes. The inhibitory interaction among cells of a proneural cluster is mediated by a group of genes called neurogenic genes, which include the signal *Delta* (*Dl*), its receptor *Notch* (*N*) and the Enhancer of split E(spl) complex, which encodes transcription factors activated by Notch (for recent review, see Artavanis-Tsakonas et al., 1995). Thus, in the typical neurectoderm, proneural gene expression promotes a state of 'neural competency' in groups of neurectoderm cells; this state is quickly resolved when, by means of Notch-Delta signaling, cells 'decide' to segregate as neural progenitors or to remain in the ectoderm as epidermal progenitors.

In the atypical neural progenitors of the head midline, expression of *l'sc* and genes of the E(spl) complex lasts for several hours (Fig. 2F,I,J). Similarly, expression of *l'sc* in the mesectoderm is significantly extended (Fig. 2C) in comparison to lateral proneural clusters. This expression pattern indicates that Notch-Delta signaling is continuously active in the head midline cells without effecting a separation between neural and epidermal progenitors. As a result, the midline cells do not differentiate as neural cells, as evident from the fact that they remain epithelial for several hours and do not express any neural-specific markers.

Genes involved in the EGFR pathway are expressed in the head midline

Both the Egfr and its ligand Spi are ubiquitously expressed in the embryo. However, the genes involved in locally activating and restricting EGFR signaling, i.e. *rho* and *aos*, are expressed in specific embryonic domains in which EGFR signaling is required (Schweitzer et al., 1995a,b; Golembo et al., 1996a,b; Gabay et al., 1996; Xiao et al., 1996). Around the stage of gastrulation (stage 5-8), *rho* is expressed in the mesectoderm and approximately four rows of ectoderm cells on either side of it ('ventromedial ectoderm'). *rho* expression leads to activation of the ligand Spi that has been postulated to form a gradient with high concentrations medially and low concentrations laterally (Golembo et al., 1996a). In support of this notion, a graded activation of EGFR in the mesectoderm and ventromedial ectoderm has been recently shown by using an antibody against activated ERK (Gabay et al., 1997a,b). The two medial rows of ventromedial ectoderm cells receiving the highest concentration of Spi activate the expression of *aos*, a gene encoding a secreted protein that has been shown to inhibit EGFR activation by Spi in the lateral part of the ventromedial ectoderm. Thus, following *aos* expression, the domain exhibiting ERK phosphorylation narrows.

The ventromedial domain of EGFR activation, as monitored by *rho*, *aos* and phosphorylated ERK expression, continues into the head ectoderm. As shown in Fig. 3A, the ventromedial stripe of *aos* expression bifurcates at the level of the stomodeal plate and continues dorsally. Approaching the dorsal midline, it turns posteriorly and continues all the way to the posterior boundary of the head neurectoderm. There is an anterior-median patch of *aos* expression corresponding to the anlage of the stomatogastric nervous system. At later stages (stage 10-12) expression of *aos* in head midline structures becomes more complex; however, a high level of expression persists in large

parts of the optic lobe, SNS, VMD and DMD (Fig. 3C). The domain of *rho* expression (not shown) and ERK phosphorylation (Fig. 3B,D) matches closely the *aos* expression domain. Fig. 3E,F shows ERK phosphorylation in the SNS and optic lobe of a stage 13 embryo. Also *pnt* and *yan*, two transcription factors executing the signal passed to the nucleus by the EGFR signalling cascade, are expressed in structures of the head midline (data not shown).

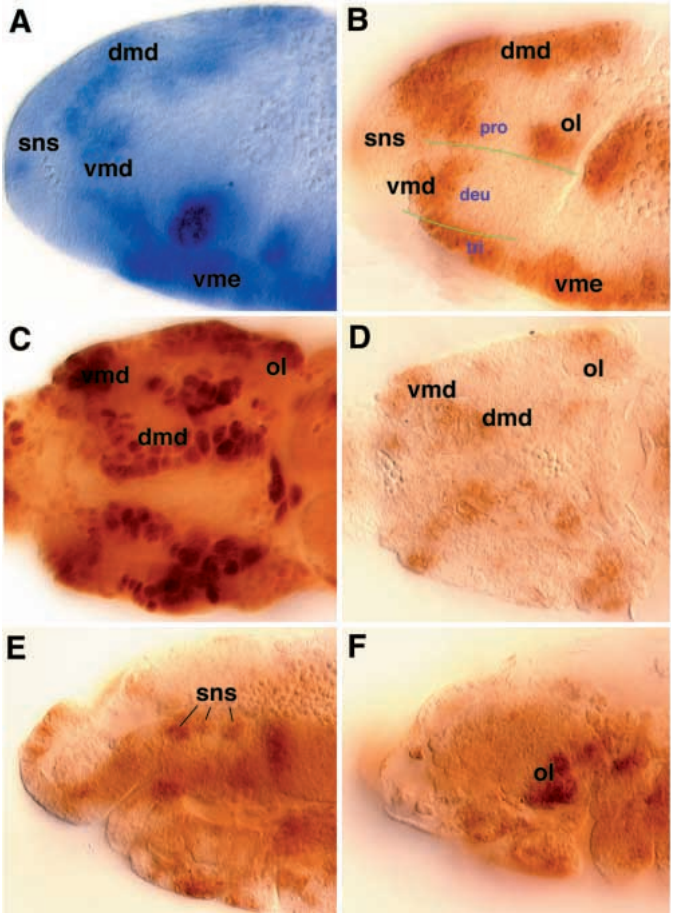


Fig. 3. Pattern of EGFR signaling in the embryonic head. (A) Lateral view of stage 8 embryo labeled with *aos* cDNA. *aos* is expressed in the ventromedial ectoderm (vme) of the trunk, as well as a continuous stripe that includes the anlage of the ventromedial brain (vmd) and dorsomedial brain (dmd). *aos* is also expressed in the anlage of the stomatogastric nervous system (sns). (C) *aos* expression at stage 11 (dorsal view), visualized by P*lacZ* insertion into the *aos* gene. Labeling is still seen in the medial domains of the brain (vmd, dmd), as well as the posterior lip of the optic lobe (ol). (B,D) Views similar to those shown in A and C of embryos labeled with anti-dp ERK to visualize domains in which the Ras pathway is activated. The fact that EGFR is responsible for Ras activation in the head was verified by the absence of the corresponding dp-ERK expression pattern in Egfr mutant embryos (not shown; see also Gabay et al., 1997b). Domains of dp-ERK expression are very similar to domains of *aos* expression shown in A and C, except that dp-ERK is activated in optic lobe (ol) earlier than *aos*. (E,F) Lateral view of stage 13 embryo showing dp-ERK expression in invaginating stomatogastric nervous system (E; deep focal plane) and optic lobe (F; superficial focal plane). pro, protocerebrum; deu, deuterocerebrum; tri, tritocerebrum.

The EGFR pathway is required in the head midline for proper cytodifferentiation and epithelial maintenance

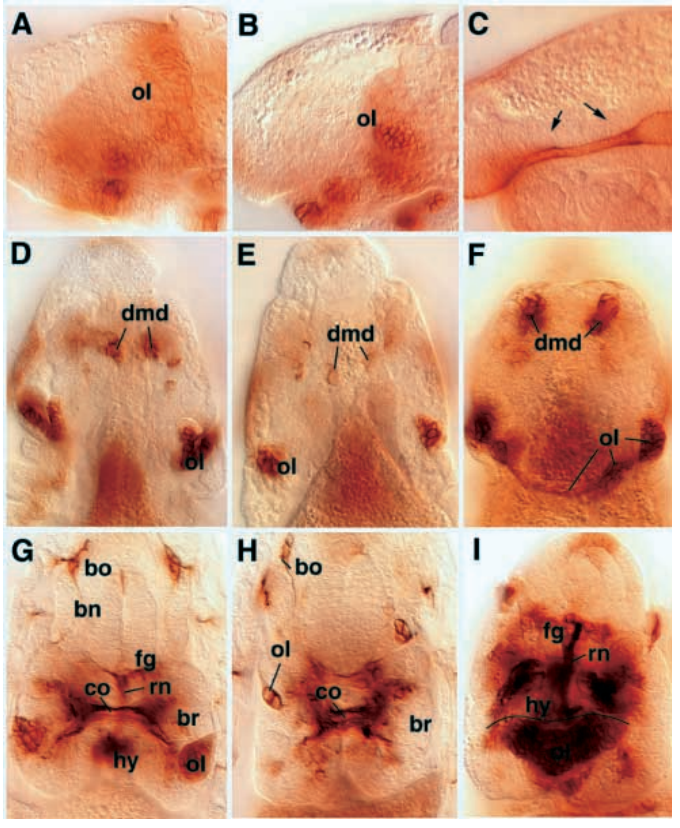
Phenotypic analyses have been carried out for all genes involved in EGFR signaling, using markers for the different partitions of head midline (see Material and Methods). In late embryos, loss of EGFR signaling results in almost total loss of optic lobe and Bolwig's organ (Fig. 4B,E,H), as well as severe reduction in other components of the head midline (dorsomedial and ventromedial brain, SNS; Fig. 4E,H). The phenotype of *Egfr* nulls is most severe; phenotypes caused by the loss of *rho*, *spi*, *S* or *pnt* (collectively called the Spitz-group of genes) are milder and resemble each other closely. In all Spitz-group mutants, abnormalities become first apparent at a stage when head midline cells would normally begin to segregate from the surface ectoderm. The number of head midline cells (shown for SNS, DMD and OL in Fig. 4) that turn on differentiation markers such as the FasII antigen is reduced and normal morphogenesis (e.g., invagination of the optic lobe or SNS, shown in Fig. 4E,C) does not take place. At the same time, apoptotic cell death sets in, resulting in the disappearance of most cells of the head midline. Fig. 5B shows masses of cellular debris filled macrophages (labeled with the anti-peroxidasin antibody; Tepass et al., 1994) in an early stage 12 *Egfr* null embryo. Many macrophages are located at the surface, due to the degeneration of surface ectoderm cells. In wild-type embryos of a comparable stage, the amount of cell death is very low, as indicated by the fact that most macrophages have not yet taken up cellular debris (compare insets of Fig. 5A and B for size of macrophages). Cell death in the optic lobe of *rho* mutant embryos can be appreciated in Fig. 5D, in which a P*lacZ* insertion in the *sine oculis* (*so*) gene was used as a marker of OL cells. Most of these cells, in particular those of more dorsal regions, have disintegrated and appear as small *lacZ*-expressing fragments at the surface or inside macrophages.

The early expression of cell fate markers, such as *so* in Spitz-group mutants, is unaltered. Thus, as evident in Fig. 5D, the overall region of *so* expression is at least as large as in wild type (Fig. 5C), indicating that a normal number of cells had originally turned on the gene. Likewise, the pattern of cell division in the early optic lobe anlage of *Egfr* and Spitz-group mutants is unchanged. Cells normally undergo two postblastoderm divisions during stage 8 and early 10 (mitosis 14 and 15; Green et al., 1993); however, shortly after mitosis 15, abundant figures of cell death become apparent in the optic lobe placode (data not shown). By late stage 12, only a few optic lobe cells remain (Fig. 4E,H). The fact that loss of EGFR signaling in the embryonic visual system primarily causes cell death is particularly evident by crossing *Egfr* to embryos carrying the deletion *Df(3L)H99* that takes out the *reaper* complex of genes (*rpr*, *hid* and *grim*). In *Df(3L)H99* mutant embryos, apoptotic cell death is prevented. In the double mutant *Egfr*;*Df(3L)H99*, many phenotypic aspects of loss of *Egfr* function are rescued (Fig. 5E,F). The optic lobe is of approximately normal size and it expresses markers that can normally be recognized in wild type, including FasII. Precursors of the Bolwig's organ do not separate from the optic lobe and express the neuronal marker 22C10 (Fig. 5G,H). Similar results are observed in the double mutant *spi;Df(3L)H99* (data not shown). These findings suggest that

EGFR signaling is not required for the initial determination of cell fate in the head midline, but for the morphogenesis and maintenance of this region.

Overactivity of EGFR signaling, as achieved by heat-shock-driven activation of a wild-type *rho* construct (Sturtevant et al., 1993), as well as loss of function of *aos or yan*, results in a complex deformity of the visual system. The optic lobe is enlarged and it invaginates earlier and at a more dorsomedial position than in wild type (in wild type, there is a pronounced ventral displacement of the presumptive optic lobe and Bolwig's organ during stage 6-11). In many instances, the optic lobes of either side are fused in the dorsal midline, resulting in a 'cyclops'-shaped visual system with a single median optic lobe (Fig. 4F,I). The differentiation of the larval eye (Bolwig's organ) is also severely abnormal; in many cases, the Bolwig's organ does not separate from the optic lobe and fails to form axons. Loss of function of *yan* and, to a lesser extent, *aos*, also leads to a significant hyperplasia of the stomatogastric nervous system and DMD domain (Rogge et al., 1995). In case of the DMD, cells continue proliferating beyond a stage when they normally stop to do so. A similar overproliferation of the SNS placode has not been observed, suggesting that an increased number of SNS progenitor cells is responsible for the hyperplasia.

We carried out a series of 1 and 2 hour heat shocks on staged collections of embryos carrying the heat-shock-inducible *rho* construct to determine the phenocritical period at which *rho* overexpression causes abnormalities (Fig. 6). The results indicate that activation of *rho* between 5 and 7 hours postfertilization has the strongest effect. This time period coincides with the stage when head midline cells normally start to invaginate. By contrast, heat pulses before 5 hours and after 7 hours caused visual system defects at a much lower frequency. These findings support the conclusion derived from the loss-of-function studies described above that EGFR signaling is required for the morphogenesis and maintenance of head midline cells.



DISCUSSION

The findings reported in this paper show that EGFR signaling is required for a specialized population of neural progenitors located along the midline of the procephalic neurectoderm. Loss of function of EGFR signaling results in the failure of these cells to turn on certain differentiation markers (e.g., FasII) and to undergo normal morphogenetic movements (e.g., invagination), and in the eventual death of these cells.

The function of EGFR signaling has been investigated in considerable detail for the ventral midline structures of the embryo where EGFR signaling is required during multiple steps of epidermal and neural development. Two main primordial structures with different developmental fates can be distinguished in the ventral midline: the mesectoderm and the

Fig. 4. Defects in head development resulting from the absence and overexpression of EGFR signaling. (A,D,G) Heads of wild-type embryos labeled with anti-FasII antibody which stains specific groups of neurectoderm cells. (A) Stage 11 embryo in lateral view. A large procephalic domain, which includes the entire future optic lobe primordium (ol), expresses FasII at a low level. At stage 12 (D; dorsal view, anterior to the top) the FasII expression level has increased in the ventral part of the optic lobe invagination (presumptive Bolwig's organ) and its posterior lip. In addition, several Fas II-positive clusters have appeared in the dorsomedial domain (dmd). (G) Stage 16 embryo, dorsal view, anterior to the top. The optic lobe (ol) has invaginated and is attached to the ventral surface of the brain hemispheres (br). Note labeling of anteriorly shifted Bolwig's organ (bo) and Bolwig's nerve (bn), commissural axons (co) formed by Fas II-positive clusters, and stomatogastric nervous system, consisting of frontal ganglion (fg), recurrens nerve (rn) and hypocerebral ganglion (hy). (B,E,H) Heads of embryos mutant for the *spi* gene. Stages and orientations correspond to those shown for wild type in left column. (B) At stage 11, wide area of low level Fas II expression can be seen in posterior procephalon. (E) By late stage 12, only small cluster of Fas II-positive cells is left of the optic lobe; the remainder of the cells have been eliminated by cell death (see Fig. 5). Also the Fas II-positive clusters in the dorsomedian domain (dmd) are reduced in size. (H) Stage 16 embryo; Fas II-positive remnants of the optic lobe and Bolwig's organ can be distinguished. The recurrens nerve and hypocerebral ganglion are absent. Note also loss of dorsomedian brain structures, as evidenced by shortening of the commissure (co). (C) Lateral view of stage 12 *spi* mutant embryo, showing absence of SNS invaginations (should appear at location in between arrows; compare to D of Fig. 2, which shows view of wild type). (F,I) Dorsal views of stage 12 (F) and stage 16 (I) *aos* mutant embryos. At stage 12, Fas II-labeled posterior lip of optic lobe (ol) reaches all the way up to the dorsal midline. Dorsomedian clusters (dmd) are increased in size. At stage 16, enlargement and fusion of optic lobes ('cyclops' phenotype) is evident (black line demarcates boundary between two focal planes; optic lobe posterior to black line is located deeper (i.e., ventral) than structures shown anterior of black line). Note also increased number of Fas II-positive neurons in frontal ganglion (fg) and hypocerebral ganglion (hy) and thickened recurrens nerve (rn).

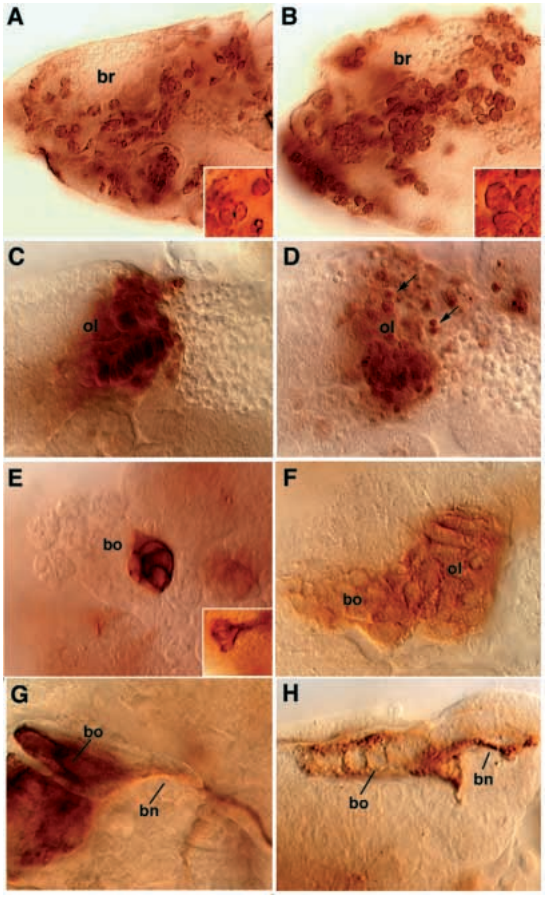


Fig. 5. Cell death following lack of EGFR activation. (A,B) Lateral view of whole mounts of stage 11 embryos (A, wild type; B, *Egfr* mutant) labeled with anti-Peroxidasin antibody to visualize macrophages. In wild type, the antibody labels numerous hemocytes that, in the absence of large-scale cell death, have not yet phagocytosed cellular debris. In *Egfr* mutant of similar stage, due to massive cell death in the head, all hemocytes have become phagocytic macrophages, as clearly seen by their large size and internalized cell fragments (compare cells shown in insets of A and B). (C,D) Lateral view of late stage 12 embryos (C, wild type; D, *rho* mutant) in which a P*lacZ* insertion in the *sine oculis* (*so*) gene visualizes cells of the optic lobe (ol). In mutant, domain of *so* expression is of roughly normal size and position, but most *so*-expressing cells have fragmented during the course of apoptosis (arrows point at examples). (E,F) Lateral views of stage 15 embryos (E, *Egfr* mutant ; F, *Egfr;Df(3L)H99* double mutant) labeled with anti-Fas II; (G,H) Same stages and orientations as in E and F stained with mAb22C10 are represented (G, wild type; H, *Egfr;Df(3L)H99* double mutant). Insert in E represents *Egfr* mutant stained with the neuronal marker mAb22C10. In the *Egfr* mutant (E), the visual system is almost entirely absent, except for a small group of cells that also express mAb22C10 and thereby can be recognized as Bolwig's organ (inset in E). In the double mutant, *Egfr;Df(3L)H99*, in which cell death is inhibited the visual system is rescued to about its normal size. However, there appear structural abnormalities in both optic lobe (failure to invaginate) and Bolwig's organ (photoreceptor cells retain an epithelial morphology and do not separate from the optic lobe).

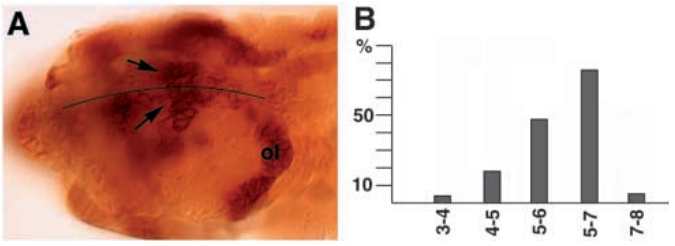


Fig. 6. Stage 13 embryo labeled with anti-FasII, following heat-shock-induced overexpression of *rho* between 5 and 7 hours (dorsolateral view; solid line demarcates dorsal midline). Note dorsomedial expansion of optic lobe (ol) and expansion/dorsal fusion of FasII-positive DMD clusters (arrows). Histogram showing percentage of *hs-rho* embryos with head midline phenotype (as shown in photograph) following heat shock given at the indicated times.

adjoining ventromedial ectoderm. In embryos lacking *Egfr* function, the ventromedial epidermis, a large fraction of CNS neurons and cells derived from the mesectoderm (in particular, midline glia cells) are absent. The development of this phenotype and how it relates to EGFR activation at different times is complex and will be briefly discussed separately for the three different tissues, in order to establish a basis on which to compare the head phenotype introduced in this paper with the phenotype in the trunk.

The loss of ventromedial epidermis is mainly caused by a transformation of cell fate. According to temperature-shift experiments, using a temperature-sensitive allele of the *Egfr* (Raz et al., 1992), the phenocritical period for the loss of ventromedial epidermis lasts from approx. 2.5 to 6 hours (stage 6-11). Markers for ventromedial ectoderm (e.g., *FasIII*) are not expressed in *Egfr*-deficient embryos; they are replaced by markers normally expressed more laterally (e.g., *dpp*). Furthermore, overexpression of secreted Spi (Schweitzer et al., 1995a) results in a significant widening of the ventromedial ectoderm, as assayed by molecular markers expressed in this region, such as *orthodenticle*. In contrast, it has been reported that cell death may also be in part responsible for the absence of ventromedial ectoderm markers in *Egfr* mutant embryos, given the abundance of pycnotic cells in this region even before markers such as FasIII are expressed (Clifford and Schüpbach, 1992; own unpublished observation).

The development of the *Egfr* mutant phenotype in the CNS has not been followed in great detail. Ventromedial neural cells (including neuroblasts and their progeny) that normally express the FasIII marker are absent from stage 11 onward. Since *Egfr* is not expressed in neuroblasts or neurons (Raz et al., 1992), this deficit has been interpreted to represent a consequence of the phenotype in the ventromedial ectoderm. Thus, as a result of the early transformation of the ventromedial ectodermal fate to a more lateral fate and/or cell death, certain neuroblasts from this region are lacking and/or do not do not produce their normal number of progeny.

In the mesectoderm and its derivatives, there are clearly at least two time periods at which EGFR signaling is required. It has been shown that mesectodermal cells express *Egfr* at an early stage and then turn it off (Zak et al., 1990; Katzen et al., 1991). This early expression is required for the formation of mesectodermal derivatives, such as midline glia cells; rearing embryos carrying the $Egfr^{ts}$ allele at the restrictive temperature between 3 and 6 hours resulted in a loss of midline glia cells (Raz and Shiloh, 1992). Similarly, loss of the signal Spi or its

activator Rho results in a phenotype in which at least part of the midline glia cells are missing from the earliest time onward at which they can be recognized by specific markers (Sonnenfeld et al., 1991). The mechanism that results in loss of midline glia cells in *Egfr* and *spi* mutants is apoptotic cell death. In addition to this early phase, *Egfr* is expressed and required in the midline glia cells also at a later stage. Thus, in *Egfr* ^{ts} mutant embryos reared at the permissive temperature up until 6 hours, then shifted to the restrictive temperature for the rest of embryogenesis, midline glia cells fail to form regular sheaths around the commissures, resulting in a fused commissure phenotype (Raz et al., 1992).

The phenotype in the head midline structures of *Egfr* mutant embryos can be most easily compared with the above summarized abnormalities of the mesectoderm and its derivatives. Thus, in both mesectoderm and head midline, EGFR signaling is activated around gastrulation (stage 7). Incubating embryos with an antibody against dp-ERK, a marker that serve as indicators of activated EGFR signaling, show uninterrupted stripes of labeling in the ventromedial ectoderm, head midline, and mesectoderm. At later stages (after stage 9), this labeling becomes generally weaker and more restricted to clusters of cells. In the mesectoderm, labeling disappears transiently, to be reactivated in specific cell types around stage 12. Similarly, among the head midline structures, strong labeling reappears in most cells of the invaginating SNS, optic lobe and several clusters of medial brain precursors. In both mesectoderm and head midline, EGFR signaling is required for proper morphogenesis to occur and to prevent cell death. During early stages of development (until stage 10), no abnormalities can be noticed in *Egfr*-deficient embryos. Cell division and the expression of early 'determination' markers, such as *single minded* (*sim*) in the mesectoderm and *so* in the optic lobe primordium, proceed normally. Then between late stage 10 and stage 12, massive cell death sets in, reducing the number of cells in these regions.

The *Egfr* loss-of-function phenotype in the head midline and the mesectoderm is well compatible with the postulated function of EGFR signaling in the *Drosophila* compound eye and in various vertebrate systems where EGFR signaling also promotes differentiation and prevents cell death. In the head midline, we do not observe any evidence for a transformation of cell fate similar to the one shown by Raz and Shiloh (1992, 1993) for the ventral neurectoderm of the *Drosophila* embryo. A transformation of fate would imply that the structures missing (e.g., optic lobe, dorsomedial brain) are replaced by other structures, which then would have to expand in size in order to occupy the space normally allotted to them, as well as the additional space normally taken by the midline structures. However, there does not appear to be an expansion of the lateral neurectoderm; for example, the number and pattern of neuroblasts delaminating from the lateral head neurectoderm in *Egfr* or *Spi* mutant embryos is normal (V. H., unpublished). Thus, it appears that the loss of head midline structures observed in EGFR-signaling-deficient embryos is effected mainly by cell death.

The gain of function of EGFR signaling causes a complex phenotype. It has been already reported in a previous study (Rogge et al., 1995) that, in *yan* loss-of-function mutants, a hyperplasia of both dorsomedial brain and stomatogastric nervous system can be observed. In case of the latter, cells of the DMD clearly overproliferate; ultimately, these cells express neural markers and become incorporated into the brain. The optic lobe phenotype is more difficult to interpret. Thus, cells located medially of the optic lobes express *fasII*, resulting in a single optic lobe ('cyclops' phenotype) overgrown by the enlarged brain. The abnormal shape and location of the optic lobe in *aos* mutants has been noted previously by Sawamoto et al. (1996). One might interpret this phenotype as a cell fate transformation: dorsomedial cells, which would normally not express *fasII* (and would not become part of the optic lobe), turn on this gene if the output of EGFR signaling is increased. However, to clearly interpret this phenotype, one would have to know much better what normally happens to the dorsomedial cells located between the optic lobes. At the blastoderm stage, the anlagen of the optic lobe map to a dorsomedial position (Hartenstein et al., 1985). Shortly thereafter, they move laterally, generating a thin membrane of amnioserosa like cells in between them. A lot of cell death takes place in this region, prompting one to speculate that cell death is important for the lateral shift of the optic lobe primordia. Supporting this idea, the optic lobe primordia in embryos in which no cell death occurs due to a deficiency that removes the *reaper* complex of genes (Nassif et al., 1998) are frequently extended towards the midline, similar to what can be observed in *yan* or *aos* loss of function. It is therefore possible that increased activity of EGFR signaling, rather than inducing another fate in the dorsomedial cells, rescues dorsomedial cells from cell death; the expression of FasII might reflect the fate that these cells would normally show if they were permitted to live. Clearly, more experimental studies are required to grasp the effect of EGFR signaling in this region.

In head midline structures, in particular the optic lobe and SNS, there may be a late phase of EGFR signaling (as assayed by the expression of aos and activated ERK) whose significance is not yet known. The fact that proneural and neurogenic genes are concomitantly expressed in these structures raises the possibility of an interaction between the EGFR and Notch signaling pathway. More specifically, our data suggest that EGFR signaling could be involved in modifying the inhibitory feed-back loop between neurogenic and proneural genes that exists in other neurectoderm cells. In the lateral neurectoderm, proneural gene expression triggers in clusters of ectodermal cells (proneural clusters) the 'competence' to become neural progenitors. At the same time, proneural genes switch on the signal Delta (Dl) that ultimately leads to the restriction of proneural gene expression and neural competence to a single neuroblasts. Thus, binding of Dl to its receptor N sets in motion a signal transduction cascade, which results in the activation of the E(spl)-C genes (Artavanis-Tsakonas et al., 1995). E(spl)-C genes cause the inhibition of proneural gene expression (Martin-Bermudo et al., 1995; Heitzler et al., 1996).

In the head midline neurectoderm, regulation of proneural and neurogenic genes has to be different. Thus, instead of a short burst of proneural gene expression in proneural clusters that is resolved into expression in individual neuroblasts, proneural genes are expressed for a long period of time; at the same time, the expression is never restricted to single neuroblasts. Since genes of the E(spl) complex are expressed in the same cells that express *l'sc*, the inhibitory loop between

E(spl)-C and proneural genes must be interrupted at some level. It is possible that EGFR signaling is causing the interruption of this inhibitory loop. Based on genetic studies of N and EGFR signaling in the compound eye, it has been speculated that one of the consequences of EGFR activation (which ultimately is required for all ommatidial cell types to differentiate) is to inhibit N signaling, since constituitively active N inhibits ommatidial cell differentiation (Schweitzer and Shilo, 1997) by preventing response to differentiative signals. However, the same effect could be achieved if EGFR signaling, similar to what is proposed here for the midline neurectoderm, interrupts the inhibition of proneural genes by E(spl). Although this would not prevent N signaling, it would cancel the effect of N signaling on downregulating proneural genes and thereby keep cells in a state of competency to respond to signals.

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