„Investigations on formation and specification of neural precursor cells in the central nervous system of the *Drosophila melanogaster* embryo“

Dissertation
Zur Erlangung des Grades
„Doktor der Naturwissenschaften“

am Fachbereich Biologie
der Johannes Gutenberg-Universität in Mainz

vorgelegt von Nirupama Deshpande
geb. in Nagpur, Indien

Mainz, 2001

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Tag der mündlichen Prüfung:

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1. INTRODUCTION

1.1 The question

According to Aristotle, the first embryologist known to history, science begins with wonder: "It is owing to wonder that people began to philosophise, and wonder remains the beginning of knowledge". The development of an animal from an egg has been a source of wonder through history. Multicellular organisms do not spring forth fully formed. Rather, they arise by a relatively slow process of progressive change that we call development. Therefore one of the fundamental questions in developmental biology has been how does a single cell, the fertilised egg, give rise to a vast amount of different cell types? This generation of cellular diversity is called differentiation. Since each cell of the body contains the same set of genes, we need to understand how this same set of genetic instructions can produce different types of cells. Towards this aim the basic question I have addressed in this study is how unique cell types are specified during the construction of the central nervous system.

The nervous system regulates all the aspects of bodily function and is staggering in its complexity. It can carry out extraordinary functions such as higher order thinking which is one of the most intricate tasks in animal development. Millions of specialised nerve cells (neurons) process information from the internal and external world and then instruct accordingly to the body to respond. Therefore, studying how the nervous system is constructed can give a better understanding to how it functions. The model system used in this study has been *Drosophila melanogaster* as its embryo has a relatively simple nervous system, which is useful for investigating the mechanisms that generate and pattern complex nervous systems. Central to the generation of different types of neurons by precursor cells (neuroblasts) is the initial specification of neuroblast identity. Thus, a complex array of different cell types is formed from relatively few precursor cells. Therefore, understanding the molecular mechanisms that control many developmental aspects like proliferation,

specification and differentiation of cells in the central nervous system (CNS) poses many challenges. The basic work of my study was to investigate mechanisms that lead to the specification of neuroblast identity. In this chapter I shall briefly describe how the central nervous system (CNS) is constructed in *Drosophila* and introduce various genes known so far which contribute to its development.

1.2 *Drosophila* as a model system for CNS development

Considerable progress has been made in answering many questions from studies involving the *Drosophila* nervous system as it has smaller number of cells compared to the vertebrates. The strength of *Drosophila* as a model system lies in its genetics that can eventually provide answers to questions for fundamental developmental mechanisms. As mutant phenotypes help reveal the parameters underlying any specific developmental process, several insights can be obtained to understand complex regulation of a given process. In their classical studies Nüsslein-Volhard and Wieschaus deduced the principles of early pattern formation in the *Drosophila* embryo based solely on the different mutant phenotypes they identified (Nüsslein-Volhard and Wieschaus, 1980). The phenotypic analysis of such mutants help to reveal the numbers of developmental steps and of genes required at each step. Finally, the analysis of double and triple mutations allows one to define functional interactions between the different genes and thus the networks of genetic functions underlying the process are gradually uncovered. Since then, the genetic approach has been used in several other organisms to dissect developmental processes (Hedgecock et al., 1985; Mullins and Nüsslein-Volhard, 1993; Driever et al., 1994).

In this study the process of cell fate specification in the CNS was the centre of investigations. The CNS of *Drosophila* provides an attractive model system for an unbiased genetic approach aimed at unravelling the genes required for CNS development since its morphology has been extensively studied. As different tools such as antibody probes and markers became available to visualise specific morphological aspects, genetic screens were employed to analyse the development of various parts of the nervous system, i.e. the visual system, the motor neurons, the peripheral nervous system, and the CNS axon pattern (Seeger et al., 1993; Vactor et al., 1993; Salzberg et al., 1994; Kolodziej et al., 1995; Martin et al., 1995; Schmucker et al., 1997). Further, individual cell types in the nervous system can be identified by

a large number of molecular markers. By using several techniques such as cell labelling and cell transplantation, the fate of individual cells can be traced and its lineage identified (Doe and Technau, 1993).

1.3 The embryonic central nervous system of *Drosophila*

The zygotic body wall of all organisms is subdivided into the three basic germlayers, the ectoderm, the mesoderm and endoderm. All structures and organs essentially stem from these germlayers by cell-cell interactions that trigger signalling processes leading to differentiation (See Fig.1). The ectoderm gives rise to the nervous system, the epidermis, the trachea and the fore and hindgut. The prospective ectoderm is subdivided into a ventral neurogenic (giving rise to neural cells) and a dorsal non-neurogenic portion by the antagonistic activity of the secreted molecules Decapentaplegic (Dpp) and Short gastrulation (Sog). The neurogenic ectoderm (neuroectoderm) starts as a simple epithelium composed of proliferative cells. This region gives rise to the neural progenitor cells (neuroblasts). Thus, cells of the neuroectoderm have a choice to develop as neuroblasts or as progenitor cells of the epidermis, and each cell has apparently the same probability of making either choice. Finally about 1500 of the neuroectodermal cells develop as epidermoblasts and only 500 develop as neuroblasts (See Fig.2).

In the late *Drosophila* embryo, the nervous system is composed of the ventral nerve cord (VNC), the peripheral nervous system (PNS) and the brain proper. In this study I have concentrated on cell specification in the VNC. The VNC consists of a sequence of 14 repeated units called neuromeres of which 8 are abdominal, 3 are thoracic, and 3 are gnathal neuromeres. A specialised population of cells called the midline cells divides each neuromere into two hemineuromeres, which demarcates the plane of bilateral symmetry between the two halves of the neuroectoderm. During neurogenesis about 30 neuroblast cells (NB) in each hemisegment delaminate from the neuroectoderm. NBs delaminate from the surface in five successive waves (S1-S5) along the mediolateral (M-L) and anterior-posterior (A-P) axes in rows and columns in a stereotyped spatiotemporal pattern (See Fig.3B). NBs are given numerical designations based on their final position in the NB pattern. The numbers consist of two digits; the first digit indicates the AP position, and the second digit indicates the

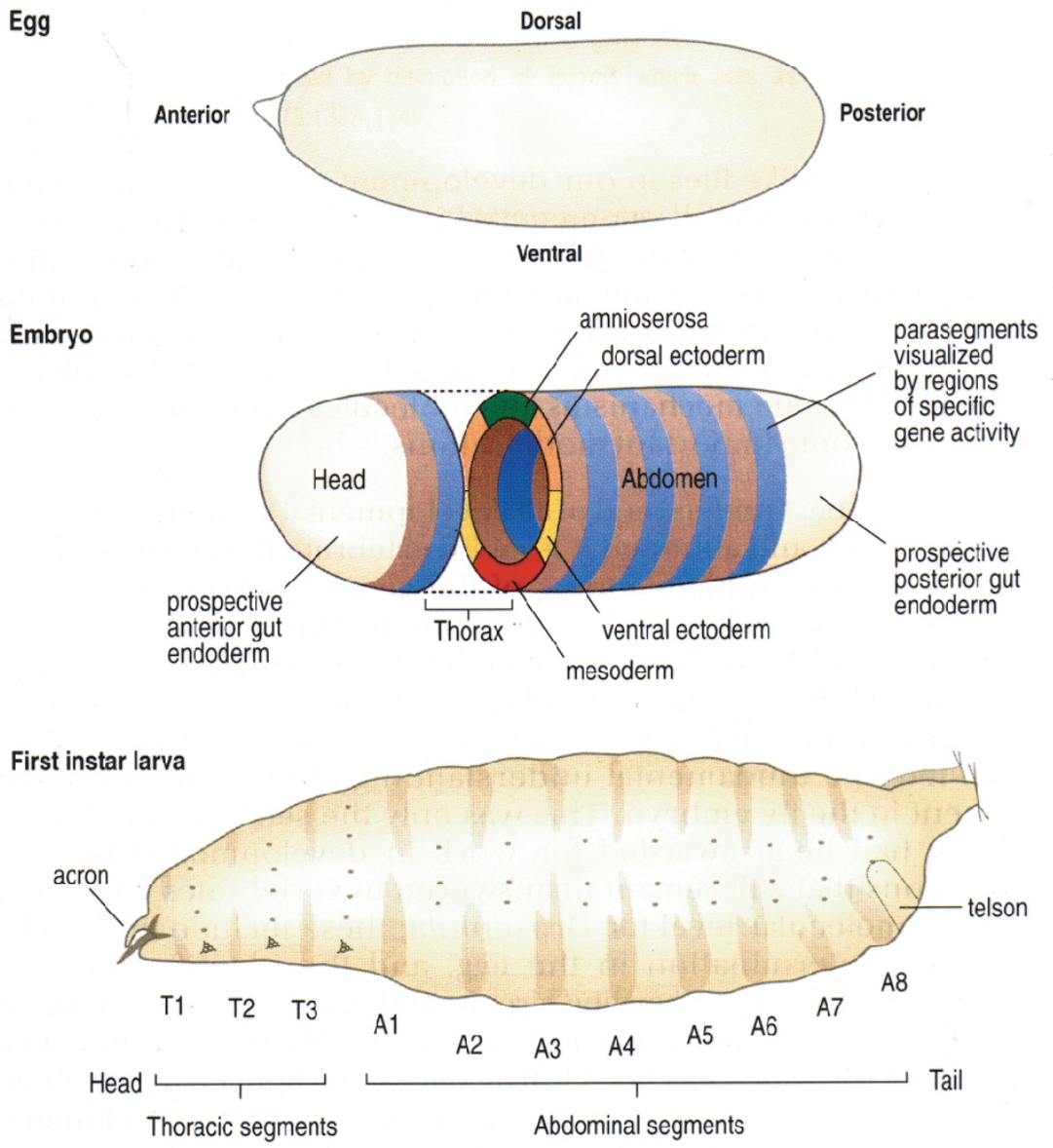


Figure 1. Different stages of *Drosophila* **development.** A) Shows an egg with its dorso/ventral and anterior/posterior orientation B) After the egg is fertilised, it develops the three basic germlayers which are: the endoderm (not shown), the mesoderm (shown in blue) and the ectoderm which is divided into the dorsal and the ventral ectoderm. The CNS stems from the ventral ectoderm. C) The embryo, which is patterend in a segmented fashion, gives rise to a larva that is also segmented.
(Scheme taken from "Developmental Biology " 5th edition, S. Gilbert)

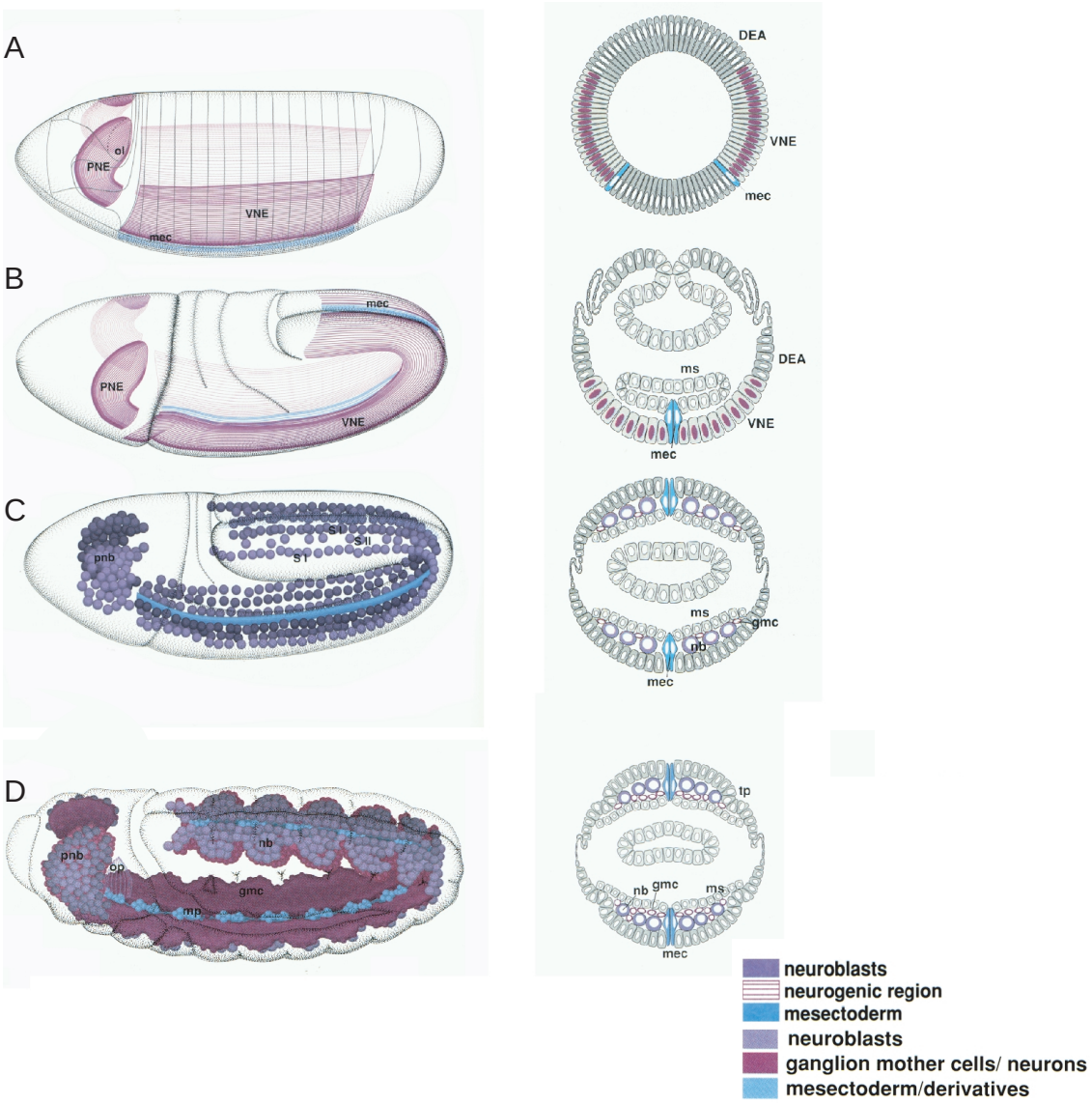


Figure 2. Development of the embryonic central nervous system of *Drosophila.*

A) The precursors of the CNS (NBs) derive from the neurogenic region of the ectoderm in the ventral region (VNE; purple stripes) as seen in a stage 5 embryo. B) Shortly after gastrulation at stage 8 the cells of the VNE swell, whereas the primordium of the dorsal epidermis (DEA) undergoes its first division. C) Starting at stage 9, NBs delaminate from the neuroectoderm. For the ventral neurogenic region, delamination occurs in 5 waves (S1-S3 during stage 9 and S4-S5 during stage 10-11). D) Shortly after their segregation, NBs start dividing with a perpendicularly oriented spindle. During stages 9-13, NBs undergo eight waves of mitosis. Their progeny, called ganglion mother cells (GCM), are placed between the NBs and the mesoderm (ms). Each GMC performs one equal division yielding two neurons or glia. GMCs and neurons/glia form an irregular layer of increasing thickness on top of the NBs. (Scheme taken from "Atlas of *Drosophila* Development" by V. Hartenstein, 1993)

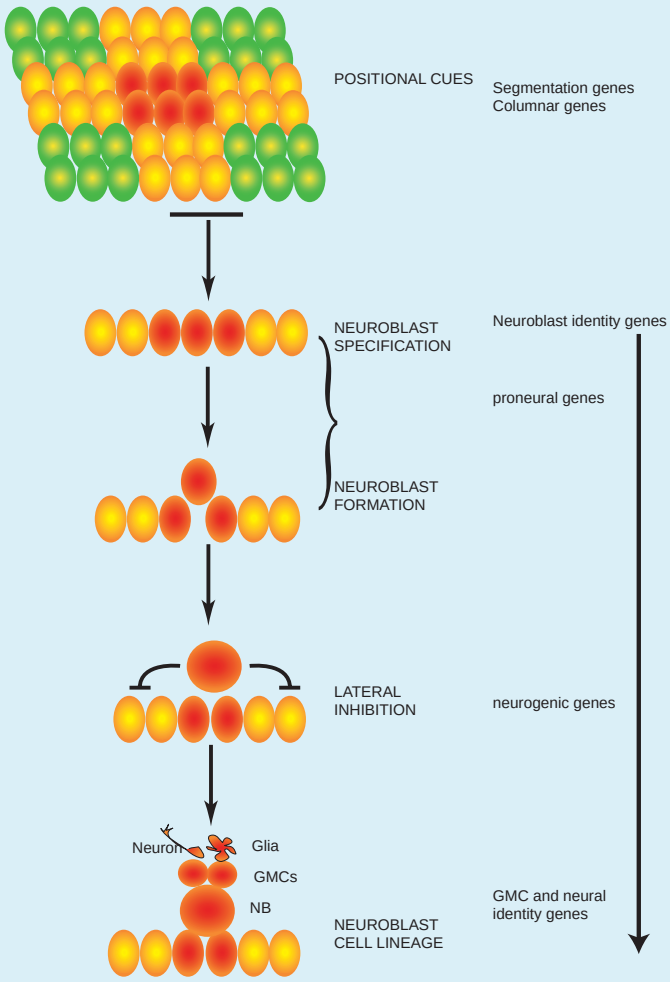


Figure 3. A) Formation and specification of neuroblasts. Proneural clusters form in the neuroectoderm by positional cues from the patterning genes like the segment polarity genes and the columnar genes. Only one cell out of the proneural cluster delaminates as a neuroblast by the process of lateral inhibition. Once an neuroblast has delaminated, it divides to give rise to ganglion mother cells (GMC) which have the ability to give rise to either neurons and/or glia.

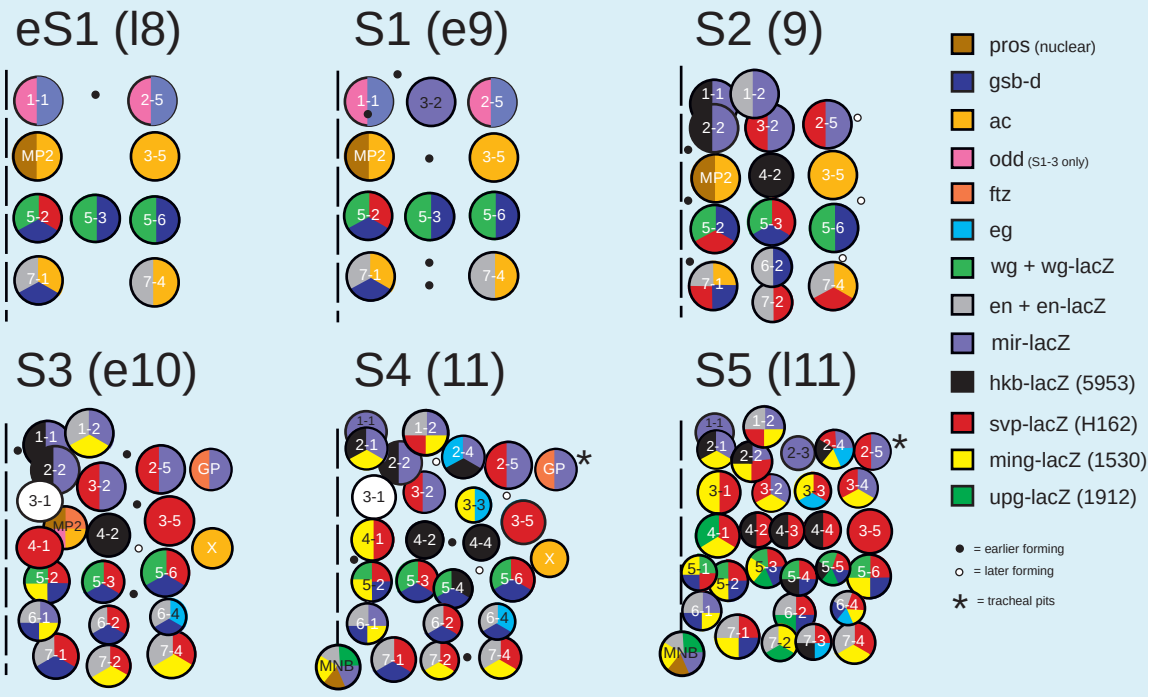


Figure 3. B) Neuroblast map. Each NB has a unique identity, which is revealed by the time of its birth, the specific set of molecular markers it expresses and the production of a characteristic cell lineage. Shown here is a schematic representation of all the NBs arranged in rows and columns in a hemisegment with the time of delamination and the expression of molecular markers for each NB (Scheme from Doe, 1992).

mediolateral position of the NB. Thus, e.g. NB 1-1 is the most medial NB of row 1, and NB 5-6 is the sixth NB from the ventral midline in row 5 after all NBs have formed.

NBs undergo a series of asymmetric cell divisions to self-renew. As the NB renews itself with each division it produces a chain of secondary neuronal precursor cells called ganglion mother cells (GMCs). A GMC does not self-renew instead it divides to generate two distinct neurons, which then undergo cyto-differentiation. Although the majority of NBs generate only neurons, some CNS precursor cells (neuroglioblasts) generate also glial cells, the other principal cell type in the CNS. The neuroblasts bud off towards the inside of the body cavity while dividing where they give rise to neural progeny. This leads to a multilayered appearance of the neuroectoderm with neural cells of different developmental state arranged from outside to inside of the embryo (Vaessin et al., 1991; Goodman and Doe, 1993; Hirata et al., 1995; Spana and Doe, 1995). Finally, each NB is characterised by a typical position in space, time of delamination and a specific set of molecular markers it expresses (Doe, 1992; Broadus et al., 1995). Additionally, the unique identity of each NB is revealed by the production of a characteristic cell lineage (Bossing et al., 1996; Schmidt et al., 1997) which means the specific cell type and number of neurons and/or glia it produces.

Thus, the process of neurogenesis in the *Drosophila* embryo takes place in three basic steps - formation of a neuroblast, specification of identity of a neuroblast and elaboration of a neuroblast lineage. In each of these steps during neurogenesis, cell-cell signalling plays a crucial role in ultimately generating a complex nervous system consisting of many different types of neurons and glial cells. The emphasis of my study has been on the first two steps of which are described in detail in the following parts of this chapter.

1.4 Neuroblast formation

As mentioned above the cells in the neuroectoderm have the potential to take either a neural or an epidermal fate. The correct separation of neural and epidermal progenitor cells is controlled by two groups of genes, the so-called ‘neurogenic genes’ and the so-called ‘proneural genes’. Most known proneural genes encoding basic

Helix-Loop-Helix (bHLH) transcription factors are the various members of the *achaete-scute* complex (AS-C), *daughterless*, and probably other as yet unidentified genes (Lee, 1997). These genes are responsible for instructing an equivalence group of cells known as 'proneural clusters' to become competent for the neural fate in the early neuroectoderm (Hinz et al., 1994; Kunisch et al., 1994). In the absence of one or more of these proneural genes a hypoplasic CNS with less cells is formed. In WT only one cell in the proneural cluster segregates as a neuroblast which is accomplished by the process of lateral inhibition, operating via the products of the *Delta* and *Notch* genes which belong to the family of the 'neurogenic genes' (See Fig3A; Campos-Ortega, 1993; Chitnis, 1999). Mutations in the neurogenic genes normally lead to a hyperplasic CNS with too many cells, which is termed as the "neurogenic phenotype".

The two genes *Notch* and *Delta* function as counterparts in a cell-cell communication cascade during the formation of neuroblasts. Both genes code for transmembrane proteins. The *Notch* gene encodes a transmembrane receptor while *Delta* encodes one of its ligands. The activation of *Notch* by *Delta* is thought to inhibit the ability of uncommitted epithelial cells in the proneural cluster to delaminate and adopt a neural fate (Struhl et al., 1993). The competence to get a neural fate is conferred on proneural clusters of neuroectodermal cells by the dynamic expression of one or many bHLH transcriptional activators encoded by the AS-C gene complex. It is believed that the cell that has the highest level of AS-C gene activity delaminates as a large distinct neuroblast cell (Martin-Bermudo et al., 1991; Skeath and Carroll, 1992; Skeath et al., 1992) whereas the level of AS-C proteins remains low in the non-neural cells as the result of Notch negative signalling. Thus, the commitment to neuroblast fate by one of the cells in a proneural cluster involves downregulation of the *Notch-Delta* signalling pathway in that cell. Loss of function for *Delta* or *Notch* leads to an excess number of neuroblasts (Artavanis-Tsakonas et al., 1995). Genetic analysis have identified a few loci that encode putative components of the *Notch* signalling pathway, including the *Suppressor of Hairless* [*Su(H)*] and *Enhancer of split Complex* [*E(spl)*] loci. *Su(H)* encodes an evolutionarily conserved sequence-specific DNA-binding protein which mediates directly the *Notch*-dependent transcriptional activation of at least one of the *E(spl)* genes which encode transcription factors belonging to the bHLH family of proteins (Schweisguth and Posakony, 1992; Brou et al., 1994). The

cell fate choice towards an epidermal fate in the neuroectoderm is determined by the activity of *E(spl)* gene products. Homozygous mutant embryos derived from mutant maternal germ-line clones of both *Su(H)* and *E(spl)* exhibit a neurogenic phenotype, indicative of a failure in lateral inhibition (Schweisguth and Posakony, 1992; Schweisguth,1995).

1.5 Specification of neuroblast identity

Apart from lateral inhibition conferred by Notch and Delta, inductive signalling also plays a role in NB formation as well as NB specification. Definitive progress has been made recently in this subject by studying several of the genes, which function during *Drosophila* body patterning (Patel et al., 1989; Bhat, 1999). Positional cues exist in the neuroectoderm from which neuroblasts delaminate, and these cues at least in part determine the identity of individual neuroblasts (Bhat, 1999; Skeath, 1999). As the identity of a given NB correlates with a certain time of delamination, temporal cues may play a critical role for NB specification in additional to positional information. Thus, to understand pattern formation within the CNS, it is critical to dissect the genetic regulatory mechanisms that specify the identity of individual neuroblasts and to determine how a combination of positional and temporal cues give a neuroblast its specific identity in a field of cells. Candidates for genes controlling neuroblast identity have been identified by virtue of their expression in neuroblasts or by screening for mutations that alter gene expression in identified GMCs. Two groups of genes recently have been demonstrated to govern the individual fate specification of neuroblasts. One group, the segment polarity genes, enables neuroblasts that develop in different antero-posterior positions to acquire different fates. The second group referred to as the columnar genes, ensure that neuroblasts that develop in different dorso-ventral domains assume different fates.

The embryonic epidermis is composed of several segments along the anterio-posterior (AP) axis, which are designed by the striped expression of the segment polarity genes. These genes have been shown to play a fundamental role in patterning cells within each metamere in the embryo. These patterning cues in turn also impart positional information along the anterior/ posterior axis for NB specification. Most of the segment polarity genes were identified in the genetic screens of Nüsslein-Volhard

and Wieschaus. Some of these include the signalling molecules Wingless (Wg) and Hedgehog (Hh), the transmembrane receptor Patched (Ptc) and genes which interact with these signalling pathways such as *gooseberry* (*gsb*), *engrailed* (*en*), *invected* (*inv*), *naked* (*nkd*) and *smoothened* (*smo*) (See Fig.4). Many other segmentation genes exist as well but shall not be mentioned as it is out of scope in this study. Molecular and genetic studies demonstrated that segment polarity gene activity subdivides each segment into an identical pattern of parallel transverse rows in the neuroectoderm as well. The AP limits of segment polarity gene activity correspond precisely to the AP limits of S1 and S2 proneural clusters.

Segment polarity gene function can largely explain how NBs that develop in the same mediolateral column but in different transverse rows acquire different fates. Segment polarity gene action, however, does not explain how NBs of the same row but different mediolateral columns acquire different fates. Until, recently the genetic regulatory mechanisms that create NB diversity along the dorso-ventral (DV) axis were unknown. Four factors have been identified that control NB fate along the D/V axis which are termed the columnar genes (Skeath, 1999). These include the EGF receptor (DER), a receptor tyrosine kinase, and three homeodomain containing transcription factors: Ventral nerve cord defective (Vnd), Intermediate nerve cord defective (Ind); and Msh. The activities of the columnar genes subdivide the early neuroectoderm into a parallel array of three adjacent longitudinal columns. Vnd is expressed in the medial column (McDonald et al., 1998), Ind is expressed in the intermediate columns (Weiss et al., 1998) and Msh in the lateral most column. This precise tripartite subdivision of the neuroectoderm arises well before S1 NB formation and largely disintegrates after S2 NBs form. Thus, although columnar gene activity can largely explain how S1-S2 NBs in different columns acquire different fates, other factors must contribute to S3-S5 NB diversity (See Fig.5).

Thus, the expression patterns and phenotypes of the segment polarity and columnar genes suggest that these genes play similar roles to pattern, form, and specify the fate of individual NBs along the AP and DV axes, respectively. Segment polarity gene activity dissects the neuroectoderm into four transverse rows per hemisegment (2/3,4,5,6/7) and ensures that NBs that develop in different rows acquire different fates. Columnar gene activity subdivides the neuroectoderm into three longitudinal columns (medial, intermediate and lateral) and ensures that NBs that

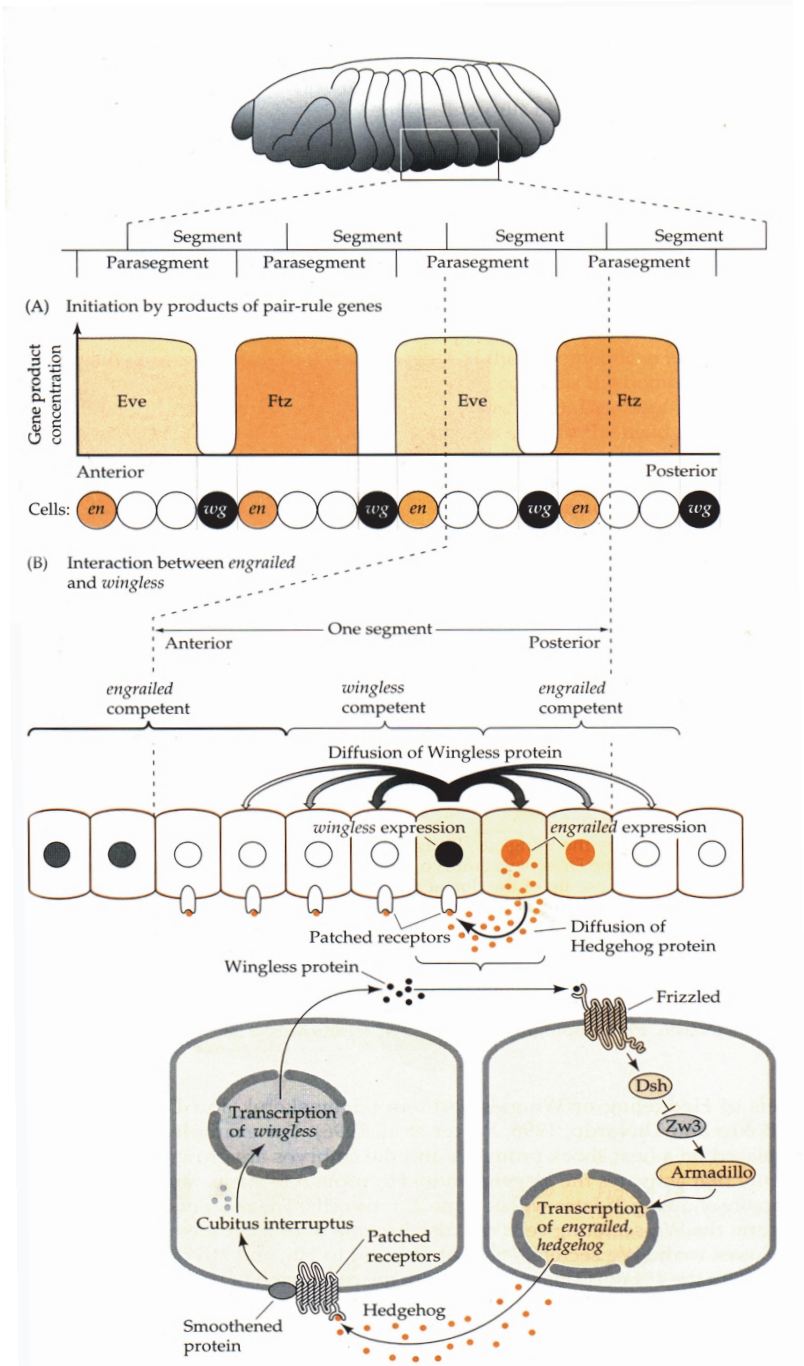


Figure 4. The segment polarity genes. Model for the transcription of the segment polarity genes *engrailed* (*en*) and *wingless* (*wg*). A) The expression of Wg and En is initiated by pair-rule genes. En is expressed when the cells contain high concentration of either Even-skipped (Eve) or Fushi-tarazu (Ftz) proteins. *wg* is transcribed when neither *eve* or *ftz* genes are active, but a third gene (probably *odd-paired*) is expressed. B) The continued expression of Wg and En is maintained by interactions between the En and Wg expressing cells. Wg protein is secreted and diffuses to the surrounding cells. In those cells competent to express En (having Eve or Ftz proteins), Wg protein is bound by the Frizzled receptor, which enables the activation of the *en* gene. En protein activates the transcription of the *hedgehog (hh)* gene and also activates its own transcription. Hh protein diffuses from these cells and bind to the Patched protein. This binding prevents the Patched protein from inhibiting the Smoothened protein signalling which enables the transcription of the *wg* gene and the subsequent secretion of the Wg protein. (Scheme taken from "Developmental Biology" 5th edition, S. Gilbert)

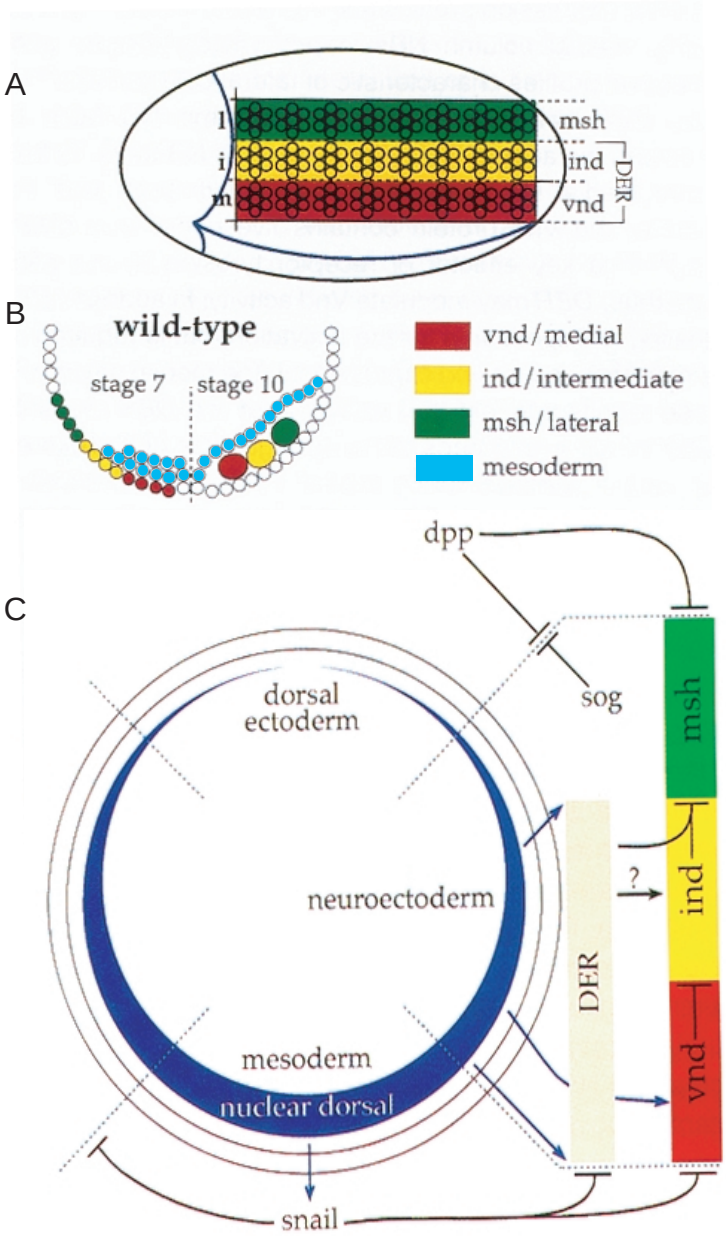


Figure 5. Gene activity domains of the dorso/ventral genes. A) A schematic lateral view of a wildtype embryo. B) Schematic views of transverse sections through wildtype embryo at stage 7 (left), to highlight the neuroectoderm, and stage 10 (right), to highlight NB fate. The *vnd* (red), *ind* (yellow), and *msh* (green) genes are expressed, respectively, in the medial, intermediate, and lateral neuroectodermal columns (A,B) and the S1 and S2 NBs thereof. C) Genetic regulatory hierarchy that control NB formation along the dorsoventral axis. Shown left, establishment of the proper spatial domains of Vnd, Ind, DER, and Msh activity. *dorsal* is thought to activate *vnd*, *DER*, *ind* and *msh.* Inhibitory interactions between these genes as well as negative regulation by *snail* in the mesoderm and *dpp* in the dorsal ectoderm limit the activity of each gene to its appropriate spatial domain (Scheme taken from Skeath, 1999).

develop in different columns acquire different fates. The superimposition of the activities of the columnar and segment polarity genes creates within each hemisegment a Cartesian co-ordinate system in which each quadrant or cluster possesses a unique combination of columnar and segment polarity gene activity. Each different combination of columnar and segment polarity genes then directs the activation of a unique set of genes (e.g., the AS-C proneural genes, *seven-up*, *huckebein*) within individual proneural clusters.

1.6 Aim of this study

In this study I have investigated aspects NB formation and specification for late segregating NBs (S3-S5) since all the work previously performed deals with respect to early NBs. However, about half of the NBs delaminate in the later segregation waves S3-S5 and acquire a different identity despite the fact that many originate from similar positions as the early NBs. Additionally, the three column-arrangement pattern is only transitory during early stages of neurogenesis and is obscured by late emerging neuroblasts (Doe and Goodman, 1985; Goodman and Doe, 1993). To identify novel and yet unidentified genes in the process of late neuroblast formation and specification, a genetic screen was conducted in collaboration with Prof. Christian Klämbt (Münster, Germany). The gene *anterior open* (*aop*) or *yan* was one of the genes picked up in this screen. Chapter 3 deals with my results with section 3.1 describing the whole screening procedure whereas section 3.2 describes my findings about the role of *yan* in the process of neurogenesis. I will show that the gene *yan* is responsible for maintaining the cells of the neuroectoderm in an undifferentiated state by interfering with the *Notch* signalling mechanism. Secondly, to understand how late delaminating NBs are specified I have concentrated on studying the function and interaction of segment polarity genes within a certain neuroectodermal region, namely the *engrailed* expressing domain. Section 3.3 describes my finding with respect to the fate specification of a set of late neuroblasts, namely NB 6-4 and NB 7-3. I have dissected the regulatory interaction of the segment polarity genes *wingless* (*wg*), *hedgehog* (*hh*) and *engrailed* (*en*) as they maintain each other's expression to show that En is a prerequisite for neurogenesis in this area and show that the interplay of the segmentation genes *naked* (*nkd*) and *gooseberry* (*gsb*), both of which are targets of

wingless (*wg*) activity, leads to differential commitment of NB 7-3 and NB 6-4 cell fate. I have shown that in the absence of either *nkd* or *gsb* one NB fate is replaced by the other. However, the temporal sequence of delamination is maintained, suggesting that formation and specification of these two NBs are under independent control.

2. MATERIALS AND METHODS

2.1 Fly stock maintenance

Fly stocks were maintained in vials containing standard *Drosophila* food media. Stocks maintained at 25°C were transferred to fresh vials every two weeks and the ones maintained at 18°C were transferred every 4-5 weeks.

2.2 Fly stocks

The following fly stocks listed in Table A were used for the work of this thesis. All the mutant alleles used were carried over a blue balancer chromosome (a balancer chromosome carrying a P-element insertion that drives the expression of β-Galactosidase under the control of a specific promoter). Thus a homozygous mutation in an embryo could be identified via antibody stainings by their lack of *lacZ* expression.

TABLE A: List of all fly stocks used.

Name and Genotype	Origin	Reference
aop^{1}/CyO	Umeå no. Z258	
aop^{2}/CyO	Umeå no. Z718	
aos $^{w11\Delta}$/TM6b Tb		C Klämbt
arm^{1}/FM7c	Bloomington no. 3378	
arm^{4}/FM7c *ftz-lacZ*	Bloomington no. 616	
Balancer-3rd chromosome: TM3/TM6b Tb TM3/TM6b Tb *ftz-lacZ*	Mainz Stocks no. 285 no. 135	O Vef

Balancers four fold Pm/CyO *wg-lacZ*; CXD/ TM6b Tb *abdA- lacZ*	Mainz Stocks	O Vef
Balancers-2nd Chromosome: Pm/ CyO Pm/ CyO *ftz*-lacZ Pm/ CyO *wg*-lacZ	Mainz Stocks no. 56 no. 57 no. 33	O Vef
$ci^{Ce\text{-}2}/sv^{spa\text{-}Cat}$	Bloomington no. 4343	
gsb^{IIX62}: Df(2R) gsb^{IIX62} / CyO	Tübingen no. Z736	
Df(3R) awd-KRB / TM3 Sb	Umeå no. 55401	
dsh ^{477}, *FRT101* / FM7		K Basler
eg ^{P289}	Mainz Stocks	(Dittrich et al., 1997)
en^{E} : Df(2R) en^{E} / CyO	Mainz Stocks	
enGal4	Mainz Stocks no. 119	A Brand
flb^{11W74} / CyO	Mainz Stocks	
ovo^{D1}, *FRT101* / c(1)Dx	Bloomington no. 1813	
fz ^{k21}		K Bhat
fz ^{r52}		K Bhat
fz^{1}		K Bhat
fu ^{513} / C(1)B		
gsb^{IIX62} / CyO *wg-lacZ*; *nkd* ^{2}/ TM6b *abdA-lacZ*		N Deshpande
*HEP*1 / FM6		E Martin Blanco

hh^{AC}		(Sanson et al., 1999)
hh^{IIO} / TM3, Sb	Tübingen no. Z853	
hs-en (2nd chromosome)	Bloomington no. 3516	Kornberg
hs-en (2nd chromosome)	Mainz Stocks no. 164	Heemskert
med^{1}		S Cohen
N^{55e11} / FM6 *grh-lacZ*; *yan*^{2}/ CyO *wg-lacZ*		N Deshpande
nkd^{2},	Mainz Stocks no. 85	
ovo^{D1} , *FRT101* (1st chromosome)		
ovo^{D1} , *FRT40A* (2nd chromosome)		K Basler
pan ^{2} / *ey*^{D}		K Basler
pnt^{Delta88} / TM3 Sb	Bloomington Stocks no. 861	C Klämbt
ptc^{H8}		J Hooper
scaGal4 (2nd chromosome)	Mainz Stocks no. 217	M.C Ellis
smo^{3} , *FRT 40A* (2nd chromosome)		K Basler
ttk $^{55.3}$/TM6B		(Lai et al., 1997)
ttk ^{B330} / TM6b Tb (*ttk*69)		C. Klämbt
ttk $^{D2-050}$ (*ttk*69 amorp)		C. Klämbt
ttk^{1e11} / TM3 Sb	Bloomington no. 4164	
ttk^{1} / TM6b Tb		(Lai et al., 1997)
UAS-Gsb (2nd chromosome)	Mainz Stock no. 5	

UAS-Nkd (2^{nd} chromosome)		(Zeng et al., 2000)
UAS-Notch ^{activated} (2^{nd} chromosome)		J Urban
UAS-Notch ^{ECN} (2^{nd} chromosome)		J Urban
UAS-Wg (2^{nd} chromosome)		(Sanson et al., 1999)
UAS-Wg, *hh* ^{AC}		(Sanson et al., 1999)
UAS-Yan ^{activated} (2^{nd} chromosome)		(Rebay and Rubin, 1995)
UAS-Yan ^{wildtype} (2^{nd} chromosome)		(Rebay and Rubin, 1995)
yan ^{XE18}, *FRT 40A* / CyO		(Lai et al., 1997)
wg^{CX2} / CyO	Umeå no. 43080	
wg^{CX3} / CyO	Umeå no. 43085	
wg^{CX4} / CyO	Umeå no. 43090	
Wildtype: *Oregon R*	Mainz Stocks no. 140	(Lindsley and Zimm, 1992)
jun^{2}, *FRT 42* / CyO		E Martin Blanco
hs-flp (1^{st} chromosome)		K Basler
zw3 or sgg^{m1H}, *FRT101*		K Basler

2.3 Genetic crosses

For setting up crosses between two strains, female virgins were always crossed to males of the other strain and allowed to mate for 2 days. If the embryos of this cross were needed for antibody stainings the flies were then placed in a cage for egg laying and if the progeny of the cross were needed, the parent flies were allowed to lay eggs in a vial with food and then discarded.

2.4 Embryo collection

To collect embryos flies were placed in a cage, at the bottom of which a changeable plate was placed. The plates contained apple juice with 2% Agar. Embryo collections were made on these plates according to the stage (st) at which the embryos were required.

2.5 Ectopic expression of a gene in embryos

For a number of experiments the *Gal4/UAS* System (Brand and Perrimon, 1993) was used for tissue targeted expression. This system consists of two components- the flies that expresses the yeast transcriptional activator *Gal4* under the regulation of an endogenous promoter (driver stock), and flies that carry a transgene of interest whose expression is regulated by the *Gal4* Upstream Activation Sequence (*UAS*; *UAS* stock). When the driver and *UAS* stock was crossed together, the transgene of interest was expressed in the same pattern as the *Gal4* protein. Thus, ectopic expression of the transgene depends on the enhancer that regulates *Gal4* expression. In addition to the *Gal4*/*UAS* system, the heat shock system was also used to drive ectopic expression. Here the transgene of interest is under the regulation of the heat shock (*hs*) promoter, which is activated by giving a heat pulse (37˚C) to the embryos.

2.6 Generation of germline clones

The production of female germline chimeras is invaluable for analysing the tissue specificity of recessive female sterile mutations as well as detecting the maternal effect of recessive zygotic lethal mutations. This technique uses germline-dependent dominant female sterile mutation ovo^{D1} as a selection for the detection of germline recombination events and the FLP-FRT recombination system to promote site-specific chromosomal exchange. The FRT element (FLP Recombinase Target) is a target for the yeast FLP recombinase (FLP; Chou and Perrimon, 1996). Thus, the these fly stocks help give rise to germ cells lacking the gene of interest thereby making it possible to analyse embryos which are mutant for the gene. To generate germline clones of a particular mutation, the FRT element was recombined onto the mutant chromosome. Virgin females carrying the mutation and FRT element along with an *hs-flp* construct were crossed to males carrying the ovo^{D1} mutation along with FRT element that is present on the same site and chromosome as that of the mutation. This

cross was subjected to a 24hr egg lay in normal food vials and aged at 25°C for 55hr till mid to late 2nd instar larval stage. The 2nd instar larvae in food vials were subsequently heat shocked to activate Flp expression at 37 °C in a incubator for a 2hr period. The progeny virgin females carrying the mutation-FRT over *ovo*^{D1}-FRT were collected and mated with males carrying the mutation. The embryos of this cross were then subjected to analysis (See Fig.6A).

2.7 Mass scale antibody stainings for a screen

During the process of screening, embryos from many lines have to be examined simultaneously; egg collection from individual cages can be time consuming and a laborious process. Therefore, for the purpose of the screen a rapid egg collection and staining device was used (Hummel et al., 1997). The device consisted of a block containing 25 mini cages. These blocks had a mesh at one end and holes at the other end for each mini cage. Therefore 25 fly stocks could be subjected for egg lay simultaneously also facilitating easy transfer of flies to new blocks. The usage of more than one block could facilitate egg lay from 150 line simultaneously. Four egg collections of 0-8hr (at 25°C) were pooled together for each line. For fixing of these collected embryos, the whole block containing the eggs were immersed first in a tray containing liquid bleach for 2min and washed thoroughly with tap water. The block was then immersed in a tray of fixative (for fixative see section 2.14) for 25min following which the block was immersed in a container with a lid containing 1:1 solution of Heptane: Methanol. The whole container then is shaken vigorously manually with the lid tightly shut for devittelinising of the embryos and then rinsed once with Methanol followed by a rinse in 100% Ethanol. The fixed embryos were then aliquoted into individual eppendorf tubes. These embryos were subjected to antibody staining (Alkaline Phosphatase) described in section 2.15. Embryos from each line were initially analysed under the dissecting microscope and the actual phenotypic analysis was subsequently done using a Zeiss Axiophot microscope.

2.8 Complementation analysis

This method is used to identify or narrow down the region of the chromosome where in an unknown mutation is situated. The requirement for this method is that the

unknown mutation has to be homozygous lethal. The principle behind a complementation analysis is that fly stocks carrying a chromosomal deletion in an identified region of the chromosome is crossed to the unknown mutation. Subsequently, if no progeny from the resulting cross with genotype of the mutation over the deletion is obtained then the mutation is situated in the region of the deletion (See Fig. 6B).

2.9 Genetic recombination

Fly stocks when subjected to a mutagen, multiple sites on a chromosome get mutagenised. A phenotype observed in such a stock could be due to a single mutation on the chromosome or a combined effect of multiple mutations. To decipher which of the mutations is responsible, each mutation has to be isolated and studied individually, therefore the sites on the chromosome has to be recombined out. In addition, this technique is used to create a clean stock without any other disruption on the chromosome for a line obtained from a mutagenesis screen. To follow recombination events on a chromosome the mutant chromosome is crossed to a multiple marker chromosome. The markers position are well mapped, therefore in an event of a recombination, knowledge of which part of the chromosome is recombined out can be identified easily (See Fig. 6C).

2.10 Mounting of embryos

Embryos were staged either prior to or after mounting. In both cases embryo staging was according to standard morphological markers (Hartenstein and Campos Ortega, 1985).

2.11 Analysis of embryos and documentation

The analysis of embryos was carried out on an Axioplan microscope mainly using Normaski optics. Embryos labelled with fluorescent dyes were analysed with a Leica TCS confocal microscope. Quantitative analysis such as cell counts was done using 63X or 100X oil objectives. Non fluorescent images were digitally recorded with a CCD video camera. Figure composition was carried out using Adobe Photoshop 5.1 preserving the integrity of the images. In cases were cells were seen in different focal

planes, cells from focal planes were combined into a single plane to give a more comprehensive impression of the specimens.

2.12 Chemicals used

All chemical used were from Roth / MERCK.

2.12.1 Buffers: The following buffers were used for most of the experimental procedure unless stated otherwise.

1) 20X PBS: (pH7.4)

H_2O	500 ml
NaCl	75.97 g
Na_2HPO_4	9.94 g
NaH_2PO_4	4.14 g

2)PBT: PBS+0.3% TritonX-100

3)PBTween: PBS+0.1% Tween-20

4) Detection Buffer:

5M NaCl	10 ml
1M $MgCl_2$	25 ml
1M Tris pH9.5	50 ml
Tween-20	0.5 ml
H_2O	412 ml

5)Blocking Solution: PBS+ 10% Calf Serum

6)DAB Solution:

PBT/PBTween: 20 ml

3,3'-Diaminobenzidin Tetrahydrochloride (DAB): 10 mg/ml

7) NBT /X Phosphate solution:

Detection Buffer	1ml
NBT (50mg/ml in 70% DMF)	4.5µl
BCIP (50mg/ml in 70% DMF)	3.5µl

(DMF: Dimethylformamid)

8) PEMS Buffer:

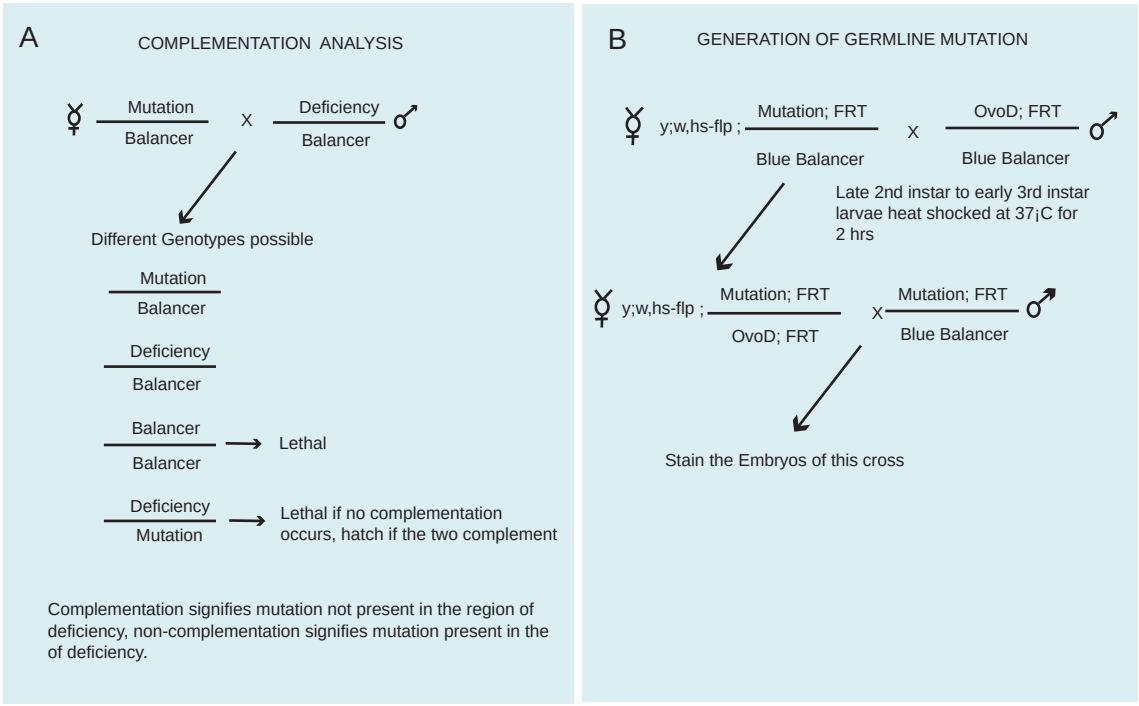
0.1M Pipes (pH 6.9)

1mM $MgSO_4$

2mM EGTA

4% Formaldehyde (freshly added)

(Stock solution should not exceed more than 0.2M)



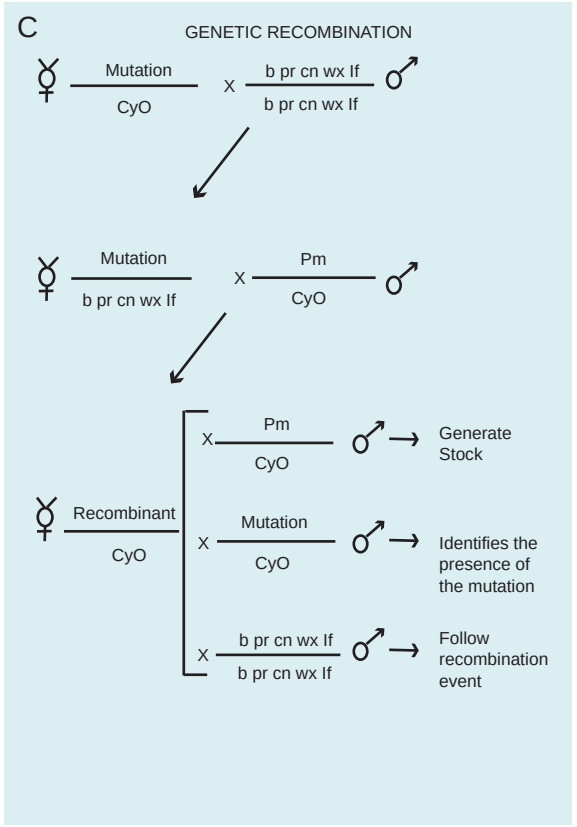


Figure 6. Schematic representation of genetic crosses. A) Complementation analysis: Mutant virgin female flies are crossed to deficiency males and the resulting progeny is tested for complementation B) Generation of germline mutation: The desired mutation is first recombined to an FRT site on the chromosome. Females of this stock are crossed to *ovo*^{D1}; FRT males. The late 2nd instar larvae of this cross are subjected to a heat shock. Subsequently female virgins with an *ovo*^{D1} chromosome are collected from this cross to be mated with mutant male flies. If the *ovo*^{D1} FRT system has worked embryos which are deficient for the desired mutation in the germline will be obtained. C) Genetic Recombination: This technique is applied to get rid of additional mutant sites on a chromosome apart from the desired mutation. It is also used for mapping of an unknown gene. Female virgins from the mutant stock are crossed to males with multiple markers on its chromosome. With the aid of this multiple marker the exact chromosome sites can be mapped and subsequently recombined out.

2.12.2 Antibodies used: Following are the list of antibodies used with the dilution used for a staining reaction. All primary antibodies were diluted in 10% Calf serum in PBT with 0.1% Sodium Azide to prevent bacterial growth. The secondary antibodies were diluted in PBT only.

TABLE B: List of antibodies used.

Antibodies	Animal raised in	Dilutions used in µl	Reference or origin
Alkaline Phosphatase conjugated anti-DIG		1 in 1000	Boehringer Mannheim
Alkaline Phosphatase conjugated anti-rabbit or anti-mouse	Goat	1 in 250	Jackson Immuno Research Laboratories
Anti- Reversed Polarity	Rabbit	1 in 1000	D Halter
Anti-Engrailed	Mouse	1 in 3	Bank-DSHB
Anti- Achaete (990)	Mouse	1 in 20	J Skeath
Anti- Gooseberry	Rat	1 in 3	D Holmgren
Anti-Eagle	Rabbit	1 in 1000	R Dittrich
Anti-Enhancer of Split (323)	Mouse	1 in 2	S Bray
Anti-Enhancer of Split (729)	Mouse	1 in 2	S Bray
Anti-Even-skipped	Rabbit	1 in 1000	M Frasch
Anti-Eyeless	Rabbit	1 in 1000	U Waldorf
Anti-Hunchback	Rabbit	1 in 100	
Anti-Ladybird	Mouse	1in 3	C Jagla
Anti-Ming (Castor)	Rabbit	1 in 1000	W Odenwald
Anti-Odd-skipped	Rabbit	1 in 5000	J Skeath
Anti-Pox-neuro	Mouse	1 in 20	C Dambly-Chaudiere
Anti-Runt	Rabbit	1 in 500	J Skeath
Anti-β-Galactosidase	Rabbit	1 in 1000	Cappel

Anti-β-Galactosidase	Mouse	1 in 250	Promega
Anti-Yan	Mouse	1 in 2	Z Lai
Biotinylated anti- rabbit or anti-mouse	Goat	1 in 250	Jackson Immuno Research Laboratories
Cy^3-conjugated anti-rabbit or anti-mouse	Goat	1 in 250	Jackson Immuno Research Laboratories
FITC-conjugated anti rabbit or mouse	Goat	1 in 250	Jackson Immuno Research Laboratories
Rodamin-conjugated anti rabbit	Goat	1 in 250	Jackson Immuno Research Laboratories

2.13 Fixation of embryos for antibody staining

Embryos were collected in a basket with a sieve and dechorionated for 2min in 15% bleach (Sodium Hypochloride) and rinsed thoroughly in running tap water. The embryos were then fixed for 20min in an eppendorf tube containing Heptane and 10% Formaldehyde in PBS in a ratio of 1:1. For certain antibodies the usage of this fixative could be too harsh for the antigen. In such cases the PEMS fixative was used which consisted of 4 parts of PEMS buffer with 5 parts of Heptane. After 20min of fixing the lower phase was removed and 1ml Methanol was added to devittelinise the embryos with vigorous shaking or vortexing for 30-60sec. Then the embryos were subjected to 2-3 rinses with Methanol after which the embryos were either used directly for antibody staining or stored at -20°C for future use.

2.14 Antibody stainings

Embryos which have been fixed and stored in Methanol were rinsed several times with PBT and then blocked for minimum of 1hr in 10% Calf Serum in PBT. Primary antibody of appropriate dilution was added and incubated overnight at 4°C. Next day the embryos were washed with PBT 3 times for 20min each to get rid of excess

primary antibody. The secondary antibody (coupled with fluorescent dye, Biotin or Alkaline Phosphatase) of appropriate dilution was added and incubated overnight at 4 °C or for 2hr at room temperature. The secondary antibody was then washed off with PBT 3 times 20min each. At this stage the embryos were treated differently depending on the type of conjugate being used i.e. fluorescence, Biotin or Alkaline Phosphatase.

2.14.1 Fluorescence staining: After secondary antibody incubation, the embryos were rinsed with PBT 3 times 20min each following 2 rinses with PBS of 15min each. This step is critical as TritonX-100 interferes with fluorescence. Finally the embryos were stored in dark at –20°C in 70% Gycerol in PBS. To observe under the fluorescence microscope the embryos were mounted in Vectashield fluorescent mounting media (Vector Laboratories)

2.14.2 Biotin Staining: DAB staining is done when the secondary antibody used is conjugated with Biotin and gives a brown colour reaction up on staining. For DAB staining during PBT washes simultaneously the AB complex (from the Vector ABC Elite kit-Vector laboratories) was prepared by adding 4µl each of solution A and B to 300ul of PBT and left to stand for 1hr. The Vector ABC Elite kit forms a complex of Biotin and Streptavidin coupled to horseradish peroxidase, which helps to amplify the signal. The AB complex was then added to embryos and incubated at room temperature for 1hr after which the AB complex was rinsed off with PBT 2-3 times following the addition of DAB solution with freshly added hydrogen peroxide to a final concentration of 0.1%. The reaction was allowed to take place for 2-3min then stopped by rinsing with PBT 2-3 times.

2.14.3 Tyramide signal amplification kit (TSA): In cases where the detection of stainings with Biotin secondary antibodies was weak the TSA kit (NEN Life Science Product, Catalogue No. NEL 700A) was used to enhance the signal. After incubation with primary antibody overnight the embryos were subjected to the same steps as for Biotin staining mentioned above. After the incubation of AB complex on embryos, the embryos were rinsed briefly with PBT twice and then incubated in Biotinyl solution (1µl of Biotinyl in 50µl dilution buffer provided in the TCS kit) for 20min. These

embryos were rinsed again in PBT briefly and incubated for 1hr in a solution of Streptavidin HRP (1µl of Stretavidin in 100µl of PBT) and then subjected to DAB staining as mentioned above.

2.14.4 Alkaline Phosphatase staining: This staining is done when the secondary antibody used is an Alkaline Phosphatase conjugated one and gives a blue/black colour reaction up on staining. After incubation of the secondary antibody the embryos were rinsed with AP detection buffer for 2-3 times. Then in another eppendorf cap to 1ml of AP detection buffer 3µl of BCIP and 6.6µl NBT was added, this constitutes the staining solution for Alkaline Phosphatase reaction. This solution was added to the embryos for staining and allowed to develop for 15-20min depending on the intensity of staining. When the staining was weak, the reaction was prolonged up to 1hr. The reaction was then stopped by rinsing the stained embryos with PBT and then fixed in 10% formaldehyde solution in PBT for 15min. After fixing embryos were rinsed in Methanol for 20min followed by a PBT and then a PBS rinse. Stained embryos were then stored in 70% Gycerol in PBS. For double staining the embryos were first subjected to DAB and then Alkaline Phosphatase staining.

2.15 RNA hybridisation on embryos

For all steps throughout out this protocol care was taken not to have any RNAase contamination by using new chemical reagents, sterile tips and eppendorf tubes and wearing gloves while handling.

Solutions for RNA *in situ* hybridisation: All solutions were made with DEPC (Diethyl pyrocarbonate) treated water. The DEPC treatment consisted of adding 0.1% DEPC to double distilled water and allowing this to stand for at least 12hr at room temperature and then autoclaved.

1) PBS: NaCl 130mM
Na_2HPO_4 7mM
NaH_2PO_4 3mM

2) PBT: PBS + 0.1% Tween-20.

3) Hybsolution: 50% Formamide in 5X SSC and 0.1% Tween-20

4) 20X SSC: NaCl 3M
Sodium Citrate 300mM

5) DIG-RNA-labelling-Mix
(Boehringer Mannheim, #1277073)

6) T7-RNA-Polymerase (Boehringer Mannheim, #881767)

7)DNAseI, RNAse free (Boehringer Mannheim,#776785)

2.15.1 Fixation of embryos for hybridisation: Embryos were dechorionated in 15% bleach for 2min and then rinsed thoroughly with tap water. Embryos were fixed for 30min in a solution of 500µl Heptane, 350µl DEPC-PBS and 150µl Formaldehyde (from 37% stock). The embryos were devittelinezed by the same procedure mentioned in section 2.13 of this chapter and stored in Ethanol at –20°C till further use.

2.15.2 Hybridisation: The embryos which were stored in Ethanol were rinsed with PBT 2-3 times then fixed again in PBT with 5% Formaldehyde for 15min. To wash off all the fixative solution the embryos were rinsed several times with PBT followed by a wash of PBT/Hybsolution in a ratio of 1:1 for 5min. Subsequently a wash was given in Hybsolution only, for 5min. The embryos were subjected to prehybridisation in 1ml Hybsolution containing 10µl of 10mg/ml sonicated salmon sperm DNA at 55ºC in a heating block for a minimum of 1.5hr (Agitation or rotation of the tubes was unnecessary). This prehybridisation solution was removed and a denatured probe in 50µl Hybsolution containing 10µl of 10mg/ml sonicated salmon sperm DNA was added and incubated overnight at 65ºC in a heating block. The next day the embryos were subjected to a wash in Hybsolution for 30min at 65ºC, followed by a wash of Hybsolution/ PBT in a ratio of 1:1 for 30min at 65ºC. After which the embryos were rinsed 5 times in quick succession in PBT at 65ºC followed by a final wash in PBT for 10min at room temperature. The embryos were then incubated with anti-DIG antibody (Alkaline Phosphatase conjugated) at a dilution of 1:1000 in PBT for 1.5hr. The staining reaction was done similar to the Alkaline Phosphatase staining reaction as described in section 2.14.4 of this chapter. If an additional antibody staining was to be continued the embryos were fixed again in 100µl Formaldehyde + 900µl PBT for 15min followed by blocking in 10% calf serum in PBT for 25min, rest of the steps

were similar to the antibody staining protocol described in section 2.14.2 of this chapter.

2.15.3 Generation of riboprobes: 10µg of plasmid DNA of interest was digested 5′ of the cDNA with an appropriate enzyme. A typical restriction digest was performed in a volume of 100µl containing 10µl 10X restriction buffer provided by the manufacturers, 5-8µl of the restriction enzyme and rest of the volume made up by sterile distilled water. Reactions were incubated at 37°C for 2-3hr. The reaction mix was subjected to an extraction of Phenol: Chloroform: Iso-Amyl alcohol by adding in a ratio of 25:24:1 followed by vigorous vortexing and then centrifuging 13000 rpm for 5min. The upper aqueous phase was carefully transferred to a fresh eppendorf tube. The digested DNA from this solution was subjected to Ethanol precipitation by adding sodium acetate to a final concentration of 300mM and two volumes of ice cold 100% Ethanol. This mix was stored in -20°C for a minimum of 30min or overnight. This solution was then centrifuged for 30min at 4°C at 14000 rpm. The supernatant was discarded and the DNA pellet obtained was dried in a Speed Vac for about 30min. This step is critical as any trace amount of Ethanol interferes with the labelling reaction. The pellet was then resuspended in a suitable volume of DEPC treated water. To about 1.5µg of linearised plasmid DNA 2µl of DIG-RNA labelling mix, 2µl 10X transcription buffer, 2µl of RNA-polymerase (T7/T3 RNA polymerase) was added and the volume made up to 20µl with DEPC treated water. This reaction mix was incubated for 2hr at 37ºC, after which 2µl RNase free DNaseI was added and incubated for 15min at 37ºC. The reaction was stopped by adding 2µl 0.2M EDTA. The newly synthesised RNA from this reaction mix was precipitated by adding 2.5µl 4M LiCl and 75µl of 100% ice cold Ethanol, followed by an incubation of 30min at -70ºC. This was then subjected to centrifugation at 13000 rpm for 30min. The dried pellet was then dissolved in 100µl of DEPC treated water. 0.1-2µl of probe was aliquoted in 50µl of Hybsolution with salmon sperm DNA and stored at –20°C. Before use, the probe and Hybsolution mix was always denatured by boiling for 2min and then added to embryos after the prehybridisation step.

Riboprobes generated from all the cDNA are given in Table C on the next page. See *Appendix I* detailed plasmid map containing the cDNAs.

TABLE C: List of all vectors used for RNA probes.

Name of cDNA and the plasmid carrying it.	**Linearised with**	**Transcribed for antisense mRNA with**
Dpp cDNA in pBSK	KpnI	T3
Hh cDNA in pBSK	NotI	T7
Gcm cDNA in pBSK	SacI	T7
dSerT cDNA in pGEM-HE	EcoRI	SP6
Smo cDNA in pBSK	NotI	T7
Nkd cDNA in pCS2	HindIII	T7
E(spl) cDNAs		
M3 in pNB40	NotI	T7
m5 in pNB40	NotI	T7
m7 in pRSET	NotI	T7
m8a in pNB40	-	-
mβ in pNB40	-	-
mδ in pBKS	-	-
mγ in pBKS	-	-

3. RESULTS

3.1 Screening for mutations affecting late neuroblasts

To unravel new genes involved in neuroblast determination and formation in the embryonic CNS, a genetic screen offers an ideal solution. The efficacy of the genetic analysis depends on the phenotypic traits that can be detected. In the case of the development of the nervous system, antibodies have proven to be extremely powerful in revealing a number of those phenotypic traits. Therefore, in this study an antibody against the Eagle (Eg) protein was used. Eg is expressed in four distinct NBs and their progeny, all of which delaminate late (S3-S5) during embryogenesis. Thus this screen offers a possibility to detect genes which are necessary for 'late NB' development. The *eg* gene encodes a transcription factor and is a member of the steroid receptor superfamily in *Drosophila* (Higashijima et al., 1996). Eg expression is observed in NB 2-4, NB 3-3, NB 6-4 and NB 7-3. For schematic representation of Eg expression pattern see Fig.7.

About 900 independent lethal fly lines carrying unknown or unidentified mutations were screened. These lines were generated in the laboratory of C. Klämbt from a saturating F2- EMS (Ethylmethylsulphonate) mutagenesis and were pre-selected for nervous system defects with aid of antibodies which recognise axonal projections in the embryonic CNS. In addition to the Eg protein, the embryos were also stained against β-Galactosidase to identify those which carry the "blue" balancer chromosome. For analysis, the embryos staining for β-Galactosidase were rejected, as they are heterozygous for the mutation.

All lines showing an abnormal Eg expression patterns were selected. The phenotypes were divided into 7 classes namely, a) too many cells, b) less cells, c) D/V

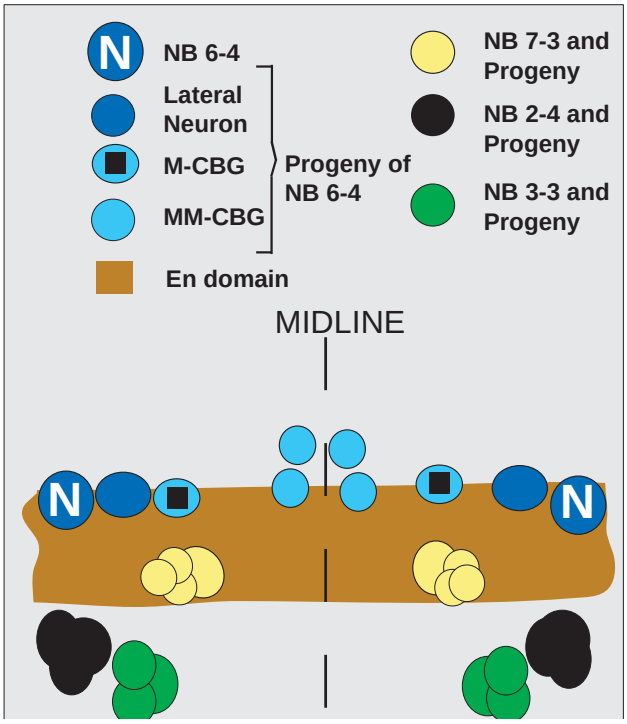
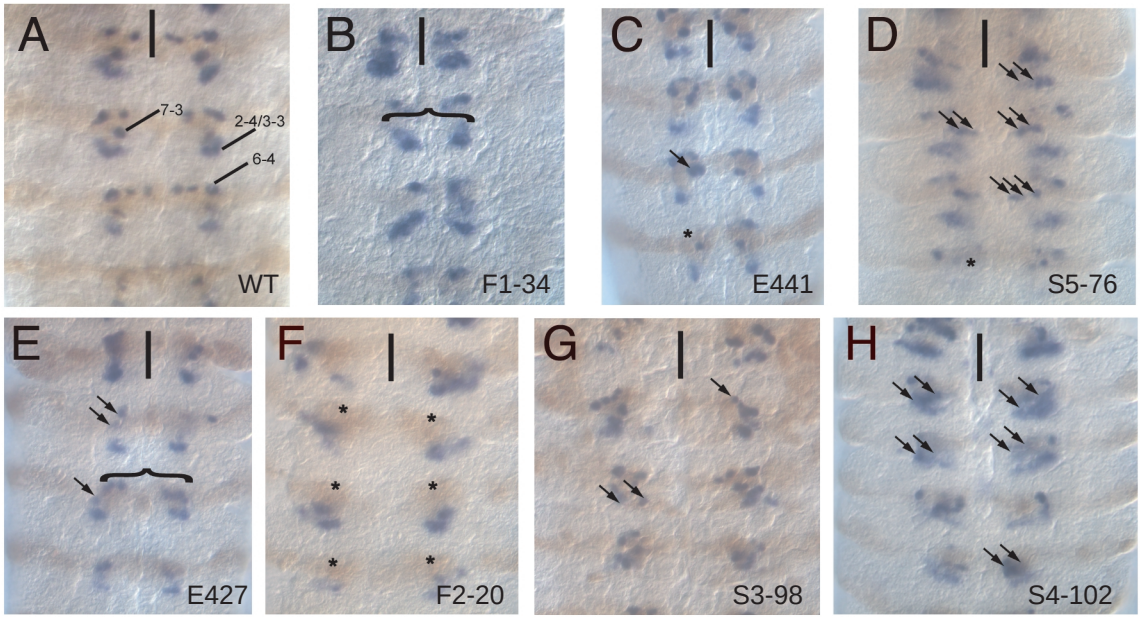


Figure 7. Eg expression pattern belonging to each phenotypic class.

Shown to the left is a schematic representation of wildtype Eg/En expression pattern: Seen here are two thoracic hemisegments on either side of the midline. Eg stains four NBs and its progeny belonging to NBs 6-4 (shown in shades of blue) and 7-3 (yellow cells) in the En-domain and NBs 2-4 (black cells) and 3-3 (green cells) outside the En-domain. NB 6-4 in the thoracic segments characteristically produces 3 glial cells (two MM-CBG glia which migrate towards the midline and one M-CBG glia) and a cluster of laterally located neurons. Below in figures A-H are Flat preparation of embryos at st 12, anterior up, Eg expression is seen in blue and En expression in brown with the first three segments from the top being thoracic. The black bar represents the midline.



Figures B-H represent each phenotypic class on which the screen was done.

A) A wildtype embryo shows Eg expression (blue) in two cell clusters in the En-domain (brown): anteriorly located is NB 6-4 and its progeny, posteriorly located is NB 7-3 and its progeny. Outside the En domain Eg expression is seen in two cell clusters namely in progeny of NBs 2-4 and 3-3. B) A mutant embryo showing a narrowed CNS, as NB to NB distance is reduced (horizontal curly bracket), this phenotype is typical of mutant genes belonging to the family of dorso/ventral patterning genes C) Seen here is a mutation of a gene causing an duplication of an NB namely, NB 7-3 (black arrow), in addition to missing of NB 6-4 (black star) probably due to mutations in cell cycle genes. D) A phenotype of Eg positive cells arranged in rows for NBs 7-3 and 3-3 clusters (black arrows). E) Mutations of genes involved in patterning show various defects like a narrowed CNS (curly bracket) to less Eg positive cells within an NB cluster (black arrows). F) A phenotype of missing of Eg positive NBs namely in the position of NB 6-4 (black stars). G) Mutations showing less cells within the Eg positive NB cluster, like NB 6-4 and NB 3-3 (black arrows). H) A typical phenotype of too many Eg positive cells within an NB cluster perhaps due to duplication of NB fate.

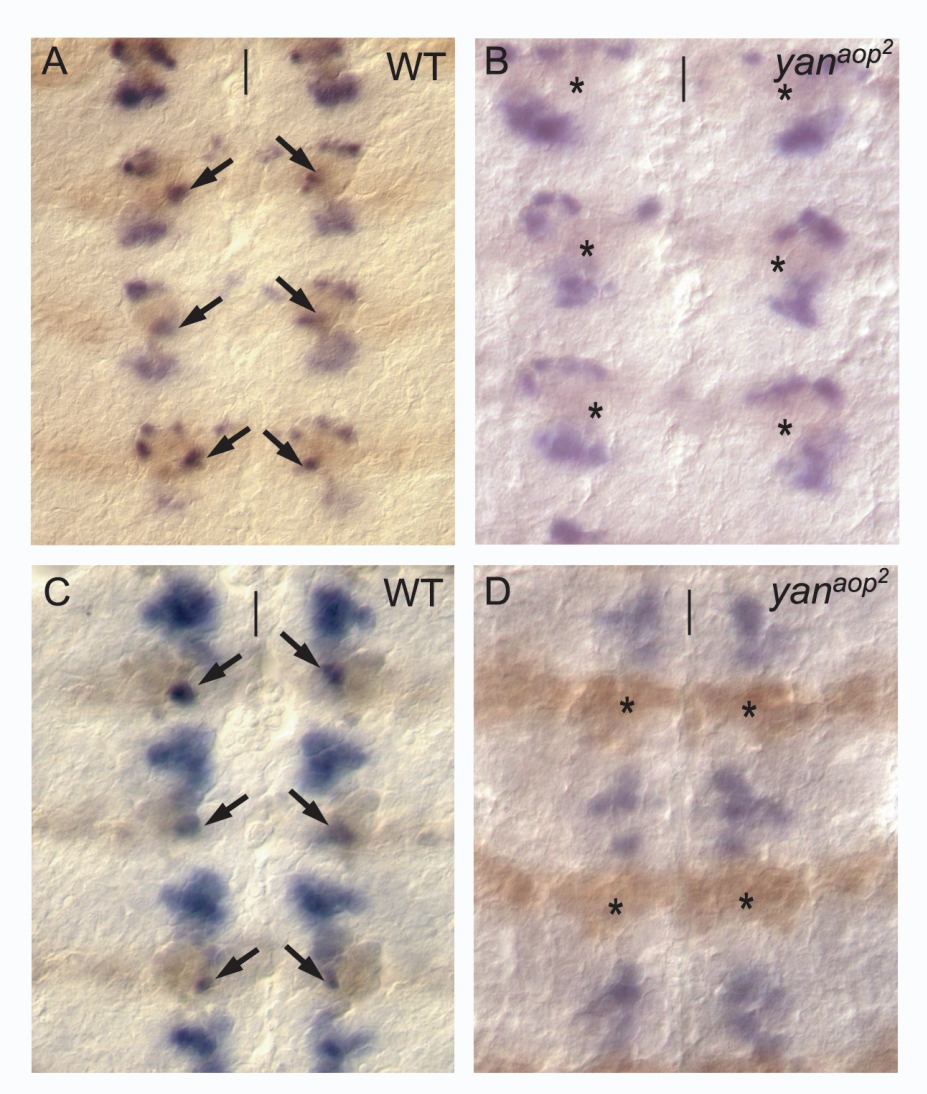


Figure 8. Eagle and Eyeless expression pattern in *yan* mutation. Flat preparation of embryos at st 12, anterior up, Eg and Ey expression is seen in blue and En expression in brown with the first three segments from the top being thoracic. The black bar represents the midline.

A) Wildtype embryo shows Eg expression in two cell clusters in the En-domain: anteriorly located is NB 6-4 and its progeny, posteriorly located is NB 7-3 and its progeny (black arrows).

B) *yan* mutant embryos lack Eg expression in the position of NB 7-3 (black stars) in 98% (n=100) of the hemisegments counted.

C) In the En-domain of wildtype embryos Ey expression is seen only in the position of NB 7-3 (black arrows). Ey is expressed additionally in 5 NBs and their progeny outside the En-domain.

D) In *yan* mutant embryos Ey expression is absent at the position of NB 7-3 in 98% (n=100) of the hemisegments counted (black stars).

patterning defects, d) cell cycle defects, e) missing NBs f) cells in rows and g) other patterning defects (Fig.7). Some of the mutants lines showed more than one of these features. About 95 lines (See Tables 1, 2 and 3 in Appendix II) with abnormal Eg staining could be found. Since the aim of the screen was to find genes involved in late NB formation and specification lines with missing NBs were selected. Four of these lines were chosen for detailed analysis as they showed a clean and reproducible phenotype. These lines were K1-66, X3-17, U6-35 and E427.

3.1.1 K1-66 and X3-17 are alleles of the *yan* gene

Further studies in this thesis was done concentrating on two out of the four lines, K1-66 and X3-17 both of which had the phenotype of NB 7-3 missing in 98% (n=100) of the hemisegments counted in the embryonic CNS. Studies on the other two lines were conducted by Lisa Meadows (personal communication and Lammel et al., 2000). Since both K1-66 and X3-17 showed exactly the same phenotype, they were crossed to each other for a complementation test. The result revealed that the two lines were allelic to each other probably carrying mutations for the same gene. To map the region of mutations, both K1-66 and X3-17 were subjected to complementation analysis with a collection of deficiency lines from the Bloomington stock centre. Two deficiency lines in combination with K1-66, three deficiency lines with X3-17 and one deficiency line in combination with both showed no complementation. This phenomenon suggests that the chromosome of lines K1-66 and X3-17 have accumulated more than one mutation during the process of mutagenesis. Since both lines showed no complementation with the deficiency line 3133, this deficiency mostly likely covered the gene of interest. Staining the 5 deficiency lines against the Eg protein showed only line 3133 had the same phenotype of missing NB 7-3. Thus, the same is gene affected in the two mutant lines which lies in the region of deficiency 3133 covering region 22A01-02 to 22D05-E01 of the 2nd chromosome.

This deficiency covered a large numbers of genes, the mutant stocks of which were available at the Bloomington stock centre. K1-66 and X3-17 were tested for complementation against each of these stocks. The stock carrying the mutation in area of the gene *anterior open* (*aop*) also known as *yan* showed no complementation. Two different available alleles of *yan*: *aop*^{1} and *aop*^{2}, showed exactly the same phenotypes

as K1-66 and X3-17 with Eg antibody stainings (Fig.8). Finally, to confirm that it is indeed the mutation in the *yan* gene causing the phenotype, the alleles aop^1 and aop^2 (in the following collectively called as yan^-) were crossed against the deficiency 3133 and the established clean stocks of K1-66 and X3-17 (see section 2.9).The result of all the crosses was no complementation. The heterozygote embryos (yan^-/deficiency, yan^-/K1-66, yan^-/X3-17) of all of the crosses showed the expected phenotype. Thus, the mutation in *yan* leads to loss of Eg staining in NB 7-3 that might be due to a loss of this NB. Since both the alleles of *yan* showed exactly the same phenotype, the allele aop^2 was used here after for further analysis and will be referred as *yan* mutant.

The *yan* gene was independently identified in a screen for embryonic lethal mutations where it was named *anterior open* (Nüsslein-Volhard C, 1985; Lindsley, 1992) but in this thesis it shall be mentioned as *yan*. Yan, is a transcription factor, which is known to be a nuclear target of receptor tyrosine kinase (RTK) signalling cascades. Yan encodes an E twenty six (ETS) DNA binding motif, a pointed (PNT) domain, 8 mitogen-activated protein (MAP) kinase phosphorylation sites, and several putative PEST (Pro, Glu, Ser and Thr) signals. The putative Yan protein is characterised by the presence of an ETS-domain and three glutamine rich regions. This ETS-domain has been shown to have sequence specific DNA binding activities in a number of nuclear proteins (Gunther et al., 1990; Klemsz et al., 1990; Urness and Thummel, 1990; Thompson et al., 1991) and these proteins are likely to function as transcriptional regulatory factors (Gunther et al., 1990; Klemsz et al., 1990; LaMarco et al., 1991; Thompson et al., 1991). A lot of work done previously has shown Yan to a negative repressor of transcription (Lai and Rubin, 1992; O'Neill et al., 1994; Rebay and Rubin, 1995; Rogge et al., 1995) The function of Yan protein could be evolutionary conserved as within the 85 amino acid ETS-domain, *yan* is 50% identical to another *Drosophila* ETS-domain protein *E74*, 51% identical to mammalian ETS-domain protein *elk1* and 50% identity with the human *tel* gene (Price and Lai, 1999).

3.2 The role of *yan* in the development of the embryonic nervous system

3.2.1 Embryonic phenotype in the CNS of *yan* mutants

The screen done above with anti-Eg antibody suggested that NB 7-3 missing in embryos mutant for *yan*. Analysis with another marker specific for NB 7-3 (see Fig. 9), namely anti-Eyeless (Ey) also showed that Ey was not found at position of NB 7-3 in 98% (n=100) of the hemisegments counted (Fig.8). Further, the proneural cluster of NB 7-3 does not seem to be formed in embryos mutant for *yan*. The NB 7-3 proneural cluster can be identified with the marker Huckebein (Hkb). *Insitu* hybridisation analysis using the antisense mRNA of *hkb* as the probe revealed no staining in the relevant position (Fig. 10A,B). The phenotype seemed to be very specific to NB 7-3 only as antibody stainings against available markers for many other NBs showed no difference to WT (see Table D). Many NBs, which delaminate late, could not be checked due to lack of markers. For a schematic representation of the specific markers used and which NBs they label, see Fig. 9. Thus, loss of *yan* function seems to specifically affect only NB 7-3 formation or specification. However, it cannot be excluded that some of the NBs without available markers could be also missing.

TABLE D: List of all the NB markers tested in *yan* mutant embryos.

Antibody markers used	**Cells labelled**	**Presence or absence in *yan* mutations**
Pox-n	NB 2-4 and its progeny	Present
Lbd	NB 5-6	Present
Eve	Progeny of NBs 4-2,1-1, 7-1, 3-3	Present
Runt	NBs 2-2, 2-5, 3-1, 3-2, 5-2, 5-3 and their progeny	Present

Ac	MP2 , NB 3-5, 7-1, 7-4 and their progeny	Present
Ems	NB 3-3,3-5,4-4 and their progeny	Present
Gsb-d	NB 5-1,5-2, 5-3, 5-4, 5-5, 5-6, 6-1, 6-2, 6-4, 7-1	Present
Repo	Progeny of NB 5-6, 6-4, 1-3, 2-5, 1-1, 2-2, 7-4 and GP	Present
Castor/Ming	NBs 1-2,2-1,2-2,2-4,3-1 3-2, 3-3, 3-4, 4-1, 5-1 5-2,5-3, 5-6, 6-1, 6-4 7-1, 7-2, 7-4 and their progeny	Present
Ey	NBs 3-2,4-2,4-3,4-4,5-3,7-3 and their progeny	Only NB 7-3 absent
Eg	NBs 2-4,3-3,6-4,7-3 and their progeny	Only NB 7-3 absent.

3.2.2 Analysis of the function of Yan with respect to EGFR and JNK signalling pathways

To identify the molecular pathway Yan is involved with respect to the detected phenotype, genetic interactions with mutations of genes belonging to certain signalling pathway were tested. It has been previously shown that NB 7-3 is partially missing in EGFR (**E**pidermal **G**rowth **F**actor **R**eceptor) mutants, as well as another intermediate neuroblast NB 4-2 (Udolph et al., 1998). Therefore the EGFR signalling pathway was explored in greater detail. Yan has been shown to act downstream of the EGFR signalling pathway as an inhibitor (Gabay et al., 1996).

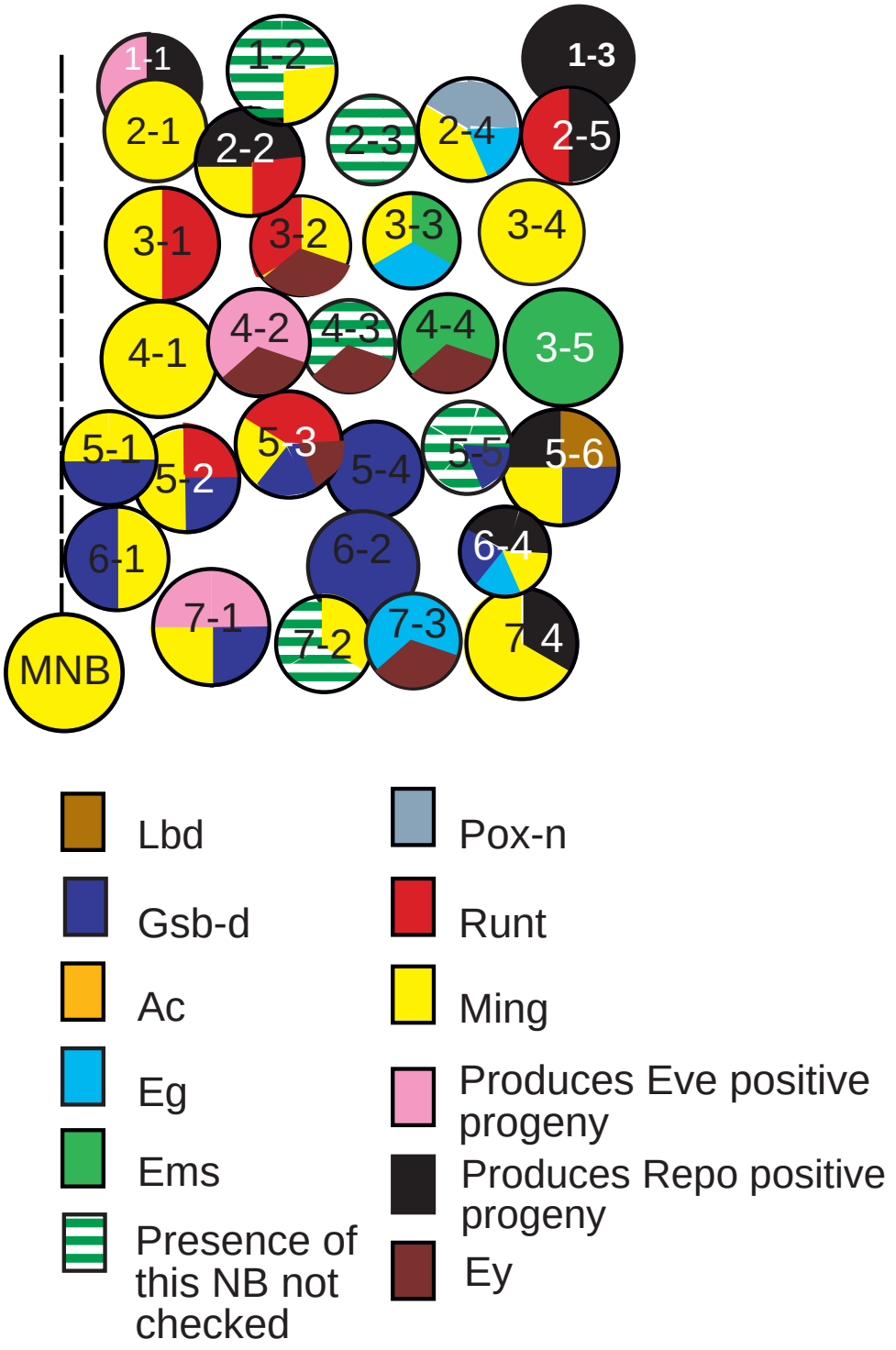


Figure 9. Marker expression in NBs. NB map showing all the antibody markers tested in *yan* mutant embryos. NBs shaded with green hatched lines were not tested for their presence due to unavailiblity of appropriate markers. Only NB 7-3 seems to be missing in *yan* mutations.

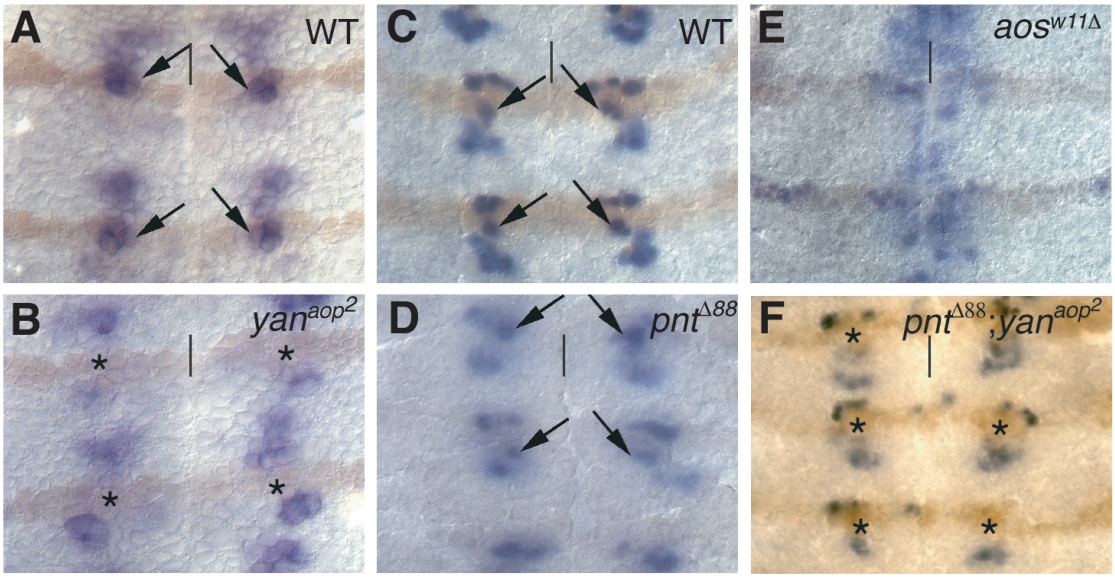


Figure 10. Hkb expression and Eg expression pattern in genes of the EGFR signalling pathway. Flat preparation of embryos with anterior up, En expression in brown with the first three segments from the top being thoracic. Figs A-B Embryos of st 10 with Hkb expression in blue and Figs C-F St 12 embryos with Eg expression in blue. The black bar represents the midline.

A) RNA *insitu* hybridisation against Hkb expression in wildtype embryos. In the En-domain the Hkb marks the proneural cluster of NB 7-3 (black arrows).

B) In embryos mutant for *yan*, Hkb expression is absent in the position of the proneural cluster of NB 7-3 (black stars). This indicates that NB 7-3 is missing, as its proneural cluster is not formed.

C) Wildtype embryos show Eg expression in two cell clusters in the En-domain: anteriorly located is NB 6-4 and its progeny, posteriorly located is NB 7-3 and its progeny (black arrows).

D) *pnt* mutant embryos show Eg expression like that of wildtype.

E) *aos* mutant embryos show Eg expression to be derepressed in the region of the midline. This is most probably due to secondary effects of the mutations as Aos plays a role in multiple tissue types.

F) Embryos double mutant for *pnt* and *yan* show Eg expression to be missing at the position of NB 7-3 (black stars).

Yan competes with Pointed (*pnt*, an activator downstream of EGFR signalling) binding sites to inhibit EGFR targets (Gabay et al., 1996). EGF receptor upon activation by appropriate ligand inhibits Yan activity and promotes Pnt activity through activated MAPK (**M**itogen **A**ctivated **P**rotein **K**inase). One of the known targets of Pnt activity is *argos* (*aos*) an inhibitor of EGFR signalling, thereby constituting a negative feed back loop (Gabay et al., 1996). In addition the gene, *intermediate neuroblast defective* (*ind*) is also shown to be regulated by the EGFR signalling cascade. Ind is responsible for the specification of NBs of the intermediate row (Weiss et al., 1998). Therefore, if Yan is involved in specification of NB 7-3 through the EGFR pathway, the missing of NB 7-3 in Yan mutations could be either due to hyperactivity of Aos or perhaps due to down regulation of *ind* in *yan* mutant embryo. Each of these possibilities was systematically analysed

Ind is a target and Aos an inhibitor of EGFR signalling. Indeed in embryos with *ind* mutations NB 7-3 was missing in 100% of the hemisegments counted. But analysis of Ind expression in Yan mutant embryos was observed to be like that of the wildtype situation (data not shown), so Ind could not be the reason for NB 7-3 phenotype in Yan mutations. In Yan mutant embryos, Aos is hyperactive, as Pnt activity is not inhibited. Upon anti-Eg staining NB 7-3 was found to be present in embryos mutant for *pnt* (Fig. 10D). If hyperactivity of Aos is indeed responsible for missing of NB 7- 3, embryos double mutant for *pnt* and *yan* must rescue the NB 7-3 phenotype. However, embryos double mutant for *pnt* and *yan* showed NB 7-3 to be absent suggesting that hyperactivity of Aos could not be the reason for the phenotype (Fig 10F). Embryos mutant for *aos* showed ectopic derepression of Eg expression such that it was observed all along the midline and in large clusters laterally outside the CNS (Fig. 10E). The double mutations of *aos;yan* also showed a phenotype similar to *aos* mutations (data not shown). This derepression of Eg expression cannot be explained with the current knowledge and could be most likely due to secondary effects of the *aos* mutation.

Deducing from the above experiments it is most likely that the missing of NB 7-3 in *yan* mutants is not due to interference of EGFR pathway. This was supported by the finding that NB 7-3 and the region of its delamination does not express activated MAPK as seen by antibody staining against activated MAPK in

combination with Eg (data not shown). Therefore, it was possible that the role of Yan with respect to NB 7-3 formation is in the context of another MAPK pathway.

Three different MAPK subfamilies have been identified: p42-p44 extracellular-signal regulated kinase (ERKs), p38 kinases and p46-p54 Jun amino-terminal kinases (JNKs). These major subfamilies respond to different signals, exhibit distinct substrate specificity and seem to have different roles. Thus, it could be that other MAPK may influence NB 7-3 formation. The ERKs cannot be invovled in NB 7-3 formation as the EGFR pathway exerts it effect through this kinase. So far no known pathways which use p38 have been identified. Analysis of embryos which carry mutations for p38 Kinases revealed that they are not involved in the specification of NB 7-3 (See Table E). The pathway which uses p46-p54 Jun amino-terminal kinases namely the JNK pathway was analysed in greater detail, since Yan is also known to functions as a repressor in the *Drosophila* JNK (DJNK) pathway, where in *decapentaplegic* (*dpp*) is one of the known downstream targets (Riesgo-Escovar et. al., 1997). Therefore the NB 7-3 phenotype could possibly be explained through the interference of the JNK signalling pathway leading to ectopic *dpp* activity. The activation of the JNK cascade has been reported after UV irradiation and is thought to be initiated by the phosphorylation of the serine/threonine kinase DPAK and the sequential modification of the JNKK homologue Hep (DJNKK) and the JNK homologue Basket (DJNK) (Sluss et. al., 1996). Upon activation the DJNK cascade acts through nuclear targets amongst them are the transcription factors, DJUN and Yan. DJUN is responsible for the transcription of Dpp. DJNK in addition inhibits Yan function which leads to inhibition of Dpp (Martin-Blanco, 1997 and references therein). However, the expression of Dpp in embryos carrying *yan* mutations was found to be normal with respect to the CNS. Analysis of mutations of all the genes down stream to JNK pathway did not show a *yan* phenotype (Table E). Therefore the DJNK pathway cannot be involved in the specification of NB 7-3.

TABLE E: Analysis of mutations of genes belonging to the EGFR and JNK signalling pathways

Mutants analysed	**Presence of NB 7-3 as seen by Eg/En antibody**	**Comments**
Genes of the EGFR pathway		
egfr	40% missing	
rhomboid	Present	Wild type
spitz	Present	Wild type
vein	Cannot be interpreted	Eg expression in epidermis, midline and laterally outside the CNS.
aos	Cannot be interpreted	Eg expression in epidermis, midline and laterally outside the CNS.
yan;aos	Like *aos*	Phenotype like *aos*
pnt	Present	Wild type
yan,pnt	NB 7-3 missing	Phenotype like *yan*
Genes of the JNK pathway and different Kinases		
jun^{FRT}	Present	Wild type
Df(2R)*rl*	Present	Wild type
hep	Present	Wild type
p38 (dominant negative)	Present	Wild type
jnk (dominant negative)	Present	Wild type
raf (dominant negative)	Present	Wild type

3.2.3 Yan is expressed in the neuroectoderm and not in the delaminating neuroblasts

Expression pattern analysis of a particular protein can give insights as to where and how a protein might function. Towards this step Yan expression was analysed with respect to the developing nervous system in the embryo using an antibody against the Yan protein (kindly provided by C. Lai). Previous studies by Price and Lai showed that Yan is dynamically expressed in early embryos. Initially Yan is detected in all nuclei at the periphery of the cellular blastoderm and during germ band extension. It is also detected in the presumptive ectoderm but excluded from the midline and several rows of ventral most ectodermal cells. Later Yan expression is concentrated in cells surrounding the tracheal pits in a group of cells slightly posterior and beneath the tracheal pits and in cells close to the ventral midline in the epidermal layer (Price and Lai, 1999).

Additionally, I observed that Yan is expressed in a whorl like pattern in the neuroectoderm, around delaminating neuroblasts at st 10. The staining was completely absent in the neuroblasts themselfs (Fig. 14C). It seems that during neurogenesis in a given proneural cluster the cell that is selected to segregate as a neuroblast has downregulation of Yan activity whereas the other cells of the proneural cluster show upregulation of Yan activity. This suggested that Yan could have a role in the process of lateral inhibition (see section 1.4). The *Notch* (*N*) gene product plays a key role in the process of lateral inhibition, allowing cells to choose between a determined and undetermined state Therefore, the role of *Notch* was examined in this context.

3.2.4 The *yan* mutation suppresses the embryonic *Notch* mutant phenotype

A genetic interaction between the *Notch* and *Yan* genes has been shown recently in studies concerning eye development in *Drosophila*. In the eye imaginal disc development loss of Notch function causes excessive development of photoreceptor neurons (Cagan and Ready, 1989) while ectopic activation of Notch during cluster formation causes cells to remain undifferentiated (Fortini et al., 1993). A decrease in Yan function in eye development results in an increased number of cells developing as R7 photoreceptor cells in the eye. In previous studies with respect to eye development it has been shown that loss of Notch partially suppresses and extra

Notch enhances the *yan* mutant phenotype (Rogge et al., 1995). In the embryonic neuroectoderm, the function of *Notch* is to inhibit cells from the equipotent neuroectodermal cell layer to acquire a neural fate thereby enabling the cells to develop an epidermal fate (See 1.4). In the absence of Notch too many cells in the neuroectoderm adopt a neural fate. To test whether there is a genetic interaction between Yan and Notch in the process of NB formation double mutants of *yan* and *Notch* were created. Indeed, it was observed that in yan^{aop2} mutant embryos carrying in addition a loss-of-function mutation of *Notch*, N^{55e11}, the CNS phenotype of *Notch* was partially suppressed. This result was obtained upon analysing embryos with anti-Eg and anti-Eve antibodies. Large Eg and Eve positive cell clusters are observed in embryos of N^{55e11} . In embryos with double mutant for N^{55e11} and yan^{aop2} the size of these cell clusters was drastically reduced (Fig. 11A-H). This result is suggestive of the fact that Yan and Notch may act in opposite direction with regard to cell fate specification in the neuroectoderm. Thus, it seems that while in the neuroectoderm Notch activity promotes cells in the proneural cluster to adopt an epidermal fate and Yan acts as an antagonist of this activity.

3.2.5 Ectopic expression of activated Yan leads to a neurogenic phenotype

If Yan indeed is inhibiting Notch activity, overexpression of constantly activated Yan should enhance or mimic a Notch loss of function phenotype. To investigate this, *UAS-*yan^{act} (where in all the MAPK consensus sites of the Yan protein are mutated; Rebay et. al., 1995) was expressed ectopically in the neuroectoderm by using *scabrous* (*sca*) *Gal4* as a driver. These embryos were analysed for a potential 'neurogenic' phenotype by staining with anti-Eg and anti-Pox-n antibodies both of which mark specific NBs. Anti-Pox-n antibody showed a duplication of NB 2-4 in 26% (n=90) of hemineuromeres (Fig. 12D). Anti-Eg antibody showed a duplication of NB 3-3 in 65% (n=20) of hemineuromeres (Fig. 12F).

To further confirm the duplication of NBs, markers for known progeny of certains NBs were tested. Anti-Eve antibody and an *in situ* hybridisation against dSerT mRNA (*Drosophila* serotonin transporter) were used as progeny markers. dSerT marks two progeny of NB 7-3 namely the two serotonergic neurons it produces and Eve among many other cells marks the RP2 neuron, which stems from NB 4-2

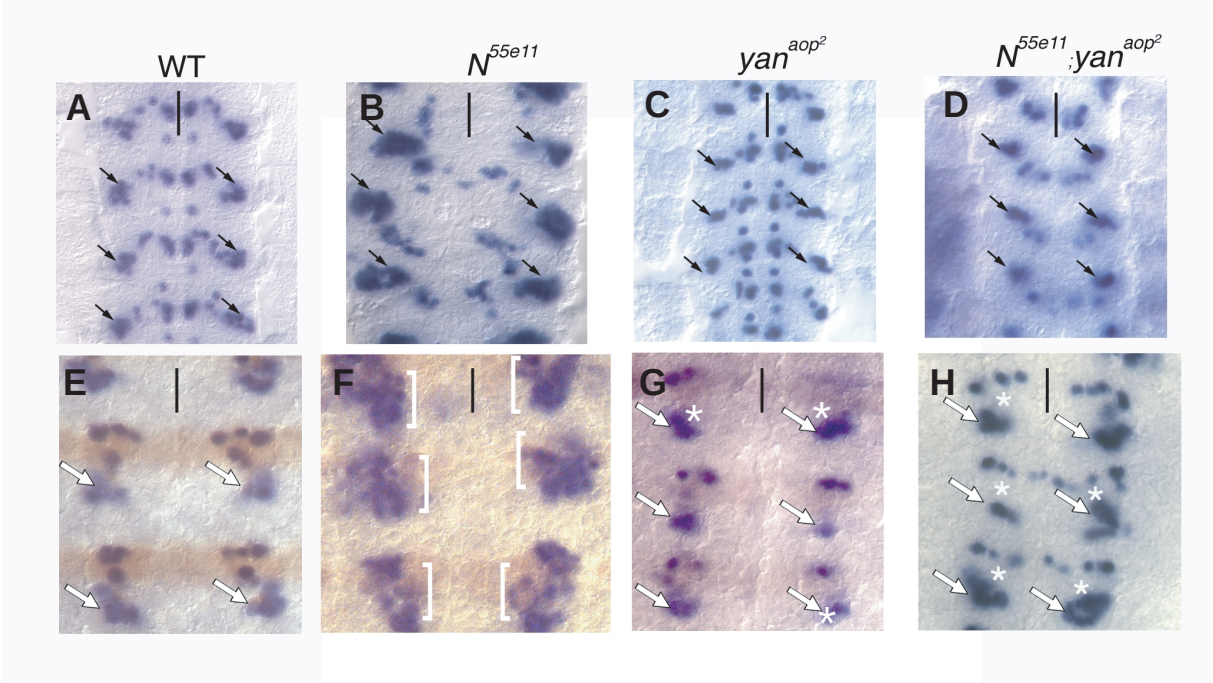


Figure 11.The neurogenic phenotype of *Notch* is suppressed in *Notch;yan* double mutants.

Flat preparation of embryos at st 15 with anterior up and the first three segments from the top being thoracic. Figs A-D show embryos with Eve expression in blue. Figs E-H show embryos with expression of Eg in blue and En in brown. The black bar represents the midline.

A) Wildtype embryos express Eve in a number of progeny cells of several neuroblasts. Marked here are progeny of NB 3-3 (black arrows).

B) Embryos of N^{55e11} a Notch null mutant show a large number of Eve positive cells in the NB 3-3 cluster (black arrows), indicating excess of NB 3-3 formation.

C) Embryos mutant for *yan* show wildtype Eve expression for NB 3-3 cluster (black arrows).

D) Less number of Eve positive cells in NB 3-3 cluster (black arrows) is seen as compared to Notch mutant in embryos double mutant for *N* and *yan*.

E) Wildtype embryos showing Eg expression in abdominal segments where it is expressed in NB 7-3 in the En domain and NBs 2-4 and 3-3 outside the En-domain. Marked here are progeny of NBs 2-4 and 3-3 (white arrows).

F) Embryos of *N* mutant show a large number of Eg positive cells (white square brackets) indicating excess of neuroblast formation.

G) In *yan* mutation NB 7-3 is missing as indicated by white stars in 98% (n=100) of the hemisegments counted and NB 3-3/2-4 cluster is wildtype (white arrows).

H) The *Notch* phenotype is suppressed in embryos double mutant for *N* and *yan* in NB 3-3/2-4 cell cluster. NB 7-3 is also missing in this double mutant (white stars).

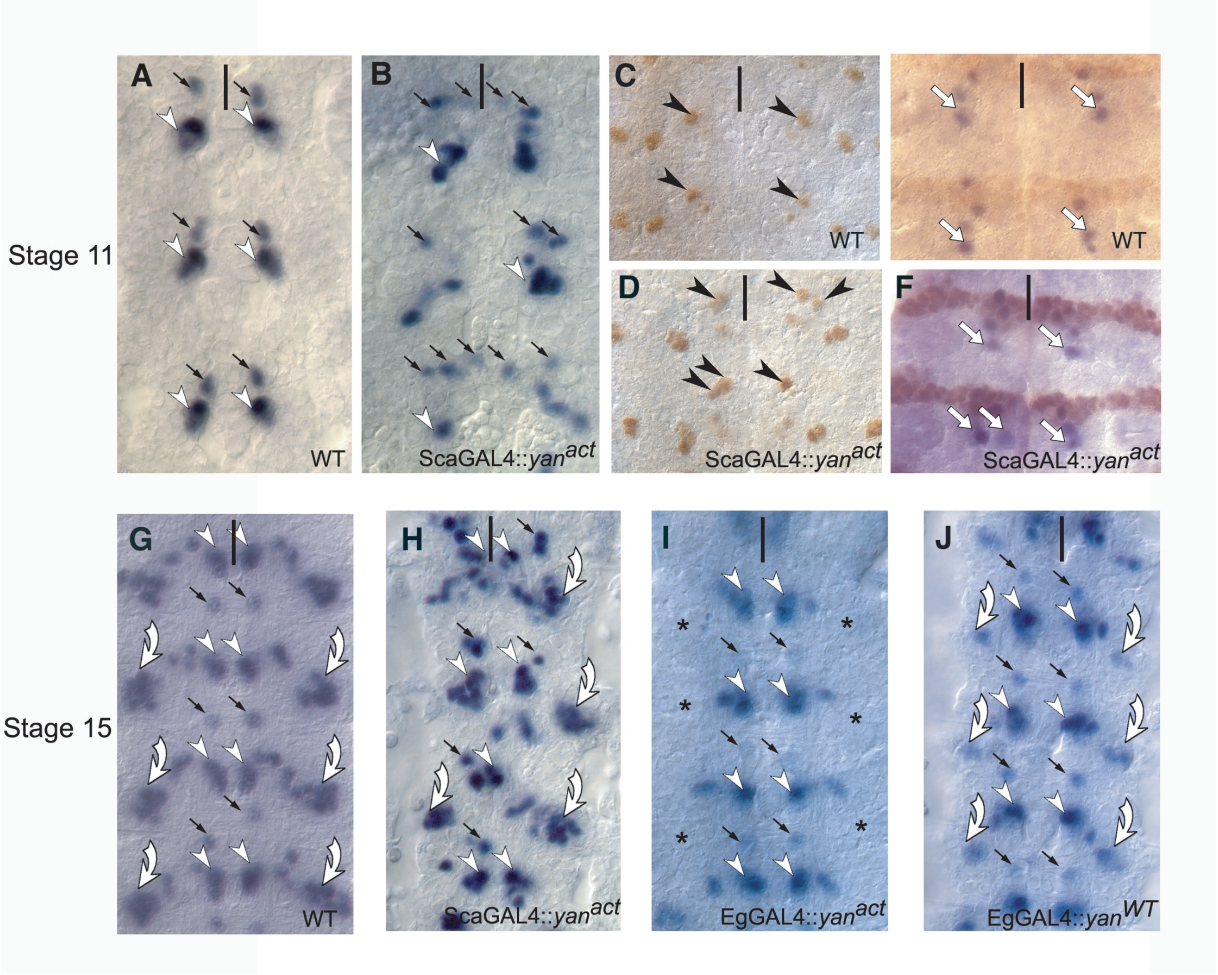


Figure 12. Ectopic expression of activated Yan leads to duplication of NBs.

Flat preparation of embryos with anterior up. Figs A-B and G-J show embryos with Eve expression in blue, with the first three segments from the top being thoracic. Figs C-D show Pox-n staining in brown and Figs E-F shows Eg expression in blue and En in brown in abdominal segments. The black bar represents the midline.

A) Wildtype embryos at st 11 show Eve positive RP2 neuron that stems from NB 4-2 (black arrows) and aCC/pCC neuronal cluster (white arrowheads). B) In embryos with *UAS-yan*^{act} driven by *scaGal4* at st 11 extra RP2 neurons are seen, the number varying between 2-3 (black arrows). C) Wildtype embryos at st 11 in the abdominal segments, show Pox-n expression in NB 2-4 (black arrowheads). D) In embryos with *UAS-yan*^{act} driven by *scaGal4* at st 11 a duplication of NB 2-4 is seen by Pox-n expression in 26% (n=90) of hemisegments (black arrowheads). E) Wildtype embryos at st 12, Eg expression is seen in NBs 2-4 and 3-3 outside the En domain. Marked here is NB 3-3 (white arrows). F) In embryos with *UAS-yan*^{act} driven by *scaGal4* at st 11 show duplication of NB 3-3 in 65% (n=20) of the hemisegments counted (white arrows). G) Wildtype embryos at st 15 show Eve positive lateral cell cluster which are NB 3-3 progeny (white curved arrows),RP2 neurons (black arrows) and aCC/pCC neurons (white arrowheads). H) In embryos with *UAS-yan*^{act}driven by *sca-Gal4* at st 15 the CNS looks narrow and Eve pattern is disrupted. I) In embryos with *UAS-yan*^{act} driven by *egGal4* at st 15 Eve expression is mostly abolished in the position of the lateral cell cluster (black stars). J) In embryos with *UAS-yan*^{WT} driven by *egGal4* at st 15 Eve expression is like that of wildtype.

Embryos with ectopic activated Yan showed more than two serotonergic neurons per hemisegment, the number varying between 3-7 cells in 66% (n=50) of the hemineuromeres indicative of additional NB 7-3s (Fig. 13B). Similarly, anti-Eve antibody stainings showed additional NB 4-2s as more than one RP2 neuron per hemisegment was observed, the number varing from 2-6 in 78% (n=50) of hemineuromeres (Fig. 12B). Thus, antibody analysis on these embryos showed additional neuroblasts, which is similar to a neurogenic phenotype observed in *Notch* mutant embryos.

3.2.6 Gain of function Yan inhibits neuronal differentiation during lineage development

Work done by Rebay et al (1995) showed that while expressing the wildtype yan protein (Yan^{act}) had no effect, the expression of Yan^{act} under the heat shock as well as the *UAS* promoter driven by *elavGal4* (*elav*: *embryonic lethal, abnormal vision*) blocks expression of neural antigens as judged by complete abolition of Elav staining. Therefore, it was hypothesised that Yan is an inhibitor of neural differentiation which is supported by the the fact that in wild type embryos Yan expression is completely absent in the developing CNS. This result contradicts my finding that ectopic Yan^{act} leads to a neurogenic phenotype. In the previous experiments (section 3.2.5) *scaGal4* was used as a driver line while Rebay et. al were using *elavGal4.* Sca expression comes on during st 8 in the neuroectoderm and begins to disappear in late satges (T Novotny, Pers. communications) but Elav is expressed very late in the developing CNS i.e. post mitotically in differentiated neurons. Therefore I hypothesised that the difference in the effect of ectopic Yan^{act} is mainly due to the different times of expression. To test this, *egGal4* was used as a driver for comparative studies. In contrast to *scaGAL4*, *egGAL4* drives expression after NB delamination in selective neuroblasts amongst them NB 3-3 and NB 7-3. Anti-Eve staining in the *egGal4*:: *yanact* embryos showed that the progeny of NB 3-3 i.e. the EL cells (Schmidt et. al., 1997) are missing in 48% (n=50; Fig. 12I) of hemisegments counted. Further dSerT RNA *in situ* hybridisation showed that the serotonergic neurons are not seen in 56% (n=50) of the hemisegments (Fig. 13D). *egGal4*::yan^{WT} embryos were found to be normal for both Eve and dSerT expressions except in a few cases. Taken together it is indicative that Yan when active late within a differentiating

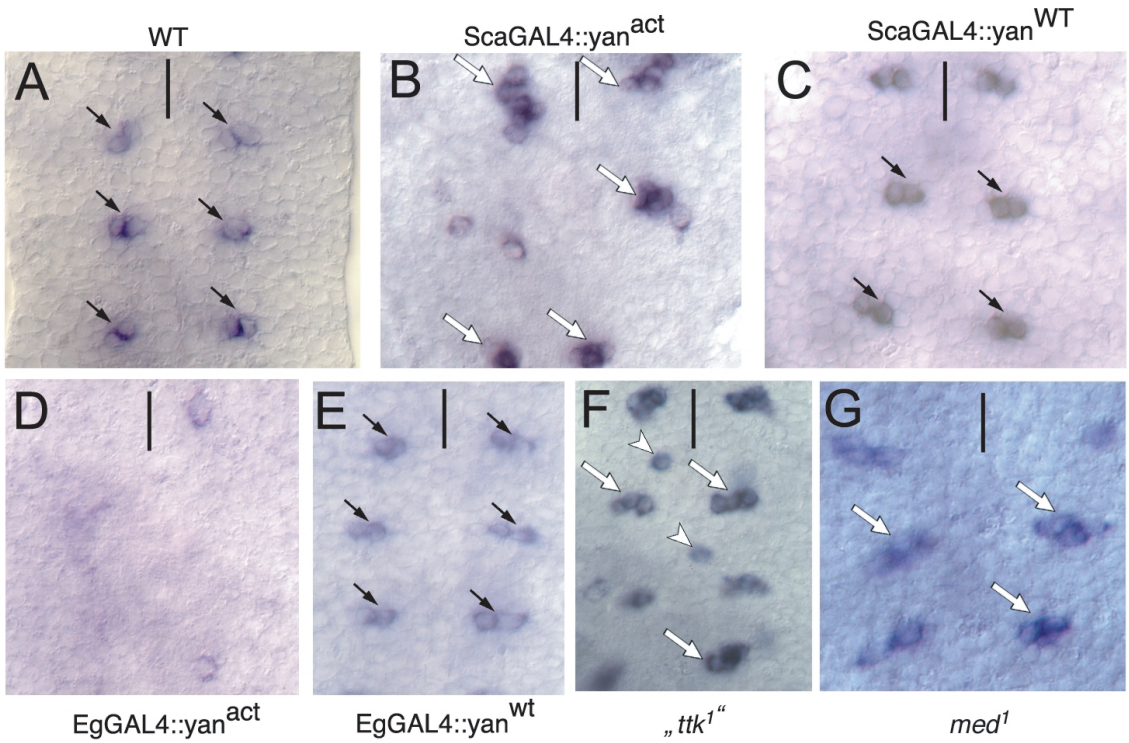


Figure 13. dSerT expression pattern in various mutants.

Flat preparation of embryos at st 17 with anterior up. RNA i*n situ* hybridisation against dSerT seen in blue. The black bar represents the midline.

A)Wildtype embryos typically shows two dSerT positive cells per hemisegment which are the progeny of NB 7-3 (black arrows).

B) In embryos with *UAS-yan*^{act} driven by *scaGal4* extra dSerT positive cells are seen, the number varying between 3-7 at the position of NB 7-3 progeny (white arrows).

C) In embryos with *UAS-yan*^{WT} driven by *scaGal4* no effect is seen on the number of dSerT positive cells (black arrows).

D) In embryos with *UAS-yan*^{act} driven by *egGal4,* dSerT expression is mostly abolished.

E) In embryos with *UAS-yan*^{WT} driven by *scaGal4,* dSerT expression is like that of wildtype (black arrows).

F) Embryos of *ttk*^{1}: this allele which carries on its chromosome a P-element inserted at the *ttk88* locus show extra serotonergic cells, the number varying between 3-7 at the position of NB 7-3 progeny (white arrows). Additional dSerT positive cells outside the position of NB 7-3 progeny are also seen (white arrowheads).

G) *med*^{1}allele shows extra dSerT positive cells occasionally at the position of NB 7-3 progeny (white arrows).

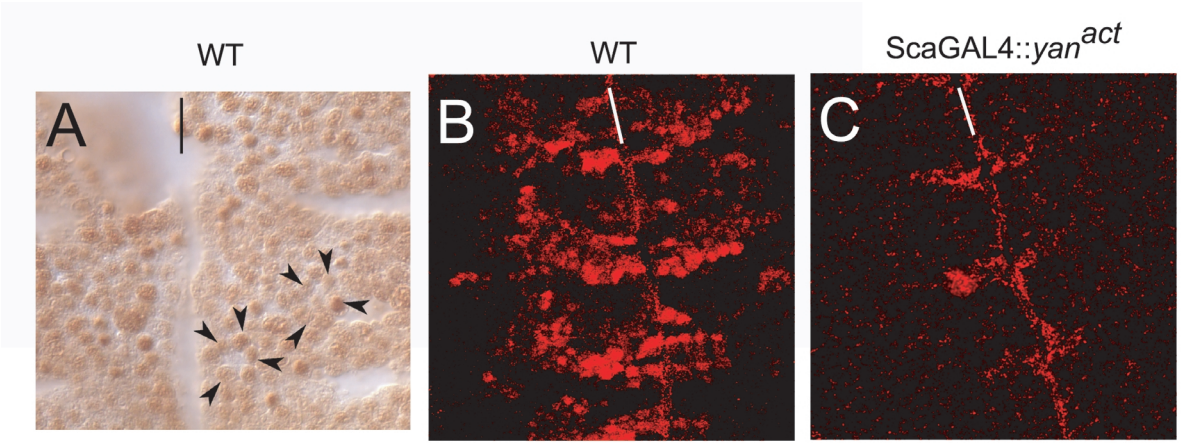


Figure 14. The E(spl) expression pattern is affected by ectopic expression of activated Yan .

Flat preparation of embryos at st 11 with anterior up. Seen here is the thoracic neuroectoderm. The black/white bar represents the midline. Fig A shows Yan expression in brown and Figs B-C confocal images of embryos showing antibody staining against most proteins of the E(spl) complex in red.

A) A wildtype embryo with Yan expression pattern seen in a whorl like pattern (black arrowheads) around a delaminating NB in the neuroectoderm. Yan expression is absent in the delaminating NB itself.

B) Wildtype embryos showing E(spl) expression in the neuroectoderm.

C) Embryos with *UAS-yan*^{act} driven by *scaGal4* the E(spl) expression is drastically downregulated.

cell inhibits the process of differentiation while Yan activity in the neuroectoderm seems to promote NB formation.

3.2.7 Ectopic expression of gain of function Yan affects Notch activity

In the neuroectoderm, neural versus epidermal fate is determined by the presence of gene products of the proneural genes of the *achaete-scute* complex (AS-C), or products of the Enhancer of split [E(spl)] complex respectively. It has been shown that the expression of the E(spl) genes suppresses cells from entering into a neural fate (Preiss et. al., 1988) whereas expression of genes of the achaete-scute complex promote neurogenesis (Cabrera et. al., 1987). Transcription of the E(spl) genes is promoted upon activation of the Notch receptor and is therefore an ideal indicator for Notch activity. *Suppressor of Hairless* [Su(H)] encodes a DNA-binding transcription factor shown to directly activate transcription of E(spl) genes in response to Notch receptor activity. The core consensus sequence GTG[G/A]GAA which is present in multiple copies in the regulatory region of the E(spl) genes was of the Su(H) protein (Lecourtois and Schweisguth, 1995). On the other hand the consensus binding sites of the six ETS-domains was shown to be 5'-GGA[A/T|-3' (Nye et al., 1992) to which Yan also is known to bind. The comparison between the Su(H) and Yan binding sites seems to suggest that both could compete to bind to the *E(spl)* enhancer, one to promote and the other to repress E(spl) gene transcription respectively. Therefore, embryos with ectopic activated Yan expression were examined with a antibody staining against the E(spl) proteins. Since the expression pattern of E(spl) is very dynamic from st 8 to st 11, I concentrated the analysis on embryos between st 10-11. At st 11, a great number of cells express the E(spl) proteins in wildtype (Fig. 14A). Strikingly, embryos with ectopic activated Yan expression at that stage showed a drastic reduction of E(spl) expression in the neuroectoderm compared to wildtype embryos (Fig. 14B). This is very indicative that Yan activity indeed is able to inhibits Notch signalling.

3.2.8 Ttk does not interact with Yan function with respect to CNS development

In the developing eye disc, before the photoreceptor precursor cells are committed to a particular fate, Yan is required to maintain them in an undifferentiated

state. Work done by Lai et al. (1997) identified *tramtrack* (*ttk*) mutations which act as dominant enhancers of Yan (Lai et al., 1997). They showed that *ttk* synergistically interacts with *yan* to inhibit the R7 photoreceptor cell fate. Therefore I tested whether *ttk* could have a similar role during Notch signalling in the embryonic neuroectoderm. Ttk encodes two proteins, Ttk69 and Ttk88, produced by differential splicing of its transcript. Ttk69 has been shown to promote glial differentiation where as Ttk88 was shown to have no role in CNS development. However, analysis of the allele ttk^{1}, which is supposed to be a specific mutation for *ttk88*, by RNA *in situ* hybridisation against dSerT revealed additional serotonergic neurons in the NB 7-3 cluster, the number varying from 3-7 (Fig. 13F). Occasionally, a single ectopic dSerT positive cell was seen away from the position of NB 7-3 cluster. In addition, anti-Eg staining also revealed expression pattern similar to that seen with dSerT (data not shown).The fly stock carrying the *ttk88* mutation is described as homozygous viable but the embryos showing the above phenotype failed to hatch and showed gross morphological defects in the head. Thus it was essential to test whether the mutation giving the phenotype is indeed in the *ttk88* gene. The mutation in the ttk^{1} allele is due to a P-element insertion which often cause additional mutations in its vicinity. Indeed I could show that the phenotype is not caused by the loss of function mutation of *ttk88* but by a mutation in a gene nearby. Embryos carrying a deficiency covering the region 100C-100D (Bloomington 3369) which removes several genes in addition to both *ttk69* and *ttk88* genes showed a phenotype similar to ttk^{1} upon RNA *in situ* hybridisation with dSerT. But embryos in trans with the deficiency and ttk^{1} showed a very weak phenotype of only one ectopic serotonergic cell in addition to the normal pair per hemisegment, hinting that a it is not the mutation of *ttk88* causing the phenotype. To identify the mutation causing the phenotype, various stocks carrying mutations in area of 100C-100D were analysed. The analysis of the stock carrying a mutation in the *medea* (*med*) gene med^{1}, revealed a similar weak phenotype only of one ectopic serotonergic cell per hemisegment(Fig. 13G). Thus, it is very likely that the observed mutation is identical to med^{1}.

3.3 The role of segment polarity genes in patterning of the CNS

The second approach used to understand how late delaminating NBs are specified was to investigate the role of genes that have been shown to play some role in early NB development. As a first step towards this goal I have concentrated on studying the interactions of segment polarity genes which confer A/P positional cues on the neuroectoderm. Therefore, I focused on two late neuroblasts situated in the domain of a segment polarity gene known as *engrailed* (*en*) which is expressed in rows 6 and 7 of the neuroectoderm. The two NBs under study were namely, NB 6-4 in row 6 and NB 7-3 in row 7 which give rise to distinct types of lineages (Bossing et al., 1996; Schmidt et al., 1997). NB 6-4 characteristically generates glia which can be identified with anti-Repo antibody (*reversed polarity*, a glial specific marker in combination with anti-Eg antibody (*eagle*, additionally stains NBs 7-3, 2-4 and 3-3 (Halter et al., 1995; Dittrich et al., 1997), whereas NB 7-3 typically generates serotonergic neurones (Lundell et al., 1996; Dittrich et al., 1997) and can be identified with anti-Ey (see also Fig. 9) in combination with anti-En antibodies. Since the segmentation genes *wingless* (*wg*) and *hedgehog* (*hh*) influence the En-domain, I analysed the individual role of these genes in detail.

3.3.1 En is a key factor for NB 7-3 formation and Hh has no independent role in this process apart from En maintenance.

The first question I wanted to address was how NBs in rows 6 and 7 NBs acquire different fates. The only factor known so far, to distinguish row 6 from row 7 is Gsb, which is expressed in row 6 neuroectoderm. It is also known that Gsb is a target of the Wg signalling cascade and specifies the identities of neuroblasts in rows 5 and 6 (Skeath et al., 1995). Wg, which is a secreted protein is expressed in row 5 and influences the specification of the fate of NBs in the adjacent rows 4 and 6 of the neuroectoderm (Chu-LaGraff and Doe, 1993). In addition Wg has been shown to maintain En expression in rows 6 and 7. Therefore, since row 7 is also under the influence of the Wg signalling, how is Gsb prevented from being expressed here? One

mechanism, which could be involved in this process, is Hh signalling. Hh, also a short range signalling molecule is expressed in rows 6 and 7 due the activity of En. Hh exerts its influence on rows 5 and 1/2 and shown to maintain Wg expression in row 5. Thus Wg, En and Hh act in concert to maintain each other's expression thereby constituting maintenance loop.

Previous work by Matsuzaki and Saigo (1996) have postulated that NB 6-4 and NB 7-3 show differences in their dependence on Wg and Hh signalling: NB 6-4, which originates from the anterior En-stripe, was missing in *wg* as well as in *hh* mutant embryos whereas NB 7-3, which delaminates around 30min later from the posterior En-stripe, appeared to be always present in the absence of Wg or Hh alone but was no longer found in a *wg;hh* double mutant. Based on these results it was proposed that Wg and Hh signalling pathways converge or compensate for each other to specify NB 7-3 fate, while both Wg and Hh are equally important for NB 6-4 formation (Matsuzaki and Saigo,1996). One important consequence of this would be that Hh could have an autocrine function in specifying NB 7-3. However, as both NBs delaminate from the En-positive neuroectodermal domain and En activity is repressing *ptc*; (Hidalgo and Ingham, 1990), the only known receptor binding to Hh directly (Marigo et al., 1996), such an autocrine function of Hh would at least need a different receptor. Therefore, I investigated in more detail the role Hh plays in the formation and/or specification of NB 6-4 and NB 7-3. I chose null mutant alleles of *wg* and *hh* for my investigations whereas in the previous work by Matsuzaki et. al. (1996) a hypomorphic allele of the *hh* gene was used. I found that the formation of NB 7-3 is affected in both *wg* and *hh* single mutants. NB 7-3 is missing in 75% (n=88) of wg^{CX4} and in 40% (n=202) of hh^{AC} mutant hemisegments counted (Fig. 15D,E). These results also show that the effect on NB 7-3 is more severe in wg^{CX4} than in hh^{AC} mutant embryos. Since En expression is fading away earlier in wg^{CX4} (~st 8) than in hh^{AC} mutant embryos (~st 10) (Bejsovec and Wieschaus, 1993) I assume that the number of remaining NB 7-3 correlates with the degree of the residual En expression. Indeed, embryos that are deficient for *en* and *inv* (*invected*, a homeobox gene that shows some functional redundancy to *en*) show that NB 7-3 is missing in 100% (n=50) of the hemisegments counted (Fig. 15C).

Additionally mutations for all the genes down stream the Wg/Hh signal transduction pathway were analysed for NBs 6-4 and 7-3 specification (Summarised

in Table F). NB 7-3 was partially missing in all mutation except in that of *pangolin* (*pan*), *patched* (*ptc*) and *fused* (*fu*). The reason for this could be due to the fact that En expression is not effected in these mutations. Taken together, these results suggest that NB 7-3 formation needs Hh indirectly for the maintenance of En expression via Wg. I confirmed this by analysing hh^{AC} mutant embryos in which Wg was ectopically expressed within the En-domain using *enGal4* as a driver of *UAS-wg*. In these embryos, the dependency of Wg expression on Hh is uncoupled and therefore En expression is rescued (Sanson et al., 1999). In accordance with my hypothesis, these embryos show a very efficient rescue of NB 7-3 to 95% (n=66) of the hemisegments counted (Fig. 15F). Thus, under these conditions NB 7-3 does not need any additional input by the Hh-signalling pathway to be formed and specified. I conclude that NB 7-3 normally requires Hh only for maintenance of Wg expression, which in turn leads to En maintenance.

TABLE F: The analysis of mutations of genes belonging to the Wg and Hh signalling pathway for the presence of NB 6-4 and NB 7-3 with anti-Eg antibody is summarised in the Table below + indicates the NB is formed, - indicates the NB is missing, -/+ indicates the NB is partially missing.

	NB 6-4	NB 7-3
Genes belonging to Wg signalling pathway		
Wg	-	-/+
Fz	-/+	-/+
Dsh FRT	-	-/+
Arm	-	-/+
Pan	-	+
Genes belonging to Hh signalling pathway		
Hh	-/+	-/+
Smo FRT	-/+	-/+
Ptc	+	+
Fu	+	+
Ci	+	-/+

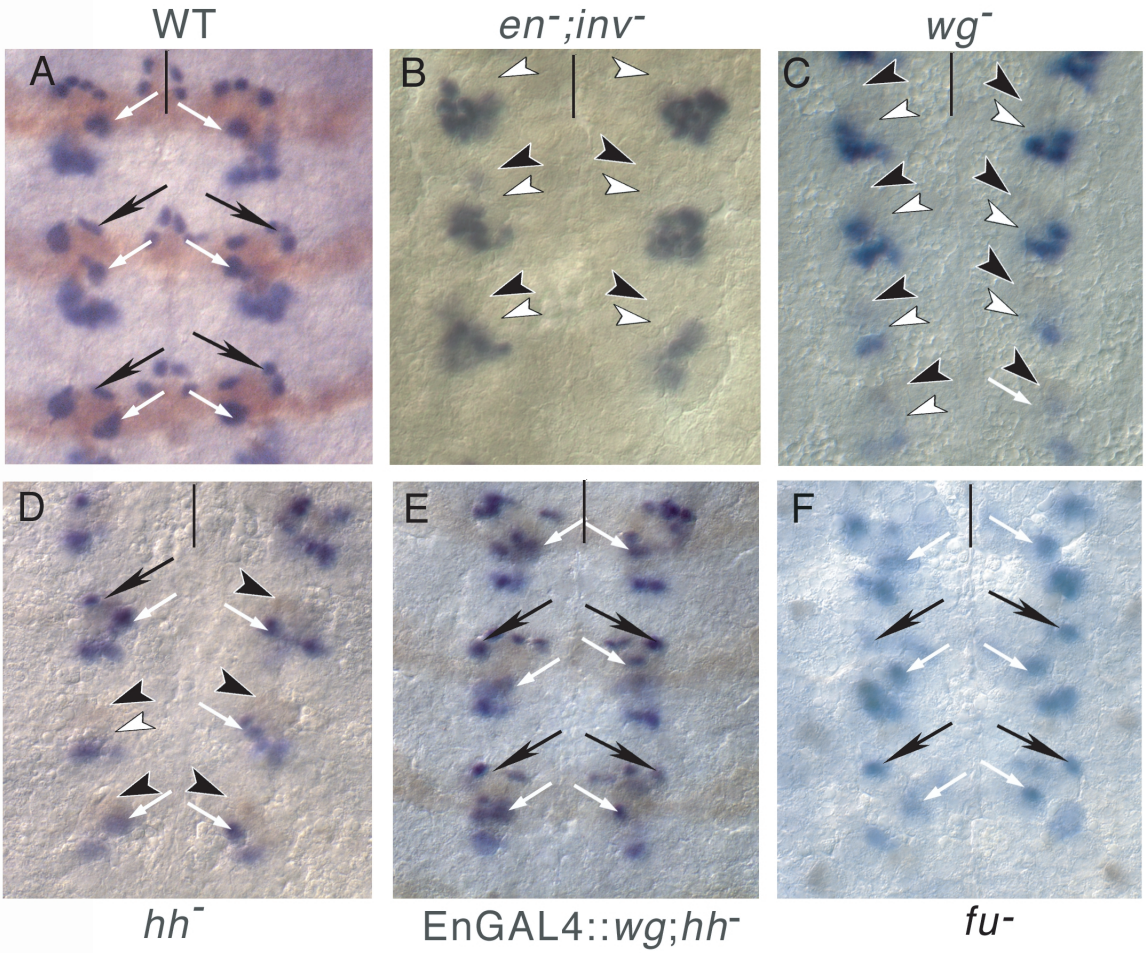


Figure 15. Loss of NB 7-3 in *hh* mutant embryos can be rescued by ectopic *wg* expression.

Flat preparation of embryos at st 12, anterior up, Eg expression is seen in blue in A-F and En expression in brown in A-E with the first three segments from the top being thoracic. The black bar represents the midline.

A) Wild type embryos show Eg expression in two cell clusters in the En-domain: anteriorly located is NB 6-4 and its progeny (black arrows), posteriorly located is NB 7-3 and its progeny (white arrows). B) en^{E} embryos which are double mutant for *en* and *inv* show no Eg expression at the position of NB 6-4 (black arrowheads) and NB 7-3 (white arrowheads) in 100% (n=52) of the hemisegments counted, En expression is completely abolished. C) wg^{CX4} mutant embryos look similar to en^{E} mutant embryos, Eg expression is absent at the position of NB 6-4 in 100% (n=54) (black arrowheads) and for NB 7-3 in 75% (n=88) (white arrowheads) of hemisegments counted. D) hh^{AC} mutant embryos show Eg expression to be missing in 40% (n=60) at NB 6-4 position (black arrowheads) and in 40% (n=202) for NB 7-3 position (white arrowheads) of the hemisegments counted. Occasionally residual En expression can be seen occasionally around the Eg positive cells clusters. E) Expression of *UAS-wg* by *en-Gal4* in hh^{AC} mutant embryos rescues the formation of NB 6-4 to 98% (n=109) (black arrows) and NB 7-3 to 95% (n=66) (white arrows) of the hemisegments counted. En expression is rescued. F) fu^{A} mutant embryos show Eg expression to be always present at the position of NB 6-4 (white arrows) and NB 7-3 (black arrows). Pox-n expression seen in brown.

3.3.2 Naked Cuticle activity is essential for NB 7-3 identity

Having shown that Hh is not involved in the differential regulation of Gsb I decided to look at inhibitors of the Wg signalling cascade, namely *naked cuticle* (*nkd*) and *shaggy* (*sgg*). Analysis with anti-Ey antibody showed that NB 7-3 specific Ey expression is missing in 83% of *sgg* mutant (n=92, data not shown) and in 81% of *nkd* mutant (n=80) hemisegments of embryos at st12-13 (Fig. 16B) although Eg-positive cells were always observed at the position of the NB 7-3 cluster using anti-Eg antibody (both NBs 6-4 and 7-3 are Eg positive). This suggests that the fate of NB 7-3 is mispecified (see below). In contrast to this NB 6-4 was found to be always present in these mutants, as judged by anti-Eg and anti-Repo antibody staining.

I selected *nkd* mutants for further analysis, as Nkd (like Gsb) is a target of the Wg signalling cascade and is thought to establish a negative feedback loop by down regulating the Wg signal (Zeng et al., 2000). As a first step I tested whether Gsb is derepressed in *nkd* mutations in regions from where NB 7-3 normally delaminates. Indeed, I found that while in the En-domain of WT embryos only row 6 NBs and NB 7-1 express Gsb (Skeath et al., 1995), in *nkd* mutants the Gsb expressing neuroectodermal region is broadened. As a result the more lateral row 7 NBs also express Gsb which must include NB 7-3 (Fig. 17). Since, in such a situation, row 7 is similar to row 6, it could have the ability to give rise to an additional ectopic NB 6-4. Staining of *nkd* mutant embryos with the glia specific anti-Repo antibody in combination with anti-Eg antibody indeed revealed an additional NB 6-4 like fate in 54% (n=40) of hemineuromeres counted (Fig. 16F). Coexpression of these markers is characteristic for NB 6-4 derived cells. To ensure that this is not due to secondary effects of the *nkd* mutation, I ectopically expressed Gsb in the En-domain using the UAS / GAL-4 system which yielded the same result as *nkd* mutations, namely a replacement of NB 7-3 by an ectopic NB 6-4 in 52% (n=40) of the hemisegments (Fig. 16E).

That Gsb expression acts a switch between row 6 and row 7 identity in the En-positive neuroectoderm is additionally supported by earlier work analysing the role of Gsb in the CNS (Patel et al., 1989; Matsuzaki and Saigo, 1996) where it was shown that in hemineuromeres of *gsb* mutant embryos, an additional NB 7-3 fate is formed. Taking this result further I confirmed that this additional NB 7-3 fate is at the cost of NB 6-4, which is converse to the situation in *nkd* mutants. Analysis of *gsb*

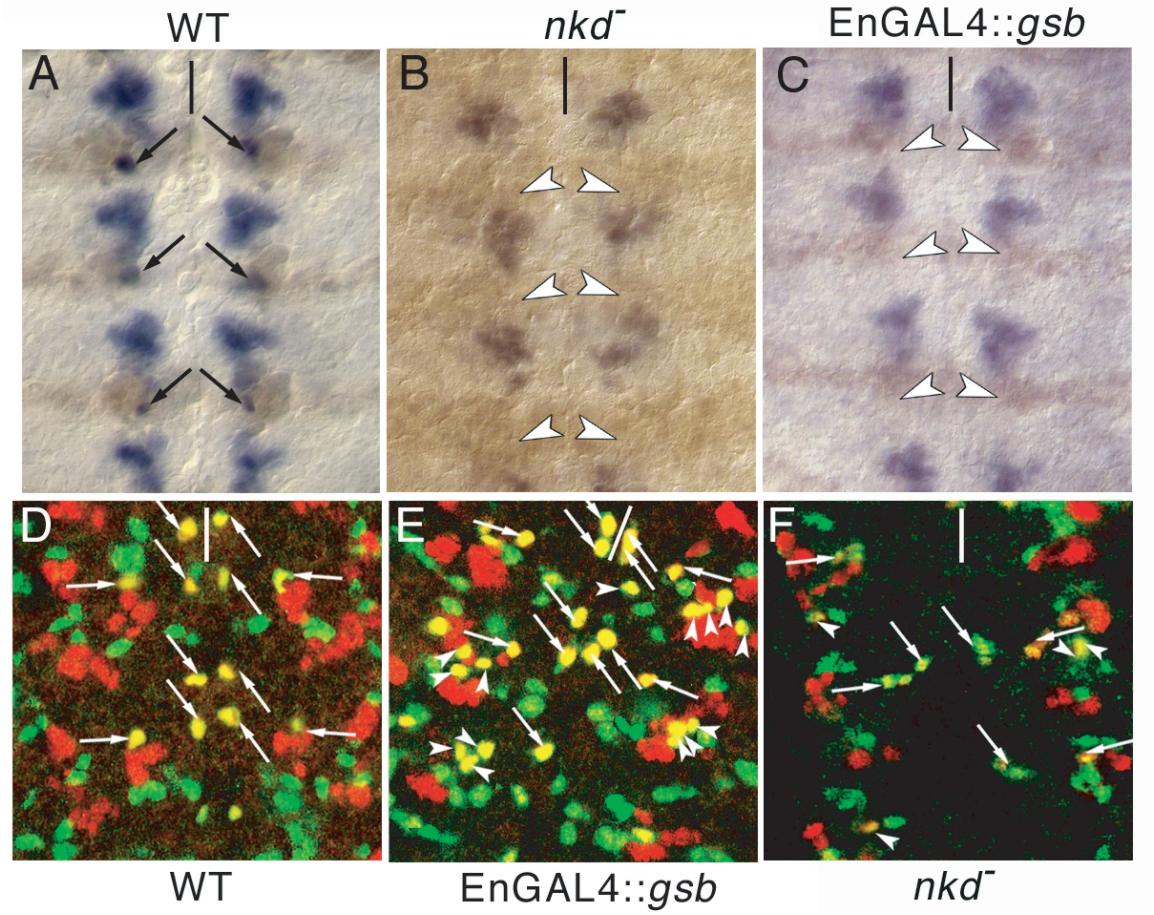


Figure 16. NB 7-3 is transformed to NB 6-4 fate in embryos mutant for *nkd* and in embryos with ectopic Gsb expression in the whole En-domain.

Flat preparation of embryos with anterior up. (A-C) show Ey expression in blue and En expression in brown at late st 12, the first three segment from the top are thoracic. The black bar represent the midline. (D-F) show combined sections of confocal images of fluoresence antibody stainings against Eg in red and Repo in green at st 13. Double labelled cells are seen in yellow with the first two segments from the top being thoracic. The white bar represent the midline.

A) In the En-domain of wildtype embryos Ey expression is seen only in the position of NB 7-3 (black arrows). Ey is expressed additionally in 5 NBs and their progeny outside the En-domain.

B) In *nkd* mutant embryos Ey expression is absent at the position of NB 7-3 in 81% (n=80) of the hemisegments counted (white arrowheads).

C) In embryos with ectopic Gsb expression in the En-stripe Ey expression is absent at the position of NB 7-3 in 84% (n=86) of the hemisegments counted (white arrowheads).

D) Wildtype embryos showing cells double labelled for Eg and Repo which are unique for the glial cells produced by NB 6-4 (white arrows). In each thoracic hemisegments two of these cells are seen along the midline (MM-CBG) and one more laterally (M-CBG).

E) In embryos with ectopic Gsb expression in the En-domain additional cells coexpressing Repo and Eg are seen in addition to the three genuine NB 6-4 progeny (white arrows) which are present in the ventral focal planes. At the position of NB 7-3 such cells are seen in the dorsal focal plane in 52% (n=40) (white arrowheads) of the hemisegments counted suggesting a tranformation of NB 7-3 to NB 6-4 fate.

F) In *nkd* mutant embryos phenotype similar to (E) is seen. At the position of NB 7-3 cells coexpressing for Repo and Eg are seen in 54% (n=40) at st 12 (white arrowheads) of the hemisegments counted suggesting a tranformation of NB 7-3 to NB 6-4 fate. For the purpose of clarity not all sections of the confocal images were combined here.

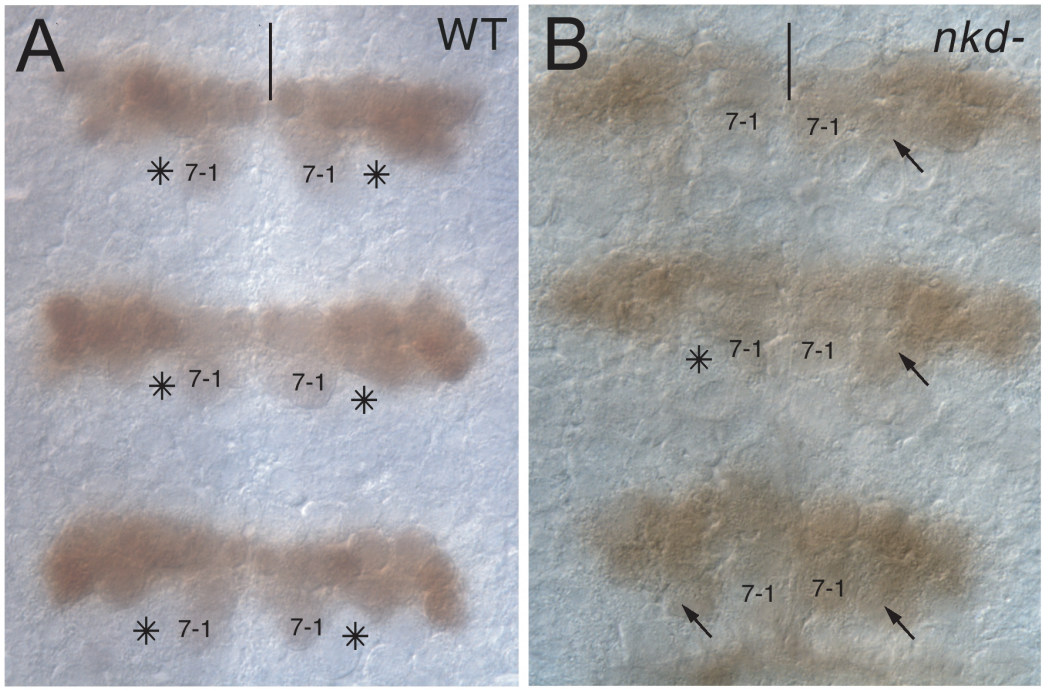


Figure 17. Gsb expression is expanded posteriorly in embryos mutant for *nkd.*

Flat preparation of embryos (early stage 12), Gsb expression as revealed by anti-Gsbd serum (brown), anterior is up. The first three segments from the top are thoracic. The black bar represents the midline.

A) Wildtype embryos show Gsb expression in all NBs belonging to rows 5 and 6. Only one NB namely, NB 7-1, belonging to row 7 is Gsb positive. The region of NB 7-3 is Gsb negative (black asteriks)

B) In *nkd* mutant embryos Gsb expression is derepressed and now expressed in additional NBs belonging to row 7 which must include NB 7-3 (black arrows).

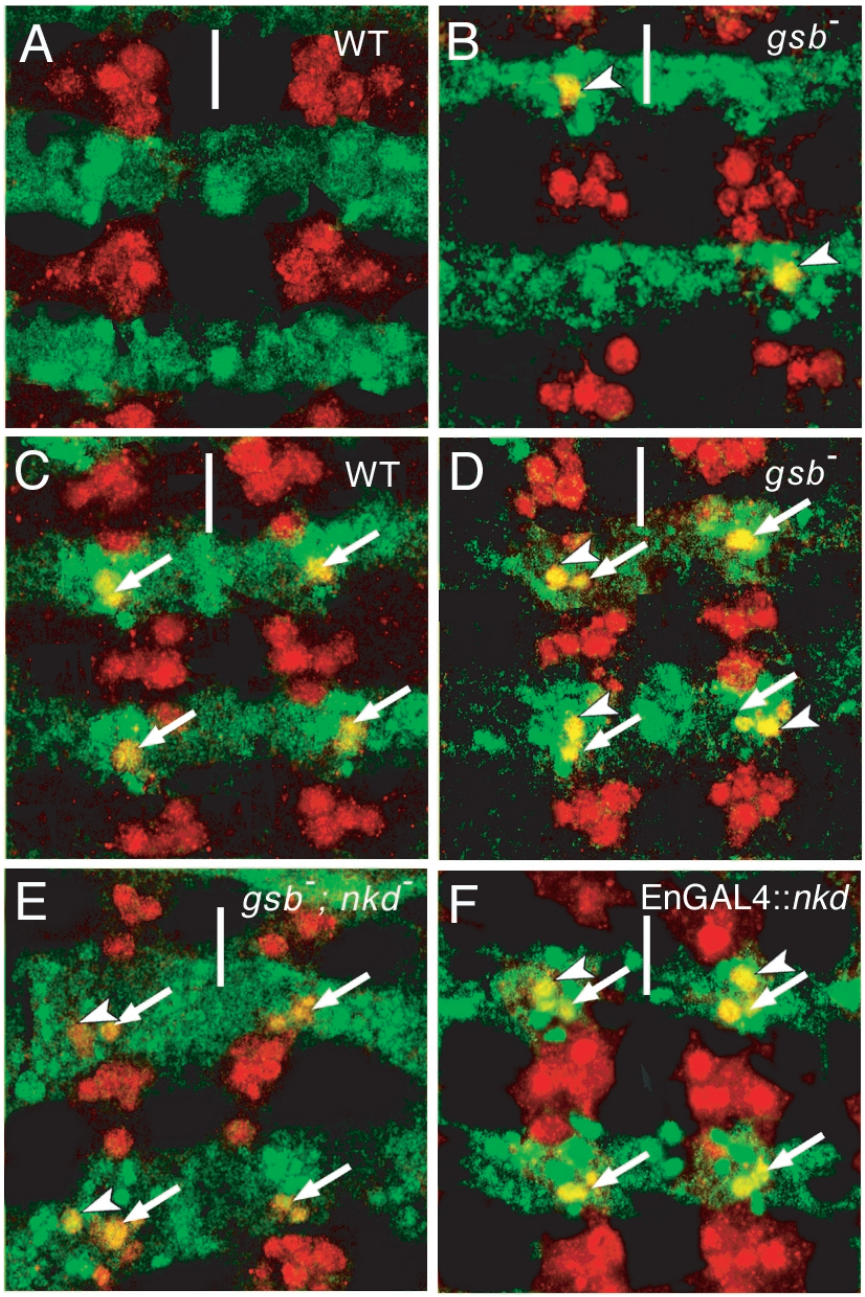


Figure 18. Loss of Gsb function as well as ectopic Nkd expression in the En-domain results in an additional NB 7-3 like fate.

Confocal images of embryos between st10-13 with anterior up. Ey expression seen in red, En in green and double staining in yellow. The first three segments from the top are thoracic. The white bar represents the midline.

A) Wild type embryo at st 10 show no Ey expression in the En-domain.

B) *gsb* mutant embryos at st 10 show Ey expression in 65% (n=60) of the hemisegments counted (white arrowheads).

C) Wildtype embryo at late st 11 show Ey expression in the En-domain at the position of NB 7-3 (white arrows) and its progeny in 100% (n=50) of the hemisegments counted.

D) *gsb* mutant embryos at late st 11 show Ey expression at the position of NB 7-3 (white arrows) and its progeny. An additional Ey positive cell cluster in a different focal plane is seen at the position of NB 6-4 (white arrowheads) in 70% (n=82) of hemisegments counted.

E) *gsb;nkd* double mutant embryos show a phenotype similar to gsb mutant embryos. Ey expression is found at the position of NB 6-4 (white arrowheads) in 76% (n=86) of the hemisegments counted.

F) *UAS-nkd* driven by *en-Gal4* results in Ey expression at the position of NB 6-4 (white arrowheads) in 40% (n=80) of the hemisegments counted.

mutant embryos with anti-Ey antibody showed that a duplicated NB 7-3 is formed in 70% (n=82) of the hemisegments counted (Fig. 18D) and NB 6-4 markers are missing in 100% of hemisegments counted (data not shown). Additionally, in the absence of both Nkd and Gsb, ectopic NB 7-3 is found in 76% of hemisegments (n=86; Fig. 18E). Similarly, 40% of the hemisegments (n=80) showed duplicated NB 7-3 fate when Nkd was ectopically expressed in the En-domain using the *UAS* / *Gal4* system (Fig. 18F). Thus, I conclude that NB 7-3 formation, as opposed to NB 6-4, requires the absence of Gsb, which is inhibited by Nkd function. Taken together the above results suggest that row 6 and 7 neuroectoderm can potentially produce NBs with the same identities and that the differential effects of Wg signalling are responsible for bringing about the different fates of the two late NBs from this region.

3.3.3 NB 6-4 and NB 7-3 specification is independent of time of NB formation.

The above results show that in *nkd* mutants an extra NB 6-4 is formed in the position of NB 7-3. Since NB 6-4 normally delaminates earlier than NB 7-3 the question arises as to when the duplicated NB 6-4 delaminates. In WT embryos, NB 6-4 delaminates during S3 (st 10) followed by NB 7-3 in S5 (st 11) from the En-domain (Doe, 1992; Broadus et al., 1995). Therefore, embryos either mutant for *nkd* or expressing ectopic *gsb* in the En-domain (EnGal4::gsb) of st 10 and st 11 were examined with anti-Eg antibody to look for the timing of NB duplication. In WT embryos at st 10, Eg is detected only at the position of NB 6-4 and never at the position of NB 7-3 (Fig. 19A). At st 11 Eg positive cells are visible in the En-domain at the position of NB 7-3 as well as NB 6-4. (Fig. 19C). Surprisingly in EnGAL4::gsb embryos this temporal sequence is maintained: Eg is first detected at the position of NB 6-4 (Fig. 19B) and later at the position of NB 7-3 (Fig. 19D). Therefore, the ectopic NB 6-4 is delaminating at S5, at the time NB 7-3 would normally appear. Conversely, in *gsb* mutants an extra NB 7-3 is formed at the cost of NB 6-4. This NB 7-3 is detected by anti-Ey antibody staining in embryos mutant for *gsb* at st 10 (Fig. 18B), whereas in WT embryos no Ey positive cell is present in the En-domain at this stage (Fig. 18A). Thus, this ectopic NB 7-3 delaminates at the time of NB 6-4. I conclude that with respect to NB 6-4 and NB 7-3 the timing of NB formation appears largely independent of NB specification and that the segmentation

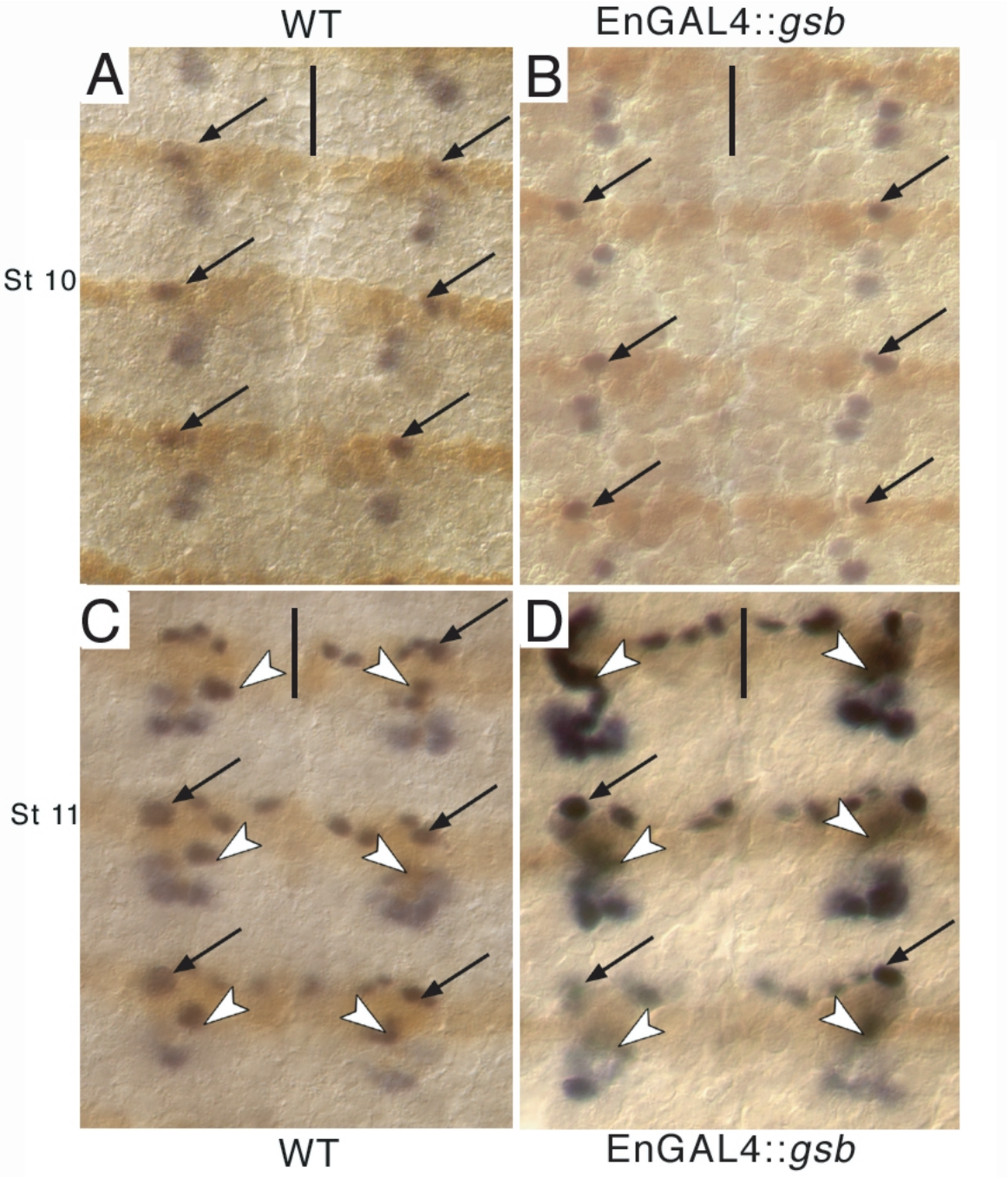


Figure 19. Duplicated NB 6-4 in embryos with ectopic Gsb expression is born at the time of NB 7-3.

Flat preparation of embryos at st 10 (A,B) and 11 (C,D) stained against Eg in blue and En in brown, anterior up. The first three segments from the top are thoracic. The black bar represents the midline.

A) Wildtype embryos at st 10 show Eg expression in the En-domain only at the position of NB 6-4 (black arrows) and not in the position of NB 7-3 as it is not yet delaminated.

B) Eg expression in embryos with ectopic Gsb expression in the En-domain at st 10 is indistinguishable from wildtype embryos.

C) Wildtype embryos at st 11 show Eg expression in the En-domain at the position of NB 6-4 (black arrows) and NB 7-3 (white arrowheads).

D) Eg expression of embryos with ectopic Gsb expression in En-domain at st 11 is again similar to that of wildtype embryos although the cells at NB 7-3 position (white arrowheads) now express characteristic markers of NB 6-4 progeny (see Fig. 16E)

genes *nkd* and *gsb* are essential to bring about the specification of the two NB fates investigated.

3.3.4 Late ectopic En expression induces an ectopic NB 7-3 fate in row 3 neuroectoderm

Since the above results suggests that the prerequisite for NB 7-3 fate specification is En in absence of Gsb expression, I examined embryos with ectopic En expression in order to see whether I can induce ectopic NB 7-3 cells outside of the normal En-domain. A heat pulse given to *Hs-en* embryos just before the delamination of NB 7-3 i.e. 5-6hr after egg laying at 25°C results in an ectopic NB 7-3 formation in 20% (n=100) of the hemisegments based on anti-Eg antibody stainings (Fig. 20C). This ectopic NB 7-3 is also able to give rise to characteristic progeny cells. I detected additional serotonergic neurons in 20% (n=68) of the hemineuromeres counted in 1st instar larvae (Fig. 20E). The ectopic NB 7-3 seems to be formed at the cost of the Eg-positive NB 3-3 of row 3 as I find a loss of the Eve-positive EL cells which are progeny of NB 3-3 (Schmidt et al., 1997). No effect is seen when the heat pulse is given between 4-5hr AEL at 25°C (Fig. 20B) although the CNS is very malformed.

Additionally, I analysed *ptc* mutant embryos, as a derepression of En in an ectopic stripe is seen in such mutants (DiNardo et al., 1988). Ptc, which is a receptor for Hh, is expressed in rows 2,3,4 and 5 of the neuroectoderm (Bhat, 1996). Ptc activity represses En expression (Hidalgo and Ingham, 1990) which results in a mutually exclusive gene expression pattern with respect to these two genes. I found that a NB 7-3 like fate is formed ectopically in *ptc* mutant hemineuromeres to 50% (n=60, see also Patel et al., 1989) which is similar to the ectopic expression experiments using *hs-en*. Again this seems to be at the cost of NB 3-3 as 60% of the EL-cells are missing in these mutant embryos as well (Fig. 20G). This is in accordance with the observation, that the ectopic En-stripe in *ptc* mutant embryos is in the region where the row 3 NBs delaminate (data not shown). Taken together these results suggest that in WT, Ptc represses En expression in row 3 neuroectoderm thereby enabling the specification of late delaminating row 3 NBs. In the absence of Ptc function at least some of these neuroectodermal cells acquire row 7 identity due to the presence of En and the absence of Gsb.

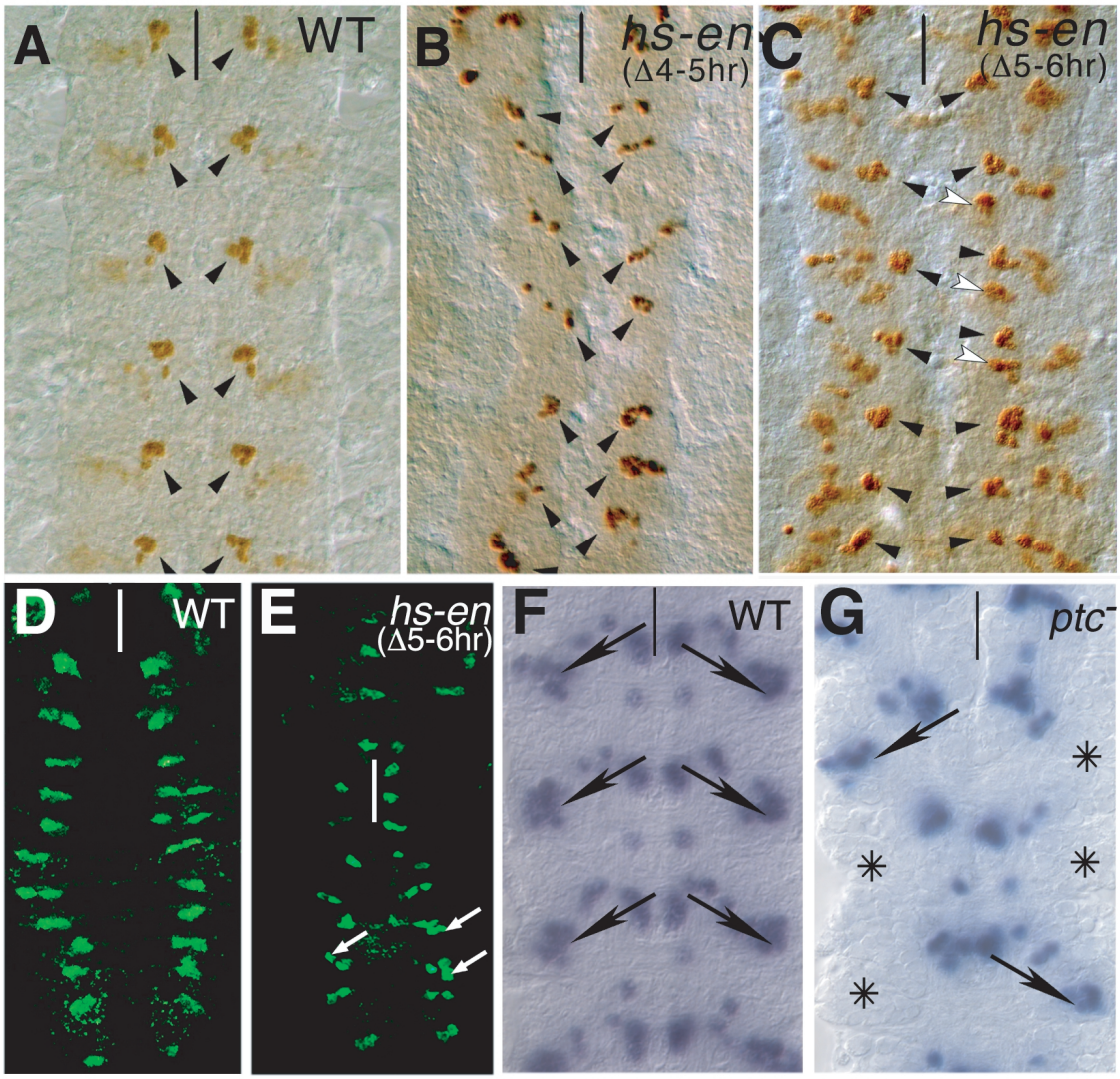


Figure 20. Ectopic En expression results in duplication of NB 7-3 fate.
Flat preparation of embryos at st 15 with the midline represented by a black bar (A,B,C,F and G) and an isolated CNS of a first instar larva (D-E) with the midline represented by a white bar, anterior is up. Figs A-C show Eg expression in brown and Figs D-E shows serotonin expression in green. Figs F-G show Eve expression in blue.
A) Wildtype embryos showing strong Eg expression in the position of NB 7-3 (black arrow heads).
B) *Hs-en* embryos subjected to a heat pulse between 4-5 hrs AEL: the CNS is malformed but NB 7-3 and its progeny (black arrowheads) can still be identified.
C) *Hs-en* embryos subjected to heat pulse between 5-6 hrs AEL: ectopic NB 7-3 like cluster (white arrowheads) can be found just below the wildtype NB 7-3 (black arrowheads) in 20% (n=100) of the hemisegments counted.
D) The CNS of 1st instar wildtype larva. Two serotonergic neurons are seen per hemisegment
E) The CNS of 1st instar *Hs-en* larva. Heat shock was applied like in (C). Ectopic serotonergic expression was found in several hemisegments (white arrows).
F) Wildtype embryos showing Eve expression in the position of EL cells (black arrows) which are the progeny of NB 3-3.
G) *ptc* mutant embryos shows Eve expression missing in the position of EL cells (black stars).

4. DISCUSSION

During development, multicellular organisms must co-ordinate the proliferation, specification, differentiation, and maintenance of many different cell types. To achieve this, each cell must continually integrate a complex array of external signals, including both inductive and inhibitory cues, and then translate these instructions into spatially and temporally appropriate developmental responses. Many of the signalling mechanisms regulating these decisions are used repeatedly, generating different cellular responses in different developmental contexts. These include the *receptor tyrosine kinase* (RTK), *Notch*, *hedgehog*, *wingless*, and *transforming growth factor*-β (TGF-β) signalling pathways. All of them play an essential role in establishing the architecture of the fly systems like the wing, eye and the CNS.

In this thesis the subject of study has been the development of the *Drosophila* central nervous system (CNS). The fly CNS serves as an ideal model system to elucidate the developmental mechanisms that link pattern formation to cell-type specification (Bhat, 1999; Skeath, 1999). The founder cells of the CNS, the NBs, are formed in a segmentally repeated pattern, each having a unique identity, which leads to the formation of a specific set of neurons and/or glial cells. Delamination of NBs occur in five waves, S1-S5 (Doe, 1992) and after S5, each hemisegment contains a subepidermal layer of 30 NBs. The early NBs, delaminating during S1 and S2, form an orthogonal array of four rows (2/3,4,5,6/7) and three columns (medial, intermediate, and lateral). However, about half of the NBs delaminate in the later segregation waves (S3-S5) and acquire a different identity despite the fact that many originate from similar positions as the early NBs. Additionally, the three column and four row-arrangement pattern is only transitory during early stages of neurogenesis which is obscured by late emerging NBs (Doe and Goodman, 1985; Goodman and Doe, 1993). As a consequence late delaminating NBs may face a quite different situation, considering that substantial morphogenetic movements take place. Therefore for the

purpose of this study firstly I was interested in unearthing novel genes which play a role in formation of late delaminating NBs. Secondly, I was investigating whether patterning genes that are known to play a role in early NB formation are also involved in late NB formation/specification. Towards this aim I asked how segmentation genes interact to confer unique identities on late delaminating NBs and whether NB formation and specification is tightly linked in this case.

4.1 Mutagenesis screen

As a first step to discover novel genes involved in segregation of late delaminating neuroblasts the method of a genetic screen was employed. About 900 fly lines, which have been generated by EMS mutagenesis, were screened (in collaboration with Dr. C. Klämbt). These lines had been preselected for CNS defects (Hummel et. al., 1997). Anti-Eagle antibody was the NB marker used for the screen as it specifically marks a subset of four late delaminating NBs namely, NBs 2-4, 3-3, 6-4 and 7-3 and thus has an easily identifiable pattern. The screen proved to be very successful as it resulted in a number of interesting phenotypes. These phenotypes were grouped into six categories namely that of too many cells, less cells, cell cycle defects, missing NBs, dorsoventral patterning defects, and other patterning defects many of them occurring simultaneously within one mutant strain. As it is difficult to exhaustively study a big number of fly lines showing potentially interesting phenotypes simultaneously, only four fly stocks were selected. The criterion for selection was to choose phenotypes that are reproducible and simple like the missing of a specific NB. One of the most interesting mutant lines identified was the gene *anterior open* or *yan* that had a phenotype of missing NB 7-3 in most of the hemisegments. This was ideally suited for the study of this thesis as NB 7-3 is one of the last NBs to segregate (S5). Therefore, in the following work, I concentrated on investigating the role of *yan* in CNS development.

Although the screen uncovered interesting genes regarding late NB development, many genes must have escaped detection. This was due to certain limitations. The usage of only Eg as a marker was one of the limitations. Not many markers are available which stain late NBs that have an easy identifiable pattern. Therefore, phenotypes manifested in other late neuroblasts could have been

completely missed out. Additionally, the fly stocks in this screen were pre-selected for axonal pathfinding defects in the CNS. Axons are formed relatively late during embryogenesis as compared to NB formation. Thus this selection process might have eliminated mutations affecting NB formation and especially NB specification. Therefore it would make sense to repeat the screen to saturate the genome for mutations in such genes.

Nevertheless, the mutations already obtained can still be further utilised to unravel additional genes, which play a role in late NB formation, as I concentrated only on one class of mutant phenotype namely missing of NBs. For example, mutants which yield too many cells that were initially classified as neurogenic mutations were rejected as they had a very low probability of being novel. However, the recent finding in our lab that genes which are involved in the specification of NB progeny are also involved in the regulation of cell division brings a new focus on these mutations (J Urban, pers. communication). Therefore it might turn out to be very fruitful to have a closer look at this class of mutations.

4.2 The role of *yan* in neurogenesis

As stated above the *yan* mutation was picked up in the screen conducted to identity putative genes involved in the formation of late delaminating neuroblasts. Several studies on Yan have reiterated the fact that it serves as a general inhibitor of differentiation. The Yan protein has to be inactivated by the process of phosphorylation for differentiation to proceed. Yan plays a critical role during early embryogenesis, as it functions as a repressor of ventral cell fate in the ectoderm. In loss-of-function *yan* mutant embryos, expression of the ventral cell markers *orthodenticle* (*otd*), *argos* (*aos*), and *tartan* (*trn*) are expanded, whereas expression of constitutively active Yan reduces expression of *otd* and *aos* (Gabay et al., 1996). Recent work has shown in addition that Yan is a repressor of cell shape changes during dorsal closure, and that this repression is relieved following phosphorylation by Jun kinase (Hou et al., 1997; Kockel et al., 1997; Riesgo-Escovar and Hafen, 1997; Noselli, 1998). Another function of Yan in the ectoderm is in the differentiation of En expressing cells, where Yan must be inactivated to specify the denticle-secreting En

cell type (O'Keefe et al., 1997). Yan has also been shown to be responsible for differentiation of the dorsal head ectoderm, a tissue giving rise to the visual system, medial brain, and head epidermis. Loss-of-function *Yan* mutations display massive head defects caused by over proliferation and subsequent cell death in these precursors (Rogge et al., 1995). Additionally, the role of Yan in regulating proliferation and differentiation in the developing eye is well established (Lai and Rubin, 1992; O'Neill et al., 1994; Rebay and Rubin, 1995; Rogge et al., 1995). It has been elegantly demonstrated that *yan* interacts with the *Notch* signalling mechanism in the eye imaginal disc. Notch in the developing eye is also involved in the process cell differentiation. Loss of Notch function causes excessive development of photoreceptor neurons (Cagan and Ready, 1989) while ectopic activation of Notch in the devloping eye causes cells to remain undifferentiated (Fortini et al., 1993). A decrease in Yan function in eye development results in an increased number of cells developing as R7 photoreceptor cells in the eye (Rogge et al., 1995). Further with it has been shown that loss of Notch partially suppresses Yan and extra Notch enhances the *yan* mutant phenotype in the eye. In this thesis I show that Yan and Notch also interact during the process of lateral inhibition within the embryonic neuroectoderm.

4.2.1 CNS phenotype of *yan* mutation is due to the interference in the Notch signalling pathway

The expression pattern of Yan is well studied (Price and Lai, 1999) and was shown to expressed in a variety of tissues. Nevertheless, Yan expression with respect to the neuroectoderm was not analysed in detail. Antibody staining against the Yan protein revealed that it is expressed in the neuroectoderm in a whorl like pattern around delaminating neuroblasts like NB 5-1 and NB 7-3 but never in the neuroblast itself. The observation by Rebay et al, 1995 that Yan is an inhibitor of neural differentiation is in accordance with the finding that the Yan protein is never expressed in NBs. This suggested that Yan could play a general role in neurogenesis by conferring its effects on the neuroectodermal cells. However, analysis of *yan* mutant embryos with various available NB markers established the fact that no other identifiable NB except NB 7-3 is missing. This was astonishing as Yan protein is expressed around a number of NBs other than NB 7-3 and is evenly distributed in the

neuroectoderm. However, the performed genetic experiments clearly showed that Yan has a function also with respect to other NBs beside NB 7-3 (see below).

What is the function of Yan during embryonic neurogenesis? The possibility that it contributes to CNS development via the known "Yan using" EGFR and JNK pathways were ruled out. As mentioned above studies conducted in *Drosophila* eye development showed that Notch and Yan interact genetically. This prompted me to test whether the function of these two genes is also involved with regard to embryonic CNS development. Loss of function Notch mutations show an excessive number of cells taking a NB fate, which is referred to as the neurogenic phenotype. On the other hand loss of function mutations in Yan show one NB missing namely NB 7-3. However, in double mutations for Yan and Notch the neurogenic phenotype is severely reduced. This strongly suggests that Yan function blocks the Notch signalling pathway during neurogenesis. Additional evidence to support this hypothesis was obtained by overexpression studies of constitutive active Yan (Yan^{act}) using the *UAS/Gal4* system. The prediction was that this should lead to some neurogenic effect. Indeed, in embryos with *UAS-*Yan^{act} driven by *scaGal4* duplication of many neuroblasts were observed. Most of these NBs belonged to S3-S5 wave of neurogenesis. A duplication of S1 NBs like NB 5-6 (tested with Anti-Ladybird) was never seen (data not shown). This could be due to the fact that *sca* expression comes up between st 8 and 9 during embryogenesis, as a result only NBs delaminating at this stage are affected. Thus a high level of repressing activity of Yan seems to negatively regulate the Notch pathway thereby creating an environment favourable for additional neuroblast formation. Interestingly this "neurogenic phenotype" was only observed after overexpression of a constitutive active Yan version, which cannot be phosphorylated.

These results let me to propose the following hypothesis: Yan functions to keep the cells of the neuroectoderm in an undifferentiated state by inhibiting the effects of the Notch pathway (Fig.21A). The CNS is derived from a bipotential neuroectodermal cell layer. These cells have the ability to take either a neural or an epidermal fate. The correct separation of neural and epidermal progenitor cells is controlled by two groups of genes, the proneural genes which instruct a equivalent groups of cells in the early neuroectoderm known as 'proneural clusters' to become competent for the neural fate (Hinz et al., 1994; Kunisch et al., 1994). Only one cell in

the proneural cluster segregates as a NB which is accomplished by the process of lateral inhibition, operating via Notch and Delta which are the 'neurogenic genes' (Campos-Ortega, 1993; Chitnis, 1999). Nevertheless, the remaining cells within a proneural cluster retain their bipotentiality for a short period of time following the delamination of a cell as NB. This hypothesis is supported by studies done in Grasshopper where ablation of a newly formed neuroblast leads to the delamination of another neuroectodermal cell from that proneural cluster as a neuroblast (Doe and Goodman, 1985; Cabrera, 1992). But the question still remains: what keeps the cells of the proneural cluster to retain their bipotentiality even when the surrounding environment contains a multitude of signals promoting cell differentiation. In other words, what counter acts the Notch signal of cell differentiation? At this juncture Yan provides a wonderful insight. In the absence of Yan, the bipotential cells of the neuroectoderm adopt an epidermal fate as Notch is hyperactive (See Fig.21C). This is based on the fact that the proneural cluster of NB 7-3 is not formed and therefore NB 7-3 is missing. Further, ectopic expression of activated Yan results in formation of extra NBs as it is sometimes able to inhibit Notch function efficiently. In the absence of both Yan and Notch, the neurogenic phenotype is drastically suppressed with regard to many NB fates. Thus, in such a situation the bipotential neuroectodermal cells by default adopt an epidermal fate leaving no cells in the pool for a neurogenic phenotype of Notch to manifest (See Fig.21D). The main drawback against this hypothesis is that if Yan is a general inhibitor then why is the phenotype of missing NB manifested only in one NB in Yan mutations? One reason could be that the early phenotypes in *yan* mutants are masked could be due to maternal contribution of the mRNA or protein. The fact that Yan is expressed in the syncitial blastoderm (Price and Lai, 1999) strongly support that Yan could have a maternal contribution although work done by Price et. al state that no detectable maternal effects were seen in *yan* mutants. Attempts to create germline mutation to get rid of the maternal contribution of the *yan* gene failed probably because Yan has a function in oogenesis. Indeed Yan is heavily expressed in the ovaries and therefore might have a crucial function there (Price and Lai, 1999). Alternatively, a partially redundant acting co-factor might rescue the phenotype since many repressor proteins have been shown to need a co-repressor for their activity. In the eye development Yan itself has been shown to need

the activity of another repressor protein, Tramtrack. Therefore, the phenotype of Yan mutants alone many not be sufficient to see many more NBs missing.

Taking together the above results clearly show that Yan is inhibiting the Notch pathway in the embryonic neuroectoderm. But how does it exert this effect? In the neuroectoderm effects of Notch signalling is transduced through its effector *Supressor of Hairless* [Su(H)] which encodes a DNA-binding transcription factor. Su(H) directly activates transcription of the genes in the Enhancer of split [E(spl)] complex, which are responsible to suppress cells from entering into a neural fate. The core consensus sequence GTG[G/A]GAA was shown to be the binding sites of Su(H) on the E(spl) enhancer (Lecourtois and Schweisguth,1995). Comparison of this sequence to that of the Yan binding ETS domain consensus binding sites which is 5'-GGA[A/T]-3' (Nye et al.,1992) suggest that both could compete to bind the *E(spl)* promoter- Su(H) to promote and Yan to repress *E(spl)* gene transcription. Indeed, in embryos which ectopically express gain of function Yan expression of E(spl) is almost abolished. To confirm this, further biochemical analysis has to be done to show that Yan can indeed bind *E(spl)* enhancer. Finally, the question remains what blocks the activity of Yan because at a certain point during development the non segregating cells differentiate into epidermal cells. Since the Yan activity is blocked by the process of phosphorylation and the ectopic expression of wildtype Yan in contrast to activated Yan does not give rise aditional NBs, it is most likely that Notch may be utilising a phosphorylating agent such as a kinase that inhibits Yan function. So far no such kinase has been described in the Notch pathway. As a next step, an enhancer or a suppression screen in conjecture with the gain of function phenotype of Yan could help reveal such potential interactors of the Notch/Yan pathway.

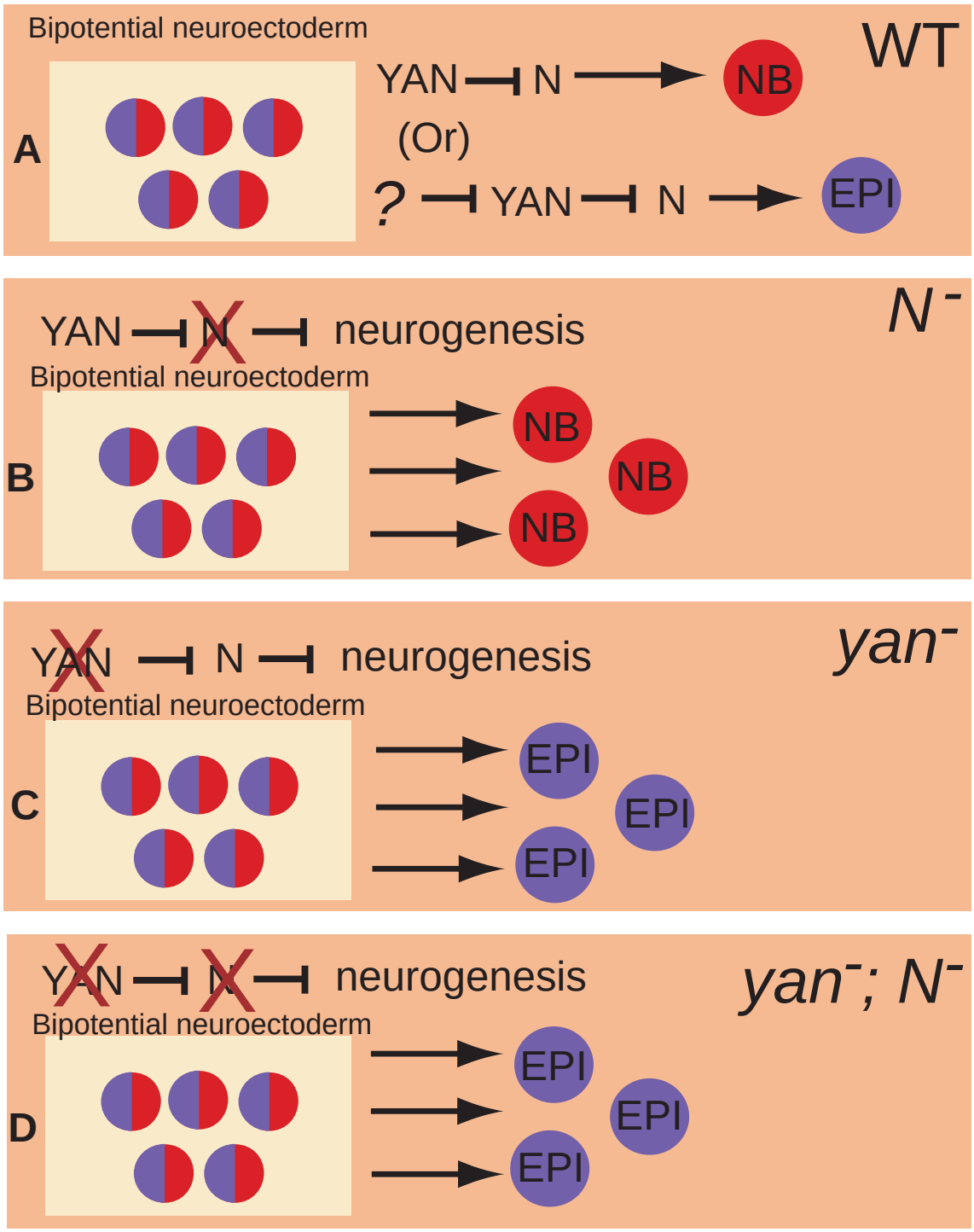


Figure 21. A model for the role of Yan in the neuroectoderm.
A) A wildtype situation where in Yan activity represses Notch activity in the neuroectodermal cells, thereby giving rise to a neuroblast fate (NB). When Yan activity is suppressed by unknown mechanisms, Notch is active, leading to an epidermal fate (EPI).
B) In *Notch* mutants too many cells of the neuroectoderm adopt a NB fate.
C) In *yan* mutants, Notch activity is not restricted, so extra EPI fates could be formed at the cost of NB fates as seen with respect to NB 7-3.
D) In *yan* and *Notch* double mutants, by default an EPI fate is formed. Yan is upstream Notch, therefore all the cells in the Neuroectoderm are used up in forming an EPI fate thus a Notch phenotype cannot be seen.
The situation in C and D is simplified for the purpose of the scheme as NBs do form in both *yan* and *yan*;*N* mutant CNS.

4.2.2 The relevance of Notch-Yan interaction with respect to vertebrate development

The most striking aspect of Yan protein is that its function could be evolutionary conserved although *yan* itself seems to have no very close relative among the vertebrate ETS-domain genes (Price and Lai, 1999). The only gene segregating with *yan* was the human *tel* gene. This in itself is very interesting given the role of Tel in hematopoesis and leukemia. The *tel* locus was first identified as the site of chromosomal rearrangements associated with leukemias of myloid and lymphoid origins (Golub et al., 1994; Stegmaier et al., 1996; Poirel et al., 1998). The normal function of *tel* appears to be in the establishment and maintenance of hematopoetic precursors within the bone marrow (Wang et al., 1998) which suggests that despite primary structural differences, *yan* and *tel* might be functionally related.

On the other hand, the Notch genes of *C. elegans*, *Drosophila melanogaster* and vertebrates have consistently been shown to be responsible for cell fate decisions during development. Most importantly, these Notch receptors and their ligands, Delta and Jagged, have been implicated in several human diseases. Truncated constitutively active mutant forms of the Notch receptor appear to be involved in human T-cell leukemia, mammary carcinomas in mice, and a tumorous germline in *C. elegans*. Since activated Notch induces solitary tumours in transgenic mice, it is very likely that collaborating genetic events are required for tumour formation. Previous studies have suggested that active signals from the Erk/MAP kinase and PI-3 kinase pathways downstream of Ras in concert with activated Notch play a role in malignant transformation (Fitzgerald et al., 2000 and references there in). Therefore, since the degree of *yan* function seems to be conserved across species, insights provided with regard to Notch-Yan interaction in *Drosophila* could be a powerful tool to investigate the molecular events leading to malignancy in human diseases.

4.2.3 Identification of *medea* as a gene involved in neural development

Since the product of the *tramtrack* (*ttk*) gene had been shown to synergistically interact with Yan to inhibit R7 cell fate in the eye (Lai and Rubin, 1992 and 1997), I wondered if this could be also the case during embryonic neurogenesis. There are two different Ttk proteins in *Drosophila* and both are transcriptional repressors. The *ttk* mRNA is alternatively spliced, giving rise to two proteins, one is a 69 kDa (Ttk69) and the other a 88-kDa protein (Ttk88; Read and Manley, 1992). Ttk69 appears to play a

dual function by serving as a negative as well as a positive regulatory role at different stages of photoreceptor development (Lai and Li, 1999). In addition it represses neuronal fate during glial development in the CNS (Brown and Wu, 1993; Xiong and Montell, 1993). Analysis of embryos mutant for *ttk*69 were wildtype with regard to NB 7-3 formation but *ttk*88 mutation (ttk^1) showed ectopic NB 7-3 derived cells which was further confirmed with *in situ* hybridisations against the mRNA of the *Drosophila* Serotonin Transporter (dSerT). dSerT is a marker for the serotonergic cells derived from NB 7-3. This phenotype was puzzling as Ttk88 is not expressed in the CNS and was previously reported to be a viable allele, which has no detectable phenotype in the CNS (Hummel et al., 1997). Therefore it was very likely that the observed phenotype was due to a second site mutation.

Subsequent complementation analysis indeed showed that an additional mutation in the vicinity of the *ttk* locus was responsible for the phenotype. This is most likely due to a defect in the *medea* (*med)* gene since the independent analysis of med^1 mutant embryos showed additionally serotonergic cells in the position of NB 7-3, although I did not find any extra serotonergic cells outside the NB 7-3 cluster. However, this could be due to the fact that med^1 is a very weak allele (a single amino acid replacement). Nevertheless, this result opens new vistas in exploring how unique lineages are built based on the signalling inputs the parent NB receives. Additionally it gives an opportunity to investigate how serotonergic cell fates are specified in the CNS. *med* has been shown to be downstream of the *decapentaplegic* (*dpp*) signalling pathway (Dale, 2000). *dpp* is a member of the TGF-β superfamily of signalling molecules, which are known to have important roles in patterning and differentiation during vertebrate and invertebrate embryogenesis. With respect to the CNS, *dpp* has been shown to have a strong anti-neurogenic function that makes it very unlikely to be the ligand involved in the TGF-β pathway which was uncovered by the *med* mutation. However, recently two new members of the TGF-β superfamily, *myoglianin* and *activin* were characterised in *Drosophila* (Lo and Frasch, 1999). Both of these genes could be potentially involved in CNS cell fate specification since both are expressed in neural cells during development. Clearly to investigate the role of *med* in CNS development is a very interesting project for the future.

4.3 The Role of Segment Polarity genes is specification of NBs 6-4 and 7-3

Worked carried out so far suggests that specification of NBs is based on a combination of positional information along the anterio-posterior (A/P) and the dorso-ventral (D/V) axis (reviewed in Bhat, 1999). To understand how late delaminating NBs are specified I have concentrated on studying the interactions of segment polarity genes which confer A/P positional cues on the neuroectoderm. The segment polarity gene *engrailed* (*en*) which is expressed in a specific region in the neuroectoderm was the focus of investigation. This domain gives rise to row 6 and row 7 NBs and is under the influence of segmentation genes *wingless* (*wg*) and *hedgehog* (*hh*). I selected a pair of late segregating NBs in the Engrailed-domain, namely the S3 neuroblast NB 6-4 in row 6 and the S5 neuroblast NB 7-3 in row 7 for analysis.

Wg and Hh are both signalling molecules, which influence the expression of En in rows 6 and 7. Therefore the signalling pathways of these genes were intensively analysed. Wg, which is a secreted protein is expressed in row 5 and influences the specification of the fate of NBs in the adjacent rows 4 and 6 of the neuroectoderm (Chu-LaGraff and Doe, 1993). Secreted Wg interacts with its receptor, the transmembrane protein dFrizzled2. This activates a signalling cascade in the receiving cell that involves the cytoplasmic protein Disheveled (Dsh), which leads to stabilisation of Armadillo (Arm), the *Drosophila* homologue of β-catenin. Arm is then translocated into the nucleus in association with the TCF-1/LEF-1 homologue Pangolin (Pan). The Arm-Pan complex activates downstream target genes such as En, which encode a homeodomain containing transcription factor. Thus, the maintenance of En expression in rows 6 and 7 is dependent on the Wg signal.

The En gene product in turn triggers Hh expression in its domain, which is responsible to maintain Wg expression. Hh is also a short-range signalling molecule. Hh interacts with its receptor, Patched (Ptc) which and releases a transmembrane protein Smoothened (Smo) from a Ptc-Smo complex. Smo signals through the activity of Cubitus interuptus (Ci) in the nucleus, which then activates expression of target genes such as Wg or Ptc. Within the ventral ectoderm, Hh signals both to the anterior

and posterior directions from the En-domain: anterior flanking cells express the Wg and Ptc both of which are Hh targets genes, whereas the posterior flanking cells express only Ptc. This directional partiality of Hh activity is brought about by Fused kinase (Fu). Fu is required autonomously in anterior cells neighbouring Hh in order to maintain Ptc and Wg expression. Fu activity has been shown to be not necessary for Hh responsive cells posterior to the En expression domain. Thus, I was interested in clarifying how late neuroblasts in row 7 become specified differently than those in row 6, since both rows stem from the same environment, i.e. the En/Hh domain where in both Wg and Hh signalling mechanisms impart their influence.

4.3.1 Segment polarity genes separate NB 6-4 and NB 7-3 fates

In the En-domain Wg plays a role both in NB formation and NB specification (Chu-LaGraff and Doe, 1993). The homeodomain transcription factor En is a prerequisite for the formation of the NBs 6-4 and 7-3 because in its absence both NBs fail to form (Lundell et al., 1996; Matsuzaki and Saigo, 1996). Since Wg signalling is necessary for maintaining En expression (Hidalgo and Ingham, 1990), it is also essential for the formation of these two NBs. In addition Hh is coexpressed in the En-domain but I found no evidence for a direct function of Hh with respect to the formation and specification of these NBs as opposed to a previous report (Matsuzaki and Saigo, 1996). En maintains Hh expression in row 6 and 7, and Hh in turn is essential for Wg expression in row 5 thereby constituting a maintenance loop (Heemskerk et al., 1991; Hidalgo, 1991; Bejsovec and Wieschaus, 1993). Analysis of all the downstream components of the Wg and Hh signalling pathway more or less supported the above results. Interestingly, in *fu* mutations, NB 7-3 formation is not affected even as Wg activity is abolished leading to a new front of investigations. Nevertheless, it can be said for late NBs in row 6 and 7 the expression of En is critical and Hh is required to maintain En expression via Wg. However, for the separate specification of NB 6-4 and NB 7-3 differential regulation of two Wg targets, *nkd* and *gsb*, is essential (Fig. 22).

Wg is a diffusable molecule expressed in row 5 and acts on neighbouring rows, which include rows 6 and 7 (Chu-LaGraff and Doe,1993). However, row 6 differs from row 7 as it expresses *gsb*, which is a target of Wg signalling (Fig. 22A). The fact that row 7 is not expressing *gsb* despite being under the influence of Wg raises the

question of how this differential regulation is brought about. In this work I could show that Nkd is essential for this regulation. Recently, Nkd has been identified as a negative regulator of the Wg signal transduction pathway, itself being a target of this pathway (Zeng et al.,2000). Here we have found that in the absence of Nkd, Gsb is derepressed due to Wg hyperactivity in row 7 leading to the generation of an ectopic NB 6-4 like fate (Fig. 22C). Thus, the distinct identities of NB 6-4 and NB 7-3 are brought about by the interplay of Gsb and Nkd. For NB 6-4 specification, Gsb is an essential factor in the absence of which it fails to be specified (Skeath et al.,1995; Matsuzaki and Saigo,1996) and instead takes the identity of NB 7-3 fate (Fig. 22B). Conversely, for NB 7-3 specification a Gsb free environment is essential which is created by the activity of Nkd. In summary, NB 6-4 needs the expression of Gsb and En, whereas NB 7-3 needs En but the absence of Gsb.

However, the fact that *gsb* as well as *nkd* are targets of Wg signalling makes it difficult to explain why *gsb* is repressed by *nkd* only in the posterior region of the En-stripe. The posterior En-domain is further away from the Wg source than the anterior En-domain and therefore should receive a lower signalling input as compared to the anterior region. As a consequence this should lead to higher Nkd activity in the anterior En cells leading to a stronger Gsb repression in this region which is the opposite of what I observe. A careful analysis of the expression pattern on the transcriptional level did not give any obvious clues to solve this apparent paradox (data not shown). I confirmed that during early germ band extension (St. 8-9) *nkd* transcription is nearly ubiquitous with higher RNA levels in the 2-4 cell rows posterior to the En-stripe (Zeng et al.,2000). At late phase of germ band extension *nkd* expression is most abundant anterior to, and lower just posterior to the En-stripe (St. 10-11; (Zeng et al.,2000). No significant difference between the anterior and posterior En-domain could be detected (own unpublished observation). One explanation for the differential regulation of *gsb* could be that due to earlier pair rule gene activity of *paired* (Bouchard et al.,2000), the level of Gsb protein at the time of NB 6-4 delamination in the anterior En region is high enough to override repression by Nkd activity. Alternatively, a direct differential regulation of the two Wg targets due to the different levels of Wg signalling could be responsible for the observed regulatory differences. It could be that the regulation is such that the amount of Wg signalling within the En-stripe causes a relatively homogenous level of *nkd* expression in this

region. At the same time the transcriptional activation of *gsb* could be more sensitive to Wg signalling levels resulting in a very strong activation especially near to the Wg expressing cells. As a result the relatively low Nkd activity in the whole En-stripe might be able to inhibit *gsb* expression in the region of low *gsb* activation only, namely the posterior En-domain. A hint that a differential regulation of Wg targets indeed exists comes from the Wg-dependent En-regulation: it seems that a lower Nkd activity is sufficient to repress *gsb* but not to inhibit *en* expression. This conclusion was drawn from our finding that overexpression of *nkd* within the En-stripe using an *enGal4* driver line leads to a selective repression of *gsb* with no obvious effect on *en* expression itself. Clearly, additional work has to be done to clarify these points.

4.3.2 Row 3 has the potential to generate a late row 7 neuroblast

Besides row 6 neuroectoderm, row 3 neuroectoderm also has the potential to generate an ectopic NB 7-3. It has been shown previously that in embryos mutant for *ptc*, neuroectodermal cells in the area of row 3 begin to express En and additional serotonergic neurons can be found in these mutant embryos which suggests the presence of an ectopic NB 7-3 like fate (Patel et al., 1989). I now show additionally that when En is ubiquitously expressed, only row 3 has the ability to give rise to an ectopic NB 7-3 fate. In all cases this occurs at the cost of row 3 NBs such as NB 3-3. I think that this might reflect that row 3 neuroectoderm, which is right in the middle of the segment, represents something like a "ground state" in the neuroectoderm. In this area neither Hh nor Wg signalling may take place. Therefore the decision to specify late row 3 or late row 7 NBs seems to be only dependent on the absence or presence of En, respectively.

4.3.3 Temporal aspects of NB specification

Previous work has indicated that genes expressed in proneural clusters are involved in specifying the individual fates of NBs that develop from these clusters (Chu-LaGraff and Doe, 1993; Skeath et al., 1995; Matsuzaki and Saigo, 1996). Our finding that NB 6-4 and NB 7-3 can be mutually transformed while the sequence of birth does not change suggests that the mechanism for the timing of late NB delamination is independent from mechanisms which regulate NB identity. This might

be reminiscent of early NBs. Initiation of S1 NB formation requires the activity of proneural genes which have been shown to be dependent on pair rule genes (Skeath et al., 1992). The identity of the NBs delaminating from these clusters however is dictated by the activity of segment polarity genes (Chu-LaGraff and Doe, 1993; Skeath et al., 1995). Thus, the control of proneural gene expression enabling NB formation and the control of segmentation genes conferring NB identity, occurs in parallel. At later stages pair rule gene expression vanishes and can no longer be responsible for NB formation (Skeath et al., 1992). How is NB formation regulated in the following segregation waves? One possibility would be that after the first segregation wave NB formation and identity are more tightly linked and the finding that certain NBs like NB 4-2 are sometimes not transformed but missing in *wg* mutant embryos (Chu-LaGraff and Doe, 1993) seem to support this idea. However, our finding that the transformed NB 6-4 and NB 7-3 are delaminating according to the "old identity" shows, that at least in these cases NB formation and specification is independent. Our results favour the idea that the timing of the formation of proneural clusters within the neuroectoderm is generally independent of the segment polarity genes investigated here. This does not exclude permissive functions like that of En, which enables the proneural cluster formation as such. According to this hypothesis, intrinsic or extrinsic factors present in the position of the proneural cluster at the time of delamination govern the identities of the NBs. This might be not only true for the positional regulation of NB identity but also for the determination of NB identity along the temporal axis. Indeed, heterochronic transplantation experiments recently performed in our laboratory (Berger et. al, 2001) strongly support that one or more extrinsic factors exist which lead to stage specific NB identities. It will be a challenge for the future to identify these factors and to investigate whether similar mechanisms exist in higher organisms.

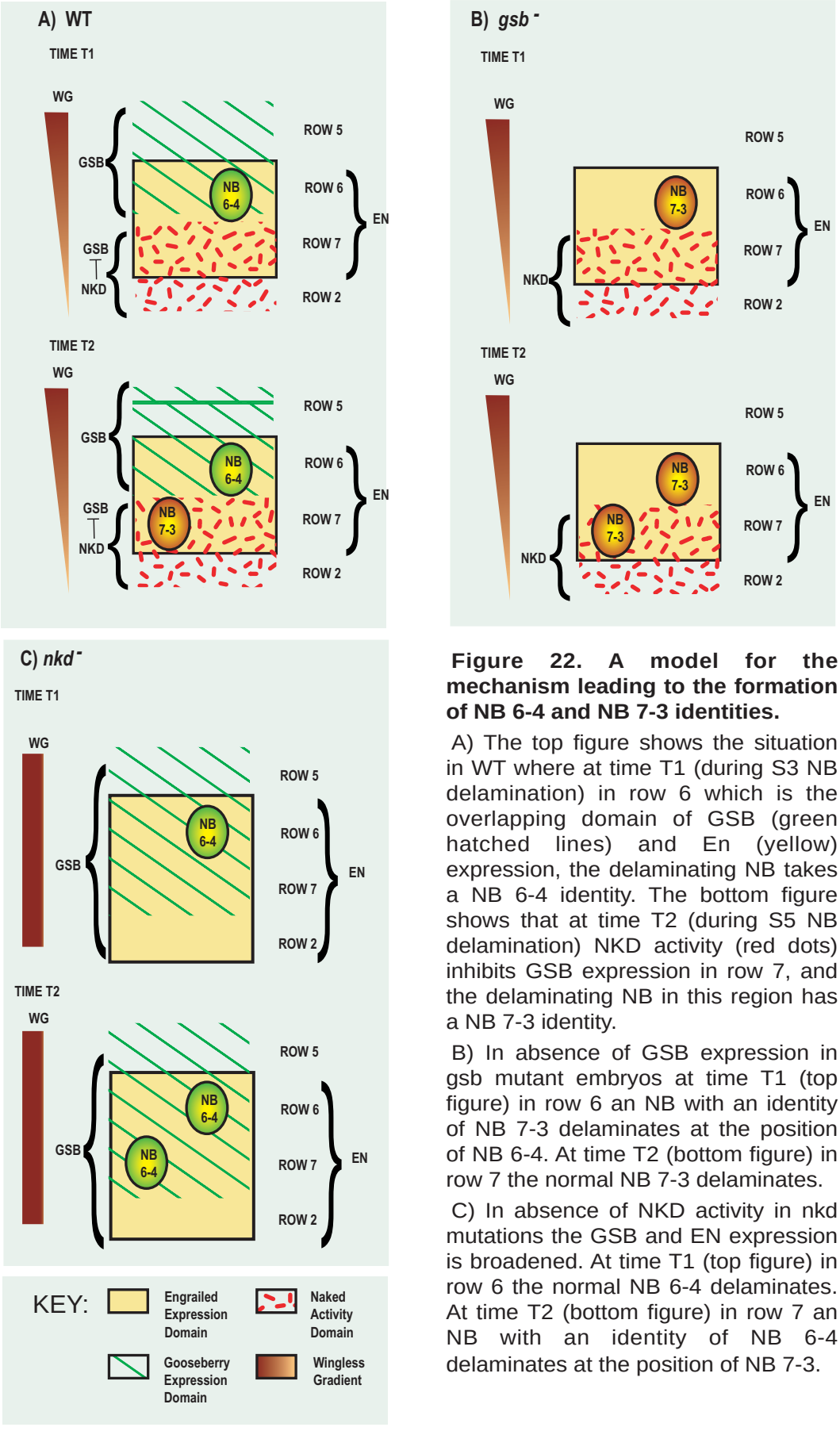


Figure 22. A model for the mechanism leading to the formation of NB 6-4 and NB 7-3 identities.

A) The top figure shows the situation in WT where at time T1 (during S3 NB delamination) in row 6 which is the overlapping domain of GSB (green hatched lines) and En (yellow) expression, the delaminating NB takes a NB 6-4 identity. The bottom figure shows that at time T2 (during S5 NB delamination) NKD activity (red dots) inhibits GSB expression in row 7, and the delaminating NB in this region has a NB 7-3 identity.

B) In absence of GSB expression in gsb mutant embryos at time T1 (top figure) in row 6 an NB with an identity of NB 7-3 delaminates at the position of NB 6-4. At time T2 (bottom figure) in row 7 the normal NB 7-3 delaminates.

C) In absence of NKD activity in nkd mutations the GSB and EN expression is broadened. At time T1 (top figure) in row 6 the normal NB 6-4 delaminates. At time T2 (bottom figure) in row 7 an NB with an identity of NB 6-4 delaminates at the position of NB 7-3.

SUMMARY

Specification of a unique cell fate during development of a multicellular organism often is a function of its position. The *Drosophila* central nervous system (CNS) provides an ideal system to dissect signalling events during development that lead to cell specific patterns. Different cell types in the CNS are formed from a relatively few precursor cells, the neuroblasts (NBs), which delaminate from the neurogenic region of the ectoderm. The delamination occurs in five waves, S1-S5, finally leading to a subepidermal layer consisting of about 30 NBs, each with a unique identity, arranged in a stereotyped spatial pattern in each hemisegment. This information depends on several factors such as the concentrations of various morphogens, cell-cell interactions and long range signals present at the position and time of its birth. The early NBs, delaminating during S1 and S2, form an orthogonal array of four rows (2/3,4,5,6/7) and three columns (medial, intermediate, and lateral) . However, the three column and four row-arrangement pattern is only transitory during early stages of neurogenesis which is obscured by late emerging (S3-S5) neuroblasts (Doe and Goodman, 1985; Goodman and Doe, 1993). Therefore the aim of my study has been to identify novel genes which play a role in the formation or specification of late delaminating NBs.

In this study the gene *anterior open* or *yan* was picked up in a genetic screen to identity novel and yet unidentified genes in the process of late neuroblast formation and specification. I have shown that the gene *yan* is responsible for maintaining the cells of the neuroectoderm in an undifferentiated state by interfering with the *Notch* signalling mechanism. Secondly, I have studied the function and interactions of segment polarity genes within a certain neuroectodermal region, namely the *engrailed* (*en*) expressing domain, with regard to the fate specification of a set of late neuroblasts, namely NB 6-4 and NB 7-3. I have dissected the regulatory interaction of the segment polarity genes *wingless* (*wg*), *hedgehog* (*hh*) and *engrailed* (*en*) as they maintain each other's expression to show that En is a prerequisite for neurogenesis and show that the interplay of the segmentation genes *naked* (*nkd*) and *gooseberry* (*gsb*), both of which are targets of *wingless* (*wg*) activity, leads to differential

commitment of NB 7-3 and NB 6-4 cell fate. I have shown that in the absence of either *nkd* or *gsb* one NB fate is replaced by the other. However, the temporal sequence of delamination is maintained, suggesting that formation and specification of these two NBs are under independent control.

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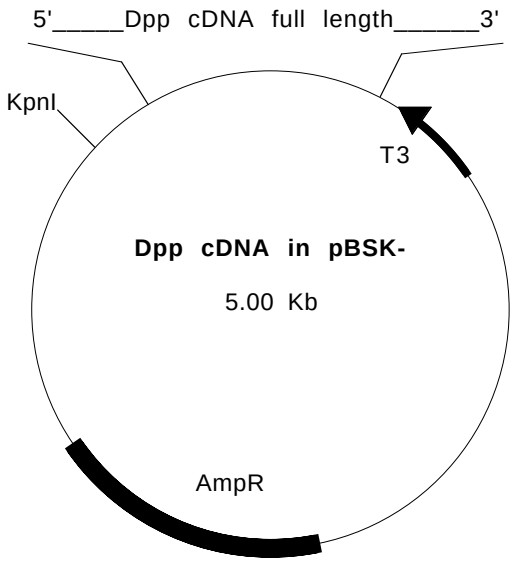
ABBREVIATIONS AND UNITS

%	Percentage
°C	Centigrade
µg	Microgram
µl	Microliter
ß-Gal	*ß- Galactosidase*
*abd*A	Abdominal A
aop	*anterior open*
aos	*argos*
A-P	Anterio-posterior
arm	*aramdillo*
ASC-C	Achaete-scute complex
bHLH	Basic helix loop helix
cDNA	Complementary Diribonucleic acid
CNS	Central nervous system
CyO	Curly derivative of Oster
DAB	Diamnio benzidin tetrahydrochlorid
DEPC	Diethyl pyrocarbonate
Df	Deficiency
DMSO	Dimethyl sulfoxyd
dsh	*dishevelled*
D-V	Dorso-ventral
E(spl)-C	Enhancer of split complex
eg	*eagle*
EGFR	Epidermal Growth Factor Receptor
en	*engrailed*
eve	*even-skipped*
Fig.	Figure
FLP	FLP Recombinase
FM7	First Multiple 7
FRT	FLP Recombinase Target
ftz	*fushi-tarazu*
fz	*frizzeled*
Gal4	Transcription factor from Yeast
GMC	Ganglion mother cell
gsb	*gooseberry*
hh	*hedgehog*
hkb	*Huckebien*
hr	Hours
hs	heat shock
Hyb	Hybridisation
inv	invected
Kb	Kilobases
lacZ	ß- Galactosidase
M	Molar
mg	Milligram
min	Minutes
ml	Milliliter
M-L	medio-lateral

mM	Millimolar
mRNA	Messenger Ribonucleic acid
NB	Neuroblast
nkd	*naked*
no.	Number
ovo	Ovarian tumours
pan	*pangolin*
PBS	Phosphate buffered saline
PBT	Phosphate buffered saline with TritonX-100
PBTween	Phosphate buffered saline with Tween-20
Pm	Plum
PNS	Peripheral nervous system
pnt	*pointed*
ptc	*patched*
repo	*reversed polarity*
rpm	Revolutions per minute
ry	*rosy*
Sb	*Stubble*
sca	*scabrous*
sec	second
spi	*spitz*
st	stage
Su(H)	Supressor of hairless
TM3	Third Multiple 3
TM6b	Third Multiple 6b
ttk	*tramtrack*
UAS	Upstream activating sequence
Vn	*vein*
VNC	Ventral nerve cord
wg	*wingless*
Yan^{act}	Yan activated
Yan^{wt}	Yan wildtype
zw3	*zeste white 3*

LIST OF FIGURES

APPENDIX I



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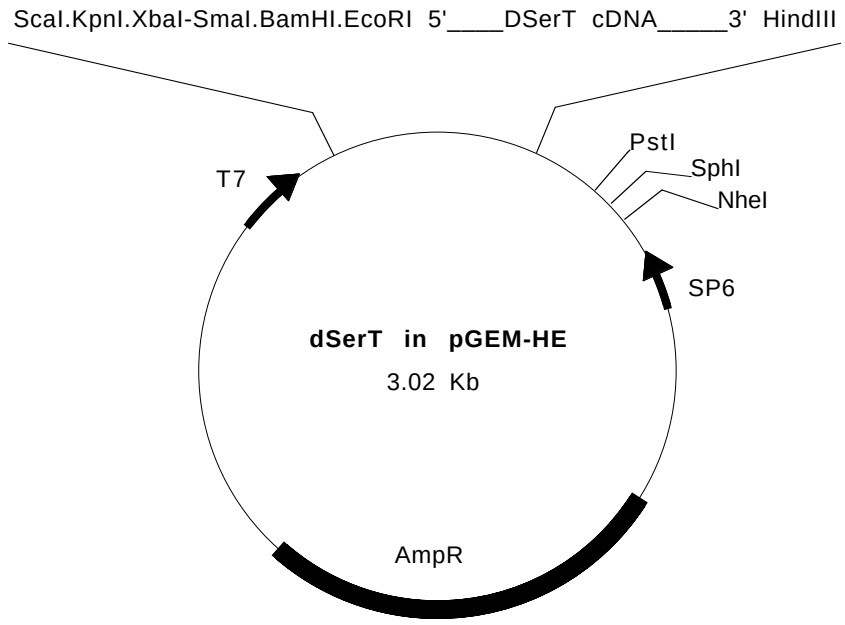
Constructed by: Konrad Basler, University Zürich
Construction date: -
Comments/References: Jay Groppe, University of Basel



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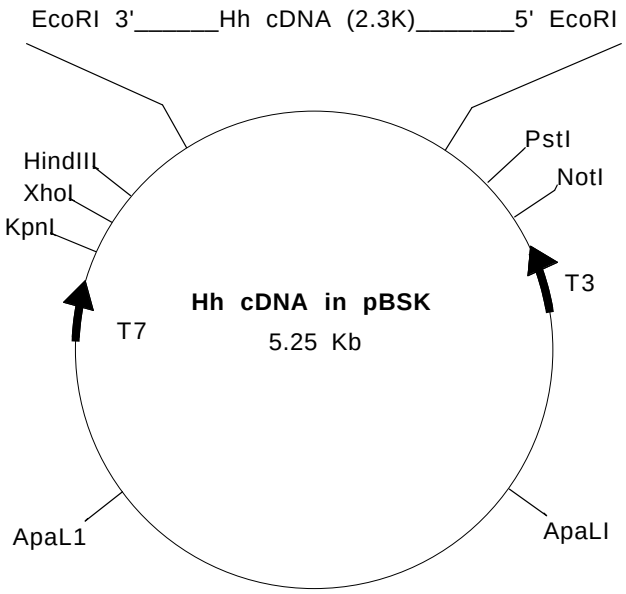
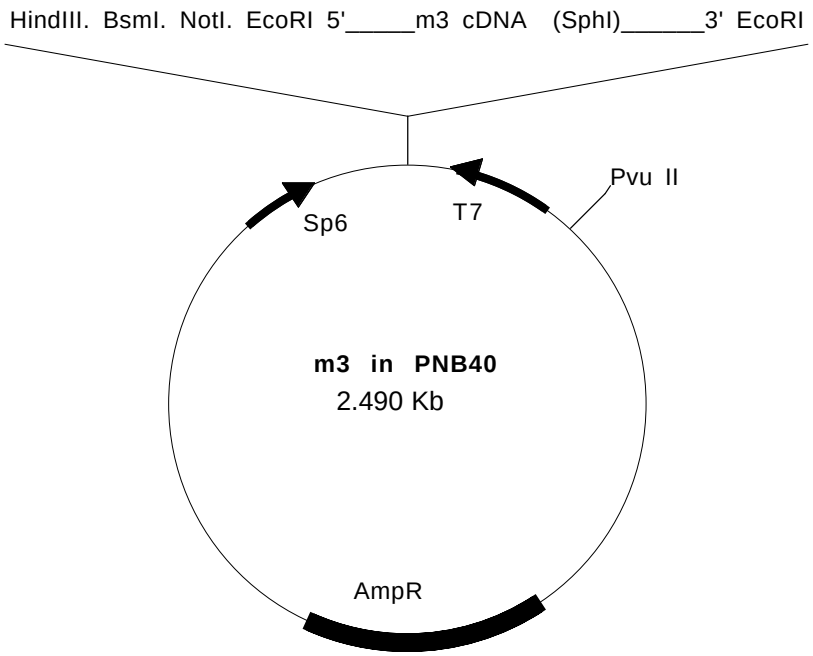
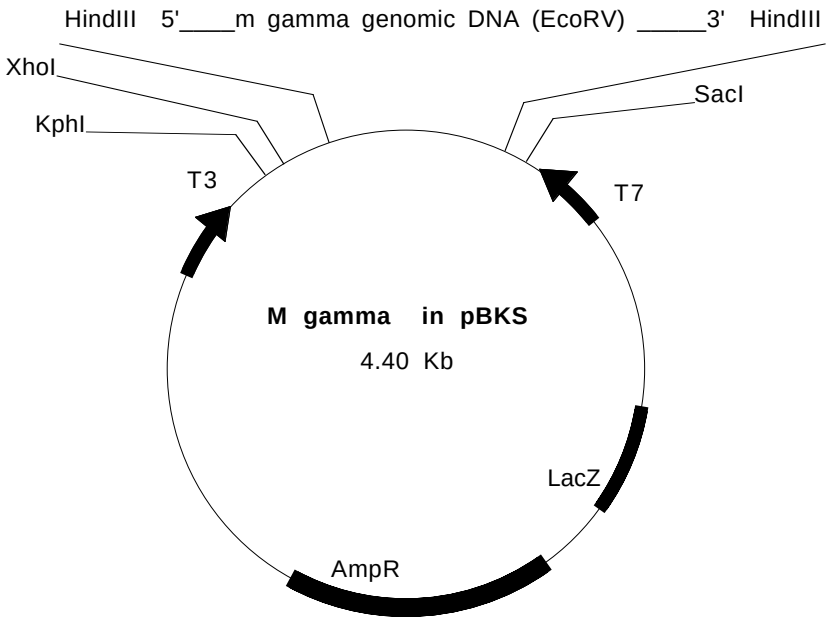
Constructed by: Michael Quick
Construction date: -
Comments/References: University of Alabama, Birmingham.



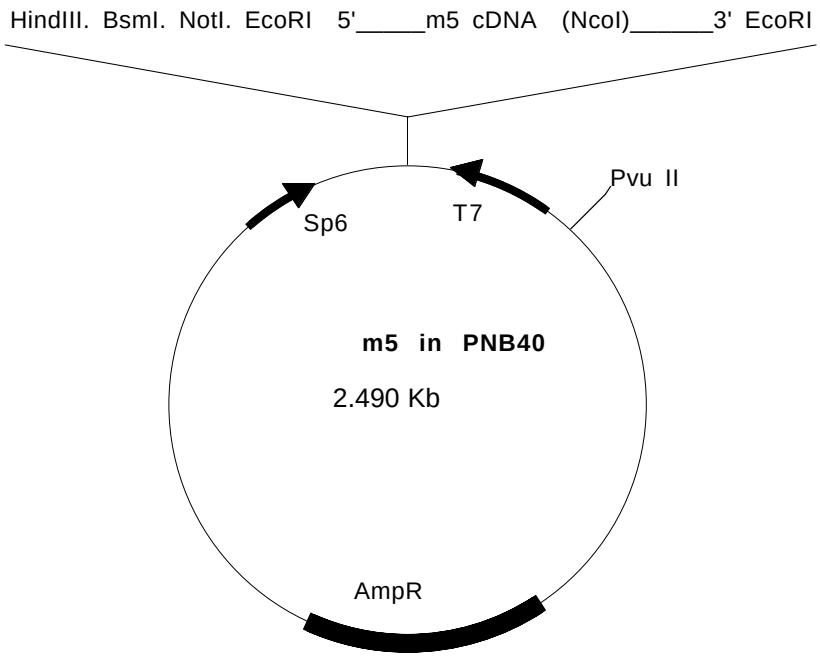
Plasmid name: Hh cDNA in pBSK
Plasmid size: 5.25 kb
Constructed by: Cohen Lab, EMBL
Construction date: -
Comments/References: Natalie Denef



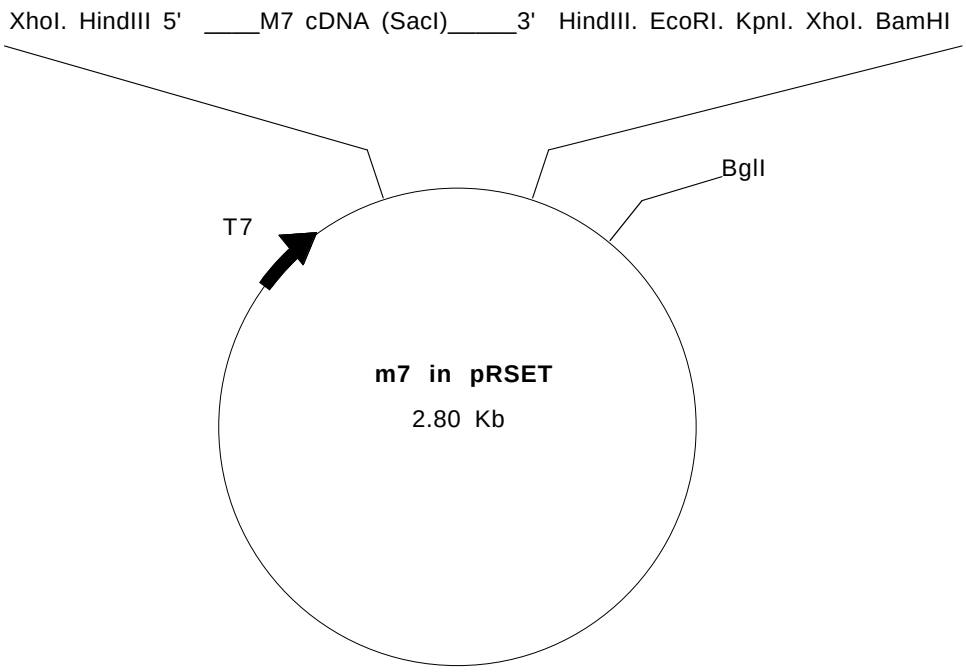
Plasmid name: m3 in PNB40
Plasmid size: ;2.490 Kb
Constructed by: Bray Lab
Construction date: -
Comments/References: Sara Bray



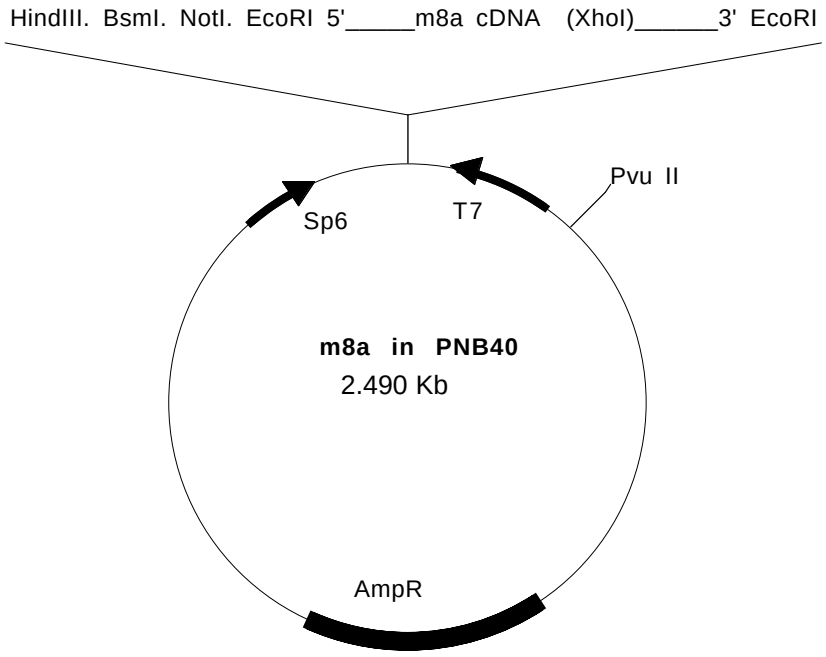
Plasmid name: M gamma in pBKS
Plasmid size: 4.40 kb
Constructed by: Bray Lab
Construction date: -
Comments/References: Sara Bray



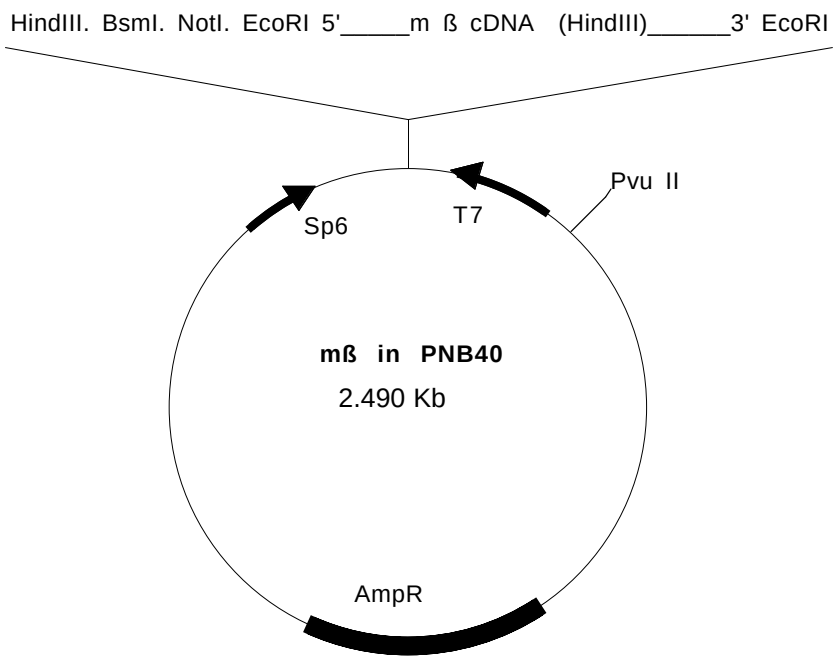
Plasmid name: m5 in PNB40
Plasmid size: 2.490 Kb
Constructed by: Bray Lab
Construction date: -
Comments/References: Sara Bray



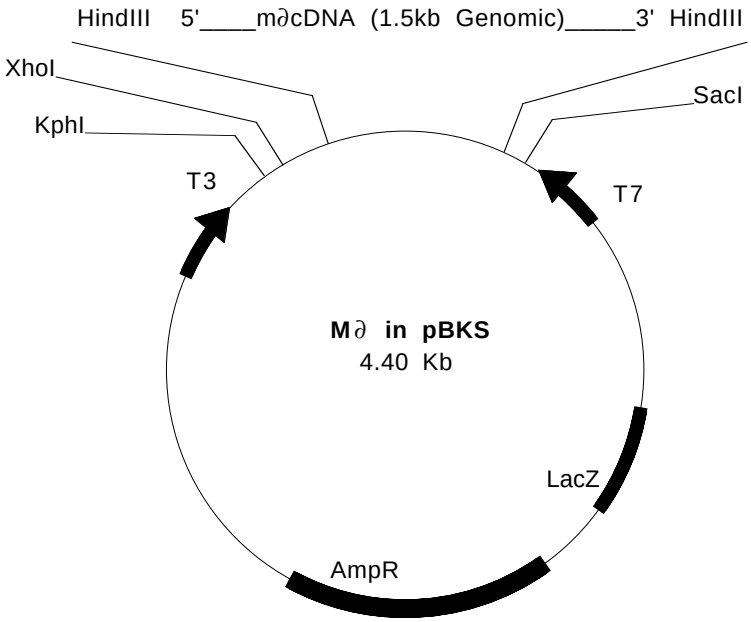
Plasmid name: m7 in pRSET
Plasmid size: 2.80 kb
Constructed by: Bray Lab
Construction date: -
Comments/References: pRSET is a derative of pBKS plasmid



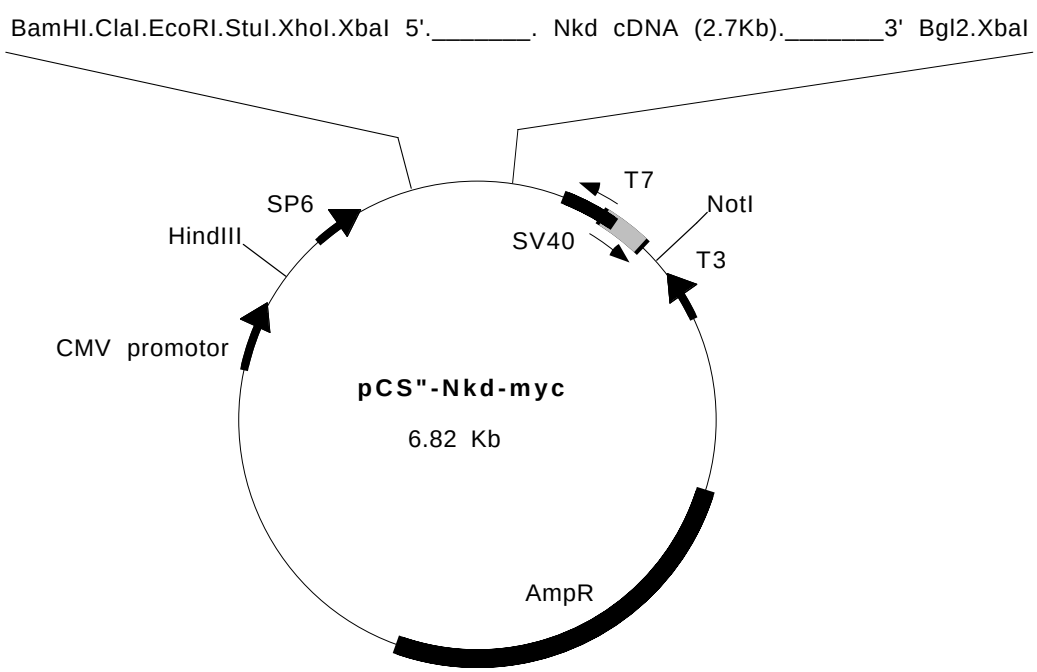
Plasmid name: m8a in PNB40
Plasmid size: 2.490 Kb
Constructed by: Bray Lab
Construction date: -
Comments/References: Sara Bray



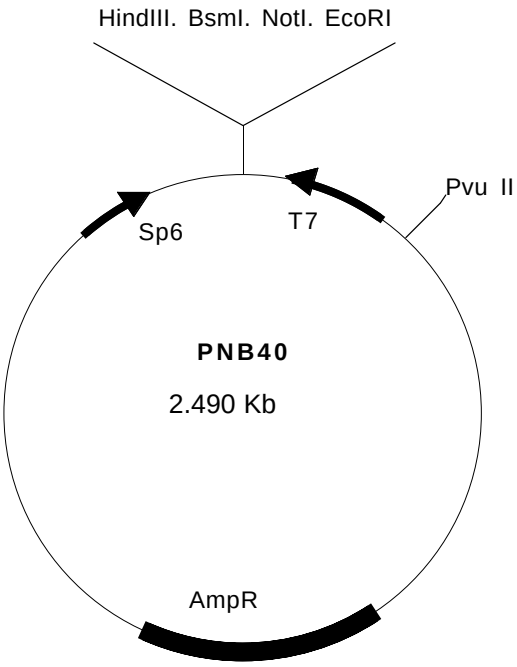
Plasmid name: mß in PNB40
Plasmid size: 2.490 Kb
Constructed by: Bray Lab
Construction date: -
Comments/References: Sara Bray



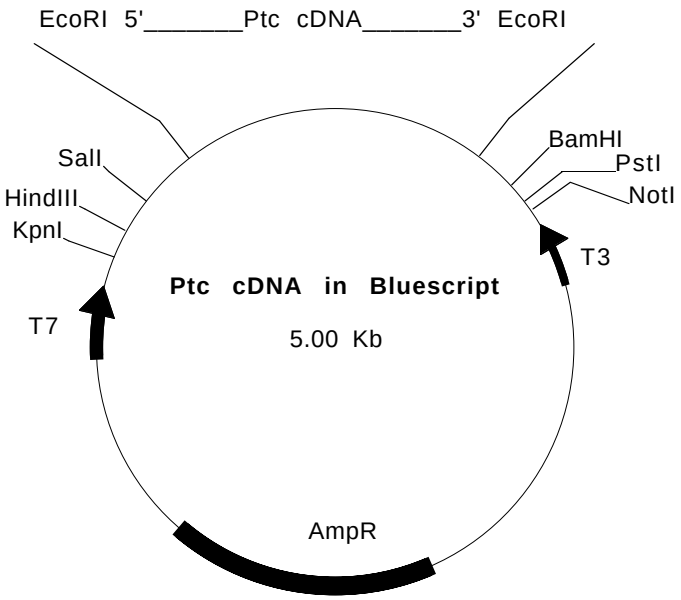
Plasmid name: Mθ in pBKS
Plasmid size: 4.40 kb
Constructed by: Bray Lab
Construction date: -
Comments/References: Sara Bray



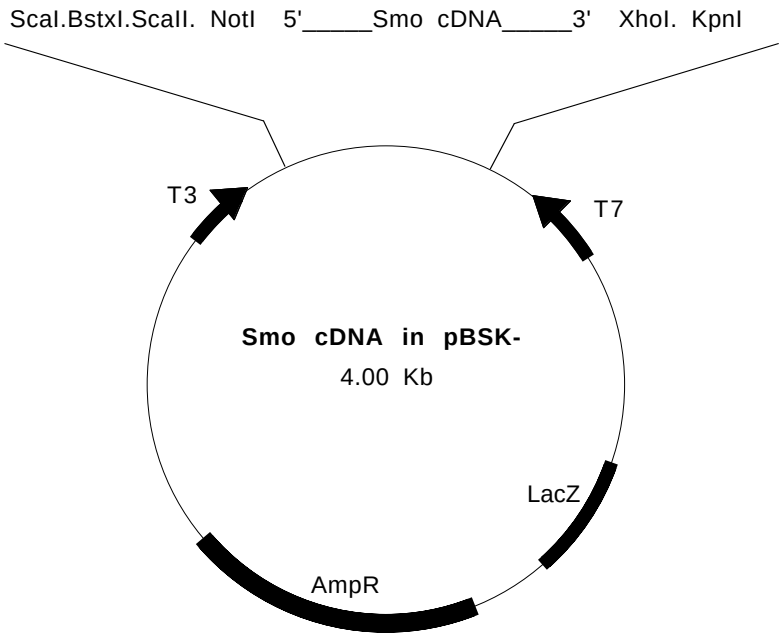
Plasmid name: pCS"-Nkd-myc
Plasmid size: 6.82 kb
Constructed by: Mattew Scott, Stanford, USA
Construction date: -
Comments/References: Nature 403, 789-95,2000



Plasmid name: PNB40
Plasmid size: 2.490 Kb
Constructed by: Nicholas Brown
Construction date: -
Comments/References: Kafatos Lab



Plasmid name: Ptc cDNA in Bluescript
Plasmid size: 5.00 kb
Constructed by: Joan Hooper
Construction date: ?
Comments/References: none



Plasmid name: Smo cDNA in pBSK-
Plasmid size: 4.00 kb
Constructed by: Markus Noll Lab, Uni Zürich
Construction date: -
Comments/References: Joy Alcedo

APPENDIX II

Table 1: List of 2nd chrosmosomal mutations showing a phenotype

STRAIN	Too many cells	Less cells	Cell Cycle	D/V Axis	NBs missing	cells in rows	Patterning	Remarks	Priorty
B1-32						X	X	NB 7-3 too near to ML->ML problem	
B2-34				X				Twisted embryo	2
B2-64				X			?	Some twisted embryos. Ectopic NBs in CNS?	2
C1-17				X			X	Ectopic cells on dorsal side of the embryo	4
E1-38					X			Only 1 embryo! NB 7-3 missing	1
F1-34				X	X			NB rows too narrow! Ventral NBs missing (incl.7-3!)	4
F2-20					X		X	Lineages unidentifiable. St11: NB 7-3 missing?	4
F2-7							X	NB 6-4 glia missing. NB 7-3 duplication	4
H1-201		X	X					NB 7-3 has 2-3 cells only	
J1-53							X	Embryo screwed up	
J2-202	X								
K1-44		X	X					Like H1-201	1
K1-66		X			X			NB 6-4/7-3 missing	4
L1-101					X			NB 2-4/3-3 missing-NB 6-4/7-3 still present	4
L1-72				?				Cells shifted dorsally-could be prep artfact	
L2-107				?		X		NB 7-3 cells too near to ML	2
L2-300					?			Embryos screwed up. One of NB 2-4/3-3 are missing?	3
L2-68		X					X	Pair Rule phenotype	
N1-101					X			NB 7-3 occasionally missing	4
N1-49		X			X			Strange pattern each NB seem to be absent in some seg	4
N1-52			X					NB dont produce GMCs	1
N1-62					X			NB 2-4/3-3 missing-NB 6-4/7-3 still present	4
P3-50							X	Pair Rule phenotype	
P3-68								Embryos screwed up.	
Q1-117				?				Ectopic eg-cells outside the CNS?	4
Q1-4					?			NB 6-4 missing?	4
R1-14	X								
R1-45	X							Ectopic eg cells	2

R3-26					X			NB 6-4/7-3 missing and duplication of 2-4/3-3?	4
R4-19	X			?				Too many cells near ML	3
R4-9							?	NB pattern not normal?	3
S2-26		?			?			NB 2-4/3-3 missing?	4
S2-29						X			1
S3-18	X			?				Similar to R4-19	3
S3-50	X								
S3-98		X						NB 7-3 duplicated	4
S4-102	X							NB 7-3 cluster too large	4
S5-57						X			
S5-76					X	X		NB 6-4/7-3 missing occasionally	4
T3-10		X						cell death	
T3-23	X								
T5-71				X	X			NBs tooo near to ML; NB 7-3 missing	4
T5-82				?				Embryo is screwed up, Cell too near to ML?	1
T6-92					X			NB 6-4 missing	4
U1-14						X			
U3-69		X			X			Pair rule phenotype	3
U4-22					X			en-like phenotype	4
U4-47				?	X			NB 7-3 missig, ectopic cells outside CNS (like Q1-117)	4
U6-18				X	?			Cells too near to ML. 7-3 evtl missing	4
U6-42				X	X		X	Cells too near to ML. 6-4 /7-3 missing, 100%	4
V3-37		?						Pair rule phenotype	3
W3-100					X			NB 6-4/7-3 missing. Pattern completely change	4
X-59					X			NB 2-4/3-3 missing! Maybe also 6-4	4
X2-24		X			?			NB 7-3 missng	4
X3-10		X			X			NB 6-4/7-3 missing.	4
X3-17					X			NB 7-3 missing, also 6-4	4
X4-14		X					X	NBs not identifiable	4
X4-46		X			X			en-like phenotype	4
X4-68		X					X	Less 7-3 cells	1
X4-7		?			?				

X5-2							X	segment polarity like pattern	4
X5-61						X	X	Duplication of NB 6-4	3
Y3-39				X				dpp like embrys, maybe ectopic cells on dorsal side	4
Y3-44					X			NB 7-3 missing, also 6-4	4
Y6-24		?				X		less cells in 7-3	1

Table 2: List of 3rd chromosomal mutations showing a phenotype

STRAIN	Too many cells	Less cells	Cell Cycle	D/V Axis	NBs missing	cells in rows	Patterning	Remarks	Priorty
7	X							Thoracic 6-4 cells in abdomen?	3
9	X						?	Ectopic eg cells within CNS. Too many 6-4 glia	2
12	X							1st abdominal seg looks thoracic, homeotic?	3
50	X							Thoracic 6-4 cells in abdomen?	3
52								Embryos heavily screwed up	
56			X					Very large cells, also outside the CNS	1
59								CNS splitted. Clusters not identifiable	
82	X								
91					X			Embryos heavily screwed up.	
94							X	NBs not identifiable.	4
105	X							Too many 7-3 cells	4
109	X							Thoracic like NB 6-4 cell cluster	4
119	X	X						Less 7-3 cells, Too many 6-4 neurons	3
134		X			X			NB 7-3 missing	4
154	X							Too many 7-3 cells; Too many 6-4 glia	3
156				?				CNS splitted	2
157		X	X					Cells form tight clusters	2
160		X					X	Pair Rule phenotype	
163							X	NB Patterning disturbed	4
183		X	X					NBs with few progeny	1
186		X						2 cells at 7-3 position, also few cells in other position	2
197				?	X		X	Pattern completely changed, unidentifiable cells in ML	4
203					?		X	Too many cells at 7-3 position	4
211	X							NB 6-4 and 7-3-too many cells	4

Table 3. List of X chromosomal mutations showing a phenotype.

STRAIN	Too many cells	Less cells	Cell Cycle	D/V Axis	NBs missing	cells in rows	Patterning	Remarks	Priorty
366		X						Less NB 7-3 cells	1
369		X		?			X	NB 7-3 missing? DV-pattern defect	4
410							X	Eg-NBs are present but misplaced	1
415		X		?				NB 6-4 missing?	2
427		X		X			X	en-like phenotype	4
441			X	?				NB 7-3 missing? Cells too close to ML	4

ACKNOWLEDGEMENTS

First of all I would like to thank my supervisors, Joachim Urban (Joachim Von Schwanheim) for his constant guidance, encouragement and advice and Gerd Technau for all the tremendous amount of paper work it took to make it possible for me do to a PhD in the Institute für Genetik at Uni-Mainz.

A special word of thanks to Olaf Vef and Christof Rickert (Lichi) for providing immense help anytime I choose to disturb them for fly genetics and computation work respectively. Special thanks to Thomas Kehrein for helping me establish and introducing the German System of functioning during my early phase of stay in Germany. I thank our fly food makers Frau Oda and Frau Böll for providing a constant and unending supply of vials. I am ever grateful for the company of my friend and Collegue Tanja Novotny for introducing me the technique of relaxation via sports and Georg Vogler for providing an extremely cheerful atmosphere.

Special regards to my father Suresh Deshpande for his constant moral support and words of encouragement when my spirits have been low. I would like to thank my mother Mrunalini Deshpande for sending in care packets all the way from India during my PhD years. I thank my brother Nachiket Deshpande for putting up with me. I deeply indebted to my friends C. U Revathy and M.S Sunanda not only for their warm friendship but also for providing a sympathetic ear for all my woes and their constant moral support without which my PhD would have been very difficult. I would like to thank Heike Urban for the efforts she took in giving me a warm welcome when I first landed in Germany on a cold wintry night. I thank my flatmate and friend, Birgit Kraft for helping me out many a times.

Lastly but not the least I would like to thank all my labmates Michael Mende (Mende-Shende), Andreas Prokop, Rolf Urbach, Christian Berger, Natalia Sánchez-Soriano, Robert Löhr, Ulrike Kalkowski, Benjamin Altenheim, Diana Rechtenbach, Barabara Küppers and the ex-members of this Institute Lisa Meadows, Anja Berger (Anja-Pirhana), Ruth Wartenberg, Hartmut Schmidt, Regina Eiselt, Itziar Canamasas, Bernd Weil, Ralf Shrank for making my stay a pleasant and memorial one

DECLARATION

Versicherung gemäß Paragraph 11, Absatz 3d, der Promotionsordnung

a) Ich habe die jetzt als Dissertation vorgelegte Arbeit selbst angefertigt und alle benutzten Hilfsmittel (Literatur, Geräte, Materialien) in der Arbeit angegeben.

b) Die vorliegende Arbeit wurde nicht als Prüfungsarbeit für eine staatliche oder andere wissenschaftliche Prüfung eingereicht.

c) Ich habe weder die jetzt als Dissertation vorliegende Arbeit, noch Teile einer Abhandlung davon, bei einer anderen Fakultät bzw. einem anderen Fachbereich als Dissertation eingereicht.

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