REGULATION OF SEGMENT-SPECIFIC NEUROGENESIS IN *DROSOPHILA*

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ABSTRACT

Following *Drosophila* embryogenesis, the morphology of the central nervous system (CNS) becomes dramatically remodelled to reflect the different locomotive and sensory requirements of the adult relative to the larva. The late embryonic onset of segment-to-segment differences in the number and mitotic capacity of neural stem cell-like precursors (termed neuroblasts: NBs) is critical to this resculpting process. My studies address the roles of homeodomain and other transcription factors in regulating thoracic and abdominal-specific patterns of neurogenesis, focusing on one embryonic and one larval example.

In the embryo, dividing NBs sequentially express a temporal series of transcription factors required to link birth-order to neuronal identity. The study of a group of neurons expressing the early NB-sublineage determinant Hunchback in a thorax-specific pattern (termed the THBs) was initiated. I developed a lineage-labelling method to show that the THB-producing NBs appear to undergo a delay in their sub-lineage transition from Hunchback-positive to negative status, relative to most other NBs. Importantly, gene skipping in the canonical transcription factor series is also observed. Genetic analysis reveals that this thorax-specific pattern of neurogenesis requires *homothorax* but surprisingly not the thoracic Hox genes. However, in the abdomen, the Hox genes *Ultrabithorax* and *abdominalA* are required to suppress the THB phenotype.

In the larva, clonal analysis and CNS-specific mutants were used to test several candidate factors potentially regulating NB divisions. This approach identified critical roles for Polycomb group genes and the transcription factor Grainyhead (Grh). I find that Grh, previously implicated as the ultimate NB-sublineage determinant in the embryo, has a differential effect on larval neurogenesis in the thorax versus the abdomen. Individual *grh* NB lineages in the thorax are smaller than wild type and stop dividing prematurely whereas those in the abdomen are larger and divide for an abnormally long period. Underlying the latter effect is an inability to respond to the normal NB-apoptosis inducing burst of the Hox protein AbdominalA. Thus, these studies identify Grh as a context-factor for Hox function in the larval NBs, linking late but not early AbdominalA expression with NB-apoptosis.

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NAMASTE

PREFACE

The research reported in this thesis was carried out in the Division of Mammalian Development at the National Institute for Medical Research (Mill Hill, London) under the supervision of Dr. Alex Gould.

This thesis describes my own original work with the exception of Figure 3.1, Figure 3.2 and Table 3.1. These show the preliminary work on THB neurons carried out by Pierre Fichelson and were included to provide the necessary background information for the studies described in Chapter 3 of this thesis.

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ABBREVIATIONS

A1-A8	abdominal segments 1 to 8
AbdA	Abdominal-A
AbdB	Abdominal-B
ana	*anachronism*
Antp	Antennapedia
AP	Anterior Posterior
AS-C	achaete-scute complex
Asx	*Additional sex combs*
β-gal	β-galactosidase
bHLH	basis Helix-Loop-Helix
BrdU	5-Bromodeoxyuridine
BRM	Brama
Cas	Castor
CdC	Cell division Control
CNS	Central Nervous System
Ddc	*Dopa decarboxylase*
DEA	Dorsal Epidermal Anlagen
DHR78	Drosophila Hormone Receptor-like in 78
DiI	1-1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate
dl	dorsolateral
DNA	Deoxyribonucleic acid
DV	Dorso Ventral
e-L3	early third instar larval stage
EcR	Ecdysone Receptor
EGFR	Epidermal Growth Factor Receptor
Elf-1	Element 1 binding factor
ESC-E(Z)	Extra Sex Comb- Enhancer of Zeste Complex
exd	*extradenticle*
Fas II	Fasciclin II
FLP	Flip recombinase

FRT	Flip Recombinase Target site
GFP	Green Fluorescent Protein
GMC	Ganglion Mother Cell
grh	*grainyhead*
GST	Glutathione S Transferase
H3	Histone 3
H3p	phosphorylated Histone 3
hb	*hunchback*
HCl	Hypochloridic acid
hr	hour
HRP	Horse Radish Peroxidase
hs	heat shock
hth	*homothorax*
L1-L3	instar larva stages 1 to 3
ind	*intermediate neuroblast defective*
INK4a/ARF	Inhibitor of cyclin dependent Kinase 4a/ Alternative Reading Frame
insc	*inscutable*
Kr	Kruppel
M-phase	Mitotic phase
mRNA	messenger ribonucleic acid
MARCM	Mosaic Analysis with a Repressible Cell Marker
Mira	Miranda
MP	Midline Precursor
msh	*muscle segment homeobox*
NB	Neuroblast
nls	nuclear localisation signal
NTF-1	Neurogenic element binding Transcription Factor 1
PBS	Phosphate Buffer Saline
PBT	Phosphate Buffered Saline with TritonX-100
PcG	Polycomb Group Complex
Pcl	*Polycomb-like*

Pdm	POU domain transcription factor
Pins	Partner of Inscutable
pNB	post-embryonic Neuroblast
POU	Pit-1 Oct-1 Unc-86 domain
PRC1	Polycomb Repressive Complex 1
pros	*prospero*
PS	parasegment
Psc	*Posterior sex-combs*
S-phase	DNA Synthesis phase
sca	*scabrous*
SOP	Sensory Organ Precursor
svp	*seven up*
T1-T3	Thoracic segments 1 to 3
TAC1	Trithorax Acetyltransferase Complex 1
TF	Transcription Factor
THBs	Thorax-specific Hb expressing neurons
trol	*terribly reduced optic lobes*
trxG	Trithorax Group Complex
UAS	Upstream Activating Sequence
Ubx	Ultrabithorax
usp	*ultraspiracle*
vl	ventrolateral
vm	ventromedial
VNC	Ventral Nerve Cord
vnd	*ventral nervous system defective*
VNE	Ventral Neuroectoderm
20HE	20-hydroxyecdysone

CHAPTER 1 INTRODUCTION

CHAPTER 1 INTRODUCTION

One of the most interesting questions that researchers in the field of developmental biology aim to address concerns how cell number is regulated. The fine coordination between cell division and cell differentiation is essential for the successful execution of most developmental programs in multicellular organisms. These programs define when and where progenitor cells are going to divide or differentiate. In this manner, the correct composition of cell number and cell-type is achieved.

The work described in this thesis takes advantage of the well-characterized model system of the fruit fly *Drosophila* to answer the following general question: What controls the final number of cells formed during the development of the central nervous system? More specifically, this thesis focuses on some of the factors that regulate neural stem cell properties in a region-specific manner.

The following introduction gives a general overview of fly neurogenesis and the role of the Hox genes in aspects of this process.

A.DEVELOPMENT OF THE EMBRYONIC CENTRAL NERVOUS SYSTEM

Much of the basic knowledge about neurogenesis in invertebrates initially came from studies in the grasshopper. This insect has a very large embryo with cells that are easy to visualize and manipulate. These first studies, involving laser cell ablation experiments and intracellular dye injections, have defined the mechanisms of early neural specification and identified individual progenitor cells and their lineages on the basis of their position within the developing tissue (Doe and Goodman, 1985b; Doe and Goodman, 1985a; Goodman and Doe, 1993). In this regard, this early analysis of the grasshopper Central Nervous System (CNS) has been very insightful for the study of the relatively smaller but highly related fruit fly CNS. The embryonic CNS of *Drosophila* consists of the developing brain and the segmentally repeated units (neuromeres) of the Ventral Nerve Cord (VNC). These are both generated by the divisions of neural progenitor cells called Neuroblasts (NBs). These neural stem-like precursors derive from a monolayer of ectodermal cells: the procephalic neuroectoderm, generating the brain hemispheres, and also the Ventral

NeuroEctoderm (VNE), from which the VNC will develop (Figure 1.1A). Extensive analysis of the VNE has been crucial in understanding the genetic basis of early neurogenesis (Campos-Ortega, 1993b; Skeath and Thor, 2003).

At stage 8 of embryogenesis (Campos-Ortega and Hartenstein, 1997), NBs start delaminating from the VNE into the inner part of the embryo. This process is invariant along most segments of the Anterior-Posterior (AP) axis and eventually results in the formation of about 30 NBs per neural hemisegment (Figure 1.1B). NB segregation lasts for approximately 3 hrs at 25°C and proceeds in five partially overlapping waves of NB delamination, termed S1 to S5 (Hartenstein and Campos-Ortega, 1984; Doe, 1992). At stage 11, an additional group of neural progenitors develops from the mesectodermal cells: these are the Midline Precursor cells (MPs) and are positioned at the borders between adjacent neuromeres (Campos-Ortega, 1993a). Once NBs have segregated from the neuroectoderm, they enlarge and divide asymmetrically, self-renewing and producing a smaller cell, called the Ganglion Mother Cell (GMC) that divides once to generate two post-mitotic progeny known as ganglion cells (Campos-Ortega, 1993b, Figure 1.1 C). These generate final cell diversity by differentiating into neurons (motorneurons, interneurons or neurosecretory cells) and/or glia (non-neuronal cells that surround, support and signal to the neurons, Jones, 2001). Each NB, will give rise to a specific progeny lineage (Bossing et al., 1996; Schmidt et al., 1997; Schmid et al., 1999). During divisions, there is a tendency for the NB to retain its proximity to the ventral epidermis, such that first-born progeny cells are pushed more dorsally while the remaining ganglion cells are generated (Kambadur et al., 1998; Udolph et al., 2001).

During 22 hrs, the time span of embryogenesis (at 25°C), sequential NBs divisions transform a simple neuroectodermal monolayer into a three-dimensional complex and functional CNS.

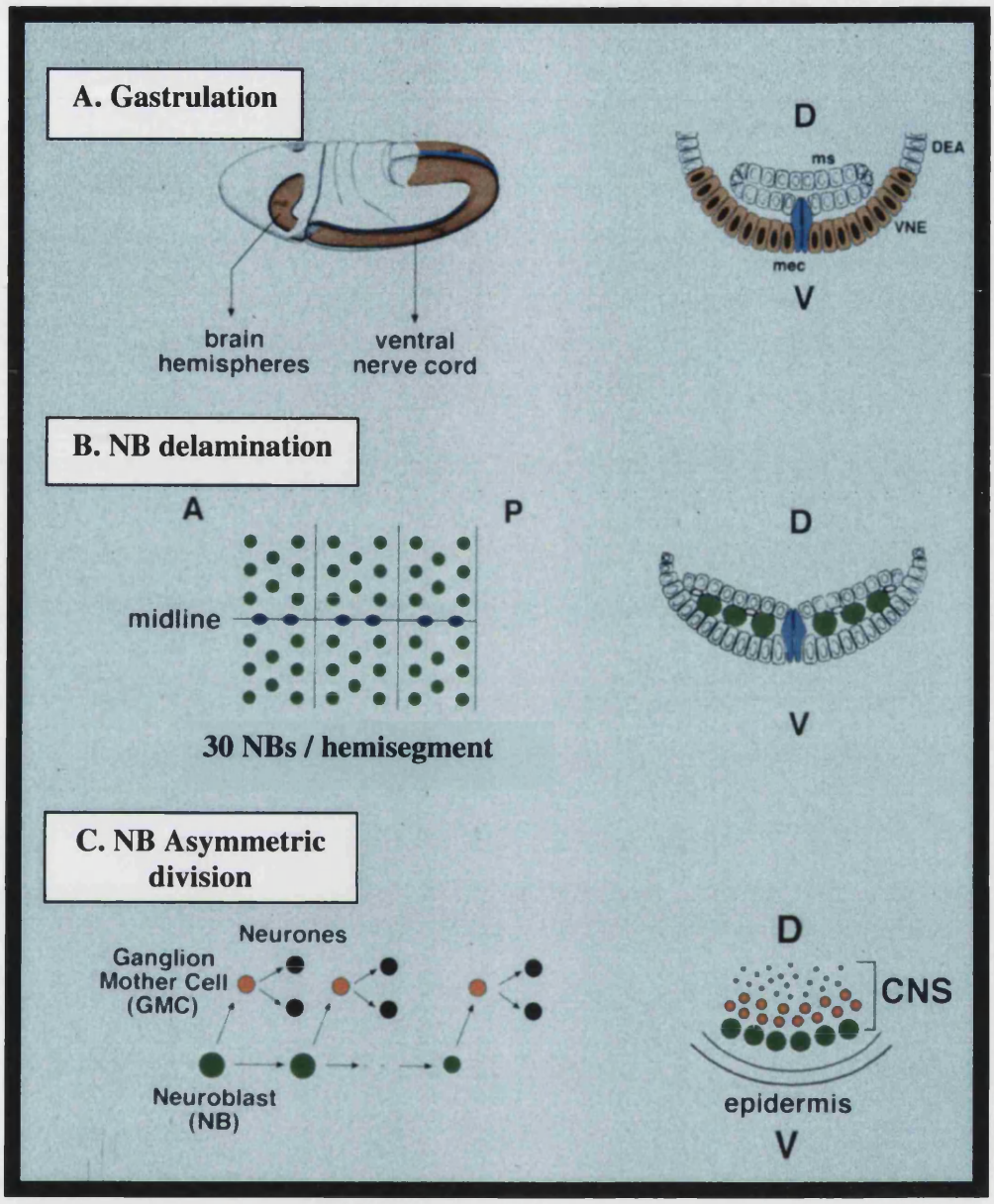


FIGURE 1.1 EMBRYONIC DEVELOPMENT OF THE *DROSOPHILA* CNS

(**A**)The anlagen of the brain hemispheres and the ventral nerve cord in a gastrulating embryo are shown in a lateral view. A: Anterior, P: Posterior, D: Dorsal, V: Ventral. The transverse section on the right shows the ventral neuroectoderm (VNE) surrounded by the Dorsal Epidermal Anlagen (DEA). The VNE will give rise to ventral epidermis and neuroblasts (green) at later stages. The mesoectodermal cells (mec) will give rise to the midline precursors (blue). (**B**) In stage 8-11 embryos, 30 NBs per hemisegment segregate from the VNE. This process is invariant from hemisegment to hemisegment. (**C**) Schematic representation of NB asymmetric divisions at stage 10-14. Following NB mitoses, the larger cell remains close to the ventral epidermis as a NB, the smaller Ganglion Mother Cell (GMC), only divides once to generate ganglion cells that then differentiate into neurons and/or glia.

1.1 Genetic control of early Neurogenesis

The underlying basis for the generation of neural diversity is set at the very early stages of embryogenesis, when the NBs are formed. During segmentation, a hierarchic genetic network determines the expression of segmental stripes of proteins along the AP axis (Akam, 1987). Proteins encoded by the segment-polarity genes, such as *engrailed*, *gooseberry* and *wingless*, are expressed in ectodermal stripes one segment-wide; in the ventral regions, these stripes of expression include the neuroectodermal cells that delaminate to form NBs. Simultaneously, a second group of genes further subdivide the neuroectoderm along the DV axis. These genes are called columnar genes and include: *ventral nervous system defective (vnd), intermediate neuroblast defective (ind)* and *muscle segment homeobox (msh)* (Figure 1.2 A). In this way, a highly controlled Cartesian coordinate system imparts unique fates to individual NBs as a function of their position and formation within the ectoderm (Doe, 1992; Goodman and Doe, 1993B37; Bhat, 1999; Skeath, 1999; Skeath and Thor, 2003). The unique combination of AP and DV gene expression defines what is referred to as an equivalence group. For example, *gooseberry* in combination with *vnd,* forms a specific equivalence group that confers a unique fate to NB5-2 (Buenzow and Holmgren, 1995; Skeath, 1999). On the top of this system of intersections that confers positional identity to the NB lineages, another genetic network is required to select neural as opposed to epidermal progenitors from the VNE. The cells of the VNE all have the potential to form both neural and epidermal progenitors, yet less than one third of ectodermal cells normally develop as NBs. This represents the first control on the final number of cells generated within the embryonic CNS. Extensive studies have led to an understanding of the molecular basis of this neuroepidermal lineage dichotomy (Campos-Ortega, 1993b). Upon homotopic transplantation, single VNE cells maintain their dual potential to develop as a NB or epidermoblast. Interestingly, heterotopic transplants show that cells from the dorso-ectodermal anlagen, that normally do not develop as NBs, can adopt a neural fate when transplanted into the VNE.

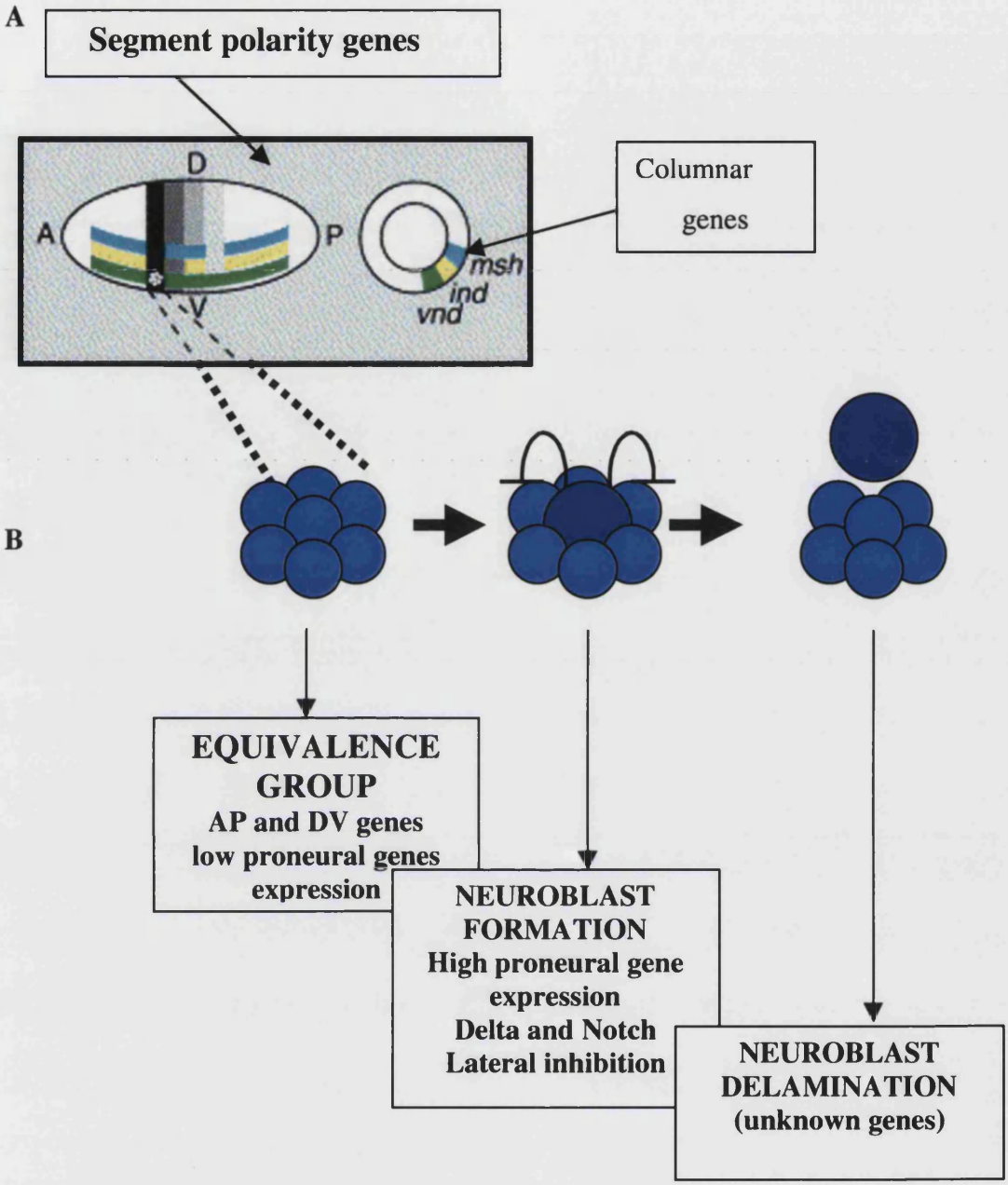


FIGURE 1.2 GENETIC CONTROL OF NEUROBLAST SPECIFICATION AND FORMATION.

(**A**) The Segment-polarity (black/grey) and the Columnar (green/yellow/blue) genes are expressed in orthogonal stripes. Only one of the 14 hemisegments is shown. Each neural equivalence group (white dots) expresses a unique combination of these genes (adapted from Skeath and Thor, 2003). (**B**) In each equivalence group the expression of the proneural genes (light blue) is initially uniform. Lateral inhibition mediated by the neurogenic genes *Notch* and *Delta* generates one Neuroblast with high proneural genes expression (dark blue) by suppressing the expression of proneural genes in the smaller surrounding cells. The genes involved in NB delamination remain unknown.

These transplantation experiments indicate the existence of cell-cell interactions requirement for commitment to one of the two developmental fates. The analysis of different mutants that affect NB formation has revealed the existence of a genetic pathway for NB selection and specification, and a similar mechanism has also been found for the specification of the sensory organ precursors (SOPs, Jan and Jan, 1993). The correct separation of neural and epidermal progenitor cells is based on the antagonistic actions of proneural and neurogenetic genes (Figure 1.2B). Mutations that result in a shortage of NBs allowed the isolation of proneural genes, these include the four transcription units of the *achaete-scute* complex (*AS-C*): *achaete, scute, lethal of scute* and *asense* (Campuzano and Modolell, 1992; Skeath and Carroll, 1992). All these genes encode nuclear proteins that bind to the ubiquitously expressed Daughterless and have a similar basic Helix-loop-Helix (bHLH) DNA-binding domain. Conversely, mutants exhibiting excess NBs led to the identification of the neurogenic genes such as *Notch* and *Delta,* required to shift the neuroepidermal dichotomy towards epithelial cell commitment. Notch and Delta are both transmembrane proteins. Upon binding to Delta present in the apposing cell, the intracellular domain of Notch is cleaved and translocates to the nucleus where it interacts with the transcription factor Suppressor of Hairless and, among many roles, represses the expression of the *AS-C* genes (Struhl and Adachi, 1998). On the other hand, the proneural genes are required to activate Delta and this creates a positive feedback loop between the AS-C genes and the Notch pathway.

In summary, all cells belonging to the VNE initially express the AS-C proteins at low levels and compete for the neural fate; however, some cells will express somewhat higher levels of these proneural factors before others and this will increase expression of Delta. This activates Notch activity in the neighbouring cell with the consequent repression of *AS-C* and adoption of the epidermal fate. This phenomenon, by which the newly formed NB suppresses the neural commitment of its neighbours is generally known as "lateral inhibition" (Figure 1.2 B). The same mechanism had been previously shown in the grasshopper embryo; when a newly

forming NB is ablated, an ectodermal cell in its proximity is released from inhibitory signalling and can enlarge and assume the neural fate (Doe and Goodman, 1985b).

The mechanisms controlling NB formation in the head differ from those in the trunk. Recently, Urbach *et al* have studied the pattern of NB formation as well as proneural gene expression during the development of the brain hemispheres and showed that, in contrast to the VNE, adjacent cells within specific domains of the proencephalic neuroectoderm develop as NBs. This suggests that in these regions there is lack of lateral inhibitory signals with epidermalising characteristics (Urbach et al., 2003).

1.2 Asymmetric division of Neuroblasts

Once it has delaminated, the NB starts dividing in an asymmetric manner; this is important for the maintenance of proliferation (via NB regeneration) and also for the generation of cellular diversity, through the formation of a smaller cell, the GMC (from which glia and neurons arise). In *Drosophila*, asymmetric division is a common theme during development, not only of NBs, but also of SOPs, and these two systems appear to share at least some of the critical molecular components (Jan and Jan, 2000; Chia and Yang, 2002).

In the past years there has been much progress in understanding the molecular basis of NB asymmetric division (for reviews see Campos-Ortega, 1997; Jan and Jan, 2000; Chia and Yang, 2002). Single NBs isolated in culture divide asymmetrically, showing that extrinsic signals are not required to maintain the process and that, instead, the information partitioning the stem cell and GMC is cell intrinsic (Broadus and Doe, 1997). Three important features characterise the events that determine asymmetric division (Figure 1.3A). The first is the uneven segregation of cytoplasmic cell-fate determinants at different cell poles, forming the basal and apical crescents. Second, changes in the orientation of the mitotic spindle allow the preferential segregation of the cytoplasmic determinants to just one daughter cell. Finally, it is thought that, by analogy with the nematode single-cell embryo, asymmetry and displacement of the mitotic spindle is necessary to generate two cells of unequal size (Cai et al., 2003).

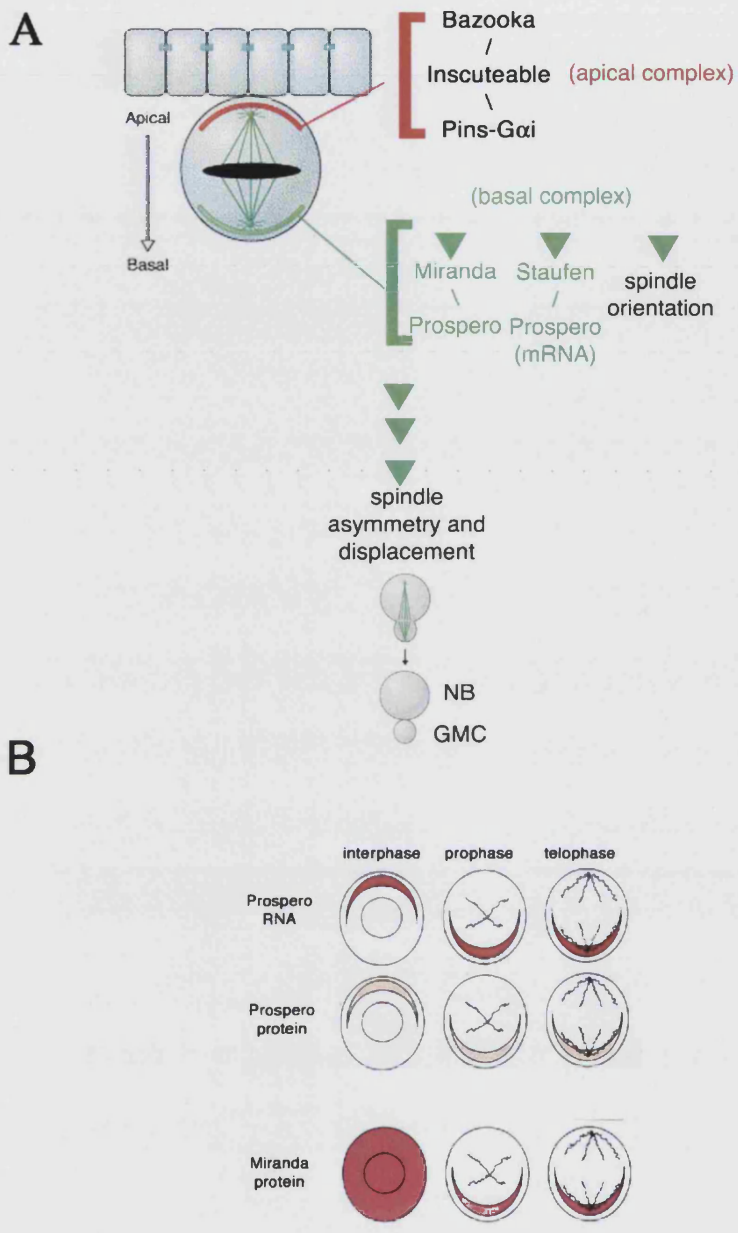


FIGURE 1.3 NEUROBLAST ASYMMETRIC DIVISION

(**A**) In the mitotic NB, Bazooka, Inscuteable, Pins and Gαi are localized to the apical cortex and form the apical complex (red crescent): Bazooka binds to Inscuteable that binds to the Pins- Gαi complex. This apical complex is required for the normal basal localisation of Miranda-Prospero and Staufen-Prospero RNA (g reen crescent, basal complex) as well as for mitotic spindle rotation. These molecules might also influence the unequal size of daughter cells, the neuroblast (NB) and the Ganglion Mother Cell (GMC) (Adapted from Chia and Yang, 2002). (**B**) Schematic representation of the localisation in neuroblasts of *prospero* mRNA and protein and of Miranda protein. The ovals represent the neuroblasts during Interphase, Prophase and Telophase (with apical to the top in each case). Interphase nuclei (circles) and M-phase chromosomes (threads) are shown (adapted from Campos-Ortega, 1997).

Importantly, the delaminating NB inherits the apical-basal polarity of the epithelial sheet from which it segregated. This is possible because the NB contains localised Bazooka from its epithelial ancestor cell (Schober et al., 1999; Wodarz et al., 1999). In the delaminating interphase NB, Bazooka localizes to the apical cortex and establishes an apical protein complex by interacting with Inscutable (Insc), Pins and GαI (Schaefer et al., 2000). In turn, Insc controls the localization of the cell determinant Prospero (Pros) to the basal pole; this is achieved indirectly through the asymmetric segregation of Miranda (Mira, binding *pros* encoded protein) and Staufen (binding *pros* mRNA) (Li et al., 1997; Shen et al., 1997). In the early mitotic NB the spindle is initially in the same orientation as for dividing epithelial cells, perpendicular to the apical-basal axis. At metaphase, however, the spindle undergoes a 90° rotation and lines up along the apical-basal axis. Cells with aberrant Insc expression fail to orient properly the mitotic spindle, indicating that Insc is required for rotation and thus for controlling the orientation of NB divisions (Kraut et al., 1996). More and more evidence is now accumulating regarding the link between molecules that mediate asymmetric cell division and cell-cycle progression (Chia and Yang, 2002). For example, the sublocalization within the NB of both apical and basal components shows cell-cycle dependence (Figure 1.3B). Mira is evenly expressed in the cytoplasm during interphase but becomes segregated to the basal pole at the beginning of mitosis, when it also allows Pros to move from an apical to basal position (Ikeshima-Kataoka et al., 1997). Indeed, perturbing the activity of mitotic regulators, such as cyclin-dependent kinases, results in the mis-segregation of asymmetric determinants (Campos-Ortega, 1997; Tio et al., 2001; Chia and Yang, 2002). Conversely, asymmetric determinants are thought to be involved in regulating cell-cycle progression. For example, the segregation of Pros into the GMC appears to be one mechanism necessary for termination of cell proliferation and onset of differentiation in progeny glia or neurons. This is achieved through the Pros-mediated activation of the cyclin-dependent-kinase inhibitor, *dacapo* (Li and Vaessin, 2000; Liu et al., 2002). This mechanism provides a simple way of balancing the equilibrium between division and differentiation. Interestingly, this role seems to

be conserved in vertebrates where *Prox-1,* the mammalian *pros* homolog, has been shown to be required for retinal cells to stop dividing and enter differentiation (Dyer et al., 2003).

Finally, at the end of embryogenesis (stage 16), with the exception of late-replicating thoracic NBs (Prokop et al., 1998), most NBs are believed to stop dividing and enter a quiescent status.

1.3 Characterization of NB lineages

As introduced earlier, each NB is formed from a unique equivalence group that determines its identity and thus the expression of particular NB lineage markers (Doe, 1992; Skeath and Thor, 2003). A detailed description of individual NBs and their progeny is now available (Bossing et al., 1996; Schmidt et al., 1997; Schmid et al., 1999) where the nomenclature applied to the different NB lineages derives from their apparent homologues in grasshopper (Broadus et al., 1995).

The identification of the parental NBs and MPs for all the motorneurons, interneurons, neurosecretory and glia cells of the embryonic CNS has been possible thanks to elegant *in vivo* lineage tracing techniques. Two methods have been used: isotopic transplantation of HRP-labelled neuroectodermal cells into unlabeled hosts (Bossing and Technau, 1994; Bossing et al., 1996) and injection of DiI, a fluorescent dye (Schmid et al., 1999). One very interesting conclusion from these experiments is that not all NBs undergo the same number of divisions. Clone size at the end of embryogenesis varies from a minimum of 2 interneurons per segment, generated by MP1, MP2 and MP3, up to a maximum of 37 motorneurons, interneurons and glia generated by NB7-1 (Schmid et al., 1999). Therefore, NBs differ significantly in their mitotic activity and there is an approximate tendency for NBs that delaminate early (S1 wave) to generate more cells by the end of embryogenesis than NBs born later (Bossing et al., 1996; Schmidt et al., 1997; Schmid et al., 1999). However, several exceptions to this rule have been found: for example, some NBs that are born at the same time as NB7-1 do not display such large clones. Another interesting finding is that a given NB often displays segment-specific differences in clone size. For example, NB7-1 in the abdominal neuromeres generates fewer progeny than its

thoracic counterpart. This general type of segment-specific differences in the number of NB divisions has been described for many other NB lineages such as NB1-1, NB2-2 and NB3-1 (Bossing et al., 1996) as well as NB1-2, NB2-3, NB3-3, NB3-5, NB4-1, NB4-3, NB5-3, NB5-6, NB6-1 and NB6-4 (Schmid et al., 1999). A survey of these NBs reveals that segment-specific difference in clone size can vary from 1 to 7 additional cells in thoracic lineages. Among the NB lineages that show differential segmental behaviour, NB1-1 has been extensively characterized. This NB generates glia only in the abdomen whereas, in the thorax, it gives rise to about three additional motorneurons or interneurons (Udolph et al., 1993; Prokop and Technau, 1994; Schmid et al., 1999). Other examples giving different cell types in thorax versus abdomen are NB1-2, NB6-1 and NB3-5, these NBs produce motorneurons only in the thoracic segments, with NB6-1 also giving rise to interneurons in a thorax-specific manner. NB5-6 produces a variable number of thorax-specific glial cells and fewer interneurons in the abdomen than in the thorax. The NB3-3 derived motorneuron that innervates muscle 5 forms exclusively in abdominal neuromeres. NB6-4 forms the same glial cells in thorax and abdomen but the abdominal neuromeres lack the interneuronal component. Interestingly, some NBs, such as NB2-3, do not appear to produce any progeny in the abdomen. In this case, the NB is the only cell observed in the abdominal clones, meaning either that this cell does not divide in the embryo or that all of its progeny die (Schmid et al., 1999). As a consequence of these proliferation differences, 5-Bromodeoxyuridine (BrdU) pulse-labelling of stage 16 embryos, labelling cells that are synthesising DNA, reveals rare replicating cells in the abdominal neuromeres compared with abundant cell proliferation in the more anterior segments of the ventral nerve cord (Prokop et al., 1998).

These AP differences in NB behaviour represent an early step towards the regional shaping of a functional CNS, influencing both cell number and cell diversity in a segment-specific manner. As I will introduce later in this Chapter, such AP differences in the CNS have been ascribed to the Hox/homeotic genes (Prokop and Technau, 1994; Prokop et al., 1998).

1.4 NB sublineage switching: linking birth order to neuronal identity

In addition to spatial patterning mechanisms, NBs utilise a temporal patterning system during embryonic neurogenesis. NBs sequentially express a temporal series of different transcription factors (TFs) (Kambadur et al., 1998; Brody and Odenwald, 2000; Isshiki et al., 2001). The zinc-finger protein encoded by the gap gene *hunchback (hb)* lies at the start of this cascade, the other TFs that take part in this sublineage-switching are, in order: Kruppel (Kr), Pdm-1 and Castor (Cas) (Figure 1.4A). *Kr* and *cas* both encode zinc-finger proteins while Pdm-1 belongs to the family of POU-homeodomain TFs. There is a well-orchestrated cross-regulatory network linking these factors. Each gene activates the transcription of the next factor in the cascade and in several cases it appears to repress the next-plus one (Kambadur et al., 1998; Isshiki et al., 2001; Edenfeld et al., 2002). Importantly, the GMCs maintain the expression of the TF that was expressed in the parental NB at the moment of birth. This mechanism thus provides a temporal label distinguishing early-born from late-born fates, thereby influencing post-mitotic neuronal identity. As a result of this temporal modulation in the expression of TFs, the late embryonic CNS appears as a layered structure where the early-born Hb-positive cells tend to be located dorsally whereas the later-born, Cas-positive cells are often positioned more ventrally, close to the NB (Bossing et al., 1996; Kambadur et al., 1998; Isshiki et al., 2001; Edenfeld et al., 2002). Most of the embryonic NBs appear to undergo sublineage switching in a similar manner, as has been shown for NB-1-1, NB2-4, NB4-2, NB6-4, NB7-1 and NB7-4 (Isshiki et al., 2001; Pearson and Doe, 2003). A few exceptions to the canonical cascade have also been described, such as NB7-3, which does not express Cas (Isshiki et al., 2001), and NB6-1, that instead expresses only this last factor but not the earlier sublineage determinants (Cui and Doe, 1992). The **Hb → Kr→ Pdm-1 → Cas** provides a label for birth order but does not directly determine specific neuronal-type. Thus, Hb is necessary and sufficient to specify the early-born fate of the progeny (Pearson and Doe, 2003) but precisely how this is interpreted depends upon the NB of origin. For example, in the NB1-1 and NB7-1 lineages, the Hb-positive first-born neurons differentiate into

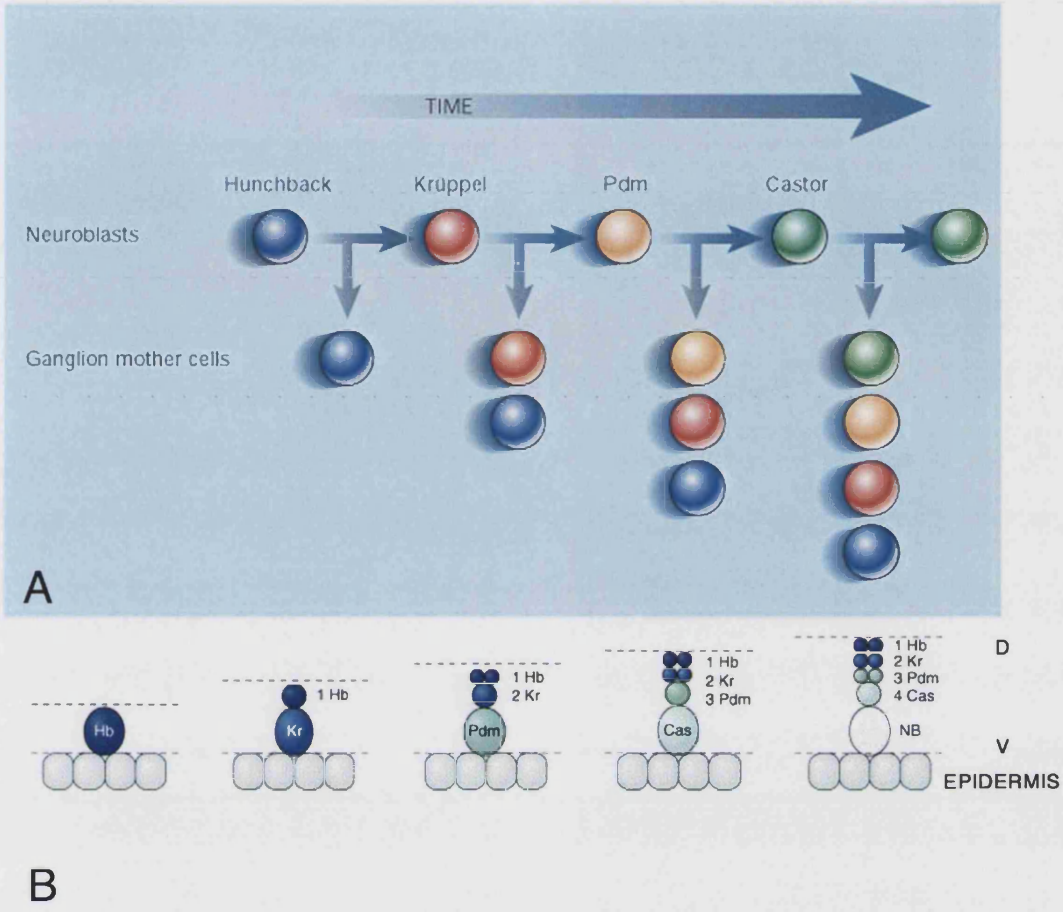


FIGURE 1.4 NEUROBLAST SUBLINEAGE SWITCHING. (**A**)From left to right, mitotic Neuroblasts express four transcription factors: Hunchback, Kruppel, Pdm and Castor, in a temporal sequence that regulates the order in which neurons are produced. Each time a neuroblast divides it gives rise to a new neuroblast and to a ganglion mother cell, which divides to generate neurons (not shown). GMCs maintain the expression of the factor expressed by the progenitor before it divided. (Experimental data from Kambadour *et al*, 1998 and Isshiki *et al*, 2001. Adapted from Livesey and Cepko, 2001) (**B**) The sequential expression of transcription factors results in a layered organization of the developing CNS (dashed lines). The first-born Hunchback (Hb) positive neurons are localised dorsally (D, top dashed line) whereas the last-born Castor (Cas) positive neurons are close to the progenitor cell, in a more ventral position, V. (Adapted from Edenfeld *et al*, 2002.)

motorneurons, whereas the early-born Hb expressing progeny of other NBs develops into interneurons (NB7-4) or glia (NB6-4) (Isshiki et al., 2001). Pearson *et al.* propose a model in which the sequential change in the expression of the determinant factors in the NB progressively restricts the NB potential to generate early-born fate cells. For NB7-1, intermediate-born sublineages are competent to make "younger" neurons in response to the over-expression of Hb but this plasticity is eventually lost in older NBs (Pearson and Doe, 2003; Isshiki and Doe, 2004). Interestingly, switching between sublineage determining TFs, is dependent upon the progression of the cell cycle. Cells that are unable to undergo the normal cell cycle also fail to exit their Hb-expression status, but when the cell cycle block is removed they carry on with the normal series without skipping any factors (Isshiki et al., 2001). This suggests that the clock specifying when TF switching should occur corresponds to the cell cycle.

It has been proposed that another TF, Grainyhead (Grh), takes part to the sequential TF series as the ultimate factor, **Hb ➛ Kr➛ Pdm-1 ➛ Cas ⇨ Grh.** When NBs are cultured for few hrs they produce Hb, Kr, Pdm-1 and Cas-positive progeny; but after an over-night culture the NB and GMC also express Grh (Brody and Odenwald, 2000). *In vivo,* Grh is expressed within the NBs starting from stage 14 (Bray et al., 1989) but whether there is direct transcriptional regulation between Cas and Grh is not known yet. Neither it is known what properties of the NB or GMC, if any, are controlled by Grh expression.

In the recent years, work on this interesting issue of sublineage switching in the NB has become intense, partly because such a temporal mechanism of cell fate determination seems to be conserved during the development of the mammalian retina and brain cortex (Harris, 2001; Livesey and Cepko, 2001a; Ohnuma and Harris, 2003).

At present, the possibility of a link between the *Drosophila* TF series and the generation of segmental differences in neuronal number and/or identity has not been raised. Moreover, the role of the TF series in regulating the overall size of NB lineages is also unclear. I address these two important issues in Chapter 3 and Chapter 5.

B. DEVELOPMENT OF THE ADULT CENTRAL NERVOUS SYSTEM

1.5 Embryonic and post-embryonic Neuroblasts share a common lineage

Drosophila is a holometabolous insect that exists in very different larval and adult forms. Consequently, neurogenesis has two distinct phases. The previously described embryonic phase generates the larval CNS and, during a post-embryonic phase, the adult CNS is generated on the top of the larval CNS (Truman and Bate, 1988). This has raised the question of which progenitor cells carry out the process of larval and pupal neurogenesis.

At the moment of larval hatching, the first instar (L1) CNS shows clear segment-to-segment differences in the number of stem cell-like precursors, referred to as post-embryonic NBs (pNBs). 23 pNBs are found in the three thoracic hemisegments, 6 in the first two abdominal hemisegments and only 3 pNBs per hemisegment can be found in the central abdomen. These three abdominal lineages can be easily detected on the basis of their position and are commonly known as dorsolateral (dl), ventrolateral (vl) and ventromedial (vm) (Truman and Bate, 1988). At larval hatching, all the pNBs in the VNC are not dividing and are termed quiescent.

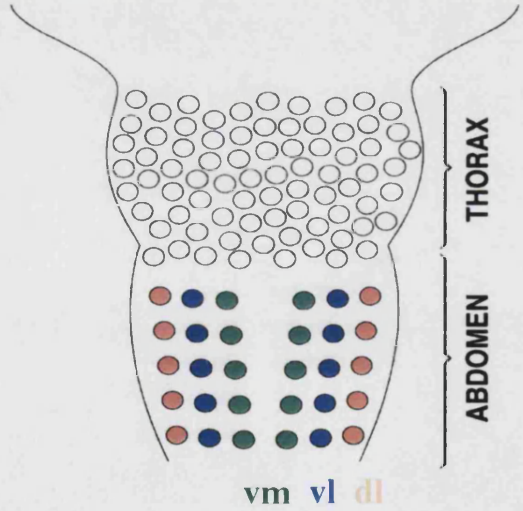


FIGURE 1.5 SCHEMATIC REPRESENTATION OF NBS PRESENT IN L1 LARVAL CNS
White circles represent the large number of NBs found in the thoracic neuromeres. Coloured circles identify the number and position of the abdominal pNBs vm (green), vl (blue) and dl (pink).

Prokop and Technau have addressed the important question of whether pNBs are related to embryonic NBs. By carrying out elegant single-cell transplantations, the authors demonstrated that pNBs share the same lineage as the embryonic NBs (Prokop and Technau, 1991). In these experiments, *LacZ* transgenic embryos, where all neural cells are labelled by the stable genetic marker (β–galactosidase, β–gal), were injected at very early stages (syncytial blastoderm) with HRP, an enzymatic marker that gets diluted out by cell division. Neuroectodermal cells from these double-labelled donor embryos were then transplanted into host wild type embryos and the clones generated were then observed at late larval stages. This technique allows the detection of two kinds of clones in the third instar larval (L3) VNC. A first type of clone is small and consists of HRP/β– gal double-labelled cells: these are neural cells that have been generated during embryogenesis and have not divided significantly during the larval stages. These small clones can also be found in specimens fixed earlier, at the late embryonic stages. The second type of clone contains more cells and consists of a group of double-labelled cells making contact with a group of β–gal single-positive cells. Among these latter cells, the large cell that is located ventrally is, by size and position, identified as the NB (Truman and Bate, 1988; Prokop and Technau, 1991). It is presumed that this NB divided during embryogenesis, producing double-labelled progeny, before entering a quiescent state. At larval stages, it has resumed several additional rounds of division, further diluting the HRP and allowing the detection of single β–gal-labelled cells. Although this experiment shows that pNBs derive from the same progenitor as embryonic NBs, it does not formally show that the two cell types are identical. However, this is generally assumed to be the case (Truman et al., 1993).

Importantly, among the 30 NBs per hemisegment that were specified at early embryogenesis, a reduced number takes part in larval neurogenesis (Truman et al., 1993). One reason for this is that, towards the end of embryogenesis, a wave of programmed cell death (apoptosis) eliminates a number of stem cells (Peterson et al., 2002). The abdominal NBs are most affected, losing 27/30 original precursors and just leaving vm, vl and dl. In the thorax, only 7/30 are lost and it is not yet clear whether this happens by the same apoptotic mechanism as in the abdomen. It is

therefore at larval rather than embryonic stages that segment-specific regulation of neural cell number becomes most dramatic. Thoracic-abdominal differences will become more and more striking as variations in the proliferative capacity of individual pNBs, as well as their number, contribute to resculpting the adult CNS from its larval predecessor.

1.6 The spatio-temporal pattern of pNB divisions

Truman and Bate studied the temporal pattern of pNB divisions throughout larval life by analysing S-phases using BrdU incorporation (Truman and Bate, 1988; Truman et al., 1993, Figure 1.6). Although NBs from most regions enter a period of quiescence at late embryonic stages, the NBs found in the mushroom bodies are an exception to this. These NBs continuously divide to produce prominent structures of the central brain necessary for olfactory associated learning (Lee et al., 1999; Jefferis et al., 2002). The BrdU experiments show that the onset of the second round of neurogenesis depends upon the position of the pNB along the AP axis. The pNBs located in the more anterior segments start dividing earlier than the ones in the more posterior positions. DNA replication can be observed in some of the pNBs of the central brain starting from close to the beginning of the first instar larva (L1). Replicating NBs are instead detectable in the thoracic neuromeres only 24 hrs later, when the second instar (L2) larval stage starts. Finally, the abdominal pNBs commence S-phase at early third instar (eL3), with a 24 hr delay relative to the thorax. The time at which pNBs cease replicating DNA also shows anterior to posterior differences. The pNBs of the central brain and the thorax continue to divide into the pupal period, whereas vm, vl and dl cells terminate proliferation at mid-L3, approximately 72 hrs after hatching. Sex-specific differences in pNBs divisions can also be observed in the posterior abdomen, where four terminal pNBs in male larvae have a longer period of neurogenesis than in females (Truman et al., 1993).

In summary, the segment-specific regulation of pNBs divisions contributes to the progressive remodelling of the adult CNS. Starting from L3, an increase in the size of the thoracic neuromeres relative to the abdominal ones becomes more and

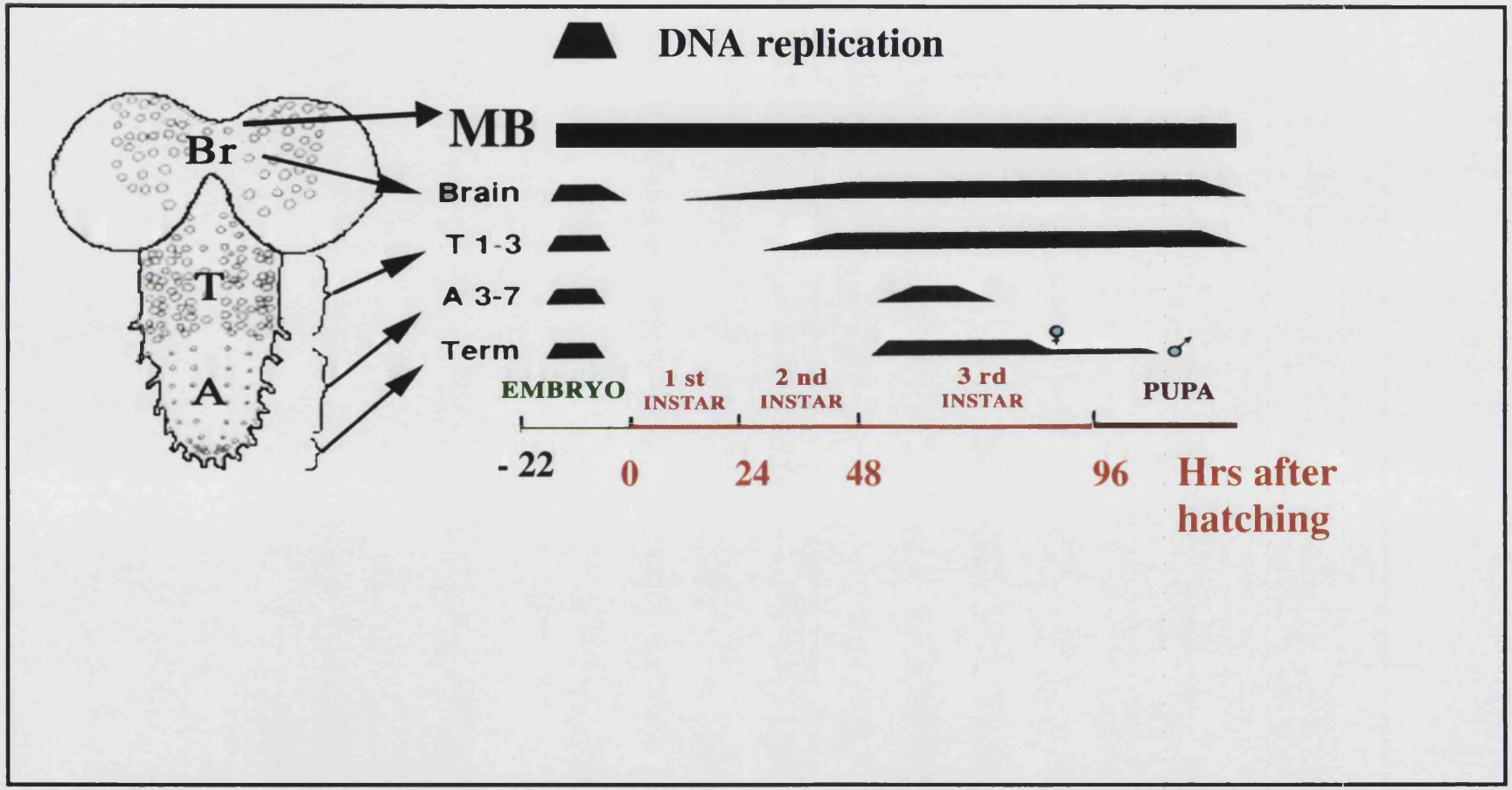


FIGURE 1.6 TIME COURSE OF NEUROGENESIS IN THE LARVAL CNS. Period of DNA replication (black bars) for the pNBs present in Mushroom Bodies (MB), central brain (Br), Thorax (T1-T3) and central abdomen (A3-A7). The terminal NBs (term) show a sex-specific temporal pattern of division. (Adapted from Truman and Bate, 1988).

more evident. This eventually results in an adult CNS where the thoracic segments display a very large size relative to the abdominal segments. This remodelling is presumably important to satisfy the different sensory and locomotor requirements of the flying adult versus the crawling larva. Two general questions rise from the Truman and Bate studies of larval neurogenesis. First, what regulates the time of transition from quiescent to dividing pNB? And second, what controls the time of cessation of pNB divisions?

1) Genes regulating the initiation of pNB divisions

Two genes have been identified that regulate the timing of transition from quiescence to proliferation in the pNBs of the central brain and VNC: *anachronism* (*ana*) and *terribly reduced optic lobes* (*trol*) (Datta and Kankel, 1992; Ebens et al., 1993). *ana* and *trol* mutants exhibit opposite phenotypes relative to the onset of NB division; *ana* loss-of-function mutants display a premature re-entry into the cell cycle, whereas *trol* mutants show a severe drop in the number of proliferating cells in the brain lobes and VNC. *ana* encodes a novel glycoprotein secreted by the glial cells whose activity is required for maintaining NBs in a quiescent status (Ebens et al., 1993). However, since Ana expression appears to remain constant once NB division is activated, a second factor, perhaps encoded by *trol*, may be needed to antagonize its activity. Consistent with this idea, *ana trol* double mutants manifest the same phenotype as *ana* mutants, suggesting that *ana* acts upstream of *trol* (Datta, 1995). Recently, the protein encoded by *trol* has been identified as the fly homolog of vertebrate Perlecan (Voigt et al., 2002; Park et al., 2003). Perlecan is a multidomain heparin sulphate proteoglycan that can influence intercellular signalling by interacting with extracellular matrix proteins, growth factors and receptors. The molecular structure of Trol is consistent with a function in releasing the NB from quiescence either by sequestering secreted proteins such as Ana or by promoting proliferation signals. Cell-cycle effectors have been identified as the candidate downstream targets of the Ana/Trol pathway (Caldwell and Datta, 1998), it is however currently unknown how this mechanism can be differentially regulated along the AP axis to produce the segment-specific features described for the

initiation of pNB divisions. The requirement of the *trol/ana* pathway is believed to regulate only the onset, not the progression of divisions; once the cell cycle has been re-initiated NBs can divide despite the lack of Trol (Datta, 1995).

Hormonal and nutritional cues have also been implicated in reinitiating pNB divisions. Much of this evidence comes from explanted cultures of larval CNS (Truman et al., 1993; Britton and Edgar, 1998; Datta, 1999). Quiescent pNBs explanted from L1 larvae can initiate cell division when cultured in the presence of fetal calf serum or larval extract. The substitution of either supplement with the steroid hormone that promotes moulting events and metamorphosis, 20-hydroxyecdysone (20HE), is also sufficient to re-activate pNB proliferation (Riddiford, 1993; Datta, 1999). Since the early larval NBs do not express Ecdysone Receptors (EcR) (Truman et al., 1994), it is likely that the steroid hormone plays an indirect role in regulating NB re-activation. Nevertheless, this activity lies upstream of *trol* function because cultures of *trol* mutant CNS fail to undergo division following treatment with 20HE (Datta, 1999).

Nutrition is another important factor regulating initiation of pNB divisions. pNBs of L3 Larvae from over-crowded or nutrient-deprived cultures do not enlarge properly and fail to start dividing, but they can be rescued by providing increased food availability. However, once pNB divisions have commenced, starvation does not halt divisions, suggesting that once initiated the mitotic programme becomes independent of nutrition (Truman et al., 1993; Britton and Edgar, 1998). It is currently not known how nutrition can be translated into signals required for pNB re-activation. However, when CNS isolated from starved larvae are co-cultured in the presence of fat body (an important endocrine tissue involved in lipid storage and metabolism) dissected from well-fed larvae, arrested NBs can be re-activated (Britton and Edgar, 1998). With this observation, Britton and colleagues suggested the existence of a novel mitogen derived from fat body. Interestingly, the nutritional regulation of pNB cell-cycle activation seems to be independent from the *ana/trol* pathway as there is NB arrest in nutrient-deprived *ana* mutants.

2) Genes regulating the speed and termination of pNB divisions

Hormonal cues also appear to have a potential role in controlling NB behaviour after the NB has been reactivated. Increased expression of the EcR isoform B1 in L3 pNBs correlates with their main proliferative period (Truman et al., 1994). When the ecdysone puparial peak required for metamorphosis is suppressed by using the temperature-sensitive ecdysone-deficient mutant *ecd*^{1}, NBs proliferate at a slower rate than normal but they eventually terminate dividing according to the normal spatiotemporal pattern and after producing the appropriate number of progeny (Truman et al., 1993). Thus, ecdysteroids seem to influence the speed of the NB cell cycle rather than affecting the final number of progeny they generate. The cessation of NBs divisions, rather than of their initiation, is the major focus of this thesis. As my findings implicate the Hox genes in this process, I will next give an overview of this gene family.

C. HOX GENES AND SEGMENTAL PATTERNING

The Hox/homeotic genes play the primary role in conferring segmental identity, or more generally AP patterning in many animal species (reviewed in McGinnis and Krumlauf, 1992; Carroll, 1995; Mann and Morata, 2000). Hox genes are expressed in different AP domains that are often overlapping (Figure 1.7A). In *Drosophila* negative cross-regulation is also observed where, the Hox gene located in a more posterior segment tend to repress the expression of the one located more anteriorly (Struhl and White, 1985). On the top of this direct transcriptional regulation, phenotypic suppression can also be observed; this is a mechanism whereby, when Hox expression overlaps, the more posterior Hox protein suppresses the function of the more anterior one at the level of the Hox target genes (Gonzalez-Reyes et al., 1992). Hox genes encode transcription factors that bind DNA via a 60 amino acid homeodomain; they are located in chromosomal clusters and expressed along the AP axis in an order that is colinear with their arrangement on the chromosome. In this way, each segment is specifically patterned by the activity of a characteristic subset of Hox genes.

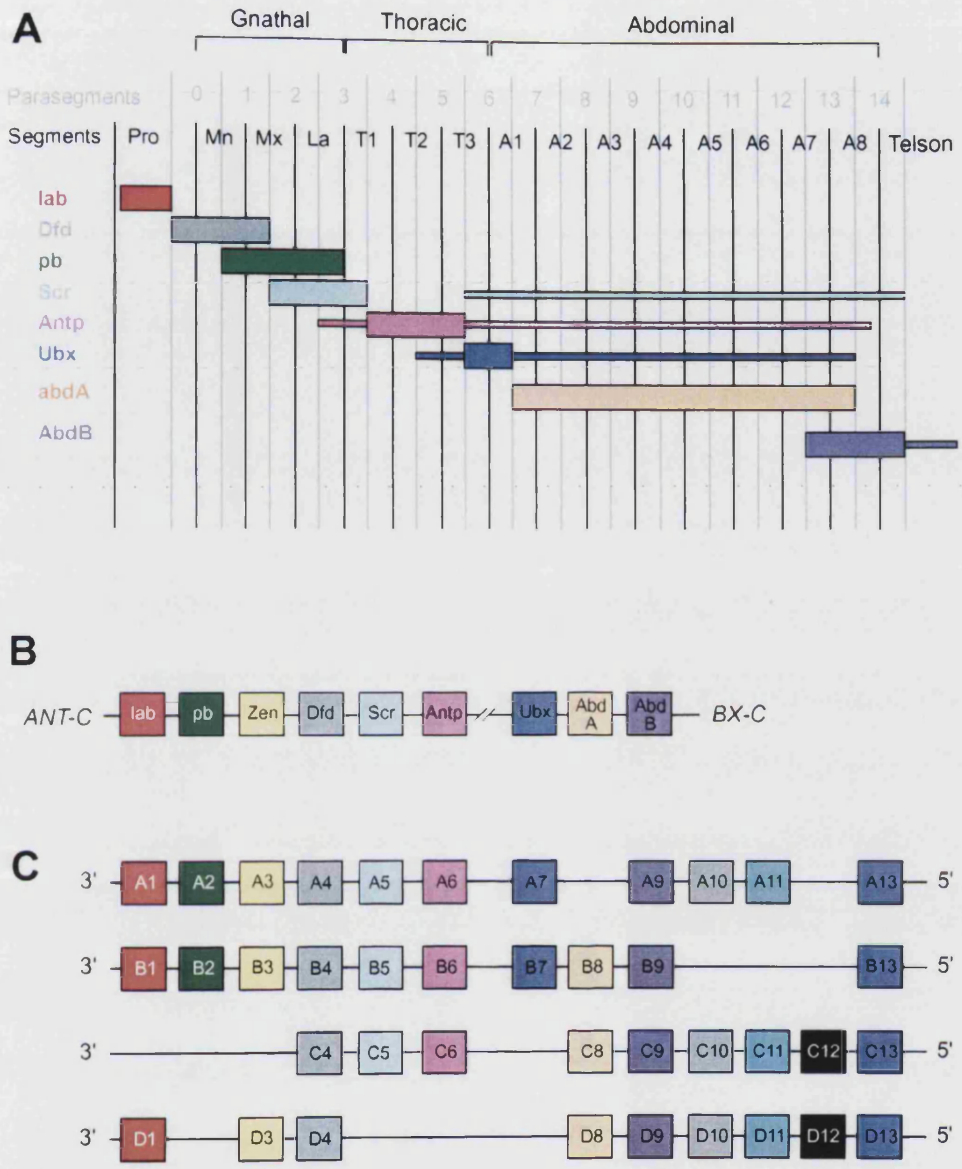


FIGURE 1.7 HOX CLUSTERS AND THEIR EXPRESSION IN THE *DROSOPHILA* EMBRYONIC ECTODERM. (**A**) Domains of Hox expression in the segments/parasegments of a stage 11 *Drosophila* embryo. The levels of expression are schematised as high (large block) or moderate (small block), but do not take into account that levels within a segment are not necessarily uniform. (**B**) Single split Hox gene cluster of *Drosophila* that comprises the Antennapedia and Bithorax complexes. Colours are as in panel A to show the collinear relationship between the position of a Hox gene in the cluster and its domain of expression along the anteroposterior axis. (**C**) The mouse genome contains four incomplete clusters of Hox genes (HoxA, HoxB, HoxC and HoxD) that are located on different chromosomes. Colours are as in panels A and B to indicate paralogous and orthologous relationships.

In *Drosophila*, the Hox cluster is present on the third chromosome and it is split into two complexes: the Antennapedia complex and the Bithorax complex (Figure1.7 B). The genes belonging to the former complex specify the head and the anterior thoracic segments and are: *labial (lab), proboscipedia (pb), Deformed (Df), Sex comb reduced (Scr)* and *Antennapedia (Antp)*. The genes belonging to the Bithorax complex specify the posterior thoracic and abdominal segments and are: *Ultrabithorax (Ubx), abdominal-A (abdA)* and *Abdominal-B (AbdB)*. In Vertebrates, four Hox complexes are present, these are believed to be the result of genome-wide duplications occurring after the evolutionary branching of the common ancestor of arthropods and vertebrates (reviewed in Carroll 2001, Figure 1.7 C).

During the very early stages of *Drosophila* embryogenesis, a complex hierarchy of segmentation genes establishes the segmented body plan and regulates Hox gene transcription in defined AP domains of expression (Simon, 1995; Wolpert et al., 1998, Figure 1.8). The first embryonic AP coordinates are defined by maternal contribution of localized mRNAs deposited into the egg by the mother. The early embryo is a syncytium, meaning that all nuclei are present in a common cytoplasm. In this environment, long-range protein gradients can form along the AP axis. The maternal genes factors, such as *bicoid*, switch on the expression of the first zygotic genes, the gap genes (for example *hunchback*), each of which is expressed in a distinct broad domain. The first sign of molecular segmentation appears with the expression of the pair-rule genes (such as *even-skipped* and *fushi tarazu*). These genes are regulated in two-segment wide stripes by the combined action of the TFs encoded by the maternal and gap genes. Pair-rule genes also encode TFs and these in turn initiate the expression of the segmental-polarity genes (such as *engrailed*). These encode a wide range of proteins such as TFs, secreted molecules and membrane receptors. As described in section 1.2, in the developing CNS, these genes also confer the AP coordinates for specifying NB fate (Skeath and Thor, 2003). Hox genes were first identified because of mutations in these loci causing the transformation of one body part into the identity of another (a homeotic transformation). For example, *Antp* gain-of-function in the most anterior part of the head results in a very dramatic phenotype in which a leg is formed at the place of the

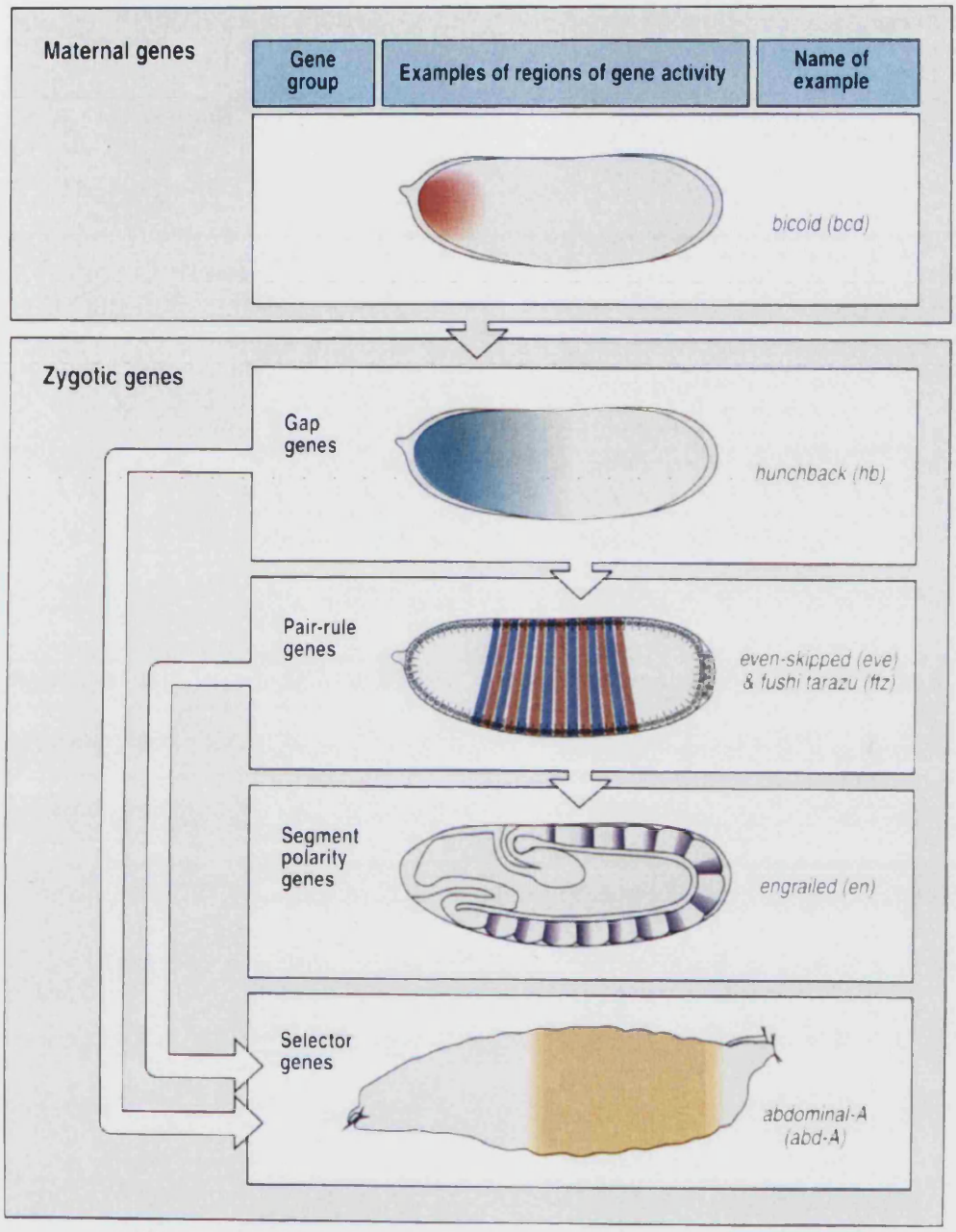


FIGURE 1.8 THE GENETIC HIERARCHY FOR ANTERIOR POSTERIOR PATTERNING AND HOX EXPRESSION DURING DEVELOPMENT. After fertilization, maternal genes laid down in the egg, such as *bicoid,* are expressed and provide the positional information required for the activation of the zygotic genes. Three classes of zygotic genes, the gap genes (for example *hunchback*), the pair-rule genes (*even-skipped* and *fushi tarazu*) and the segment polarity genes (*engrailed*) act along the AP axis. Each of these classes contributes to Hox gene expression (*abdA*). (From Wolpert *et al.*,1998).

antenna (Kaufman et al., 1990). Another very well known homeotic phenotype is consequence of a partial loss-of–function of *Ubx*. This results in the transformation of the posterior compartment of the third thoracic segment (T3), normally carrying a haltere, into the identity of the second segment (T2), where the wings develop, giving rise to a four-winged animal (Lewis, 1963). On the basis of such spectacular phenotypes, in 1975 Garcia-Bellido proposed the "selector gene hypothesis". According to this, Hox genes act within each compartment in a cell-autonomous manner to select between alternative developmental pathways (Garcia-Bellido, 1975). In the case of *Ubx*, its activity selects the developmental pathway for halteres and suppresses the one specifying wings.

1.7 Stable maintenance of homeotic decisions

The early embryonic genetic network set by the maternal and segmentation genes (Figure 1.8) determinates Hox expression only transiently. Nevertheless, the genes belonging to the Antennapedia and Bithorax complexes are required throughout development for the maintenance of the correct fate of many cell types such as the imaginal discs. In addition, it is important that Hox expression is maintained within correct segmental boundaries: for example, ectopic expression of AbdA at late larval stages results in the transformation of A1 into a more posterior identity and produces lethality (Sanchez-Herrero et al., 1994).

Extensive genetic analysis has identified a class of mutations that are not linked to the Hox complexes but do still exhibit homeotic transformations. These correspond to two gene families: genes required for the maintenance of Hox gene activation (the *Trithorax group, trxG*) and those necessary for maintaining the silencing of Hox expression (*Polycomb group, PcG*) (Paro, 1990; Paro, 1993; Pirrotta, 1995; Gould, 1997; Orlando, 2003). In general, mutations in *PcG* genes cause homeotic transformation due to the ectopic expression of Hox genes outside their normal AP boundaries. In contrast, *trxG* mutations cause loss of Hox expression within normal domains. The proteins encoded by these gene complexes are responsible for stabilising Hox expression patterns through many rounds of cell division. This cell-memory mechanism is able to maintain Hox gene expression throughout

development, even though segmentation gene products are no longer present. PcG and trxG appear to exert this function mainly by acting at the level of chromatin. Biochemical and genetic studies indicate PcG proteins exist in at least two separate complexes, the Polycomb Repressive Complex 1, PRC1, and the Extra Sex Comb-Enhancer of Zeste complex, ESC-E(Z). Two complexes have also been characterized for trxG, the Trithorax Acetyltransferase Complex 1, TAC1, and the Brama complex, BRM (Simon and Tamkun, 2002). PRC1 functions by inhibiting the events of chromatin remodelling promoted by BRM and that are required for gene activation (Figure 1.9). PRC1 also recruits proteins that take part to the basal transcription machinery, probably denying their access to promoters. A common way of modulating gene expression is given by covalent modification of the nucleosomes, such as histone acetylation, which activates gene expression. ESC-E(Z) and TAC1 play antagonising roles by exerting histone deacetylase and histone acetyltranferase activities respectively (Simon and Tamkun, 2002). Another covalent modification, histone lysine methylation, is instead implicated in gene silencing and has emerged to be a major player in regulating Hox expression (Cao et al., 2002; Czermin et al., 2002; Muller et al., 2002). In the proposed model, ESC-E(Z) mediates Histone 3 (H3) methylation and this in turn recruits and facilitates binding of PRC1, resulting in cooperation towards Hox gene silencing (Cao et al., 2002; Simon and Tamkun, 2002).

1.8 Versatility of Hox genes

In his "selector genes hypothesis" Garcia-Bellido predicted that the Hox genes do not act directly to specify the morphological differences between segments but instead they control a battery of numerous subordinate target genes (the "realizator genes") that carry out diverse cellular functions required for the various patterns of cell differentiation (Garcia-Bellido, 1975).

In the past years, many studies have been carried out in order to characterize the downstream targets of Hox genes and thus to identify the molecular links between these TFs and their effectors determining the differences in morphology between

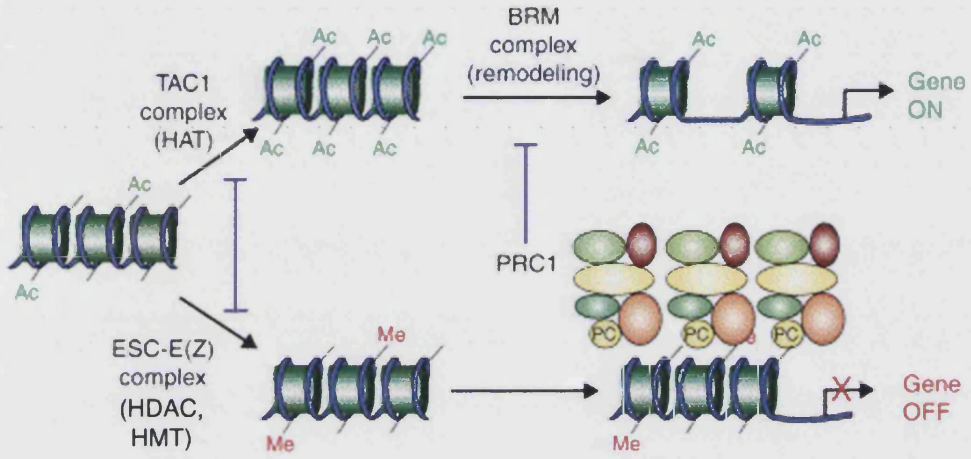


FIGURE 1.9 MODEL FOR MECHANISMS OF TRXG AND PCG CHROMATIN COMPLEXES. Representation of a nucleosome array at a target Hox gene under trxG/PcG control. The trxG activating pathway is shown at the top, the PcG repressive pathway at the bottom. Ac: acetylation; Me: methylation. TAC1 has Histone AcetylTransferase (HAT) activity whereas ESC-E(Z) has Histone DeACetylase (HDAC) activity. ESC-E(Z) also recruits a Histone MetylTranferase (HMT). The recruited PRC1 (shown as a big protein complex including Polycomb, Pc) and BRM complexes have opposite roles in nucleosome remodelling, leading to the respective outcomes of gene silencing and gene activation . Adapted from Simon and Tamkun, 2002

serially-homologous structures (Graba et al., 1997; Mannervik, 1999).

Despite these efforts, the number of Hox target genes reported until now is far too small to account completely for the numerous developmental roles of any Hox gene. In fact, it is believed that Hox genes act by controlling (directly and indirectly) the expression of a large proportion of all genes (Akam, 1998).

Several different approaches have been used to search for Hox targets: for *Antp*, a series of *LacZ* enhancer trap lines were generated to select for sites in the genome where, upon misexpression of *Antp*, there is segment-specific expression of β-gal. This led to the identification of several target genes whose expression differed from leg to antennal imaginal discs (Wagner-Bernholz et al., 1991). Another method, based on a subtractive hybridization technique was developed for the isolation of genes transcribed following Ubx mis-expression (Feinstein et al., 1995). Neither of these methods distinguishes between direct and indirect targets. However, molecular techniques such as immunopurification of chromatin fragments using anti-Ubx antibodies or a yeast one-hybrid system were instead adopted in order to identify DNA sequences directly bound by Ubx (Gould et al., 1990; Mastick et al., 1995). A description of the identified target genes is beyond the scope of this thesis, however, it is important to notice that, these different methods, have provided evidence that Hox genes are very versatile TFs, regulating the expression of many diverse target genes, among which are those encoding TFs, adhesion and signalling molecules.

More recently, a more biologically focused approach for the identification of *Ubx* target genes was carried out (Weatherbee et al., 1998). This focused on the analysis of a well-characterized developmental process, the formation of wings. Many of the signalling molecules and TFs required for the development of wings are known, and Weatherbee and colleagues asked which ones of these are repressed by *Ubx*. The authors found that many of the genes required for the initial stages of wing patterning are downregulated by Ubx in the haltere. Moreover, Ubx independently and selectively regulates genes that act at many levels of regulatory hierarchies to inhibit wing formation (Weatherbee et al., 1998). It has been calculated that, in the haltere discs, Ubx must be controlling no less than 30 genes (Akam, 1998). Finally, these studies indicate that, as opposed to TFs that are specialised in controlling one

particular cell-fate or behaviour, Hox factors appear to be very versatile proteins. These TFs modulate a wide range of morphological processes exerting their control on target genes in a highly context-dependent manner at very different levels.

How then do different Hox proteins achieve specific control of their numerous target genes? In addition to subtle DNA-binding preferences, an important contribution to specificity is given by interaction with cofactors, such as the homeodomain proteins Extradenticle (Exd) and Homothorax (Hth). Reduced levels of Exd cause homeotic transformation, largely without interfering with Hox gene expression (Rauskolb et al., 1993). Biochemical and genetic experiments have shown that Exd can co-operatively bind Hox proteins to DNA (Mann and Chan, 1996). This results in the length of the consensus binding sequence being increased from approximately 4 specific nucleotides for the Hox protein alone up to 8-10 nucleotides for the Hox/Exd heterodimer. *hth* mutants have been shown to have a similar phenotype to *exd* mutants (Jurgens et al., 1984; Rieckhof et al., 1997). This is because Hth acts by regulating Exd nuclear localization. Exd contains both nuclear import and export signals and Hth binds to it in the cytoplasm, unmasking its nuclear localization sequence, thus shifting the import/export balance in favour of import (Rieckhof et al., 1997). Once in the nucleus Hth can fulfil a second function by acting as a TF and taking part in forming a Hox/Exd/Hth ternary complex (Ryoo et al., 1999). The cooperation with these cofactors alone cannot however explain the versatility of Hox proteins, the mechanism underlying this is explained in the following section.

1.9 Hox activity is cell-context dependent

The original "selector gene hypothesis" (Garcia-Bellido, 1975) implied a uniform and continual requirement for Hox gene activity within a compartment as a whole. In sharp contrast with this definition recent works have shown that each compartment or segment contains many different Hox-dependent cell-fates and behaviours, patterned on a cell-by-cell basis and not necessarily in a cell-autonomous manner (Brodu et al., 2002; Rozowski and Akam, 2002; Bello et al., 2003). The present view is that Hox genes act according to these cell-specific contexts to "micromanage"

diverse cell fates and behaviours and that the summation of a mosaic of Hox activities constitutes what has been termed segmental identity (Akam, 1998; Lohmann and McGinnis, 2002). Studies that focus on Hox functioning at the single cell level are thus likely to be more tractable in terms of identifying all relevant Hox target genes required for a particular process.

Rozowski and Akam have dissected the role of Ubx in the modulation of the pattern of the leg mechanosensory bristles (Rozowski and Akam, 2002). These structures derive from SOPs singled out from a proneural cluster. During bristle development, SOPs divide to generate two second-order precursors. One of these will give rise to the bristle shaft and socket, whereas the other will divide to generate the neuron sheath and glia cell. The authors concentrated on particular bristles that differ between the second and the third thoracic segment (T2 and T3). These are the sternopleural and the apical bristles, formed exclusively in the legs of thoracic segment T2, and the pre-apical bristle, which is present in both segments, however its shaft is stout in T2 but fine in T3. By inducing *Ubx* mutant clones at different developmental times, the authors demonstrated that this Hox gene can act at several different steps of bristle formation rather than exclusively at the early stage of patterning the leg disc (Figure 1.10A). *Ubx* is required in the early SOPs to inhibit the formation of the T3 sternopleural bristle, later, it is necessary to abort the specification of the second order precursors of the T3 apical bristle. *Ubx* is then required to determine the shaft difference between T2 and T3 preapical bristles. These findings show that *Ubx* regulates different bristle programmes in very different manners, depending on cell context (Rozowski and Akam, 2002).

Brodu *et al* have adopted a similar single-cell approach and focused on a group of secretory cells, called the oenocytes. These cells only form in the abdominal segments and require a non-cell autonomous input from *abdA/exd* (Brodu et al., 2002). The Hox protein and its cofactor, present in a neighbouring SOP (called C1), are needed for expression of Rhomboid, the protease that is required for

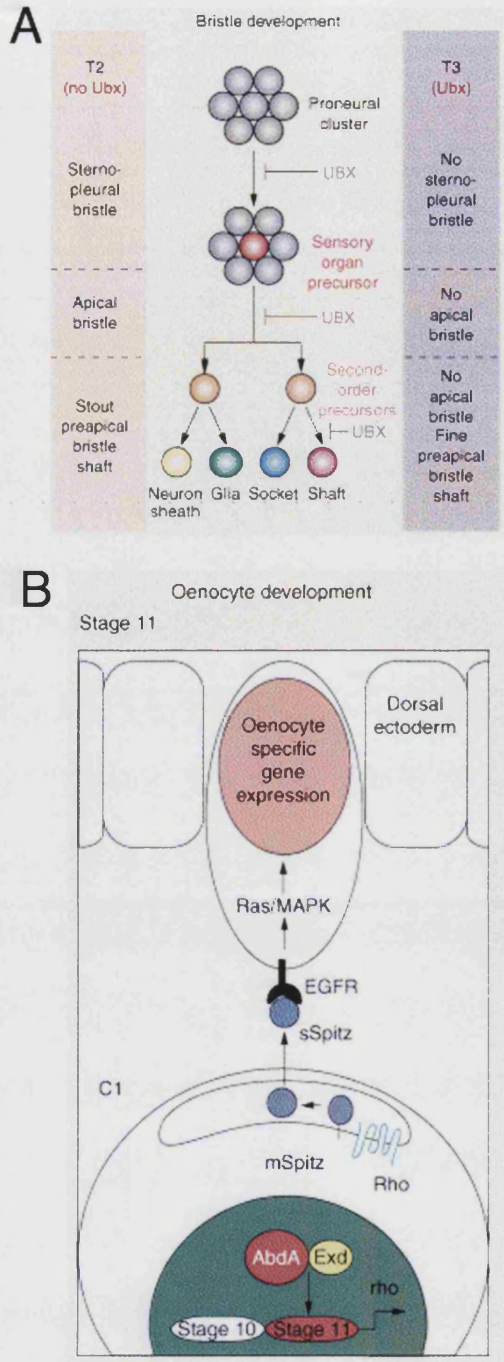


FIGURE 1.10 HOX FUNCTIONS IN TWO DIFFERENT CELL TYPES
(**A**) Schematic diagram of T2 and T3 bristle differentiation. In the T3 leg imaginal disc Ubx suppresses bristle formation at different levels. Its presence results in the complete block of the development of the sternopleural and the apical bristles. In a different cell context, the preapical bristle, Ubx modifies the morphology of the shaft (fine versus stout). When Ubx is absent, as in the T2 disc, the default developmental program generates the sternopleural and apical bristles and the stout preapical bristle shaft. (**B**) Scheme of oenocyte induction by *abdA*-mediated signalling. *abdA* and *exd* are required in the chordothonal organ precursor (C1) for *rhomboid* (*rho*) transcription and thus for keeping secreted Spitz (sSpitz) available to activate the Epidermal Growth Factor Receptor (EGFR) in the apposing dorsal ectoderm. The presence of a specific oenocyte prepattern in the ectoderm, leads to the linkage of a generic signal transduction pathway (Ras/MAPK) to a highly specific oenocyte differentiation programme. (Experimental data from Rozowski and Akam, 2002 and Brodu *et al*, 2002. Adapted from Lohmann and McGinnis, 2002).

processing Spitz ligand. This protein binds to the Epidermal Growth Factor Receptor (EGFR) on the cells of the dorsal ectoderm, activating a Ras/MAPK signalling pathway leading to oenocyte induction (Figure 1.10B). Importantly, ectodermal cells require the expression of specific factors, such as Spalt, for their specific response to EGFR activation. In the absence of such a specific context, they fail to adopt the oenocyte fate (Elstob et al., 2001). These studies indicate that the very diverse cell contexts within a single segment allow Hox genes to function in a versatile manner.

During my studies I have applied this single-cell approach to the analysis of the developing NBs and their progeny neurons; as I will describe, these cells also respond in different ways to Hox expression.

D. ROLE OF HOX GENES DURING NEUROGENESIS

Given the progressive remodelling of the fly CNS around segment-specific features, researchers have been prompted to address the role of Hox genes during neurogenesis (reviewed in Doe and Scott, 1988). In this section, I give an overview of the findings concerning the role of the Hox genes in patterning the behaviour of *Drosophila* NB lineages.

1.10 Hox gene expression in the developing CNS

The expression patterns of all of the Hox genes have been extensively analysed in the CNS (see Doe and Scott, 1988, for early studies and Hirth et al., 1998, for a detailed description of Hox expression in the embryonic brain and VNC).

The gene *Antp* is expressed in a broad domain from PS3 towards the end of the VNC, with high levels of expression in PS4-5; *Ubx* expression extend from PS5 to PS12, with the highest expression in PS6. The *abdA* domain spans from PS7 to PS13. Finally, *abdB* is expressed from PS11 to the end of the VNC, with the most intense localization in PS13 (Figure 1.11). The pattern of neural Hox expression can be very dynamic throughout development (Doe and Scott, 1988; Prokop et al., 1998; Bello et al., 2003). For example, upregulation of *Ubx* expression is observed, *in vitro* and *in vivo,* in the maturing neurons of L3 larvae and prepupae

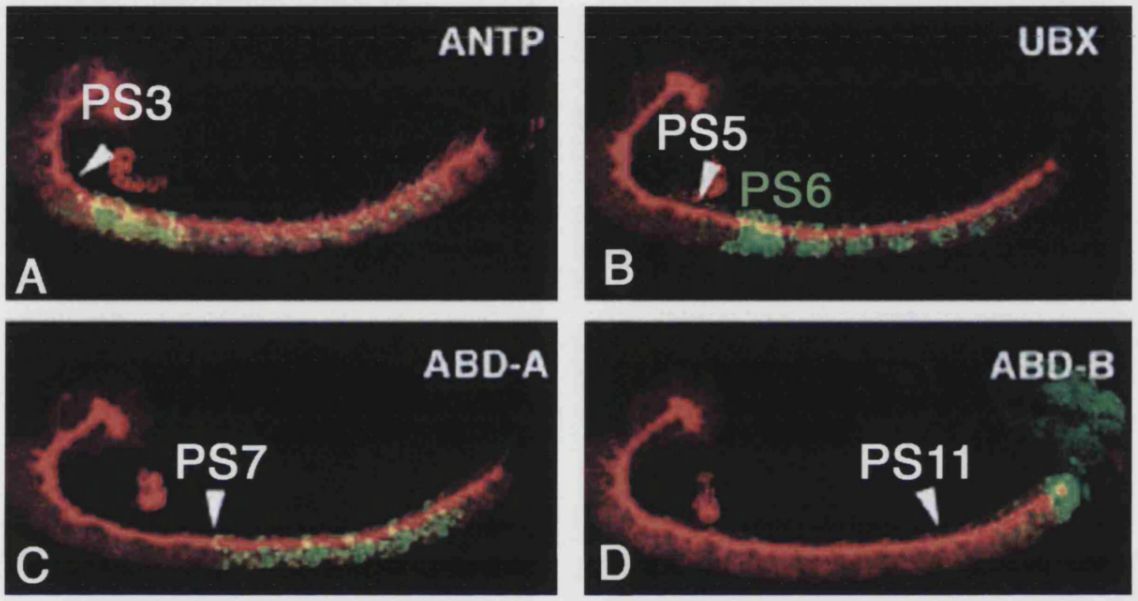


FIGURE 1.11 *ANTP* AND *BITHORAX COMPLEX* GENE EXPRESSION IN THE EMBRYONIC CNS. Lateral view of stage 14 embryos stained with antibodies that reveals the brain and VNC (red), and (in green) Antp (**A**), Ubx (**B**), AbdA (**C**) and AbdB (**D**). Arrowheads indicate the most anterior border of expression along the AP axis. The highest domain of expression of Ubx (PS6) is also indicated. *Ubx* expression differs in the mesoderm (PS6-12) and in the epidermis (PS5-13). (Adapted from Hirth *et al.*, 1998).

(Truman and Bate, 1988; Glicksman and Truman, 1990; Bello et al., 2003, see also Figure 1.13 and Figure 4.2). NBs also show a pattern of Hox expression that varies with time. During the early stages of embryogenesis, from stage 8 to stage 12, the actively-dividing NBs express variable levels of *Ubx* and *abdA*; after these stages, the embryonic NBs are believed to be no longer Hox-positive (Prokop et al., 1998). However, at late larval stages, Hox expression can be transiently re-activated in the pNBs. This has been shown for *abdA* in the abdominal lineages (vm, vl and dl) of the mid-L3 larval CNS (Bello et al., 2003). Bello and colleagues found that abdominal pNBs undergo a burst of AbdA expression 60-66 hrs after hatching. At the moment of this burst, the cells are dividing since they often express phosphorylated Histone H3, marking cells in M-phase (Figure 1.12).

1.11 Hox functions in neurogenesis

What is the function of the dynamic Hox patterns of expression during larval neurogenesis? Relatively little is known about the roles of Hox genes in differentiated neurons. For example, the significance of *Ubx* expression within the maturing late L3 neurons has not been addressed. Cultured CNS studies do however suggest a mechanism underlying this upregulation. Glicksman and Truman have investigated the role of the L3 peak of the steroid hormone 20HE. When the larval CNS is explanted before the puparial ecdysteroid peak, the increase of neuronal Ubx expression does not occur. However, addition of exogenous 20HE to the culture media rescues the expected rise (Truman and Bate, 1988; Glicksman and Truman, 1990). Although the role of Hox genes within imaginal neurons has not been well characterized, some progress has been made for the NBs of the embryonic and post-embryonic CNS. As described in section A of this Chapter, segment-specific differences along the AP axis of the VNC are first set up very early in embryogenesis, when NB lineages behave differently according to their location and additional NB divisions are often observed in the thoracic neuromeres relative to the

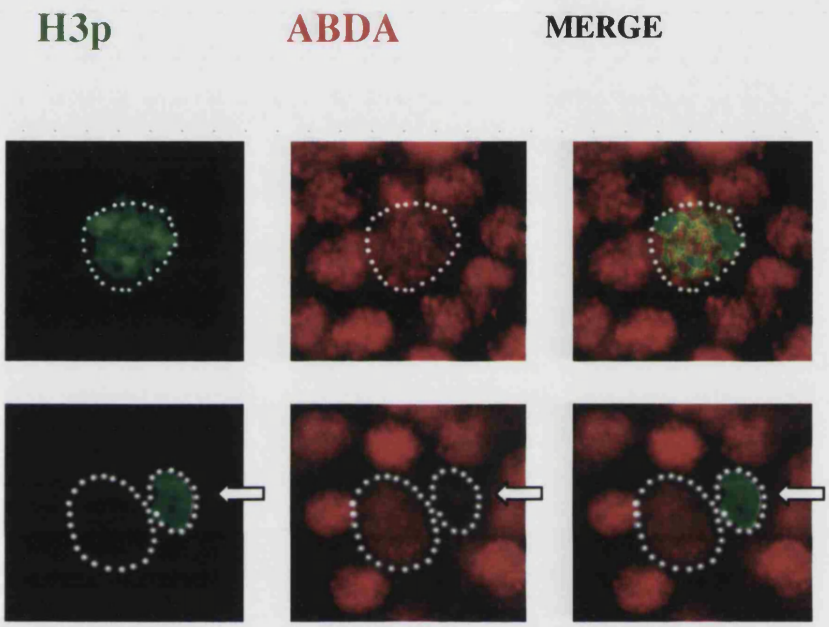


FIGURE 1.12 BURST OF ABDA IN MID-L3 ABDOMINAL PNBS.
60-66 pNBs. The pNBs (big dotted circle) and the GMC (smaller dotted circle and arrow) are labelled with an antibody recognizing mitotic cells, anti 3Hp (green), The pNB expressed AbdA (red). Notice that the GMC is not AbdA positive. (Adapted from Bello *et al.*, 2003).

abdominal ones (Prokop and Technau, 1994; Bossing et al., 1996; Schmidt et al., 1997; Prokop et al., 1998; Schmid et al., 1999). The role of *Ubx/abdA* in the specification of the different variants of the NB1-1 lineage has been analysed in detail (Prokop and Technau, 1994). This NB gives rise to an additional number of progeny in the thorax relative to the abdomen and the activity of *Ubx/abdA* is required for the generation of glia by the abdominal variant of this lineage. Ectopic induction of either *Ubx* or *abdA* overrides thoracic NB1-1 specification and suppresses the production of thorax-specific interneurons and motorneurons. In another study, Prokop and colleagues studied the pattern of BrdU incorporation in late embryos that lack *abdA* function and observed that, in the central abdomen, cells divide in patterns reminiscent of the thorax. *abdA* activity is in fact needed to repress divisions in some lateral abdominal NBs. Similarly, the authors found that *Antp* functions by suppressing replication in ventral thoracic NBs (Prokop et al., 1998).

Large BrdU incorporating clusters are observed in the abdominal neuromeres of *abdA* hypomorphic larvae, indicating that AbdA exerts a function limiting pNBs divisions. Furthermore, ectopic pNBs are found in the abdomen of these mutant larvae, presumably because extra NBs persist in the abdominal neuromeres of *abdA* mutant embryos (Prokop et al., 1998). This has suggested that this Hox gene controls the 30 to 3 reduction in the number of NBs compared to pNBs (Prokop et al., 1998). This control appears to be linked to cell-death in late embryonic abdominal NBs as embryos lacking the pro-apoptotic gene *reaper* develop into larvae that, similar to *abdA* mutants, carry ectopic abdominal pNBs (White et al., 1996; Peterson et al., 2002). Initially it was thought that AbdA was no longer expressed in the NBs of the late embryo and larva. To explain this paradox, Prokop *et al* suggested the existence of a cell-memory mechanism. According to this hypothesis, the presence of AbdA within the early embryonic NBs is sufficient to imprint a later programme of proliferation control (Prokop et al., 1998). However, in the larval CNS, a causal relationship between the burst of AbdA expression (Figure 1.12) and cell death has been demonstrated (Bello et al., 2003). By using TUNEL staining, positively labelling apoptotic cells, Bello *et al* show the presence of a burst of cell-death in the

abdominal pNBs of mid-L3 larvae. The authors have then taken advantage of a very elegant genetic mosaic technique and demonstrated a direct link between the burst of AbdA in the pNBs and induction of cell-death. The methodology used, Mosaic Analysis with a Repressible Cell Marker (Lee and Luo, 1999), allows positive labelling of wild-type or mutant clones and it will be extensively described in Section 4.1 (also see Figure 4.1 and Material and Methods). The size of L3 wild-type thoracic clones (meaning the number of cells per clone) is large, containing approximately 60 cells. This is due to extensive mitotic activity of the pNB. In contrast, abdominal clones are rare, contain approximately 6 cells and lack the pNB by late L3. In *abdA* mutant clones the pNB persists into late L3, actively dividing as indicated by the abnormally large clone size. Furthermore, *DfH99* mutant clones in which the three pro-apoptotic genes *grim hid* and *reaper* are deleted (White et al., 1994), phenocopies *abdA* mutant clones. Also, premature induction of a burst of AbdA using a heat-shock promoter results in the premature loss of pNBs. The ability of AbdA to induce cell death in the pNBs is further demonstrated by showing that ectopic expression of AbdA or other Hox proteins in the thoracic NB lineages results in a dramatic reduction of clone size and pNB elimination. Since ectopic Hox expression in thoracic *DfH99* clones fails to alter clone size or the presence of the pNB, Hox genes probably lie upstream of apoptosis (Bello et al., 2003).

In summary, the burst of AbdA expression observed in mid-L3 NBs limits the proliferative potential of these abdominal precursors by inducing apoptosis. Thoracic pNBs do not express *Ubx* or any other Hox gene at these stages and thus maintain the ability to divide throughout larval life (Figure 1.13). Apoptotic activity of Hox genes has also been described in another *Drosophila* context, where *Deformed* and *Abdominal-B* shape segmental grooves by inducing cell-death through the transcriptional activation of *reaper* (Lohmann et al., 2002).

Finally, it is important to point out two issues arising from the findings of Bello *et al.* The first issue concerns the mechanism of dynamic Hox gene regulation in pNBs. Absence of Hox expression is required for the maintenance of neuronal proliferation in the thoracic neuromeres, whereas in the abdominal pNBs expression of AbdA is

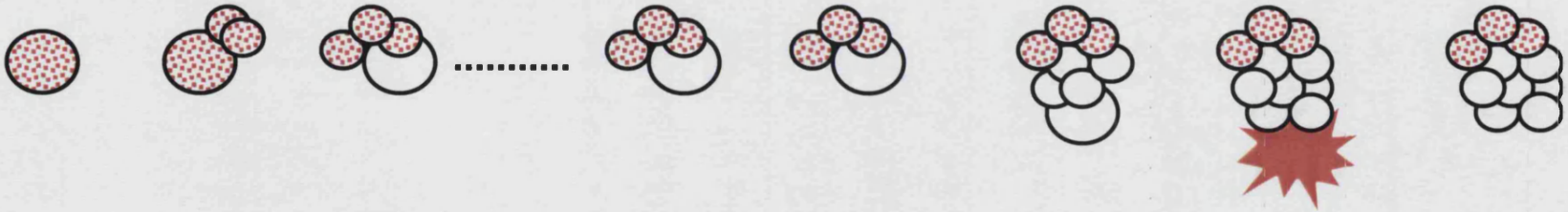
FIGURE 1.13 TIME COURSE OF *UBX* AND *ABDA* EXPRESSION WITHIN NB LINEAGES.
Schematization of a thoracic (**A**) and a central abdominal (**B**) neuroblast lineage. The NB is represented by a large circle and the progeny neurons by the smaller circles. Embryonic and larval expression of Ubx (green) and AbdA (red) are represented respectively by dotted and uniform shading. The different larval stages are indicated: first instar larva (L1), second instar larva (L2), early, mid and late third instar larva (eL3, m-L3 and lL3). The embryonic pattern of Hox expression is similar in the thorax and abdomen, with early dividing NBs and some of their progeny neurons expressing Ubx or abdA. Late in embryogenesis the thoracic NB no longer expresses Ubx, whereas it is upregulated in maturing imaginal neurons (**A**). The burst of AbdA expression at mid-L3 (60-66 hrs) causes NB apoptosis (starburst) (**B**). Dotted lines at the embryo-to-larva transition indicate the uncertainty in tracing individual identified NBs through this period. Data from Prokop *et al* 1998, Bello *et al*, 2003 and A. Gould, unpublished.

A Thorax, PS 6 Ubx

embryo — larva

L1 L2 eL3 m-L3 lL3

B Central abdomen AbdA



required to limit proliferation. A second, very interesting issue concerns cell context: whereas Hox expression in the pNBs triggers apoptosis this does not happen in two other neural contexts, early embryonic NBs and neurons (Figure 1.13)My thesis examines this issue of cell context and its relationship to segment-specific neurogenesis.

In Chapter 3, I provide evidence that NB sublineage switching can be modulated segment to segment and may involve differences in the sequence of TF series.

In Chapter 4, I use the MARCM strategy to study factors regulating the time window of NB divisions in the thorax.

In Chapter 5, I identify a cell-context factor required for the late pNB-specific response to AbdA expression.

CHAPTER 2 MATERIAL AND METHODS

CHAPTER 2 MATERIALS AND METHODS

2.1 Drosophila stocks and crosses

Flies were maintained on standard cornmeal/yeast/agar medium at 25°C. Unless indicated otherwise, fly stocks were obtained from the Bloomington stock centre.

When required, *yellow white (yw)* flies were used as a control strain. The following mutant stocks were used: $hth^{64\text{-}1}$, a strong hypomorph of *hth* (Kurant et al., 1998); svp^{1} (Mlodzik et al., 1990), Psc^{Df2}, Asx^{xF23}, Pcl^{XM3} (the combined mutations are referred to as *3Pc*). Two different *grh* alleles were used: grh^{B37} (Bray and Kafatos, 1991), affecting both the epithelial and neural isoforms and the CNS-specific, larval viable, grh^{370} (Uv et al., 1997). The deficiency $Df(2R)Pcl^{7B}$ that removes the *grh* locus was balanced over the *Cyo-GFP* chromosome.

The presence of usp^{3} true loss of function on the *FRT19A,* usp^{3} chromosome was confirmed by complementation testing with usp^{4}. The following chromosomes were used for the mis-expression experiments: *sca-GAL4* and *hsabdA* (II chromosome); *UAS-hth,* and *UAS-p35* (III chromosome). *UAS-hth,* $hth^{64\text{-}1}$ and *hsabdA,* grh^{370} were generated by meiotic recombination. The crosses for the generation of the *hsabdA,* grh^{370} stock are schematised in Figure 2.1.

For the induction of wild type and usp^{3} MARCM clones (X chromosome) the following stocks were used: *FRT19A, tub-GAL80, hsFLP; UASnlsLacZ, UASCD8::GFP; tubGAL4. FRT19A,* usp^{3}*; +;* $\lambda 10Tb$. $\lambda 10Tb$ is a rescue transgene carrying a wild type copy of the *usp* gene, thus providing usp^{+} Tb larvae as an internal control (Lee et al., 2000a).

For the *3Pc* and grh^{B37} clones (II chromosome, right arm) three stocks were used: *elavGAL4, hsFLP; FRTG13, tubP-GAL80; UAS-nlsLacZ, UASmCD8::GFP. FRTG13,* grh^{B37} and *FRTG13, 3Pc.*

For the svp^{1} clones (Chromosome III, right arm) three stocks were used: *ElavGAL4, hsFLP; UASnlsLacZ, UAS-CD8::GFP; FRT82B, tubP-GAL80. FRT82B* and *FRT82B,* svp^{1} .

For more detailed descriptions of genotypes and alleles see Flybase http://flybase.bio.Indiana.edu/ and Table 2.1

FIGURE 2.1 GENERATION OF hsabdA, grh^{370} / CyO-GFP LINE

w[+]; hsabdA/CyO; + X w[+]; grh^{370}/CyO-GFP ; +

↓

Females non-CyO selected

hsabdA/grh^{370} X w; CyO/GlaBc; +

↓

Single CyO males

(hsabdA) , (grh^{370})/ CyO X w[+]; DfPcl/CyO-GFP; +

↓

3 twin vials
N1. N2. N3

SINGLE CROSSES (N=107) and triplicated

N.1= select vials with all balanced progeny: **A** grh^{370}/CyO-GFP and DfPcl/CyO

and **B** hsabdA, grh^{370}/CyO-GFP and DfPcl/CyO

N.2= vials heat-shocked when progeny at embryonic stage 9 and at L3 (1hr, 37°C)

➡ record 15% vials with only GFP-negative flies (**B**)(heat shocked hsabdA dies at pupal stage)

N.3= GFP-positive recombinants selected from twins of N.2 recorded vials

w[+]; hsabdA, grh^{370}/ Cyo-GFP; +

TABLE 2.1 DESCRIPTION AND ORIGIN OF FLY STOCKS

Definition	Full genotype	Origin
$hth^{64\text{-}1}$	y w; +; $hth^{64\text{-}1}$/TM6, Tb, Hu p{w[+]abdA-lacZ}	Kurant *et al,* 1998
grh^{B37}	+; dp cn grh^{B37} bw/CyO; +	Bray and Kafatos, 1991
grh^{370}	+; b pr cn grh^{370} bw/CyO-GFP	Uv *et al,* 1997
DfPcl	+; $Df(2R)Pcl^{7B}$/CyO; +	Bloomington BL 3064
usp^4	usp^4/FM7a; +; +	Bloomington BL 4660
3Pc	yw; $Psc^{Df(2)}$, Asx^{XF23}, Pcl^{XM3}/SM1, CyO; +	B. Bello
svp^1	+; +; svp^1/TM3 Sb	Mlodzik *et al,* 1990
sca-GAL4	w; sca-Gal4; +	Bloomington BL 6479
hsabdA	+; hsabdA/CyO; +	G. Struhl
Uashth, $hth^{64\text{-}1}$	y w; +; Uas-hth, $hth^{64\text{-}1}$/ TM3	Recombinant from $hth^{64\text{-}1}$ and UAS-hth
Uas-p35	w; +; p{w^+ Uas-p35}BH2	Bloomington BL 5073
hsabdA, grh^{370}	+; hsabdA, grh^{370}/CyO-GFP; +	Recombinant from grh^{370} and hsabdA see Figure 2.1

FRT19A	w, p{neoFRT19A}; +; +	Bloomington BL 1740
FRT19A, usp^3	FRT19A, usp^3; +; λ10Tb/TM3	Lee *et al,* 2000
FRTG13	W; p{w^+FRTG13}42B	Bloomington BL 1956
FRTG13, grh^{B37}	w;p{w^+FRTG13}42B, grh^{B37}/CyO;+	Recombinant from FRTG13 and grh^{B37}
FRTG13, 3Pc	w;p{w^+FRTG13}42B, Psc^{Df2}, Asx^{XF23}, Pcl^{XM3}/SM1, CyO	Recombinant from FRTG13 and 3Pc (B. Bello)
FRT82B	+; +; p{neo,ry^+, FRT82B}, ry^{506}/TM3	Bloomington BL 2035
FRT82B, svp^1	+;+;p{neo,ry^+, FRT82B}, svp^1, ry^{506}/TM3	Recombinant from FRT82B and svp^1 (B.Bello)
MARCM Driver stock (X chromosome)	FRT19A, tubP-GAL80, hsFLP, w; UASnls-LacZ, UAS-CD8::GFP/CyO; tubP-GAL4/TM6, Tb, Hu	Recombinant (B.Bello)
Driver stock (2 R chromosome)	elavGAL4, hsFLP; FRTG13, tubPGAL80/CyO; UASnls-LacZ, UAS-CD8::GFP/TM3, Sb	Recombinant (B.Bello)
Driver stock (3 R chromosome)	elavGAL4, hsFLP; tubP-GAL4/CyO; FRT82B, tubP-GAL80/TM6 Tb, Hu	Recombinant (B.Bello)

2.2 Generation of THB-GAL4 transformants

For the construction of *pWHGAL4*, the GAL4 coding sequence was excised from *pGaTB* (Brand and Perrimon, 1993) as a SpeI Klenow filled/BamHI fragment. This was subcloned into vector *pWHZ* (gift from B.Bello) prepared by PstI T4 DNA polymerase blunted/BamHI restriction to exchange the *LacZ* sequence for *GAL4*. The BamHI hsp70 basal promoter fragment, obtained from *pSKTH*, was finally subcloned into this construct. The final *pWHSpeGAL4* vector was generated by inserting an oligonucleotide containing the SpeI site (GGACTAGTGGTAC) into the BamHI site of pWHGAL4. To target GAL4 expression in a THB-specific manner, different fragments of the *hb* promoter were subcloned into *pWHSpeGAL4*. The neural specific XbaI 4Kb DNA fragment was excised from the *HZA* DNA construct (Margolis, 1992). From this, the BglII 2.8 Kb DNA fragment and the BglII/XbaI 1.2 Kb DNA fragment were obtained. All three fragments were then cloned into SpeI, BglII or BglII/SpeI cut *pWHSpeGAL4* to generate the *p4.0hbGAL4*, *p2.8hbGAL4* and *p1.2hbGAL4* constructs respectively (Figure 2.2).

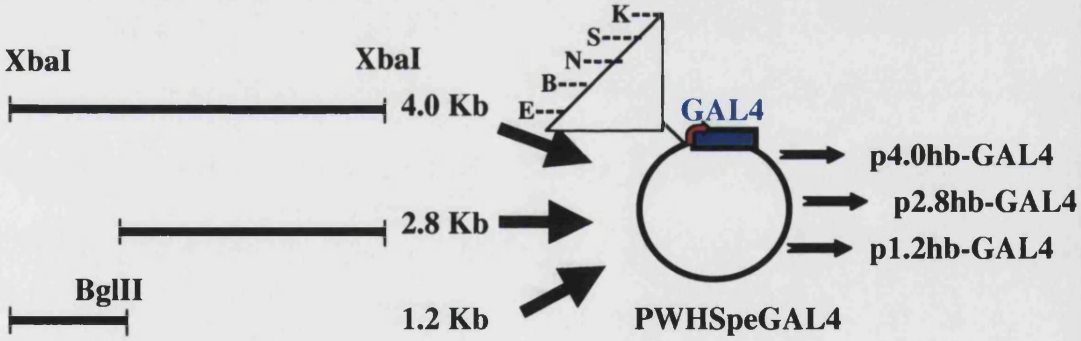


FIGURE 2.2 GENERATION OF HB-GAL4 CONSTRUCTS.
The XbaI cut 4 Kb fragment, the 2.8 Kb BglII/XbaI fragment and the 1.2 Kb BglII fragment were each cloned into pWHSpeGAL4 construct to generate p4.0hb-GAL4, p2.8hb-GAL4 and p1.2hb-GAL4 respectively. The polylinker region of the pWHSpeGAL4 construct is indicated. E: EcoRI; B: BglII; N: NotI; S: SpeI (XbaI compatible); K: KpnI. Red arrow indicates the initiation of GAL4 (blue) transcription unit.

The three vectors thus obtained were amplified in DH5α competent cells (Life Technologies) and then extracted and purified using a Maxi-prep kit (Qiagen).

Fly transformation was carried out using standard procedures (Spradling, 1986) with the following modifications. The injection mix consisted of a transposase source, 100ng/μl of pWC-TURBO helper DNA and 300ng/μl of construct DNA, made up to volume with 0.1X PBS. Host embryos were collected on yeasted grape juice-agar plates from yw flies in a population cage. Following an approximately 45 min collection at 25°C, sodium hypochlorite (50,000ppm) was poured onto the plates and the embryos dechorionated for 2 min. The contents of the plate were poured into a mesh basket and the embryos washed thoroughly with water. The mesh was removed from the basket, the embryos were aligned, all with the same AP orientation, along a cut made in the agar and then transferred onto a line of dried glue on a slide (glue was made by solubilising the adhesive from Scotch double-sided sticky tape in heptane). Embryos were desiccated for 3 min in a box containing Silica gel (BDH) before covering them with 10S Voltafel oil (Elf Atochem) and injecting them. Injectants were incubated at 18°C in a moist box and upon hatching were transferred into a food vial. Mapping of transgene insertion in flies to particular chromosomes (see Table 3.2) was performed according to J. Greenspan (J.Greenspan, 1997).

2.3 Rearing and staging of larvae

Embryos staging is according to J.A. Campos-Ortega and V. Harterstein (Campos-Ortega and Hartenstein, 1997). Larval staging is given in hours after hatching (0 hr). Mid-L3 larvae were harvested at 70-74 hr. Late-L3 larvae at 94-98 hr. For the analysis of mutant larvae that have a different rate of development than the wild type (svp^1 mosaics and grh^{370} homozygous), distinction between mid-L3 and late-L3 was performed on the basis of morphology (retracted versus protruded anterior spiracles) and behaviour (crawling in the food versus wandering) (Bodenstein, 1994).

Newly hatched larvae were collected over a 2-4 hr time window and raised at 25°C at low density on standard cornmeal/yeast/agar medium, supplemented with live yeast. For the proliferation studies, 5-Bromodeoxyuridine, BrdU (Sigma) was resuspended in 80% Ethanol and added to the food. A final concentration of 0.2 mg/ml was used when the larvae were continuously exposed to the chemical. The 1mg/ml BrdU diet was used when giving short pulses of 6 hours duration. No supplement of living yeast was

added to the BrdU diets. The analysis of *grh* CNS-specific mutants was carried out in larvae from *Df2RPcl^{7B}/CyO-GFP* X *grh^{370}/ CyO-GFP* crosses. The correct larval genotype was distinguished by observation at the MZFluoIII microscope (Leica). Balanced larvae, expressing GFP in the anterior tissues, were discarded or used as controls.

2.4 Heat shock induction of FLP and AbdA

For using the MARCM technique (Lee and Luo, 1999) to generate clones that are positively labelled, embryos of the appropriate genotype were collected on yeasted grape juice-agar plates over a 4 hrs window. Heat shock induction of FLP was performed 4 to 8 hrs after larval hatching by immersing the food vial in a 37°C heated water bath for 1 hr and 30 minutes. Once aged until the appropriate stage (at 25°C), larvae were partially dissected in 1X PBS and immunostained. After staining, the CNS was isolated and mounted in Vectashield (Vector Laboratories) on a microscope slide for further analysis.

AbdA was ectopically provided in L2 or Mid-L3 in *hsabdA, grh^{370}/ Df2RPcl^{7B}* larvae using a 1hr heat-shock. As controls heat-shock was also performed in *hsabdA/CyO. yw* and *grh^{370}/Df2RPcl^{7B}* larvae. Larvae were dissected at 36 or 24 hrs after heat shock.

2.5 Antibody labelling of embryos and larval tissue

Embryos were collected, fixed and stained according to a permanent immunostaining protocol adapted from Gould (1990). Embryo collections were made over 12 to 20 hrs. Dechorionation was carried out in 50000 ppm Chloros solution for 2 min followed by several rinses in tap water. Fixation was in 2.5 ml of fixing solution (4% paraformaldehyde, 0.1 PIPES pH6.9, 1mM $MgSO_4$, 2 mM EGTA) mixed with an equal volume of heptane.

Larvae were partially dissected in 1X PBS to expose the CNS. Fixation was performed in 2% paraformaldehyde in PBL buffer (0.1 M lysine-HCl, 0.1 Na_2HPO_4, NaH_2PO_4) for 1 hr at room temperature. Following fixation, samples were washed for 30 minutes in PBT (1X PBS, 0.5% Triton-X-100) and then pre-incubated for 30

minutes in 90% PBT, 10% Normal goat serum (Sigma). For BrdU staining, before pre-incubation, larval tissue was treated with 2N HCl in PBT for 30 min and then washed for 3 X 5 minutes in PBT.

Fixed embryos and larval tissues were exposed to primary antibody over-night at 4°C. Primary antibodies used were: Guinea Pig anti-Hb diluted 1:500 and anti-Kr diluted 1:300 (both gift of J. Reinitz and D. Kosman), rabbit anti-βgalactosidase (βgal, Cappel) 1:7000, mouse anti-βgal (Promega) 1:1000, rabbit anti-Cas (gift of W.F. Odenwald) 1:2000, rabbit anti-H3p (Upstate Biotechnology) 1:400, rat anti-AbdA (gift of J. Casanova) 1:500. The monoclonal antibodies anti-Ubx (FP3.38, gift of R. White) 1:20, anti FasII (ID4, gift of G. Tear) 1:4, anti-Mira (Mab81, gift of F. Matsuzaki) 1:50. Anti-BrdU G3G4 1:200, anti-Elav 9F8A9 1:200 and anti AbdB IA2E9 1:50, anti-pros MR1A 1:100 (all from the Developmental Studies Hybridoma Bank, University of Iowa).

Secondary antibody incubation was performed for 3 hrs at room temperature. For secondary antibodies, Alexa488 (green) and Alexa594 (red) florescent conjugates (Molecular probes) were used at 1:100 dilution.

All fluorescent images were taken using scanning confocal microscopy (Leica) with a pinhole of 1. Figures are all projections of several sections except for Figure 4.2B,C and the following figures in Chapter 5: Figure 5.2, Figure 5.5, Figure 5.6, Figure 5.7, Figure 5.8B and D, Figure 5.9 and Figure 5.10D, where single sections are shown.

RESULTS

CHAPTER 3

SEGMENT-SPECIFIC REGULATION OF EMBRYONIC NEUROBLAST SUBLINEAGES

CHAPTER 3 SEGMENT-SPECIFIC REGULATION OF EMBRYONIC NB SUBLINEAGES

3.1 Introduction

The basis for region-specific patterning of the adult CNS is established during embryogenesis, and is first observable when NBs divide differently in the thorax relative to the abdomen. To shed light on the mechanism underlying segment-specific patterning and, in particular, to explore possible links between Hox genes and NB sublineage switching, I studied a group of embryonic neural cells that are specific to the thorax. These cells have been previously identified in the lab and are referred to as THB neurons (Thorax specific Hb expressing neurons; P. Fichelson, B. Bello and A. Gould, unpublished). THB neurons express the NB sublineage switching gene *hunchback (hb)* and the neural-specific gene *elav* and are restricted to the three thoracic neuromeres (Figure 3.1A).

Hb is evenly expressed all along the embryonic CNS in the dorsal region (Kambadur et al., 1998; Isshiki et al., 2001) but, beginning at stage 12 of development, bilaterally symmetrical clusters of approximately 4 cells become visible in a more ventral position. Importantly, these clusters are only seen in the thorax and by the end of embryogenesis contain about 12 cells each. *In situ* hybridisation studies (Margolis, 1992) indicate that *hb* is transcribed within the CNS starting at stage 7 of embryogenesis but that, by the beginning of stage 15, *hb* mRNA is no longer detectable. This is consistent with my finding that, in the CNS, Hunchback protein can be observed by immunostaining until the end of embryogenesis, but it is not detected at larval stages. To characterize the identity of the THBs in more detail, a screen of other markers labelling these cells was performed (see Table 3.1). Interestingly, none of the markers analysed gave complete overlap with the THB neurons. For example, the 1D4 antibody which recognizes FasciclinII (FasII), the *Drosophila* N-CAM homologue, involved in neuronal cell recognition (Grenningloh et al., 1991; Grenningloh and Goodman, 1992), labels four to six of the most dorsal THB cells (Figure 3.1B) and thus can be used to help identify THB neurons.

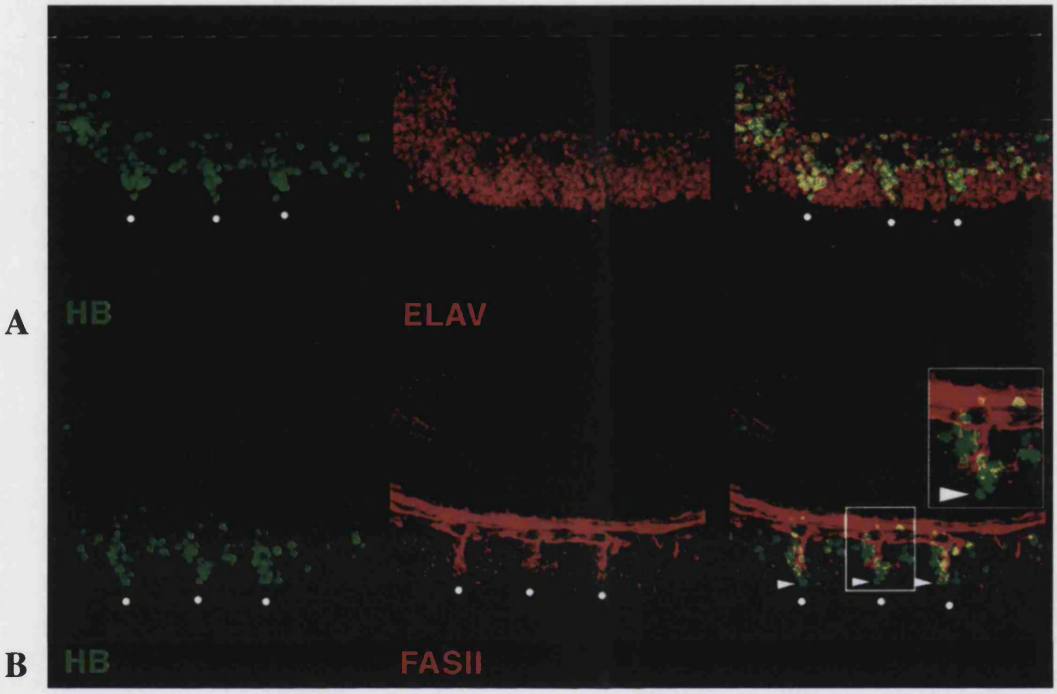


FIGURE 3.1 ALL THB CELLS EXPRESS *HUNCHBACK* AND *ELAV* AND A SUBSET ALSO EXPRESSES *FASCICLIN II.*

Lateral views of CNS of two stage 16 *Drosophila* embryos are shown. Anterior to the left, dorsal is up. In this and subsequent figures in this chapter, the white dots indicate the position of the THB neurons in T1, T2 and T3. (**A**) Hunchback (Hb) is labelled in green and is expressed evenly along a dorsal zone of the CNS. Clusters of Thorax specific Hb (THB) expressing cells project ventrally and are Elav-positive (red). (**B**) A subset of THBs also expresses Fasciclin II (Fas II, red). Arrowheads indicate a ventral THB neuron that does not express Fas II. (Adapted from P. Fichelson's data).

MOLECULAR MARKER	DESCRIPTION	POSITIVE CELLS/ TOTAL PER CLUSTER
ELAV	RNA-binding protein Neuron-specific	12/12
FASCICLIN II (FASII)	Ig superfamily Motor axon marker	5/12
CONNECTIN	Leucine-rich repeat protein	4/12
LIM-3 *	LIM-homeodomain motor neuron marker	3/12
EAGLE	Zinc-finger protein	3/12
EVEN-SKIPPED	Homeoprotein	0/12
ENGRAILED	En-Gal4 x UAS-nlacZ lineage marker for the posterior compartment	0/12
CASTOR *	Zinc-finger protein	0/12

TABLE 3.1 EXPRESSION OF SUBTYPE MARKERS IN THB NEURONS AT STAGE 16
Approximate number of THB cells expressing different markers. 12/12 cells label with Hunchback. Data is from P. Fichelson except where indicated by asterisk (my own original work).

These initial studies suggested that THB neurons might provide a useful system for studying some aspects of segment-specific neurogenesis and its regulation by the Hox genes. Embryos mutant for *Antennapedia* show no deviation from the THB wild type pattern (Figure 3.2A,B), suggesting that the positive regulators for the THB neurons do not include the thoracic Hox genes. In contrast, when *Ubx* mutant embryos are analysed, additional THB cells are detected in the first abdominal segment (P. Fichelson and A. Gould, unpublished, data not shown). More dramatically, THB neurons are found all along the abdominal neuromeres of *Ubx/abdA (Df109)* double mutant embryos (Figure 3.2C). Together, these results indicate that both *Ubx* and *abdA* are capable of suppressing THBs. Using carefully timed, heat-shock induced expression of Ubx, the temporal requirement for Hox suppression was pinpointed to between stage 5 and stage 9 of embryogenesis. Outside this temporal window, ectopic induction of Ubx does not result in THBs suppression (Figure 3.2D-F). Also, the over-expression of Antp at stages 5-9 fails to perturb the THB pattern, confirming the specific requirement for the abdominal and not thoracic Hox genes in suppressing THB neurons (data not shown).

Given this early Ubx/AbdA temporal requirement and that their expression has been detected in a subset of NBs from stages 8 through to 12 (Prokop et al., 1998), it is likely that Hox proteins act in the NBs giving rise to the THB neurons, rather than directly in their progeny. Within the NBs, Ubx and AbdA might function by limiting either the divisions or the survival of abdominal THB-progenitors. Alternatively, Ubx/AbdA may not affect cell number but could affect the timing of the NB switching from Hb-positive to Hb-negative status.

This Chapter describes my studies aimed at identifying the positive factor(s) required for THB specification, as well as the mechanism of Ubx/AbdA suppression. Lineage-labelling based evidence is also presented that there is a segment-specific modulation of the canonical TF series of NB sublineage determinants.

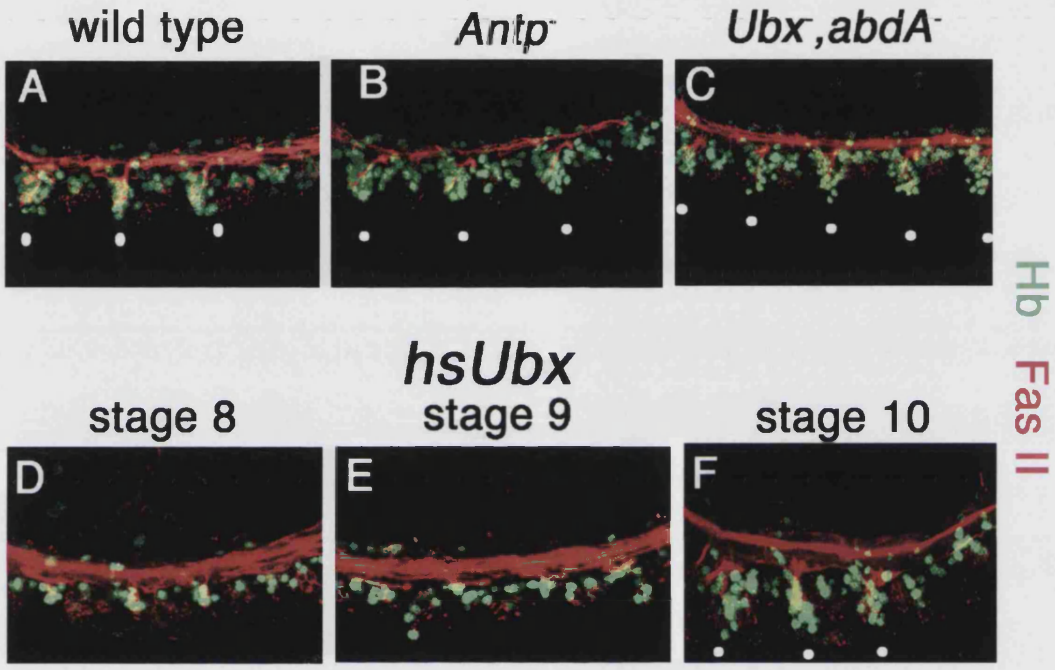


FIGURE 3.2 UBX/ABDA BUT NOT ANTP SUPPRESS THB NEURONS.
Stage 16 embryonic CNS labelled with Hb (green) and Fas II (red). Wild type (**A**), *Antp*$^{-}$ (**B**) and *Ubx*$^{-}$, *abdA*$^{-}$ (**C**) embryos. THB neurons (white dots) are still observed in *Antp*$^{-}$ embryos. Additional THBs are found in the abdomen of *Ubx*$^{-}$, *abdA*$^{-}$ embryos. **C** shows only the additional THBs found in A1 and A2. (**D-F**) Ubx is ectopically provided in *hsUbx* embryos at different stages. Suppression of THBs is observed following heat-shock at stage 8 (**D**) and stage 9 (**E**). Ubx induction after stage 10 (**F**) does not affect THB formation (adapted from P. Fichelson's data).

3.2 Results

3.2.1 Homothorax is required for THB specification and is phenotypically suppressed by Ubx/AbdA

Homothorax (Hth) is a homeodomain protein that can function as a Hox-cofactor (Rauskolb et al., 1993; Mann and Affolter, 1998); It is expressed throughout embryonic development in a complex pattern. From stage 11, it becomes progressively down-regulated in the abdominal segments by *Ubx* and *abdA*, such that the late embryo has much higher expression in the thoracic than the abdominal CNS (Kurant et al., 1998).

I analysed embryos homozygous for a strong hypomorph of *hth* ($hth^{64\text{-}1}$). Stage 17 mutant and wild type embryos were double stained with antibodies to Hb and FasII and their THB patterns compared (Figure 3.3A,B). Whereas the dorsal expression domain of Hb and FasII is not affected in $hth^{64\text{-}1}$ embryos, the ventral projections that characterize the THB neurons are lacking in all three thoracic segments. These findings identify Hth as a positive regulator of THB specification, moreover, together with the previous results, they indicate that, in this respect, Hth functions in the thorax independently from the Hox genes.

The phenotypes of *Ubx/abdA* double mutant embryos (*Df109*) and of $hth^{64\text{-}1}$ embryos suggest that abdominal Hox proteins and Hth play opposite roles in THB formation. Ubx/abdA are redundantly required to suppress THB cells in the abdomen, while Hth is necessary for their formation in the thorax. What is the nature of the suppressive mechanism of Ubx/abdA? Ubx and AbdA can transcriptionally repress *hth* in the abdominal CNS from stage 11, but since these Hox proteins appear to be required earlier to inhibit THB formation (stage 5 to 9), other mechanisms must be considered. For example *Ubx/abdA* could act by phenotypically suppressing Hth, that is, by silencing Hth function at the level of its target genes and not its transcription (Gonzalez-Reyes and Morata, 1990). In order to test this possibility, Hth expression was driven within $hth^{64\text{-}1}$ embryos by the use of the GAL4-UAS binary system (Brand and Perrimon, 1993). GAL4 is a yeast transcription activator that acts by binding a specific DNA responsive element, the Upstream Activator

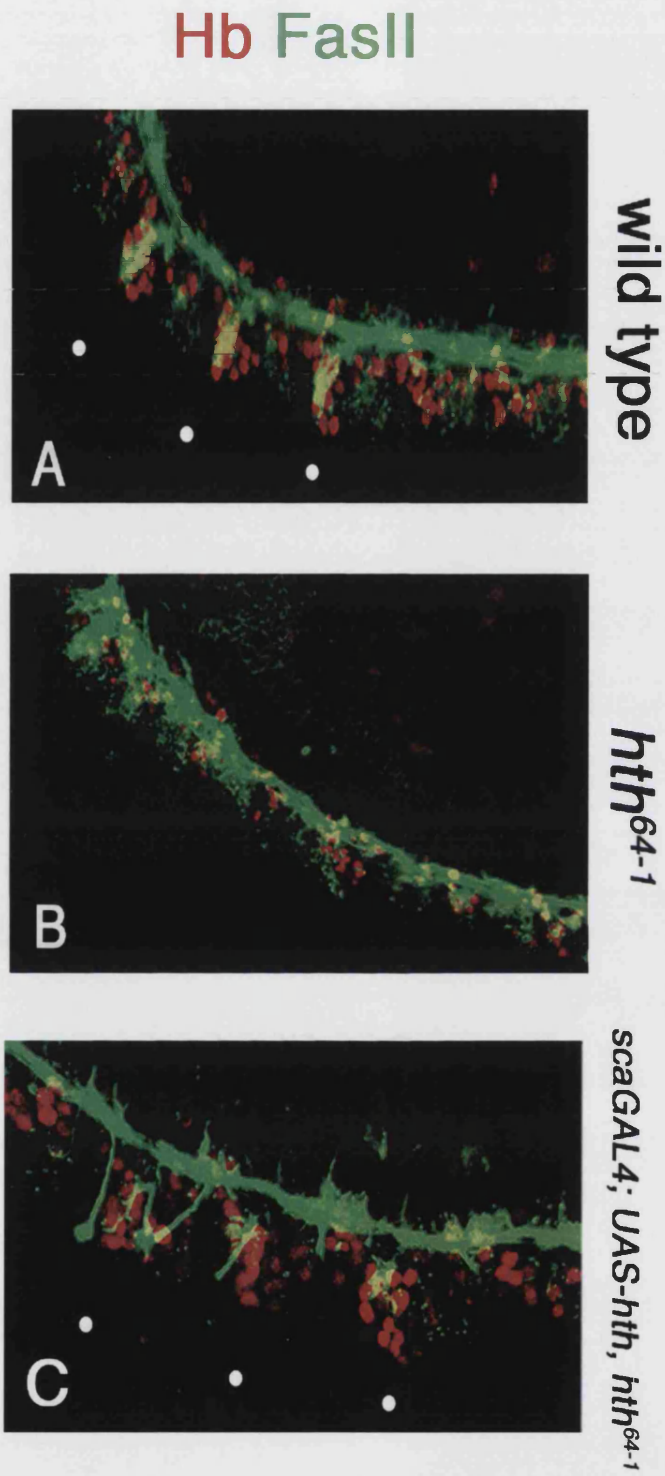


FIGURE 3.3 *HTH* IS REQUIRED FOR THB FORMATION AND CAN BE PHENOTYPICALLY SUPPRESSED BY *UBX/ABDA*. Lateral view of stage 17 embryos, double labelled with anti-Hb (red), anti-FasII (green). (**A**) Wild type embryo presenting three THB clusters. (**B**) $hth^{64\text{-}1}$ embryo showing no THB clusters. (C) $hth^{64\text{-}1}$ embryo carrying *sca-GAL4* and *UAS-hth*. THBs are rescued but FasII projections, though present, are disorganized.

Sequence (UAS). When flies expressing GAL4 under the control of a defined enhancer (driver line), are crossed to flies carrying a UAS-target gene construct (responder line), the progeny that receives both the driver and responder transgenes will express the target gene in a pattern defined by the chosen enhancer. The transgene *scabrous-GAL4 (sca-GAL4)* is ubiquitously expressed in neuroectoderm starting from stage 9 of embryogenesis and its transcription is Hox independent. When Hth expression is added back to $hth^{64\text{-}1}$ mutants using *sca-GAL4*, the THB neurons are rescued; this confirms that Hth can promote THB formation (Figure 3.3C). Nevertheless, the number of Hb expressing cells per cluster is somewhat increased (on average 3 more cells) and the organization of their FasII projections is only partially rescued. This may be explained by a specific temporal and quantitative requirement for Hth, that is not fully recapitulated by *sca-GAL4*. Importantly, no THB clusters are seen in the abdomen of these rescued embryos. Thus, Hox-independent Hth expression in the thorax and in the abdomen of mutant embryos is sufficient to rescue partially THB neurons in the thorax but not in the abdomen. These experiments allow two important conclusions to be drawn. First, Hth is required to specify THB neurons in the Thorax and second, phenotypic suppression of Hth by *Ubx/abdA* is sufficient to inhibit THB formation.

3.2.2 Lineage-labelling of THB neurons

To understand the cellular basis of the THB segmental restriction, as well as to trace the development of THBs at later stages, a specific method was developed that labels the NB lineages giving rise to these neurons. Hereafter, this method will be referred to as " THB-lineage labelling".

As a first approach, a system based on FLP-mediated recombination (Golic and Lindquist 1989) was used to positively mark lineages in the embryonic CNS. Although this has been successfully adopted by others (Buenzow and Holmgren, 1995), it did not turn out to be useful for this project. In fact, the general low frequency of clones I detected in the CNS does not allow significant labelling of THB clusters.

Then, the GAL4-UAS system was used to express a stable marker in the THB lineages (Brand and Perrimon, 1993). The delay in expression associated with this binary system, combined with the use of a very stable reporter *nls-lacZ*, encoding nuclear β-galactosidase (β-gal), allows persistent expression long after a standard enhancer-LacZ construct would have been switched off. Thus, this technique can be used as a form of lineage label (P. Elstob and A. Gould, unpublished) albeit not a permanent one. Previous studies indicate that a 4 Kb enhancer of the *hb* distal promoter directly driving *LacZ* is sufficient to recapitulate a subset of the full *hb* mRNA expression pattern in the CNS from stage 9 onwards. Throughout embryogenesis the *LacZ* expression is specific for the nervous system and it is excluded from the ectoderm where *hb* is known to act early on as a gap gene. More importantly, in stage 12 embryos, this expression recalls the THB pattern: it appears to be restricted to few NBs and to their progeny in each of the thoracic segments (Margolis, 1992). For this reason, the 4 Kb enhancer was used to build a specific THB-GAL4 driver. Three different enhancer fragments were used to generate the *hb-GAL4* lines; a 4 Kb XbaI fragment and two smaller subregions of this, a 2.8 Kb BglII fragment and a 1.2 Kb BglII/XbaI fragment. These were cloned into a GAL4 recipient vector (pWHSpeGAL4) specifically built for this purpose (see Materials and Methods). The respective vectors obtained, p4.0Hb-GAL4; p2.8Hb-GAL4 and p1.2Hb-GAL4 were injected into host embryos to establish transgenic *hb-GAL4* lines. As summarized in Table 3.2, nine lines were obtained for *4.0hb-GAL4*, four for *2.8hb-GAL4* and eleven lines for *1.2hb-GAL4*. Each line was crossed to *UAS-nlsLacZ* and tested by X-gal staining for THB-specific expression. All nine of the *4.0hb-GAL4* lines generated showed a THB-like pattern of expression in embryos. In larvae, however, no reproducible THB-like pattern was observed. As neither the *2.8hb-GAL4* nor *1.2hb-GAL4* lines showed any reproducible pattern of embryonic or larval staining, it is likely that critical THB regulatory elements lie in both the 2.8 and 1.2 Kb fragments. Line number 34, which carries the *4.0hbGAL4* transgene on the X chromosome, was selected as a THB driver for further analysis and from now on will be referred to as the *THB-GAL4* driver line.

Construct	Embryo	Larva	Chromosome
p4.0hbGAL4	9 / 9	0/9	X (line 34) II (lines 7, 26, 29, 48, 55) III (lines 16, 22, 23)
p2.8hbGAL4	0/4	0/4	_
p1.2hbGAL4	0/11	0/11	_

TABLE 3.2 Hb-GAL4 LINES SHOWING A THB-LIKE PATTERN IN THE EMBRYONIC AND LARVAL CNS.
Numerator indicates the number of lines with THB pattern and denominator the total number of lines. The chromosomal location of *4.0hb-GAL4* lines is also shown.

3.2.3 THB precursors also produce a Hb-negative sublineage

To examine the THB embryonic lineages in detail, stage 17 *THB-GAL4; UASnlsLacZ* embryos were labelled for Hb and βgal expression (Figure 3.4). An average of 18 (for n=27 clusters) thorax-specific βgal expressing cells can be observed in a latero-ventral position within the CNS. As expected, the βgal and the Hb patterns of expression are not completely overlapping and only 8 neurons are Hb-βgal double-positive. Of the remainer, 4 of the early-born THB neurons (tending to be located more dorsally, Kambadur et al., 1998) are Hb single-positive because, due to the GAL4-UAS delay, they were presumably born prior to *nlsLacZ* expression. In addition 10 out of 18 of the late-born neurons (Bossing et al., 1996; Kambadur et al., 1998; Udolph et al., 2001) are βgal positive but have switched off Hb. The 8 THB neurons that are Hb-βgal double-positive tend to be located at an intermediate position and were presumably born at an intermediate developmental time. These findings indicate that the NB precursors that produce the 12 THB neurons also produce about 10 Hb-negative cells. A first suggestion about the nature of these Hb-negative sublineages was obtained by analysing the pattern of expression of Cas, the latest embryonic sublineage marker known thus far (Mellerick DM, 1992; Kambadur et al., 1998). In the thorax of a wild-type stage 17 embryo, Cas expressing neurons can be observed forming a niche juxtaposing the THB cells (Figure 3.5).

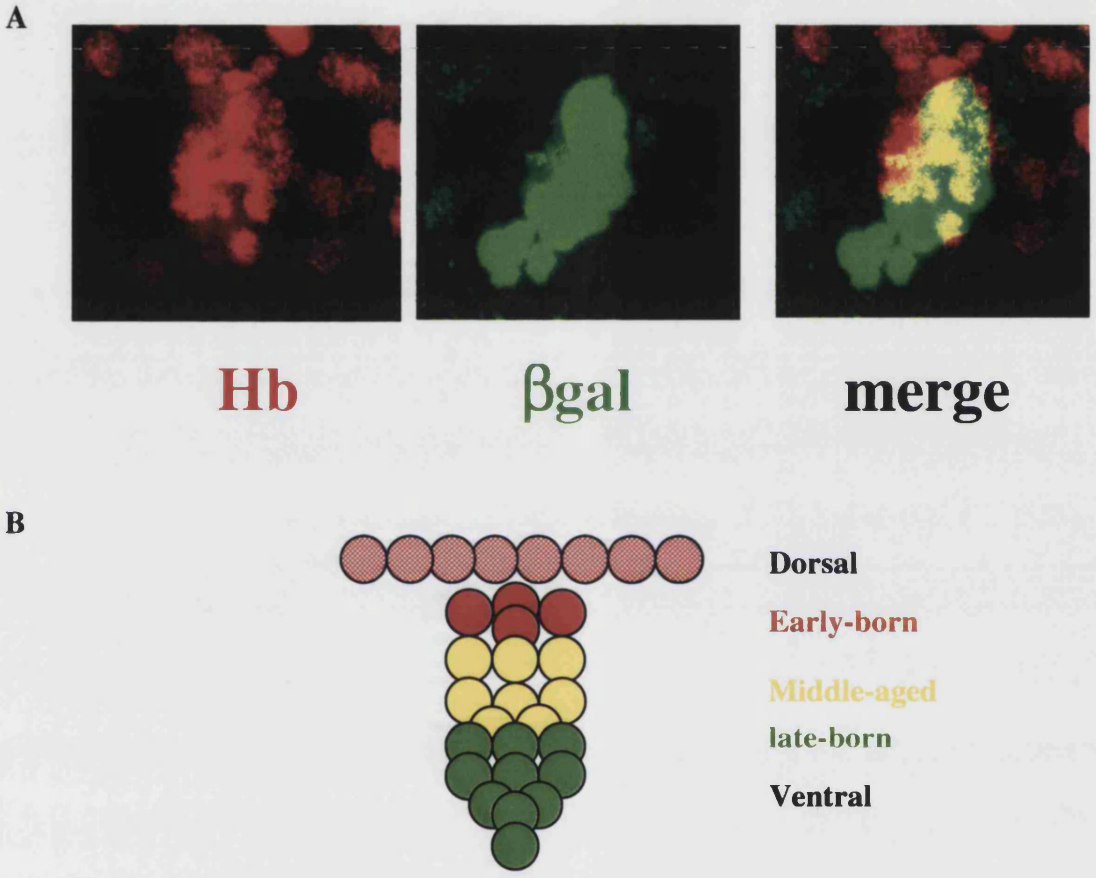
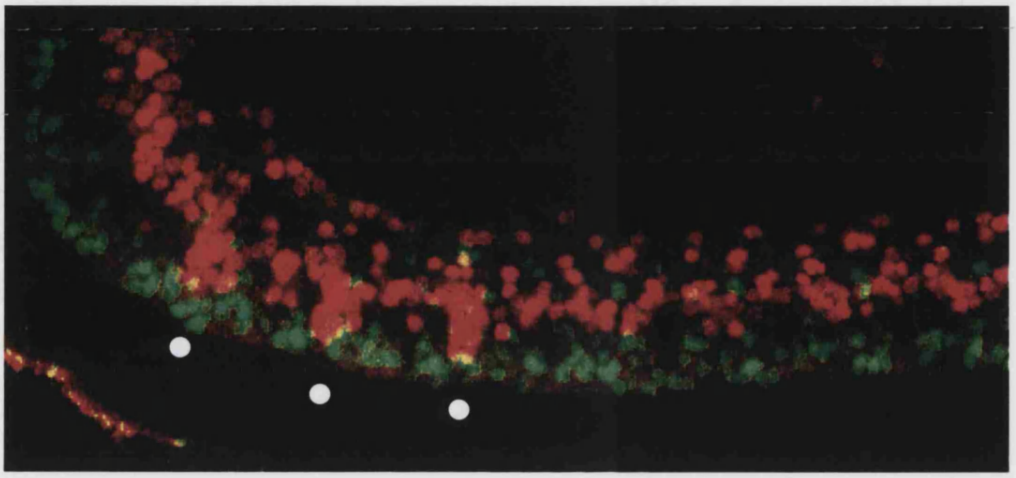


FIGURE 3.4 SUBLINEAGE SWITCHING OUT OF THE HB MODE IS DELAYED IN THE THB LINEAGES.

(A) Confocal projections of one THB cluster of a stage 17 embryo carrying *THB-GAL4* and *UAS-nlsLacZ,* double stained for anti-Hb (red), anti-βgal (green). **(B)** Cartoon of average pattern of Hb and βgal in a cluster. Approximately 4 Hb single-labeled neurons tend to be located dorsally, in proximity to the common AP Hb domain of expression (dashed red in cartoon scheme) and were probably born early. On average 10 neurons are βgal single-labelled, these are located ventrally and were late-born. 8 intermediate (middle-aged) neurons are double-labelled.



Cas Hb

FIGURE 3.5 LAYERED HB AND CAS EXPRESSION PATTERNS WITHIN THE EMBRYONIC CNS.

Stage 17 wild type embryo double stained with anti-Cas (green), anti-Hb (red). Close apposition of Cas expressing cells and Hb occurs around the thoracic THBs (white dots) but not in the abdomen.

In the abdomen, however, they are well separated from the dorsal Hb expression domain as previously reported by others (Kambadur et al., 1998; Brody and Odenwald, 2000). The close proximity of THB and Cas-positive cells raises the possibility that the THB precursors might switch from Hb to Cas with little or no time in the Kr or Pdm modes of expression.

3.2.4 *Pdm-1* is not expressed within the THB lineages

To address whether the above hypothesis could be correct, the early-born, Hb-negative cells generated by the THB precursors were tested for the expression of the sublineage markers, Kr, Pdm and Cas (Figure 3.6). Among several THB βgal-expressing clusters analysed (n=14), only one to two of the neurons located at an intermediate position showed Kr expression (Figure 3.6A). Even more interestingly, none of the βgal labelled neurons expressed Pdm-1 (n=11 clusters, Figure 3.6B). Finally, 2 to 4 of some of the late-born neurons belonging to the THB lineages (n=11 clusters) expressed Cas (Figure 3.6C). These findings are consistent with the reported evidence that both Hb and Cas act by suppressing *Pdm-1* (Kambadur et al., 1998) and suggest that the THB lineages go through a sublineage sequence that differs from the canonical cascade previously described: **Hb → Kr→ Pdm-1 → Cas** (Kambadur et al., 1998; Brody and Odenwald, 2000; Isshiki et al., 2001; Pearson and Doe, 2003). The basis for this difference lies in an extended number of early Hb-positive cell fates at the apparent expense of Pdm-1 and possibly also Kr-positive fates. Interestingly, these results provide evidence for gene skipping within the canonical cascade of transcription factors such that the intermediate sublineage determinant, Pdm-1, is not expressed but the late determinant factor Cas is:
Hb →→→→ Kr → Cas.

3.3 Discussion

3.3.1 Role of Hth and Hox genes in THB specification

THB neurons represent a good model to study the role of Hox genes in the regulation of segment-specific neurogenesis. These thorax-specific neurons do not appear to require the anterior Hox genes for their specification and therefore, they probably

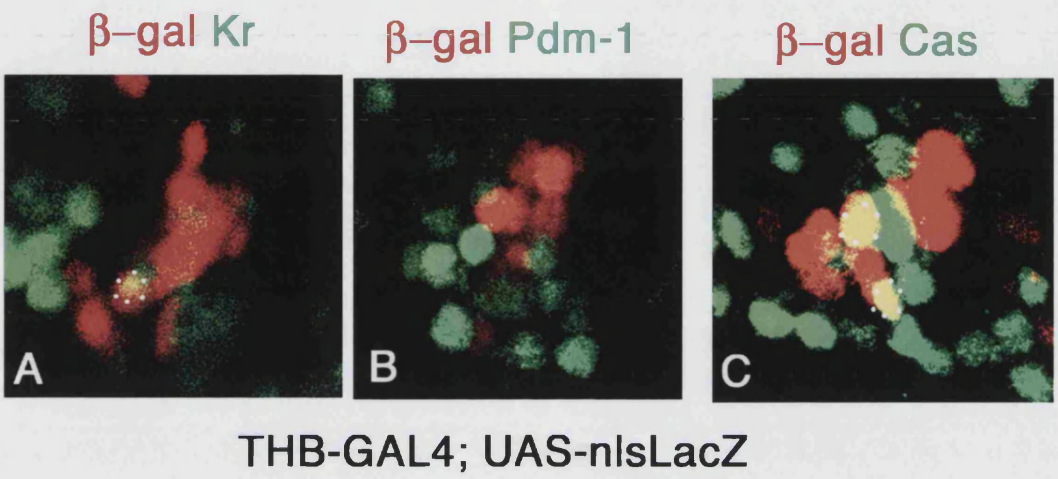


FIGURE 3.6 THB LINEAGES MAY UNDERGO GENE SKIPPING IN THE CANONICAL TF SERIES.

Confocal projections of THB β-gal labelled lineages (red) of stage 17 *THB-GAL4; UAS-nlsLacZ* embryos, also labelled with the NB sublineage TFs (green), Kr (**A**), Pdm-1 (**B**) and Cas (**C**). Pdm-1 is not expressed in the THB lineage, whereas only one cell of the lineage expresses Kr and 2 to 4 cells express Cas (dotted cells in **A** and **C** respectively). Note that some of the latest-born, most ventral β-gal-positive cells are not Cas-positive.

belong to what is defined as "the ground state" (Lewis, 1978; Struhl, 1983; Casares and Mann, 2001). In the abdomen, *Ubx* and *abdA* act on top of this common ground state to suppress THB formation. Ectopic expression of *Ubx* or *abdA* results in the complete suppression of all the THB neurons but only when provided at early stages of embryogenesis (from stage 5 to stage 9). In contrast, successively later (stage 10 onwards) induction of either of the two abdominal Hox genes results in a progressively less dramatic reduction in cell number. Such a time-restricted requirement of the *bithorax* complex genes for the repression or specification of segment-specific cell types appears to be widespread and has previously been observed for several ectodermal derivatives (Castelli-Gair et al., 1994; Brodu et al., 2002) and for some post-embryonic NB lineages (Bello et al., 2003).

I have shown that *hth* plays a positive role in promoting THB formation in the thorax. However, because *sca-GAL4* drives Hth expression ubiquitously, the rescue experiment did not allow the definition of the cell type (neuroblast, GMC or neuron) in which Hth is needed for THB specification. In addition, the temporal aspect of the Hth requirement is unknown and a time course experiments of *hs-hth* induction could be carried out to address this point. Although Hox genes do not appear to have an input to THB formation, it may be that the Hth cofactor, Exd, is also required for their specification. The ability of Hth/Exd to confer specific identity to cells without a requirement for Hox proteins themselves has already been described in the context of the antenna where *hth/exd* act as antennal selector genes (Casares and Mann, 1998; Casares and Mann, 2001). In *hth* or *exd* mutant flies, the antenna is transformed into a leg without any activation of *Antp*. Similarly, *hth* specifies THB formation without an apparent requirement for the genes belonging to the *Antennapedia* complex.

A transcriptional repression model cannot solely explain the underlying mechanism accounting for how Ubx/AbdA counteract hth activity during THB formation. This is because *Ubx/abdA* repress the transcription of *hth* at stage 11(Kurant et al., 1998), many hours after they are required to suppress the THBs in the abdomen. More conclusively, my experiments show that when Hth is expressed

in a Hox-independent manner in the abdomen, and kept at the same levels in both the thorax and the abdomen, *Ubx/abdA* can still suppress the THBs. This failure of THB induction in the abdomen indicates that phenotypic suppression (Gonzalez-Reyes and Morata, 1990) of Hth by Ubx/AbdA, acting presumably at the level of *hth* target genes, is sufficient to inhibit the THBs. However, I cannot rule out that during normal development both transcriptional repression and phenotypic suppression make redundant contributions towards preventing THB specification.

One of the *hth* target genes could be *hb* itself; experiments such as gel retardation and CAT assays could be designed to test the possibility that Hth acts by directly binding the 4 Kb *hb* enhancer to activate gene expression, and that *Ubx/abdA* might also directly interact here, perhaps in a competitive manner. The latter possibility would be rather surprising because it would invert the genetic hierarchy that is established at the syncytial blastoderm stage, when Hb expression in the thorax directly represses *Ubx* and thus defines the anterior limit of its expression (Zhang and Bienz, 1992). Hox genes are known to restrict the proliferative capacity in late embryonic CNS (Prokop et al., 1998) and to induce cell death in post-embryonic NB lineages (Bello et al., 2003). Therefore, a second possibility is that *Ubx/abdA* act indirectly to cause the disappearance of Hb-positive cells, for example by inhibiting the divisions of the THB progenitor cells and/or causing them or their progeny to be eliminated by apoptosis. In order to test this idea, *DfH99* mutant embryos, lacking the three pro-apoptotic genes required for cell-death (White et al., 1994), were analysed for THB formation. However it was not possible to draw hard conclusions from this experiment because the *DfH99* mutant embryos display a very disorganised CNS (data not shown). Given such large scale disruption, it was difficult to detect the presence of anomalies within THB cell lineages.

3.3.2 What are THBs and which precursors generate them?

Although I have shown that THBs represent a good model for examining Hox control of neurogenesis, several important questions remain unanswered. For example, which NBs generate the THBs? And what types of neurons do THBs become? To address the latter issue of the neuronal subtype of THBs, A search for

additional THB markers was made. A panel of several different CNS markers were tested (for a summary see Table 3.1) among these, Engrailed (En) a protein specific for the posterior compartment, allowed the positioning of these cells to the anterior compartments of T1, T2 and T3.

Most of the other neural markers tested showed only partial overlap with the THB neurons. For example, 3 out of 12 cells express Eagle, a zinc-finger protein necessary for the differentiation of serotonergic neurons (Higashijima et al., 1996; Dittrich et al., 1997). Four of the THBs express *connectin*, encoding a homophilic cell-adhesion molecule involved in motorneuron pathfinding and synaptogenesis, that, interestingly, has been shown to be repressed by *Ubx* and *abdA* (Gould et al., 1990; Gould and White, 1992; Meadows et al., 1994). The motorneuron markers Lim3 and Fas II are also expressed in different subsets of THBs (Lin, 1994; Thor, 1999). At larval stages, FasII labels prominent thoracic projections that make contact with the leg imaginal discs (data not shown) and therefore THB/FasII cells in the embryo might later contribute the Presumptive Leg Neuromeres of the adult CNS (Ghysen et al., 1985). This speculation that some of the THBs are leg motorneurons is consistent with BrdU incorporation studies showing that the leg motorneurons of the adult fly are generated during embryogenesis (Tissot and Stocker, 2000).

Concerning the origin of the THBs, it may be that more than one progenitor is involved in the formation of these cells; the difference in lineage size between thorax and abdomen for a single thoracic NB is never as high as 12, the number of THB neurons found at the end of embryogenesis (Schmid et al., 1999). On the basis of the description of segment-specific NB lineages characterised (Prokop and Technau, 1994; Schmid et al., 1999) it is possible that NB1-1 may contribute at least some of the candidate THB precursor cells. This NB lineage expresses FasII and derives from the anterior compartment (Doe, 1992; Schmid et al., 1999). In the abdomen, it generates a single glial cell, whereas in the thorax it generates four additional interneurons and motorneurons (Schmid et al., 1999). As with the THBs, Prokop and Technau have shown that Antp is not required for the specification of the NB1-1 lineage but that Ubx and abdA are required for the reduced lineage in the abdominal neuromeres (Prokop and Technau, 1994).

3.3.3 THB lineage labelling

Several technologies are now available for following cell lineages during development and among these, single-cell transplantations and DiI labelling have been used to characterise fly NB lineages (Prokop and Technau, 1991; Schmidt et al., 1997; Schmid et al., 1999). Both these methods rely largely on identifying which NB was labelled "in reverse" by examining its progeny. Less random labelling methods were however preferred to these in order to trace THB origin and development. Therefore, a method based on the use of the specifically constructed THB-GAL4 line and of a stable reporter protein was used. Although the THB-GAL4 lines I generated proved useful for THB-marking, some limitations were encountered. For example, THB labelling is only detected in a subset of the thoracic hemisegments, suggesting mosaic expression of the driver. For this reason, further investigation of the THB-lineages, such as following their projections or analysing them in $hth^{64\text{-}1}$ and *DfH99* genetic backgrounds, was not initiated. A second limitation of the THB-GAL4 driver was that it did not allow permanent lineage marking during larval development. Despite these two failings, the THB-GAL4; UAS-nlsLacZ method has allowed the characterization of a segment-specific and non-canonical type of sublineage factor switching. In combination with my previous results, these studies also show that Hth is required for this thorax-specific sublineage pattern and suggest that Ubx/AbdA may be suppressing it in the abdomen.

3.3.4 THB-specific modulation of NB transcription factors

Sublineage switching through sequential expression of TFs by NBs, **Hb** ➛ **Kr** ➛ **Pdm-1** ➛ **Cas** has recently been uncovered. This links the birth date of neurons with their final identity (Kambadur et al., 1998; Brody and Odenwald, 2000; Isshiki et al., 2001; Pearson and Doe, 2003). A few exceptions to the canonical cascade have also been described, such as NB7-3, that does not express *Cas* (Isshiki et al., 2001), and NB6-1, that instead expresses only this last factor but not the earlier ones (Cui and Doe, 1992). Therefore there is prior evidence for missing part of the sequence, but

not for the skipping of a TF from the normal sequence. Furthermore, the possibility of a segment-specific modulation of this TF series has never been described to my knowlege.

The over-expression of Hb within the NB7-3 or NB7-1 lineages leads to the generation of more of the cell-types specified by this TF state, possibly at the expense of the neurons with later sublineage identities (Isshiki et al. 2001; Pearson and Doe, 2003). Similarly, my work suggests that the THB progenitors remain in the Hb mode of expression such that they specify more early fated neurons than the abdominal lineages. Nevertheless, the THB progenitors (or at least some of them) do still undergo a switch in their competence and express Kr. However, from this point the overall sequence of TFs becomes alterated because THBs express Cas, apparently without passing through the Pdm-1 mode. Moreover, because some of the most ventrally located neurons in the THB-lineage do not express any of the well-characterised TFs, I now speculate that these may correspond to the late born neurons and so may be derived from progenitor expressing *grainyhead*, a NB marker that has been proposed as the next factor in the TF sequence (Brody and Odenwald, 2000; Brody, 2002).

In summary, these findings identify an example of segment-specific expression of the NB sublineage factor Hb. In addition, they suggest that there is segment-specific skipping of *Pdm-1* from the canonical TF sequence. I have shown that Hth acts as a positive regulator of this thorax-specific pattern. In the abdominal lineages (as already discussed in Section 3.3.1) Ubx/abdA could act by repressing *hb* and allowing the switch to proceed as described. Alternatively the Hox genes could negatively regulate THBs, by limiting the mitotic potential or survival of the THB progenitors or their progeny (Prokop et al., 1998). This latter scenario could be similar to one uncovered by recent studies showing that in the larval CNS AbdA induces NB cell-death and thus limits proliferation (Bello et al., 2003).

Due to the two limitations encountered with the THB-GAL4 labelling method, I next turned to exploring related issues using an alternative methodology for labelling single NB lineages in the larval CNS, as described in the next chapter.

CHAPTER 4
REGULATION OF LARVAL NEUROBLAST DIVISIONS BY THE POLYCOMB GROUP GENES

CHAPTER 4 REGULATION OF LARVAL NEUROBLAST DIVISIONS BY THE POLYCOMB GROUP GENES

4.1 Introduction

As described in the general introduction, Truman and Bate have defined the time windows of segment-specific neurogenesis during larval stages (Truman and Bate, 1988). The time of exit from the NB quiescent state in early larval life is known to be controlled by glial secreted molecules and long-range signalling factors induced by hormonal and nutritional cues (see Introduction, Section 1.6.1). Regarding the termination of NB divisions following larval neurogenesis, very little is known for most AP regions. However, recent studies in the laboratory have indicated that the pNBs of the abdominal neuromeres cease dividing at mid-L3 because of AbdA-induced cell-death (Bello et al., 2003). Interestingly, misexpression experiments carried out in this study show that the response to ectopic Hox expression is different according to the cell-type: cell death is induced only in the NBs, but not in their progeny neurons. Indeed, during normal development of parasegment 6, post-mitotic neurons express Ubx but do not die (Glicksman and Truman, 1990; Truman et al., 1993, see also Figure 1.13). Moreover, it is clear that Hox expression must be excluded from the thoracic pNBs to allow their normal survival into pupal life. In this Chapter, I explore some of the factors controlling thoracic NB divisions in the larval CNS. The roles of candidate genes were tested using a powerful genetic mosaic technique: the MARCM system (Lee and Luo, 1999; Lee and Luo, 2001, Figure 4.1). This represents a method for studying the effects of gene mutations in individual positively-marked pNB lineages, and has been successfully used for the study of neural morphogenesis (Lee et al., 2000a; Lee et al., 2000b; Hitier et al., 2001; Bello et al., 2003; Reuter et al., 2003). In this method, a constitutively expressed repressor (GAL80) is placed *in trans* to a mutant gene of interest, distal to FLP recombinase target sites (FRTs). This repressor inhibits the GAL4-mediated activation of a given UAS-marker (for my studies UAS-nlslacZ). Mitotic recombination, promoted by a heat-shock inducible Flipase (hs-FLP), generates

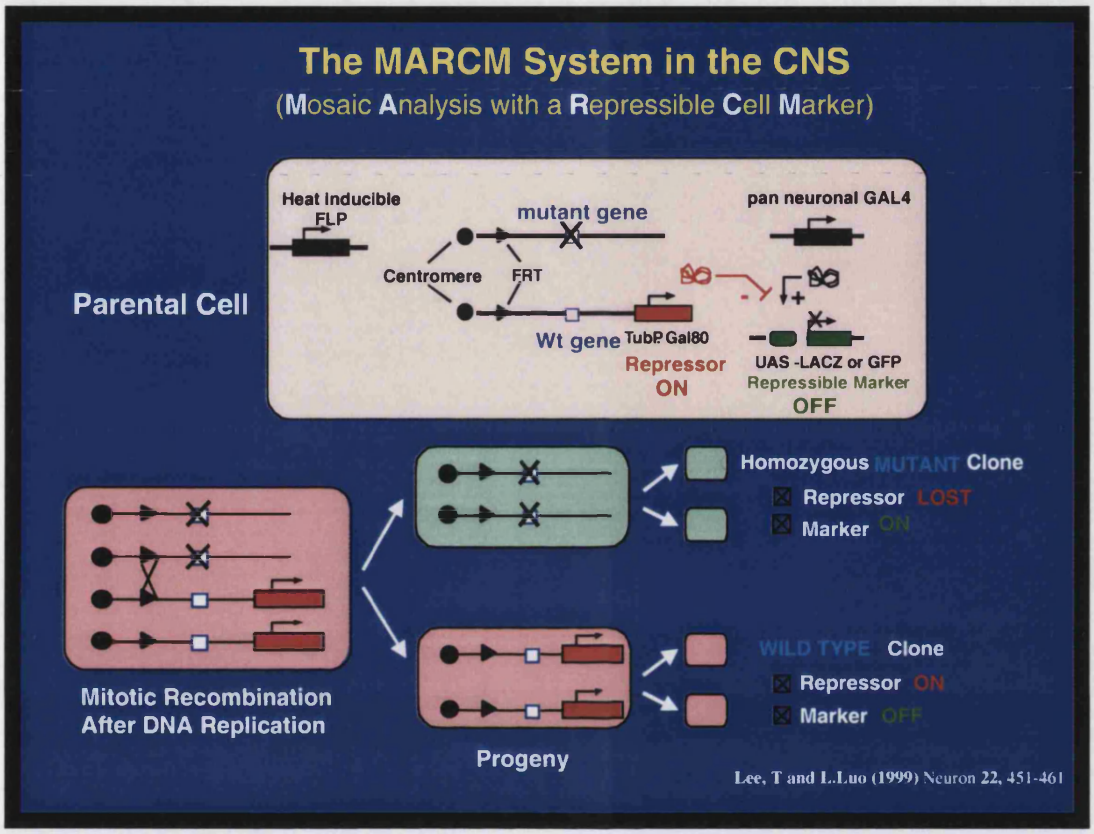


FIGURE 4.1 MOSAIC ANALYSIS WITH A REPRESSIBLE CELL MARKER (MARCM). In the MARCM system, a transgene ubiquitously encoding a repressor of Gal4 (*Tub-Gal80*) is placed distal to a site (FRT) for the Flipase (FLP) recombinase. The homologous chromosome arm contains an identical FRT site proximal to the mutation of interest. Prior to mitotic recombination, Gal80 acts by repressing a pan-neuronal Gal4, thus preventing *UAS-lacZ* and *UAS-GFP* activation. When mitotic recombination is induced in G2 by the use of a heat-inducible FLP, the homozygous mutant progeny cell loses the repressor and therefore express *UAS-lacZ* and *UAS-GFP*. In this way the expression of the markers is maintained in progeny cells. The other daughter cell receives two copies of the repressor transgene and therefore does not express the UAS reporters.

homozygous mutant cells that are positively labelled for βgal due to the loss of GAL80. If mitotic recombination occurs during G2 of the NB cell cycle, half the time the newly born NB will be devoid of GAL80 and thus positively labelled. By using the pan-neuronal driver, *Elav-Gal4* (Lin and Goodman, 1994), all the subsequent progeny neurons are labelled. The MARCM strategy can be mainly used for the production of brain lobe and thoracic clones; this is because, due to limited GAL80 dilution, small clones such as those in the abdomen cannot be detected.

Considering potential extrinsic mechanisms, peaks of 20-hydroxyecdysone (20HE), the steroid hormone that promotes moulting, are important for timing many developmental events (Riddiford, 1993). pNBs and larval neurons express high levels of the Ecdysone Receptor B1 isoform (EcR B1). Interestingly, prominent EcR B1 expression is found in pNBs during the last larval instar, correlating with main neural proliferative period (Truman et al., 1994). CNS culture experiments have indeed suggested a role for 20HE in stimulating NB division and the presence of this hormone appears to be required for Ubx induction within mature postembryonic neurons (Glicksman and Truman, 1990; Truman et al., 1993). Together, these reasons prompted me to focus on the role of two genes, members of the nuclear receptor superfamily, *ultraspiracle (usp)* and *seven up (svp)* that respectively transduce and inhibit the 20HE response (Perrimon et al., 1985; Mlodzik et al., 1990; Zelhof et al., 1995). Usp binds to the EcR and in this heterodimeric form recognizes DNA responsive elements in the promoters of 20HE responsive genes. The activation of these target genes occurs when the presence of 20HE induces the appropriate conformational changes of EcR to unmask its transcriptional activation domain (Horner et al., 1995). *svp* encodes an orphan nuclear receptor expressed in embryonic NBs (Doe, 1992). In the eye discs, it is required for eye photoreceptors development and it has been shown to antagonize the functions of EcR/Usp (Mlodzik et al., 1990; Zelhof et al., 1995).

The involvement of a cell-intrinsic mechanism was also analysed. Candidate factors for the repression of Hox expression in the dividing thoracic NBs are the genes belonging to the Polycomb group (Paro, 1993; Gould, 1997; Orlando, 2003). These well-studied factors are part of the cell-memory mechanism that maintains

Hox genes in the silenced status. I analysed the combined roles of three genes belonging to the Polycomb group: *Posterior sex-combs* (*Psc*), *Additional sex-comb* (*Asx*) and *Polycomb-like* (*Pcl*) (Duncan, 1982; Jurgens, 1985; Breen and Duncan, 1986).

4.2 Results

4.2.1 Size and Ubx expression of wild-type thoracic pNB clones

For the MARCM studies, FLP-mediated recombination was induced soon after hatching and pNB clones analysed at the third larval instar (L3). Mutant clones were analysed on the basis of three parameters:

1) Presence of the pNB
2) Measurement of clone size (number of cells derived from one pNB)
3) Analysis of Ubx expression pattern

Initially, I examined these parameters in wild-type clones of parasegment 6 at mid-L3, late-L3 and puparation stages (Figure 4.2 A-C). The presence of a large cell located at the ventral surface is detectable in 90% of the clones and at all three stages considered. This cell represents the asymmetrically-dividing NB that generates the clone of progeny neurons. The labelling of the progenitor cell within the clones turns out to be very useful for my studies and is due to aberrant expression of the *Elav-Gal4 C155* driver. In fact, the endogenous *elav* gene is normally expressed exclusively in post-mitotic neurons. Consistent with the study of Bello *et al* described in the introduction (Section 1.11, Bello et al., 2003), the NB present in parasegment 6 does not express Ubx protein at any of the three stages.

At mid-L3, the clone size is on average 32 (n=56) and none of the cells within a clone express Ubx (Figure 4.2A). At late-L3 stage, clone size has increased to an average of 60 cells (n=52). At this later point Ubx expression is detected within the clone but the fraction of Ubx-positive neurons is extremely variable, although never more than 62%. Ubx-positive neurons are normally located away from the pNB, deep within the CNS and close to the neuropil. This indicates that they were born relatively early in larval development. Superficial cells, close to the pNBs and therefore late-born, do not express Ubx at late-L3 (Figure 4.2B). However, at

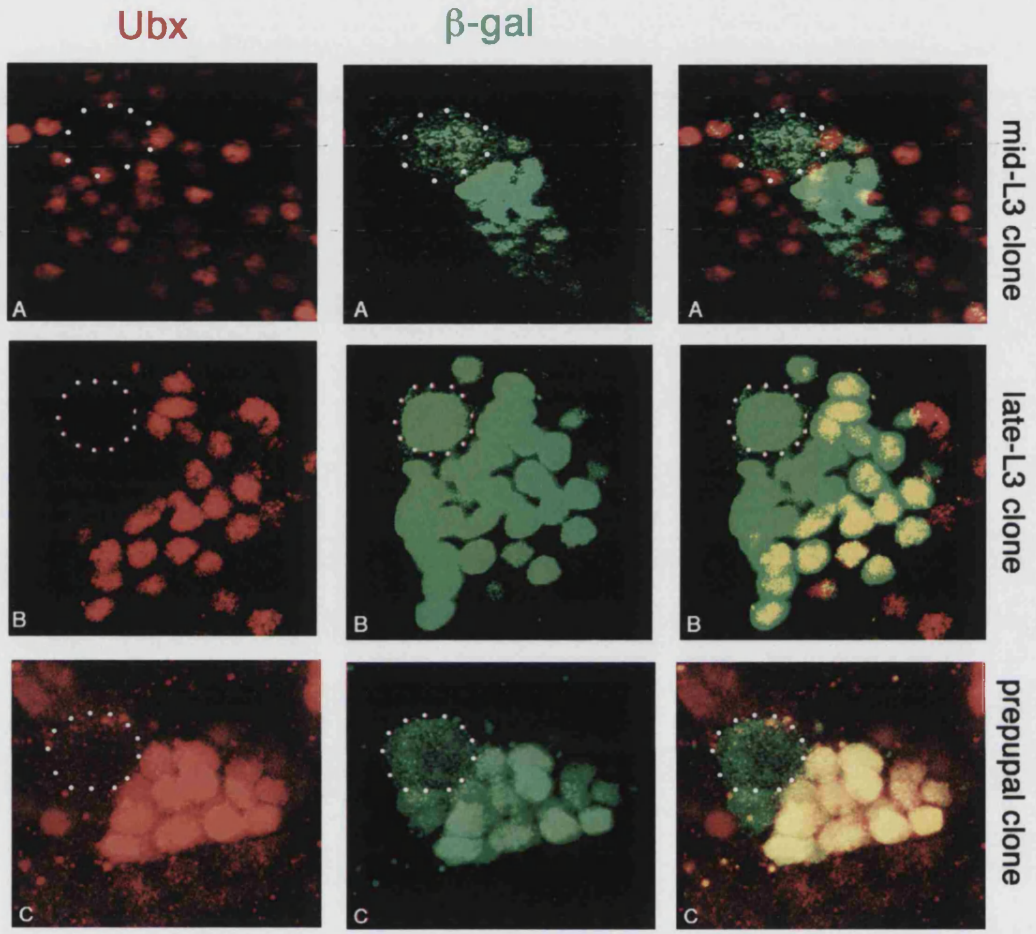


FIGURE 4.2 UBX EXPRESSION IN L 3 AND PRE-PUPAL THORACIC MARCM CLONES. Thoracic (PS6) MARCM clones labelled with anti-β–gal (green). Ubx (red) expression within the clones is shown. In this and following figures the dotted circle indicates the position of the NB. Ubx is never found in PS6 NBs but it is progressively upregulated in progeny neurons. (**A**) Mid-L3 (72 hr), all neuronal cells are Ubx-negative. (**B**) Late-L3 (96 hr) clone, some of the more dorsally located neurons express Ubx. (**C**) Pre-pupal (106 hr) clone, most of the ventral neurons now express Ubx. Panel A shows projections of confocal sections. Panel B and C show single-section confocal images of the ventral region of clones.

prepupal stages, superficial neurons do express Ubx and, in many clones, I find that all the postmitotic cells express this Hox protein (Figure 4.2C). Therefore, the upregulation of Ubx expression within post-embryonic (imaginal) neurons depends on developmental time. As clone size increases with time during the last larval instar and prepupa, a wave front of Ubx expression appears to expand from older to younger neurons.

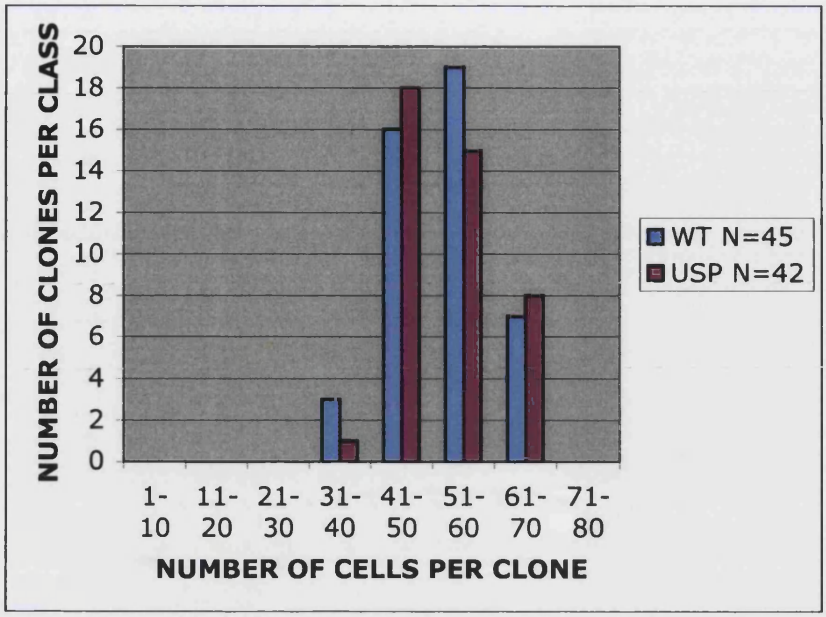
Having characterized the wild-type clones, I next analysed the same parameters in mutant clones.

4.2.2 A pNB role for *ultraspiracle* and *seven-up*?

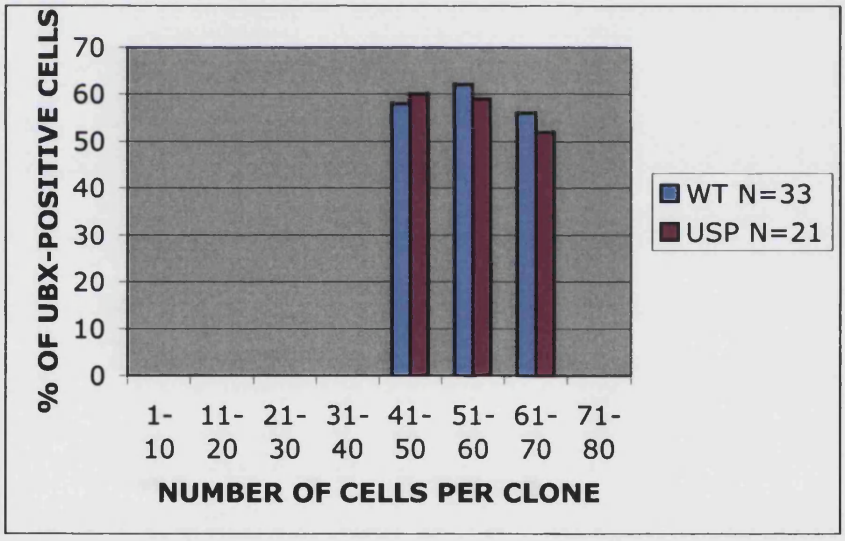
First, a possible role of *usp* in controlling the development of the NB was considered. MARCM clones homozygous for usp^3 were induced; this allele is a strong hypomorph of *usp* resulting from a missense mutation in the DNA-binding domain (Henrich et al., 1994; Zelhof et al., 1997; Lee et al., 2000a).

Thoracic usp^3 clones were analysed at late L3: these were all positive for the presence of the NB (n=42) and further analysis failed to show any significant clone size differences relative to the wild type (Figure 4.3A). This indicates that, from exit of quiescence to at least until the end of L3, Usp is not required for NB survival and divisions. When Ubx expression was assessed in usp^3 clones, again it was not possible to observe any deviation from the wild-type pattern (Figure 4.3B). These surprising findings are in apparent contradiction with what was observed in previous *in vitro* experiments where 20HE was shown to be required for Ubx upregulation in neurons (Glicksman and Truman, 1990; Truman et al., 1993). Given this result, a complementation test was carried out. This verified that the FRT chromosome used contained a true loss-of-function allele of *usp* (see Material and Methods), confirming the lack of a cell-autonomous requirement for a Usp-based 20HE trasduction pathway, either for up-regulation of Ubx in neurons or for the control of NB divisions.

The role of a second nuclear receptor, Svp (Mlodzik et al., 1990; Zelhof et al., 1995), was next considered. Svp is a known embryonic NB marker but its



A



B

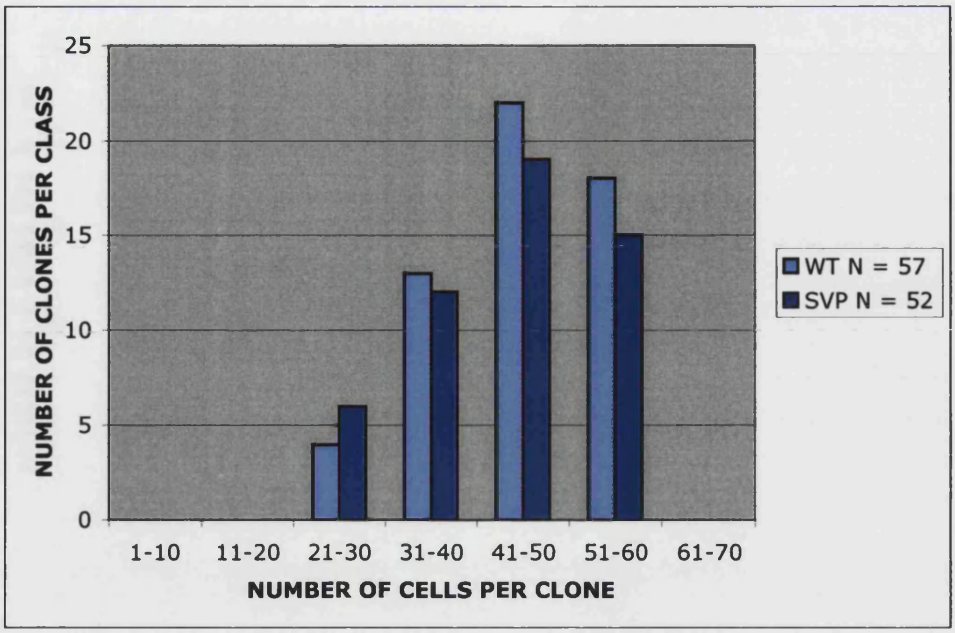
FIGURE 4.3 *ULTRASPIRACLE* IS NOT REQUIRED TO CONTROL CELL PROLIFERATION OR *UBX* EXPRESSION IN THORACIC PNB CLONES.
Graphs show (**A**) the distribution of clone sizes (**B**) the percentage of Ubx-positive cells within each size class. For both studies, clones were analysed at late-L3 (96 hr) and for Ubx expression only clones in PS 6 were scored. N: number of clones studied.

functions in the CNS are not yet clear (Doe, 1992; Younossi-Hartenstein et al., 1996). For the MARCM analysis, clones homozygous for the amorph, svp^1, were induced (Mlodzik et al., 1990). While carrying out this experiment, I noticed that svp^1 mosaic larvae have a relatively faster rate of development than wild-type: most 96 hr larvae analysed had already undergone puparation while their wild type counterparts were still at wandering stage. Although the use of the *Elav-GAL4* driver in this method allows the detection of the clones exclusively in the CNS and in the eye imaginal discs, recombination is induced in all mitotically-active tissues. This suggests that, in some as yet unidentified tissue(s), Svp negatively regulates the normal rate of larval development. As a consequence of this developmental asynchrony, I dissected wild type and mutant mosaic CNS from larvae that were staged on the basis of behaviour (wandering or puparation) and morphology of the anterior spiracles, rather than absolute time. Using these more accurate staging criteria, it can be seen that a large NB persists in the late-L3 svp^1 clones observed, and that there is no dramatic difference in clone size relative to wild type (Figure 4.4A). For this reason, I conclude that the pNB does not require Svp function from quiescence to late-L3 for its survival and division. Regarding the fraction of Hox expressing neurons, once again no significant difference from the wild type was found (Figure 4.4B).

In conclusion, I have shown that Usp and Svp, two modulators of the ecdysone pathway, appear to be dispensable in the thoracic NB clones for cell proliferation as well as Ubx regulation.

4.2.3 *Polycomb* group genes promote pNB divisions

Next, I turned to study a candidate cell-intrinsic mechanism for Hox regulation in the CNS, the *Polycomb* group (PcG). MARCM clones homozygous for all three amorphic alleles Psc^{Df2}, Asx^{XF23} and Pcl^{XM3} (*3Pc* clones) (Simon et al., 1992) were studied in the thoracic region and in the brain lobes of the L3 CNS (Figure 4.5).



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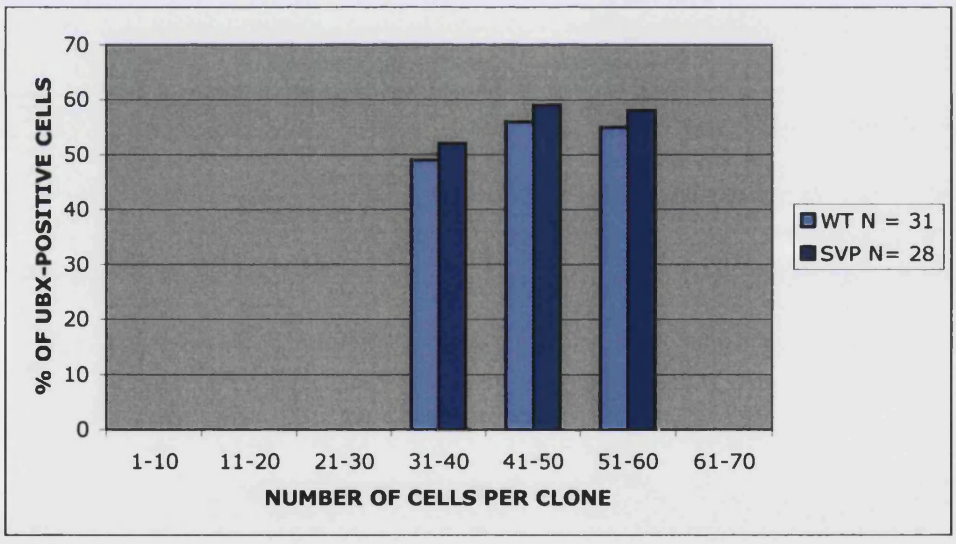


B

FIGURE 4.4 *SEVEN-UP* IS NOT REQUIRED TO CONTROL CELL PROLIFERATION OR *UBX* EXPRESSION IN THORACIC PNB CLONES.
Graphs show (**A**) the distribution of clone sizes (**B**) the percentage of Ubx-positive cells within each size class. For both studies clones were analysed at late-L3 (96 hr), for the study of Ubx expression only clones in PS 6 were scored. N: number of clones studied.

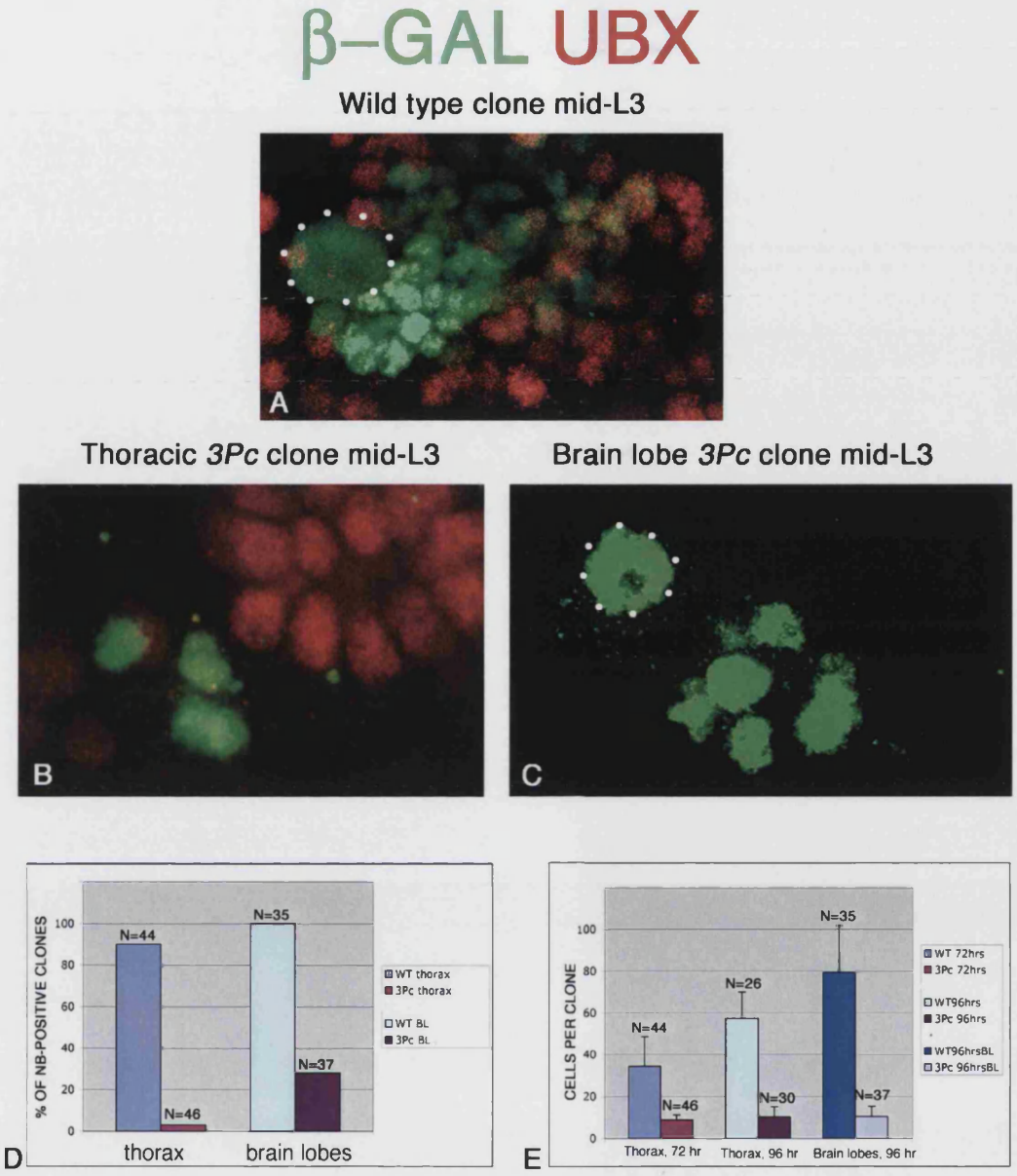


FIGURE 4.5 DISAPPEARANCE OF THE pNB AND REDUCTION OF SIZE IN *3Pc* CLONES.
(**A-C**) Mid-L3 (72 hr) MARCM clones labelled with anti-β-gal (green) and anti-Ubx (red). (**A**) Large size and presence of the pNB is observed in a thoracic wild type clone in PS6. (**B**) Dramatic reduction of clone size and disappearance of the pNB in a *3Pc* thoracic clone in PS6. Note that all cells in the *3 Pc* clone are Ubx-negative. (**C**) *3Pc* clone found in the brain lobes (BL), clone size is reduced but a pNB-like cell can be observed in 28 % of clones. (**D-E**) Graphs showing the percentage of cases in which the pNB is found (**D**) and clone sizes (**E**) for wild type and *3Pc* clones (in thorax, T, and in the brain lobes, BL). N: number of clones analysed for each genotype. Error bars in this and all subsequent figures show standard deviation (sd) from the mean (m). m and sd of clone size values in **E** are: WT T 72 hr (m: 34.3, sd: 14.4) *3Pc* T 72 hr (m: 8.8, sd: 2.4). WT T 96 hr (m: 57.3, sd: 12.7), *3Pc* T 96 hr (m: 10.1, sd: 4.9). WT BL 96 hr (m: 79.3, sd: 22.7) and *3Pc* 96 hr BL (m: 10.4, sd: 4.7).

In the thorax, the NB is absent in more than 95% of the *3Pc* clones (n=46). Consistent with this observation, there is no significant increase in the size of *3Pc* clones between mid-L3 (72 hr) and late-L3 (96 hr). Importantly, clone size is dramatically reduced relative to wild-type, with *3Pc* clones being approximately six times smaller (Figure 4.5B,D,E). In the brain lobes, a somewhat different result is obtained with a large NB observed in 28 % of the *3Pc* clones (n= 37). Nevertheless, the mitotic capacity of this cell appears to be strongly reduced as clone size is about 8 times less than wild-type (Figure 4.5C-E).

In addition, there is no significant difference between the sizes of clones analysed at 72 hr and at 96 hr and, when NBs do persist, they are never labelled by H3p, a marker for actively-dividing cells (data not shown). This demonstrates that *3Pc* genes are required to promote normal NB division in both thorax and brain lobes and, in the majority of cases, loss of *3Pc* function also results in the disappearance of a recognisable NB. In this latter respect, the difference observed between the thorax and the brain may be explained by a different temporal requirement for *3Pc* activity: remaining NBs in brain lobe clones might disappear at slightly later stages.

The neurons found in the mutant clones do not appear to stay clustered together as much as in the wild type; this is particularly evident in brain lobe clones where the progeny neurons are located relatively distant from the progenitor cell (Figure 4.5 C). Bello *et al* have observed a similar scattered appearance of the cells belonging to UAS-abdA, UAS-Ubx and UAS-Antp misexpression clones (Bello et al., 2003). This prompted me to examine the pattern of expression of Ubx. However, I found no evidence for any Ubx expression in PS6 mutant clones (Figure 4.5B). Furthermore, *3Pc* clones do not appear to express ectopic Ubx, AbdA or AbdB either (Figure 4.5C and data not shown), ruling out the possibility that *Ubx* might be down-regulated by repressive interactions from more posteriorly derepressed Hox genes (Struhl and White, 1985).

The absence of Ubx expression from all neurons in *3Pc* clones is surprising as these neurons must have been born early in larval development, prior to NB

disappearance and normally would be expected to be Ubx-positive by late-L3 (see Figure 4.2) The lack of neural Ubx expression suggests a novel requirement for the *3Pc* genes in inducing Hox expression in the imaginal neurons. In conclusion, the function of *3Pc* genes is required for the division and persistence of a recognisible NB but I find no evidence that they silence Hox gene expression in NB lineages.

4. 3 Discussion

4.3.1 *usp* and *svp* clones show normal pNB divisions and Ubx expression

The functions of three different candidate factors within the pNB lineages were analysed in genetic mosaic experiments. In each case, mutant clones were induced at the beginning of first larval instar and the resulting phenotypes were analysed during L3 or pupal stages. As a first approach, I considered the involvement of two receptors that influence 20HE signalling, an extrinsic mechanism.

usp^{3} mutant clones failed to show any difference relative to the wild type ones. This is a rather surprising finding. In fact, as described in Section 1.11 of the general introduction, previous studies using cultured larval CNS preparations suggested that 20HE triggers the activation of Ubx expression within the neurons and controls the NB rate of proliferation (Glicksman and Truman, 1990; Truman et al., 1993). The apparent inconsistency of my findings, based on MARCM analysis, with Truman's *in vitro* experiments could be explained in at least three different ways. First, Usp/EcR might still be required for pNB divisions but play a non-cell-autonomous role, perhaps being necessary elsewhere in the CNS, for example in the surrounding glial cells. Second, 20HE signal transduction via EcR might use a different partner than Usp. For testing *EcR* function, because this locus is centromeric to all available FRT sites on 2R, MARCM analysis cannot be used; instead, the use of dominant-negative and RNAi forms of EcR driven by specific UAS constructs could be considered. Finally, the discrepancies between the clonal analysis and the *in vitro* findings, could also be explained by the fact that 20HE might act independently of EcR. In this respect, the orphan nuclear receptor DHR78, may have a EcR-independent role in mediating the ecdysone response in L3 larvae (Fisk and Thummel, 1998).

Svp can modulate Usp-based ecdysteroid signalling during eye development (Zelhof et al., 1995), however, thoracic svp^1 clones, like the usp^3 mosaics, are indistinguishable from wild type, both in terms of neural proliferation and neuronal Ubx expression. Therefore it seems that this early embryonic NB marker (Doe, 1992) does not play a lineage-autonomous role in the development of thoracic pNBs.

There is, however, a major limitation with the MARCM *usp* and *svp* experiments. As this method relies on at least one cell division, gene function is not removed until the exit from quiescence. Therefore it is still possible that these two genes might exert their control on neural proliferation and Ubx expression at early larval stages.

4.3.2 *3Pc* genes promote divisions of thoracic pNBs.

3Pc mutations result in a dramatic reduction in thoracic clone size and in loss of Ubx neuronal expression. The possibility that this surprising latter phenotype could be due to the phenomenon of Hox negative cross-regulatory interactions (Struhl and White, 1985) is unlikely because the most posterior Hox gene, *AbdB,* is not up-regulated in place of *Ubx* (data not shown).

I now discuss the two observed phenotypes separately.

4.3A. *3Pc* genes are required for Ubx expression in neurons

3Pc thoracic pNBs produce a small number of progeny neurons before they cease dividing (Figure 4.5). These neurons presumably correspond to wild type early-born cells that are normally located distal to the NB and that, in parasegment 6, express *Ubx* at late-L3 (Figure 4.2). However, no Ubx, AbdA or AbdB expression was found in any of the *3Pc* clones examined. This unexpected *3Pc* neuronal phenotype raises the interesting hypothesis that one or all of the *3Pc* genes are required for Ubx up-regulation and for somehow controlling neuronal maturation.

PcG and *trxG* members have been ascribed roles in the maintenance of gene repression and activation respectively (Paro, 1990; Gould, 1997, see also Figure 1.9). However, members of both families can play positive and negative roles, suggesting that both these Hox maintenance effectors interact with one another in the normal regulation of gene expression (Brock and van Lohuizen, 2001). Whereas *Pcl* and *Psc*

have been shown to repress *AbdB* expression in the embryonic CNS (Simon et al., 1992), it might still be possible that they play an opposite role in the context of the larval CNS. Mutations in individual *3Pc* genes could be tested in MARCM experiments to further characterise the effect on *Ubx*. In addition, studies of the consequences of *trxG* mutations in MARCM clones could be carried out in order to address the possible additional involvement of members of this group in the late L3 activation of Hox expression in the early-born neurons.

4.3B. *3Pc* genes promote pNB divisions, probably via a Hox-independent pathway.

3Pc mutations cause a premature halt in thoracic and brain lobe pNB divisions. In the thoracic segments and in most of the clones found in the brain lobes, this also results in the disappearance of the large stem-cell by mid-L3. This could be due to NB cell cycle exit or apoptosis. Experiments using TUNEL or anti-caspase immunostaining would address the possible involvement of the latter mechanism.

Why do *3Pc* mutations have such a dramatic effect on NB divisions? Based on the work of Bello *et al.*, and the known role of *PcG* genes, a Hox-mediated mechanism was initially favoured. However now this looks less likely as the lack of Hox expression within the progeny neurons argues against the possibility that the thoracic clones undergo widespread Hox de-repression before their disappearance. Nevertheless, to rule out Hox involvement completely, *3Pc* thoracic clones could be analysed at earlier stages in an attempt to detect Hox expression in the NB before its disappearance.

On the basis of my results thus far a Hox-independent role in pNB divisions is favoured. In addition to their well-characterized role in Hox regulation, members of the *PcG/trxG* are known to control many other processes including cell proliferation (Gould, 1997; Muyrers-Chen and Paro, 2001; Jacobs and van Lohuizen, 2002). Related oncogenic and tumour-suppressive functions have been described for mammalian *PcG* and *trxG* genes within the haematopoietic system. For example, *Bmi1*, the mammalian gene related to one of the *3Pc* genes, *Psc,* appears to be a positive regulator of haematopoietic cell proliferation (Jacobs et al., 1999; Jacobs and van Lohuizen, 2002). Interestingly, direct evidence that Hox gene de-repression

is causal to the haematopoietic abnormalities in PcG mutant mice is so far lacking and other critical target genes have been identified. For example, the *INK4a/ARF* tumour suppressor locus was identified as a downstream target of *Bmi1* (Jacobs et al., 1999). *INK4a/ARF* is a member of the cyclin-dependent kinase inhibitors responsible for regulating cell cycle exit from G1 to a G0-like quiescent state. In this regard it would be interesting to test the role of the *Drosophila* inhibitors of the cell cycle, such as *dacapo* (de Nooij et al., 1996).

In the next Chapter, I test another NB intrinsic factor, the transcription factor Grainyhead, for possible roles in regulating neural proliferation.

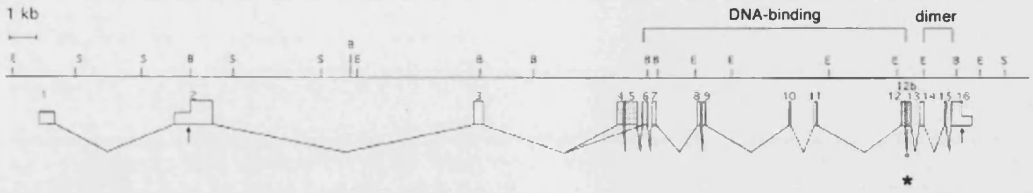
CHAPTER 5
REGULATION OF LARVAL NEUROBLAST DIVISIONS BY GRAINYHEAD

CHAPTER 5 REGULATION OF LARVAL NEUROBLAST DIVISIONS BY GRAINYHEAD

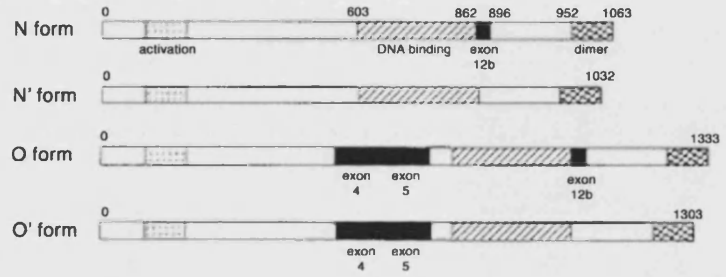
5.1 Introduction

Drosophila Grainyhead (Grh) defines a conserved family of mammalian transcription factors with highly-related activation and DNA-binding domain (Wilanowski, 2002). Grh is also known as NTF-1 (Neurogenic element-binding Transcription Factor 1) and Elf-1 (Element 1 binding factor) as it was initially identified through its ability to bind *in vitro* to regulatory DNA elements critical for the activation of the *Dopa decarboxylase* (*Ddc*) and *Ubx* genes promoters (Bray et al., 1989; Dynlacht et al., 1989; Attardi and Tjian, 1993). Early during embryonic development, maternally provided Grh is required for dorsal-ventral and terminal patterning (Huang et al., 1995; Liaw et al., 1995). Towards the end of embryogenesis (stages 14-17) *grh* expression becomes restricted to tissues derived from the ectoderm: the cuticle-secreting cells of the epidermis and the CNS. In the latter tissue, it is specifically expressed in a subset of cells that, based on their location, have been identified as the quiescent embryonic NBs (Bray et al., 1989). Later in development, *grh* expression persists in epidermal cells and in the post-embryonic NBs throughout larval life (Uv et al., 1997). The primary transcript of *grh* undergoes tissue-specific alternative splicing that results in the production of different mRNAs encoding 4 protein isoforms, the major ones being the N-form and the O-form (Figure 5.1). The mRNA of the O-form includes two additional exons (exon 4 and 5), producing an isoform with an extra 270 amino acids adjacent to the predicted DNA-binding domain. Each form has a different expression pattern, with the N-form found in epidermal tissue as well as in the optic lobes of the larval CNS. The O-form is instead a CNS-specific protein, found in the pNBs of the brain lobes and the ventral nerve cord (Uv et al., 1997).

grh must play several essential roles during development: null mutations in the gene, such as grh^{B37}, cause late embryonic lethality associated with defects in the cuticle and head skeleton, probably due to impaired epidermal expression of *Ddc*, necessary for colouring and hardening the cuticle (Bray and Kafatos, 1991).



A



B

FIGURE 5.1 STRUCTURE OF THE *GRH* GENE AND ITS PRODUCTS.
(**A**) Organization of the *grh* gene in *Drosophila*, scale is indicated at the top left corner. The horizontal line represents the genomic DNA with the restriction sites for EcoRI, BamHI and SalI indicated respectively as E, B and S above the line. Vertical arrows indicate the start and termination of the translated region. Boxes and numbers denote the position of the exons, the shaded boxes represent the exons that are alternatively used and the asterisk marks alternate exon 12b. The exons encoding the DNA-binding and dimerization domains are denoted by brackets above the genomic DNA. (**B**) The four identified Grh isoforms are represented. N-form, N'-form, O-form and O'-form mRNAs are illustrated with a horizontal box. The hatched boxes indicate DNA-binding domains, cross-hatched boxes indicate dimerization domains and dark boxes represent the alternative exons. The numbers above the boxes represent the amino acids at various positions: the N-terminal and C-terminal of each protein, the site of alternate splicing and, for the N form, each end of the DNA-binding and dimerization domains. The O form contains additional exons (exons 4 and 5) compared to the N-form. The N'-form and the O'-form lack 30 amino acids downstream of the DNA-binding domain (exon 12b) whose function remains unknown. (Adapted from Uv *et al* 1997).

Moreover, embryos that lack *grh* function display a patchy tracheal network caused by the aberrant elongation of the branches (Bray and Kafatos, 1991; Hemphala et al., 2003).

However, in contrast with these strong cuticular defects, the general development of the CNS appears normal in *grh* mutant embryos. The axonal scaffold is unaffected and *Ddc* is normally expressed in the CNS, where it is required for the synthesis of serotonine and dopamine (Bray and Kafatos, 1991). For these reasons, and also because the CNS expressed O-isoform does not exhibit any novel characteristics *in vitro,* no particular CNS function has yet been ascribed to Grh. Importantly, Uv *et al* have isolated a mutation, grh^{370}, that specifically disrupts the O-isoform producing a Grh protein that is truncated at the end of exon 5 and thus lacks DNA-binding and dimerization domains. In contrast to grh^{B37}, grh^{370} homozygotes are not embryonic lethal but survive until larval life (Uv et al., 1997). When grh^{370} is placed over a deficiency ($Df(2R)Pcl^{7B}$), more than 75% of the progeny larvae die as early third instar. However, a few survive pupation, and 1% eclose as adults that appear unable to coordinate movements and so die soon after. This adult escaper phenotype suggests that Grh may play an important role in the post-embryonic development of the CNS. Consistent with this phenotype, Grh shows an interesting pattern of expression within the developing CNS: it is expressed within quiescent NBs (embryonic and larval) and later on remains switched on in dividing pNBs in both the thorax and abdomen (Bray et al., 1989; Uv et al., 1997; Bello et al., 2003). Nevertheless, the role of Grh during these diverse moments in the development of the NB lineages remains to be assessed. In this Chapter, I analyse grh^{B37} mutant NB clones and grh^{370} mutant larvae, revealing segment-specific functions for *grh* in the regulation of larval NB divisions.

5. 2 Results

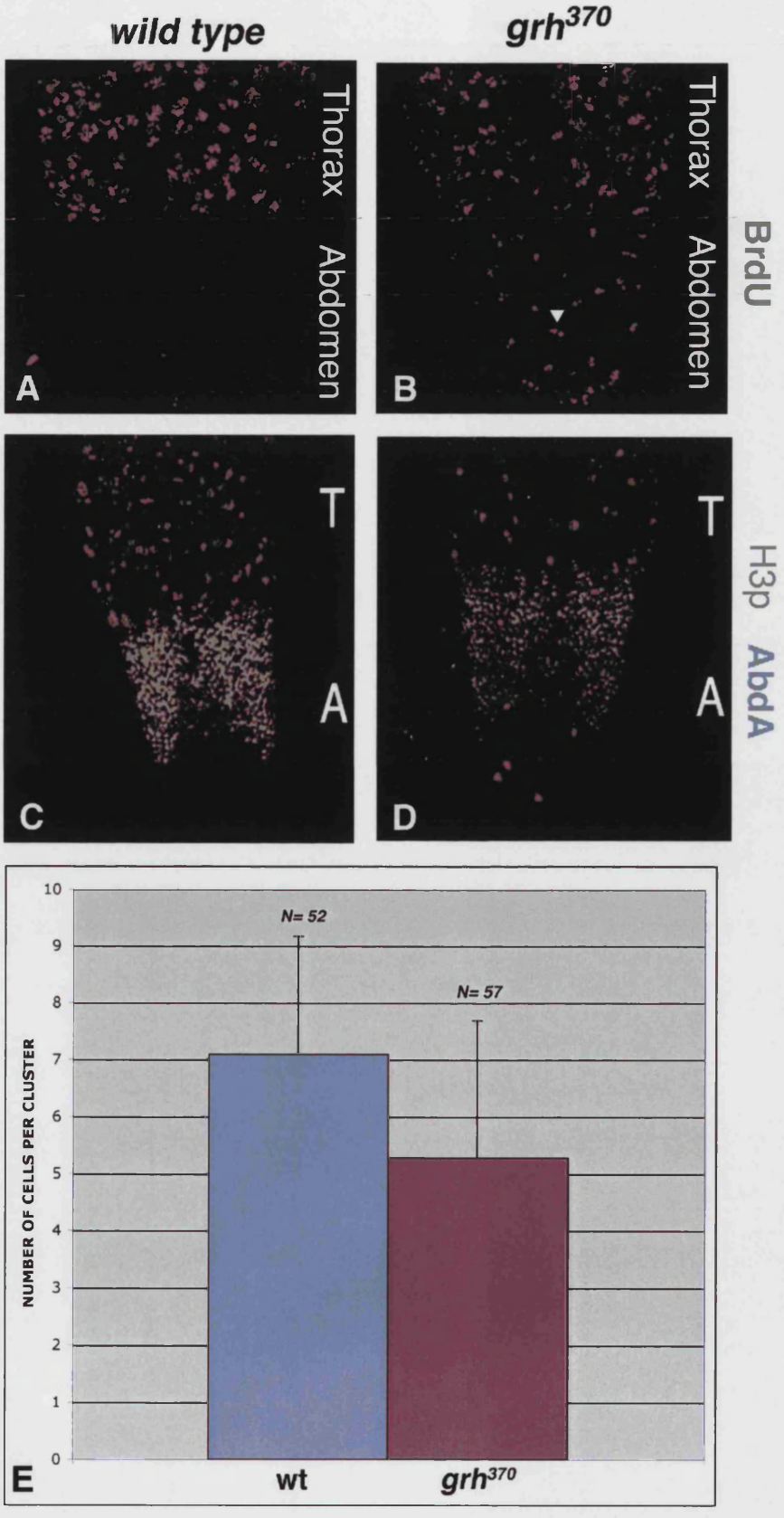
5.2.1 Segment-specific proliferation patterns in the *grh*^{370} CNS

To characterize initially Grh function in the NBs, the CNSs of wild-type and *grh*^{370}/*Df(2R)Pcl*^{7B} mutant larvae were fed with BrdU, a Thymidine analogue. In this way, the spatial pattern of all cells that had undergone S phase during the labelling period could be observed. Mid-L3 larvae were given a 6 hr pulse (72 –78 hr after hatching) of BrdU and then analysed by anti-BrdU immunostaining to observe the pattern of proliferation in the thoracic and in abdominal neuromeres (Figure 5.2 A,B).

First, I will consider the pattern of NB proliferation in the thoracic neuromeres. Thoracic clusters of cells that had undergone S phase during the 6 hr time window were found in both wild type and mutant larvae (Figure 5.2 A,B). However, the size of the *grh*^{370} clusters (number of BrdU-positive cells per cluster) is reduced from 7 to 5 cells, representing a 29% reduction relative to wild-type (Figure 5.2E). From this experiment, the average NB cell-cycle time at mid-L3 can be estimated, assuming it does not vary between 72 and 78 hrs. For the wild type this corresponds to about 1 3/4 hrs (6 ÷ (7× 0.5)), whereas for the *grh*^{370} mutant it is about 2 1/2 hrs (6 ÷ (5 × 0.5)). This demonstrates that, at mid-L3, the thoracic pNBs lacking Grh function are cycling at a reduced rate relative to their wild type counterparts. To confirm that this was also the case using a marker for M-phase of the cell cycle, an antibody to the phosphorylated form of Histone H3 (H3p), was used to stain mid-L3 (78 hr) wild-type and *grh*^{370}/*Df(2R)Pcl*^{7B} CNSs (Figure 5.2C,D). Wild type thoracic neuromeres label with frequent M-phase NBs and GMCs. In contrast, *grh*^{370}/*Df(2R)Pcl*^{7B} neuromeres show fewer H3p-positive cells. Thus, as both a cumulative marker of S-phases and an "instant" marker of M-phases show reduced labelling, I conclude that the speed of the cell cycle in thoracic pNBs is reduced in *grh*^{370}/*Df(2R)Pcl*^{7B} mutants.

Now considering the effect of *grh*^{370} mutations on mid-L3 divisions in the abdominal neuromeres. In the wild type, abdominal segments exposed to BrdU from 72 to 78 hr, no labelled cells can be observed. This is consistent with previous results

FIGURE 5.2 NEURAL PROLIFERATION IN WILD TYPE AND *GRH*^{370} L3 LARVAE.
(A-D) Single confocal sections of mid-L3 (78hr) ventral nerve cord, with the thoracic (T) and abdominal (A) regions indicated. **(A-B)** VNC from wild-type and *grh*^{370}/ *Df(2R)Pcl*^{7B} larvae exposed to a 6 hr pulse of BrdU (72-78 hr) and then labelled with anti-BrdU. Clusters of labelled cells are observed in both wild-type and *grh*^{370} thoracic neuromeres. The abdominal region of *grh*^{370} mutants but not wild-types is labelled with BrdU-positive cells. Most of these fall into the predicted positions of the vm,vl and dl. Ectopic clusters can also be observed (arrowhead). **(C-D)** VNC from wild-type and *grh*^{370} larvae labelled with anti-AbdA (green) and anti-H3p (red). AbdA marks the boundaries of the central abdomen (PS7-PS13) and H3p labels cells in M-phase. In the thorax of *grh*^{370} mutants, H3p-positive cells are less frequent relative to wild-type. In the abdomen, H3p-positive cells are found in *grh*^{370}. **(E)** Graph showing the number of BrdU incorporating cells per thoracic cluster for wild-type and *grh*^{370}. n= number of clusters analysed. Mean: wild-type = 7.09; *grh*^{370} = 5.27. Standard deviations: wild-type = 2.07 and *grh*^{370} = 2.41. Assuming a normal distribution, the wild-type and *grh*^{370} populations are statistically different at a high level of confidence ($P < 0.01$).


wild type
grh370
Thorax
Abdomen
A
B
BrdU
T
A
C
D
H3p AbdA
10
9
8
7
6
5
4
3
2
1
0
NUMBER OF CELLS PER CLUSTER
N= 52
N= 57
wt
grh370
E

indicating that the abdominal pNBs of 72 hr larvae are frequently TUNEL-positive, and thus already undergoing apoptosis (Bello et al., 2003).

However, in $grh^{370}/Df(2R)Pcl^{7B}$ abdominal neuromeres, many clusters of neurons that have incorporated BrdU can be detected (Figure 5.2A,B). On the basis of their dorso-ventral position most of these clusters appear to represent the neuronal progeny of vm, vl and dl pNBs (Truman and Bate, 1988). However, several labelled clusters are also located in positions where normally there are no pNBs, like for example in proximity to the midline. These observations indicate that abdominal pNBs lacking *grh* function persist after 72 hr and remain actively engaged in the cell cycle. To characterize further the effects of the grh^{370} mutation on abdominal pNB divisions, $grh^{370}/Df(2R)Pcl^{7B}$ and wild type larvae were fed continuously with BrdU from hatching to 72 hr (mid-L3) or 96 hr (late-L3) (Figure 5.3). In wild type CNSs, as described earlier, this protocol labels three clusters of vm, vl and dl progeny neurons in each of the central abdominal segments (Truman and Bate, 1988). The vm and vl clusters contain, on average, 4 cells and, because abdominal pNBs normally stop dividing and die at mid-L3, no significant difference in wild-type cluster size was detected at the two different developmental stages (Truman and Bate, 1988; Bello et al., 2003, compare Figures 5.3C and 5.3D). In the grh^{370} larval CNSs, continuous BrdU feeding labels clusters of neurons located in similar position relative to vm, vl and dl, in addition to some ectopic ones close to the midline. However, on average, 8 BrdU-positive cells are found in mid-L3 grh^{370} clusters in the position of vm and vl, and by the end of larval life, the size of the clusters has increased to an average of 14 cells, three and an half times the wild-type vm/vl cluster size (Figure 5.3 D). This striking result indicates that, in grh^{370} mutants, abdominal proliferation persists for at least 24 hrs beyond the normal time of 72 hr. During this extended period, pNBs must remain mitotically active, generating approximately 6 cells. For the 48-72 hr period, the average cell cycle time in the abdomen of grh^{370} mutants is approximately 6 hrs ($24 \div (8 \times 0.5)$), whereas estimates from wild-type vm/vl, give about 12 hrs ($24 \div (4 \times 0.5)$). Therefore, together with the previous BrdU and H3p labelling results (Figure 5.2), these studies demonstrate

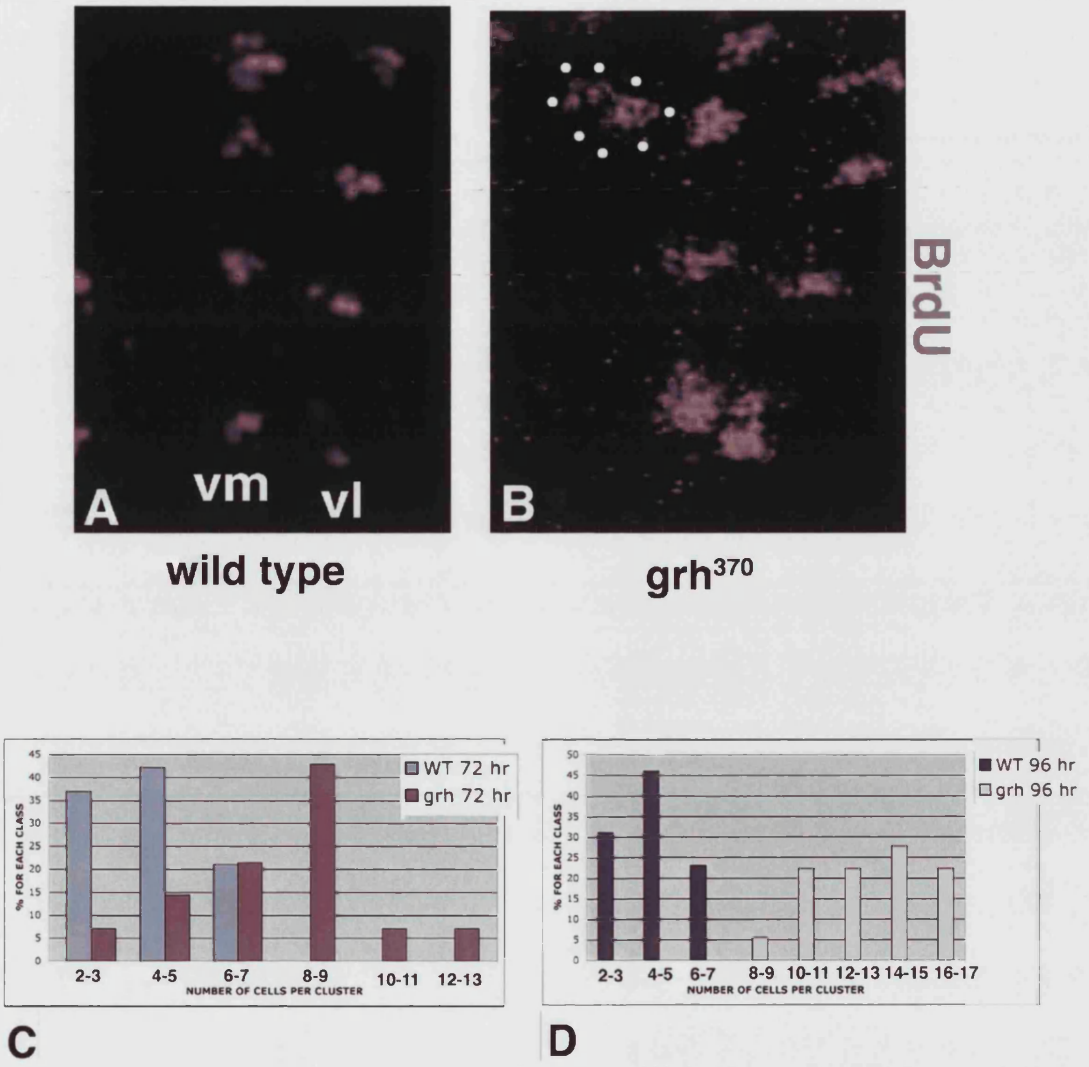


FIGURE 5.3 ABDOMINAL OVERPROLIFERATION IN *GRH*^{370} CNS. Wild type and *grh*^{370} larvae were continuously fed with 0.2 mg/ ml BrdU and the number of BrdU incorporating cells per cluster analysed at mid-L3 and late-L3. High power view of vm and vl clusters in (**A**) wild type and (**B**) *grh*^{370} late-L3 CNS. Ectopic clusters are found in *grh*^{370} mutants (white dotted circle). *grh*^{370} clusters are bigger than wild type. (**C**,**D**) Histograms showing the distribution of cluster sizes (number of BrdU incorporating cells per cluster) (**C**) at 72 hr and (**D**) at 96 hr within each size class. Wild type 72 hr N = 19. *grh*^{370} 72 hr N = 14. Wild type 96 hr N = 18; *grh*^{370} 96 hr N = 18.

that *grh*^{370} mutant pNBs in the abdominal segments divide twice as fast and remain mitotically active for at least one day longer than their wild-type counterparts. Together, these results, demonstrate that the O-form of Grh is required to regulate both the rate and the duration of pNB divisions.

Surprisingly, the O-form of Grh has an opposite effect on neural proliferation in the thorax versus the abdomen. In the former context, it shortens the cell cycle and increases proliferation, whereas in the abdomen it lengthens the cell cycle and is necessary for halting cell proliferation. For a better understanding of these segment-specific Grh functions, Grh-deficient thoracic and abdominal lineages were analysed in more detail, paying particular attention to the fate of the pNB.

5.2A. Role of Grh in thoracic pNB lineages

Single Grh-deficient thoracic NB clones were generated using the MARCM technique described in the previous chapter (see Figure 4.1). For these experiments a different *grh* mutation, *grh*^{B37} was used. This is a null allele, corresponding to a 7 bp deletion of the coding sequence that causes a frameshift resulting in the truncation of both the N and O isoforms of the protein (Bray and Kafatos, 1991).

5.2.2 Grh is required for the division and maintenance of thoracic pNBs

grh^{B37} MARCM clones were induced at the beginning of L1 and analysed at mid-L3 or late-L3 (Figure 5.4). First, I observed that, at mid-L3, *grh*^{B37} clones are smaller than wild type ones, with an average of 20 cells per mutant clone relative to 34 per wild-type lineage (Figure 5.4A-C). At this stage, most *grh*^{B37} clones also display the presence of the progenitor NB, defined on the basis of its large cell size and superficial, ventral location. However, whereas 37 % of wild-type NBs (n= 56) are found in M-phase (H3p-positive), none of the mutant NBs analysed (n= 51) was ever found to be labelled by the anti H3p-antibody. Thus, at 72 hrs, although the Grh-deficient NB is still present, it is no longer able to divide. This is consistent with what I previously observed in BrdU or H3p labelled *grh*^{370} mutant larvae (compare with Figure 5.2), where the number of M-phase cells in the thorax is reduced relative to the wild type.

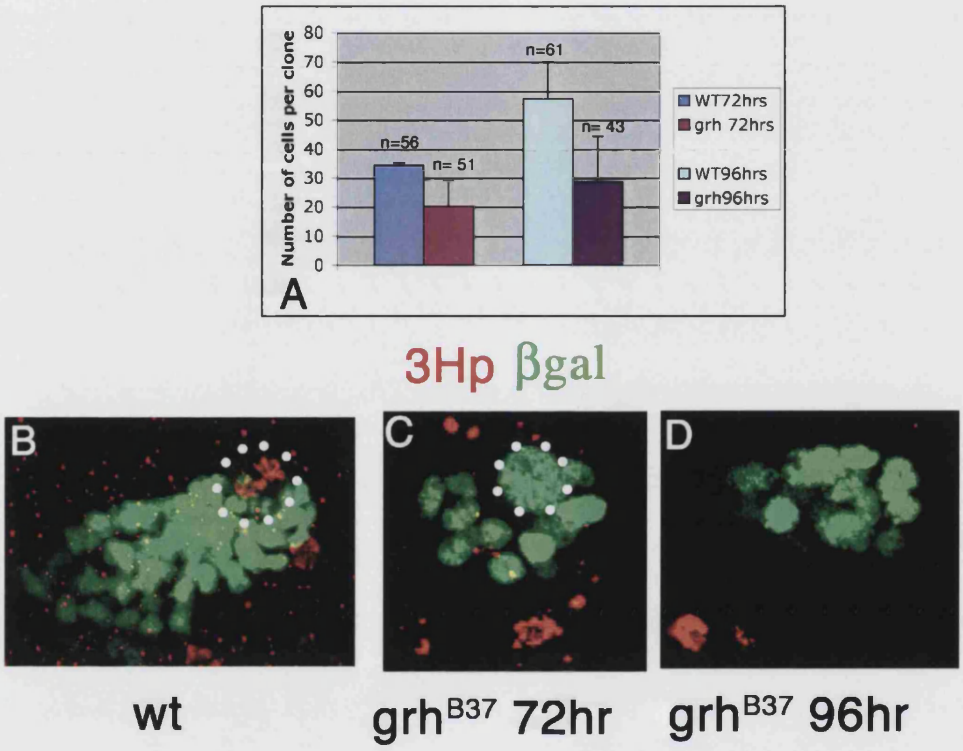


FIGURE 5. 4 GRH IS REQUIRED FOR THORACIC pNB DIVISIONS AND PERSISTENCE.
(**A**) Graph showing MARCM clone size for wild type and *grh*^{B37} larvae at mid-L3 (72 hr) and late-L3 (96 hr). Number of clones analysed for wild type at 72 hr, n = 56 mean, m = 34.3 standard deviation, sd = 0.9. Wild type clones at 96 hr n = 61 m = 57.3 sd = 15.7. For *grh*^{B37} at 72 hr n = 51 m = 20.2 sd = 8.7 and at 96 hr n = 43 m = 28.6 sd = 12.7. (**B-D**) MARCM clones immunolabelled with anti-H3p (red) and an ti-β–gal (green). When present, the pNB is indicated with a white dotted circle. (**B**) Wild type late-L3 (96 hr) clone. The pNB is shown in M-phase (3Hp-positive) (**C**) *grh*^{B37} mid-L3 (72 hr) clone. Note that the pNB is H3p-negative (**D**) *grh*^{B37} late-L3 clone, a large pNB is no longer present.

At the late L3 stage, the size of grh^{B37} mutant clones was found to be less than half the wild-type size (on average 28 cells in a grh^{B37} mutant clone, n= 43, compared to 57 for the wild type ones, n=61). Despite the observation that the mutant NB is no longer dividing at 72 hr, the clone size at 96 hr appears larger than at 72 hr. This may be accounted for by GMC divisions but, since great variability in cell number found in each clone is observed in wild-type, this difference may not be significant.

Interestingly, none of the clones analysed at 96 hr were found positive for the presence of the NB (Figure 5.4 D). In conjunction with the analysis carried out in the $grh^{370}/Df(2R)Pcl^{7B}$ larvae, the MARCM analysis indicates that removing Grh activity has a two-step effect on thoracic NB development: first, it impairs cell proliferation by reducing the speed of cell cycle progression, eventually halting it completely by 72 hr. Second, loss of Grh leads to a large NB no longer being recognisable by 96 hr.

To investigate the fate of grh^{B37} NBs in more detail, an immunostaining was performed using the NB marker Miranda (Shen et al., 1997). As described in the introduction, Mira is expressed in NBs in all the larval proliferative anlagen of the CNS. At interphase and early prophase it is evenly distributed in the cytoplasm (Shen et al., 1997, see also Figure 1.3B). During M-phase, it shows a basal distribution in the pNB cortex and is segregated to the GMC during cytokinesis where it is rapidly down-regulated (Ceron et al., 2001). The large cell in mid-L3 grh^{B37} clones expresses Mira, confirming its identity as a NB. However, the polarised Mira distribution which is typical of M-phase is never observed, instead the protein is evenly distributed in the cortical cytoplasm (Figure 5.5A,B). This is consistent with the H3p-labelling results showing that grh^{B37} NBs never occupy M-phase at 72 hr. Next, the expression of Prospero (Pros), another asymmetric-division factor was analysed (Hirata et al., 1995; Knoblich et al., 1995; Hassan et al., 1997). In embryos, ectopic expression of Pros has been shown to induce the termination of neural cell divisions (Li and Vaessin, 2000). In contrast to embryonic NB lineages, however, Pros is reported to be exclusively expressed in the neurons of the larval CNS (Ceron et al., 2001). In agreement, I find Pros expression in imaginal neurons but, as all cells in a clone apart from the pNB itself are labelled, GMCs may also express this

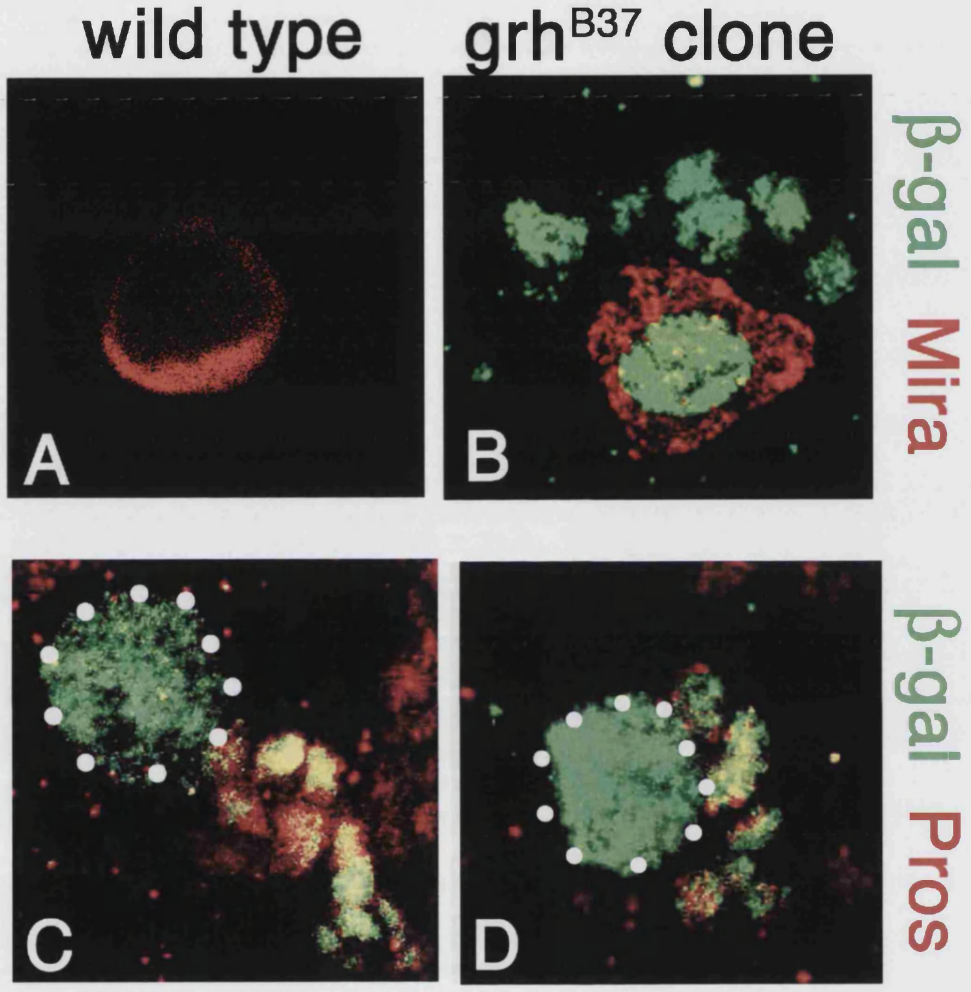


FIGURE 5.5 EXPRESSION OF MIRANDA AND PROSPERO IN *GRH^{b37}* CLONES. (**A**) Basal localization of Miranda (red) in a wild type M-phase pNB. (**B**) interphase or prophase NB of a 72 hr *grh^{b37}* clone (β-gal, green), Miranda is evenly distributed in the cytoplasm. (**C**) wild type and (**D**) *grh^{b37}* 72 hr clones (β-gal, green); Prospero (red) is found in the progeny neurons but not in the NB (white dotted circle).

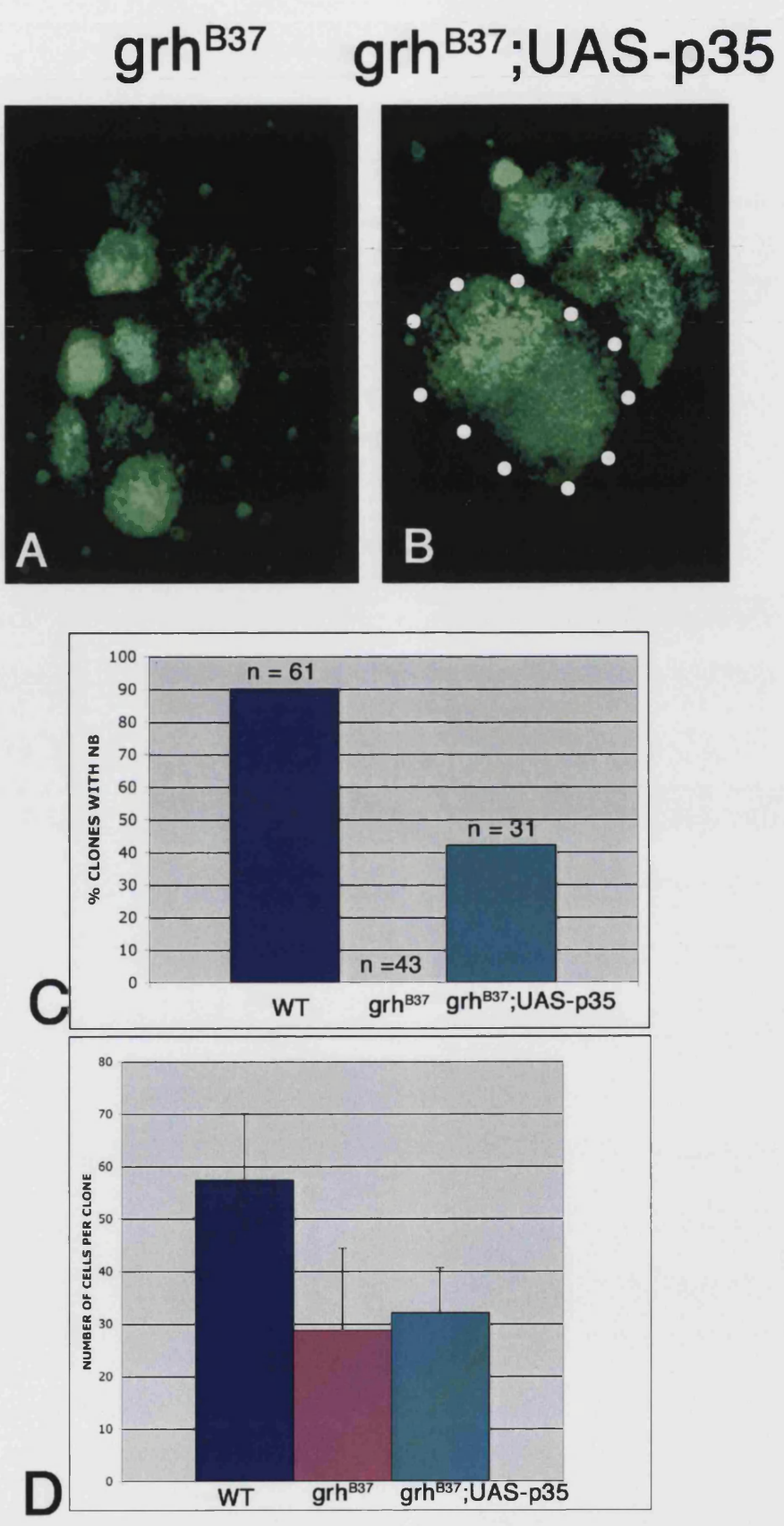
protein. This distribution of Pros is unaffected in grh^{B37} mutant pNB clones (Figure 5.5C,D). These experiments demonstrate that the premature halt of the cell cycle observed in grh^{B37}mutant NBs is not likely to be due to ectopic Pros expression in the NB.

5.2.3 Grh defective thoracic pNBs undergo apoptosis

In order to understand the cellular basis for the grh^{B37} NB disappearance in late L3 clones, I addressed the potential involvement of a cell-death mechanism. *p35* encodes for an inhibitor of caspases that trigger the apoptotic response (Hay et al., 1994) and, when over-expressed in *UAS-p35* MARCM clones, can rescue AbdA-induced cell death of thoracic pNBs with about 50% efficiency (data not shown). *p35* was over expressed in grh^{B37} clones that were then analysed at late L3, scoring both clone size and presence/absence of the NB (Figure 5.6). In grh^{B37} clones at 96 hr a recognisable NB is never observed, but in 42% of the grh^{B37} clones expressing *UAS-p35* a large NB-like cell is rescued (Figure 5.6 A-C). However the proliferative capability of this large cell is still impaired as grh^{B37} and grh^{B37}; *UAS-p35* clone sizes are virtually identical (Figure 5.6D). These findings favour the hypothesis that the disappearance of the NB in grh^{B37} mutants is a result of apoptosis.

Next, Hox expression was assessed in the grh^{B37} NBs at 72 hr, to test the possibility that, similar to the normal abdominal process (Bello et al., 2003), the disappearance of the NB might be due to Hox-mediated cell-death. However, when expression of a resident Hox protein, Ubx, was studied within the PS6 mutant clones at mid-L3, no deviation from the wild-type pattern was observed, that is no Ubx protein was found either in the NB or its progeny (Figure 5.7). This suggests that the disappearance of the Grh-deficient NB at later stages, may be via a Hox-independent apoptotic mechanism. These MARCM experiments, complement the analysis of grh^{370} larval CNSs and indicate that Grh plays an essential, cell-autonomous role in promoting thoracic pNB survival and division. A study of Ubx expression in grh^{B37}; *UAS-p35* clones at 96 hr would be needed to reveal whether the later pattern of expression of this Hox gene is also normal.

FIGURE 5.6 RESCUE OF THE PNB BUT NOT CLONE SIZE IN *GRH^{B37}; UAS-P35* CLONES. (**A**,**B**) Single confocal section (**A**) *grh^{B37}* and (**B**) *grh^{B37}; UAS-p35* MARCM clones at 96 hr. The white dotted circle in **B** indicates the rescued pNB. (**C**) Graph showing the percentage of visible NBs for wild type (n= 61), *grh^{B37}* (n= 43) and *grh^{B37}; UAS-p35* (N = 31) clones. The pNB is absent in 100% of the *grh^{B37}* clones and rescued in 42% of the *grh^{B37}; UAS-p35* clones. (**D**) Graph showing clone size for wild type (m = 57.3 sd = 12.7), *grh^{B37}* (m = 28.6 sd= 15.7) and *grh^{B37}; UAS-p35* (m = 32 sd = 8.6). All clones are analysed at late-L3 (96hr).


grh^B37
grh^B37;UAS-p35
A
B
C
% CLONES WITH NB
n = 61
n =43
n = 31
WT
grh^B37
grh^B37;UAS-p35
D
NUMBER OF CELLS PER CLONE
WT
grh^B37
grh^B37;UAS-p35

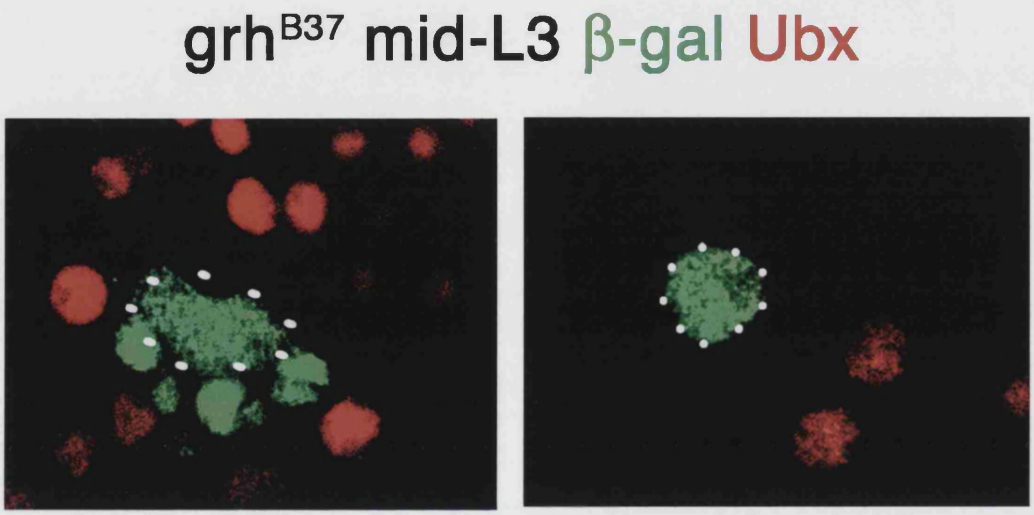


FIGURE 5.7 UBX IS NOT EXPRESSED IN *GRH^{B37}* pNBs AT MID-L3. Confocal section of *grh^{b37}* MARCM clones in parasegment 6 (β-gal, green). Ubx (red) is not expressed within the pNBs (white dotted circle).

5.2B. Role of Grh in the abdominal pNB lineages

The BrdU incorporation studies presented earlier have identified Grh as a factor that, similar to AbdA (Bello et al., 2003), is required to limit cell proliferation within abdominal neuromeres. Although Grh is reported to be expressed in the CNS exclusively in NBs (Bray et al., 1989), it is possible that it could also act at the level of the progeny GMC. Also, the regulatory relationship between *grh* and *abdA* needed to be examined.

5.2.4 *grh*^{370} pNBs survive until late L3 but still express the normal pulse of AbdA

To follow pNB fate in the abdomen, I studied the AbdA and Mira patterns of expression in *grh*^{370} mutants staged from mid-L3 to late-L3 (Figure 5.8). As expected, some Mira-positive cells can still be observed in the abdomen of 72 hr wild-type larvae. These cells are the remaining pNBs that will soon be eliminated (Bello et al., 2003). By late L3, however, no Mira-positive labelling can be detected within the AbdA domain of expression (Figure 5.8A,C). In *grh*^{370} mutants, Mira-labelled pNBs are clearly present in all abdominal neuromeres at mid-L3 and, in sharp contrast to the wild type, they survive until late L3 (Figure 5.8B,D). Consistent with the BrdU labelling studies, most of the surviving *grh*^{370} pNBs fall within the expected three abdominal rows of vm, vl and dl. However, some ectopically positioned Mira-positive cells can also be found. Particularly frequent seems to be the presence of a pair of NBs positioned close to the midline. Whether these ectopic NBs are of embryonic origin and have escaped the normal burst of abdominal death in the late embryo (Peterson et al., 2002) is not known. Another important finding is that, at the two time points analysed, *grh*^{370} pNBs do not express AbdA, indicating that Grh is not required to keep AbdA repressed in the pNBs.

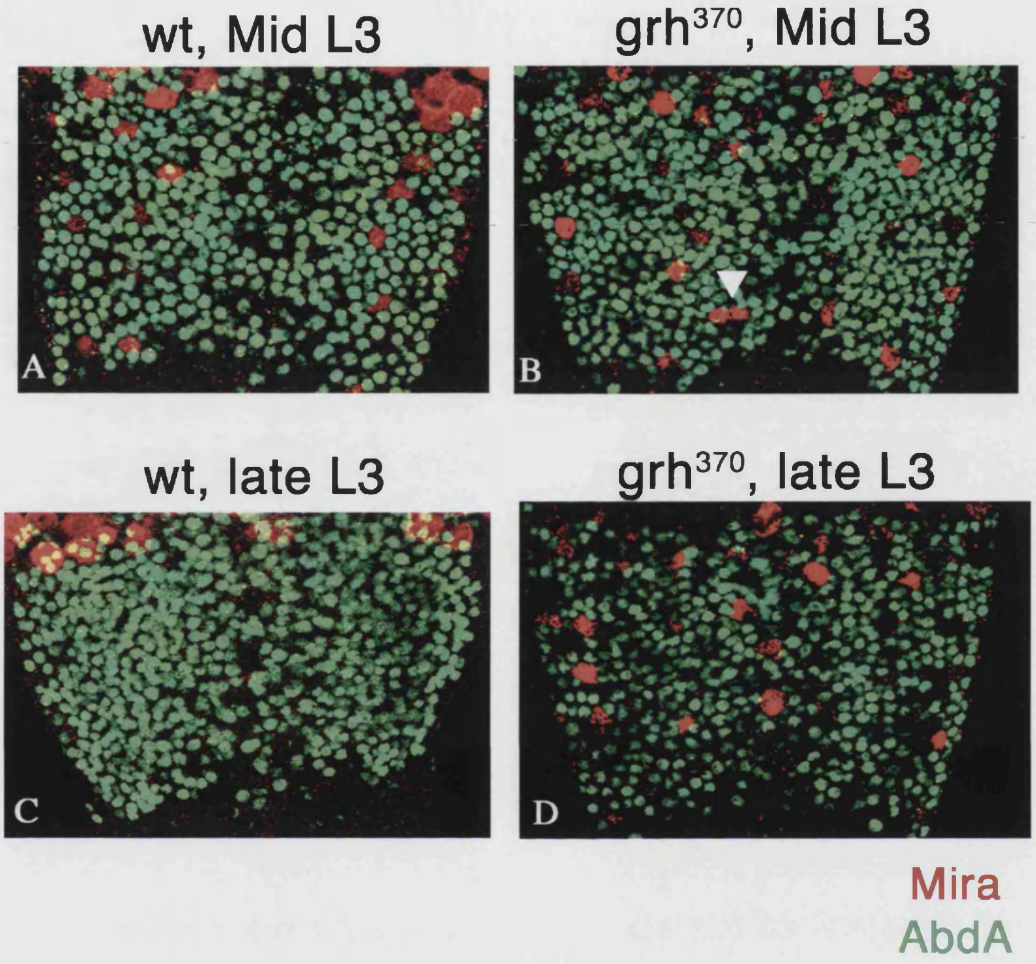


FIGURE 5. 8 ABDOMINAL pNBs PERSIST TO LATE L3 IN *GRH*^{370} MUTANTS.
Confocal sections of wild type and *grh*^{370} CNSs, immunolabelled with anti-AbdA (green) and anti-Mira (red) antibodies. (**A**) Some Mira-positive NBs remain in the wild type central abdomen at 72 hr. (**B**) Additional abdominal NBs persist in *grh*^{370} mutants at 72 hr. An ectopic pair of NBs (arrowhead) is often found in proximity to the midline. (**C**) In the wild type abdomen all NBs are absent by 96 hr (**D**) *grh*^{370} NBs persist in the abdomen until 96 hr. Note that all the NBs are AbdA-negative at these stages.

However, a positive, rather than a negative role of Grh in AbdA regulation might be envisaged on the basis of the over-proliferation phenotype observed in the grh^{370} mutants. In the abdominal CNS of 60-66 hr larvae, a subset of interphase and mitotic pNBs undergo the cell-death inducing burst of AbdA expression (Bello et al., 2003, see also Figures 1.12 and 1.13). To address the important question of whether Grh is required for the AbdA pulse in mid-L3, I examined 60-66 hr larvae by anti-Mira and anti-AbdA immunostaining (Figure 5.9A). During this time-window, I find that grh^{370} pNBs retain expression of AbdA. This is also confirmed by anti-AbdA/anti-H3p double labelling showing that, as in the wild type, the NB can be in M-phase at the moment of the AbdA pulse (Figure 5.9B). These important findings rule out the possibility that Grh is needed to activate AbdA expression in the mid-L3 pNB lineages. Together with the previous results, I conclude that Grh is not required to repress *Ubx* expression in the thoracic pNBs or to activate *abdA* expression in the abdominal pNBs. Importantly, this result indicates that in the abdomen, Grh-deficient pNBs survive and divide at least until puparation despite the fact that they undergo the normal burst of AbdA expression in mid-L3.

5.2.5 grh^{370} pNBs are insensitive to AbdA-induced apoptosis

There are several possibilities for why a Grh-deficient NB does not undergo apoptosis at mid-L3 despite a pulse of AbdA expression. First, Grh could be required for conferring Hox sensitivity to the abdominal lineages; in other words it could act as a cofactor that, in conjunction with AbdA, triggers the apoptotic pathway in the NBs. Alternatively, Grh might stabilize AbdA expression levels and/or duration of expression. This point is difficult to address by immunostaining grh^{370} mutants and so it remains possible that the burst of AbdA expression is still affected in a subtle way. I addressed this important consideration using inducible AbdA misexpression within the mutant abdominal lineages. For this experiment, *hs-abdA*, grh^{370} recombinant chromosomes were generated (see Material and Methods and Figure 2.1). *hs-abdA* expression was first induced at the end of L2 and its effects on the pNB pattern observed at mid-L3 using anti-Mira/anti-AbdA immunolabelling (Figure 5.10). I find that AbdA misexpression in the wild-type abdomen results, one

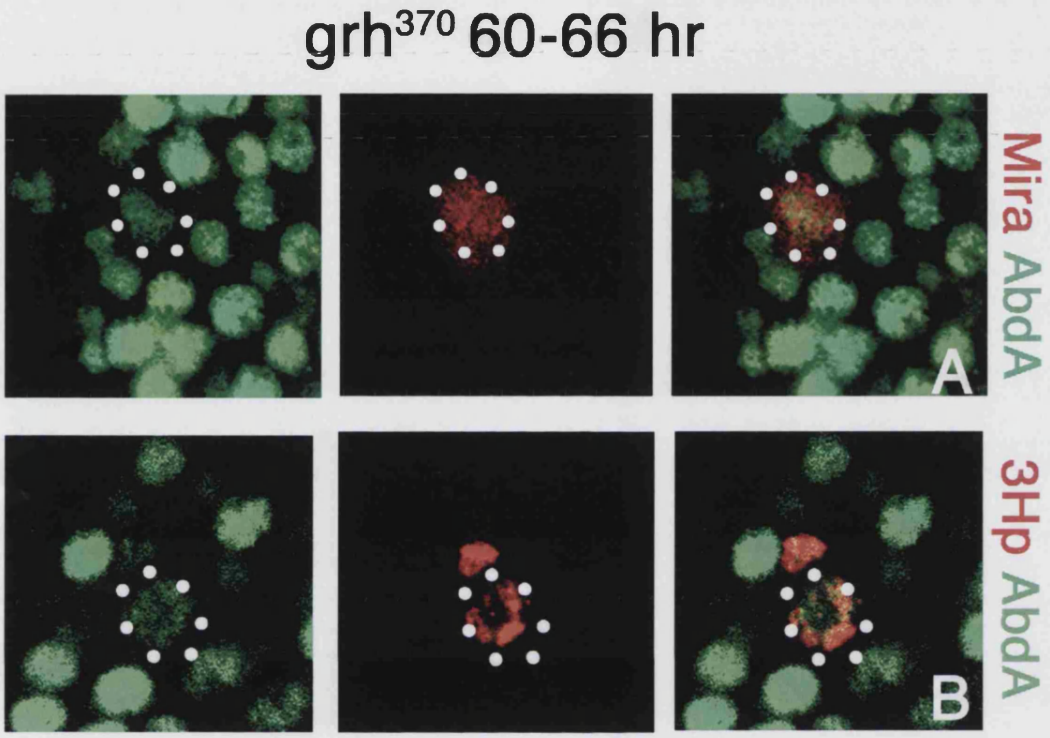


FIGURE 5.9 *GRH*^{370} pNBs STILL UNDERGO A BURST OF ABDA EXPRESSION AT 60-66 hr. (**A**) Anti-AbdA (green), anti-Mira (red) single section of a *grh*^{370} pNB. The NB (dotted circle) is AbdA positive. (**B**) anti-3Hp (red), anti-AbdA (green) single section of a dividing pNB (dotted circle) and GMC (smaller H3p-positive cell). The M-phase NB is AbdA positive.

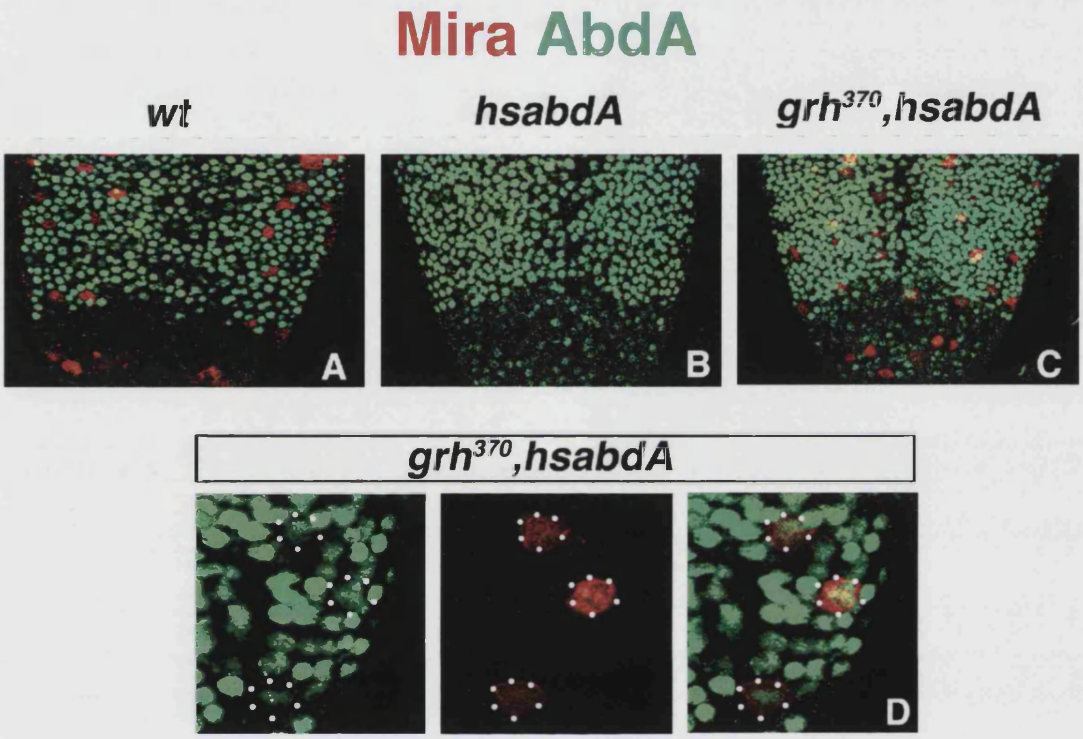


FIGURE 5.10 ABDA MIS-EXPRESSION DOES NOT KILL *GRH*^{370} ABDOMINAL pNBs. *hs-abdA* and *grh*^{370}*; hs-abdA* L2 (48 hr) larvae were heat-shocked for 1 hr and analysed one day later by anti-AbdA/anti-Mira immunostaining. (**A**) Wild type CNS. (**B**) *hs-abdA* CNS, ectopic expression of AbdA at the abdominal tip can still be detected one day after heat-shock, no Mira-positive NBs survive upon AbdA mis-expression. (**C,D**) *grh*^{370}, *hs-abdA* CNS, Mira-positive cells persist, despite expressing high levels of AbdA. (**D**) shows single confocal section of three *grh*^{370} NBs (dotted circles) mis-expressing AbdA.

day later, in the premature disappearance of all the vm, vl and dl pNBs (Figure 5.10A,B) confirming what was shown previously by Bello *et al.* using BrdU incorporation in *hs-abdA* larvae (Bello et al., 2003). However, repeating this experiment using *hs-abdA, grh^{370}/ Df(2R)Pcl^{7B}* larvae results in pNBs persisting in abdominal segments (Figure 5.10C,D). In another series of experiments, *hs-abdA* was used to boost and stabilise AbdA levels at the moment of the endogenous pulse (60-66 hr), and the larvae analysed one day later. Under these conditions, grh^{370} pNBs again persisted (data not shown). In conclusion, these experiments show that Grh does not seem to be required to stabilize AbdA expression but instead acts as a competence factor that confers AbdA-sensitivity to abdominal pNBs. pNBs that lack *grh* function are unable to respond to AbdA, even when this is supplied artificially at high levels. Moreover, after failing to undergo AbdA-dependent apoptosis, abdominal pNBs lacking Grh remain mitotically active, at least until the end of L3.

5.3 Discussion

5.3.1 Grh plays opposite roles in neural proliferation in the thorax and abdomen

Grh is expressed in the CNS throughout larval life: it is found in the quiescent as well as in the re-activated pNBs of the thoracic and abdominal neuromeres (Bray et al., 1989; Dynlacht et al., 1989; Uv et al., 1997). It is also known that, prior to apoptosis, *grh* expression is still maintained in the vm, dl and vl abdominal lineages (Bello et al., 2003). The function of Grh in the developing CNS has not been addressed in detail before; I have analysed the role of this novel TF using BrdU incorporation and immunolabelling in grh^{370} larvae and also grh^{B37} MARCM clones. Together, these experiments reveal that, in Grh-deficient larvae, the normal segmental pattern of neural proliferation is disrupted. Thoracic pNB lineages stop proliferating too early and the stem cell dies whereas abdominal ones fail to undergo apoptosis and divide for an abnormally long time. Thus, abdominal pNBs undergo a potential homeotic-like transformation in that they assume proliferation characteristics similar to, but not identical, with the thoracic pNBs. For the thoracic

lineages, an opposite transformation towards the character of more posterior lineages is observed. In other words, there is a tendency towards equalising pNB clone size in thoracic and abdominal segments. Interestingly, I showed that these phenotypes do not appear to result from altered Hox expression. The pulse of AbdA expression that normally induces apoptosis is still retained in grh^{370} abdominal lineages and the NB in grh^{B37} thoracic MARCM clones does not misexpress *Ubx* at 72 hr.

These findings are surprising in that they indicate that although Grh is not a Hox gene and is equally expressed in the pNBs of all segments, it is nevertheless producing a segment-specific effect. Furthermore, whereas it has been shown that proteins can act as activators or repressors of the cell cycle depending on the cell type they are expressed in, here, Grh is playing different roles within the same cell type in different segments. This could be achieved through direct interactions with different segment-specific proteins or via spatially-regulated post-transcriptional modifications. Interestingly, Liaw *et al* have demonstrated that in the very early stages of embryogenesis, Grh is a target for modulation by tyrosine-kinase receptors. In this context, Grh is phosphorylated by the mitogen-activated protein kinase (MAPK) that acts downstream of the *torso* pathway (Liaw et al., 1995). It is therefore possible that a similar mechanism might be at the basis of the dichotomy in thoracic versus abdominal Grh roles.

5.3.2 *grh* is required for thoracic pNB division and survival

The grh^{B37} MARCM analysis differs from the study of viable grh^{370} mutant larvae in several ways, including the fact that the mutation is only induced at the beginning of L1. Furthermore, the clonal analysis only allows us to determine cell-autonomous functions. The comparison of mid-L3 wild type and mutant clones has shown that Grh disruption has a two-step effect on the pNBs. First, the cell cycle speed is slowed down, stopping by 72 hr. Second, pNBs are eliminated by a caspase-dependent cell-death mechanism. Concerning the impairment of cell division, the expression of two well-known asymmetric cell division determinants, *pros* and *Mira* has been examined. *pros* is normally not expressed in the larval pNBs (Ceron et al., 2001) and, in the embryo, it has been shown to be required to promote exit from the

cell cycle and neuronal differentiation (Li and Vaessin, 2000). Since I find that Pros expression is not affected in grh^{B37} clones, premature cessation of divisions cannot be ascribed to the misexpression of this factor in the NB. Also, the expression of Mira remains localised in the grh^{B37} pNB, however, it is never found in the basally polarized distribution, typical of M-phase (Ikeshima-Kataoka et al., 1997). Whether Grh is directly responsible for this asymmetry phenotype or whether this is just a consequence of the fact that mutant pNBs stop dividing outside M-phase is not clear. Grh has been shown to activate transcription of PCNA (Proliferating Cell Nuclear Antigen) *in vitro* and this factor is required for DNA replication (Hayashi et al., 1999). However I find that grh^{B37} mid-L3 NBs maintain the expression of PCNA (data not shown). This indicates that the Grh-deficient NB has stopped dividing but it still retains a marker of engagement in the cell cycle. In grh^{B37}*; UAS- p35* clones, the NB is rescued from cell death. These clonal analysis experiments indicate that, after the mutant NB has prematurely terminated dividing at 72 hr, the apoptotic machinery must eliminate it before 96 hr. However *UAS-p35* is not sufficient to rescue clone size, and thus, the pNB's capability to divide. This is consistent with the observation that the P35-rescued NB often has a peculiar elongated morphology at late L3 and, in particular, is abnormally large (see Figure 5.6B). Whether the rescued NB expresses PCNA, Mira or H3p has not been tested yet.

Hox gene mis-expression is known to induce cell-death in the pNB thoracic lineages (Bello et al., 2003). However, grh^{B37} mutant NBs do not appear to undergo Hox-mediated cell death as mid-L3 mutant NBs do not express Ubx. Moreover, the expression of the other abdominal Hox genes (AbdA and AbdB) also failed to be detected in mid-L3 mutant clones (data not shown). In this way, the occurrence of a Hox cross-repressive mechanism, masking the expression of Ubx by a more posterior Hox protein (Struhl and White, 1985) seems unlikely. On the basis of these results, a cell-death pathway triggered in a Hox-independent manner, perhaps as a consequence of cell cycle withdrawal, is favoured.

5.3.3 Grh is a competence factor for AbdA-mediated pNB death in the abdomen

In strong contrast to the phenotype observed in the thorax, the abdominal neuromeres of grh^{370} mutant larvae display over-proliferation. This is due to the persistence of dividing vm, vl and dl at least until late L3. To determine whether and when divisions cease, pupal and adult stages would need to be analysed. However, due to the very high rate of lethality of the grh^{370} mutant at puparation (Uv et al., 1997), this analysis was not practical. In addition to vm, vl and dl, ectopic NBs can also be observed in the abdomen of grh^{370} mutants. Their identity has yet to be determined but they are likely to correspond to NBs that have failed to undergo cell death in the late embryo (White et al., 1994; Prokop et al., 1998; Peterson et al., 2002). Consistent with what was found in the thoracic NBs, *grh* is not required to regulate Hox expression, in fact the normal pulse of AbdA expression can still be observed in mid-L3 grh^{370} lineages. Importantly, the mutant NBs are insensitive to this and fail to undergo apoptosis. This central result is confirmed by mis-expression experiments: exposing the NB at different times to high and relatively long-lived doses of AbdA is still not sufficient to induce cell death. Ectopic AbdA expression can still be detected one day after induction in the surviving mutant NB, also ruling out the possibility that Grh is required for maintaining Hox expression in the NB. These experiments demonstrate that, once activated in the NBs (by a still not identified factor), AbdA requires Grh to trigger the apoptotic response. Although the molecular basis of this requirement remains to be addressed, it might be interesting to explore whether pro-apoptotic genes such as *reaper* are regulated directly by Grh and AbdA inputs.

Brody and Odenwald have proposed that Grh might be the ultimate TF that follows Cas in the temporal sequence of NB TFs or sublineage factors (Brody and Odenwald, 2000). They observed that, when cultured for only a few hrs, NBs sequentially generate Hb-Pdm and Cas expressing sublineages but over-night cultures express Grh. This indicates that *grh* is activated during late NB lineage development *in vitro* but whether *in vivo* there is a direct transcriptional relationship between Castor and Grh remains to be characterized. In addition, unlike the well-

characterized NB sublineage factors, Grh does not seem to be expressed in the progeny neurons. Therefore, I propose a model where Grh acts as a late determinant, perhaps not for neuronal identity, but to define the pNB window of sensitivity to AbdA and thus to make it competent to cease dividing at the appropriate time. In this model, exclusion of Grh from post-mitotic neurons might also provide them with the ability to express Hox proteins such as Ubx without dying.

CHAPTER 6 DISCUSSION

CHAPTER 6 DISCUSSION

Neurogenesis requires a delicate balance between cell proliferation and cell differentiation to yield the appropriate final number of cells in the brain. In the fly, this balance is different in the different regions of the CNS. In particular, this is achieved through segment-specific regulation of the initiation and termination of progenitor cell divisions. During the course of my PhD, I have studied segment-specific aspects of neuronal specification and NB divisions in the context of the embryonic and imaginal CNS.

NB divisions and neuronal fate both appear to be linked to the temporal series of sublineage transcription factors (Ohnuma and Harris, 2003). In the embryo, dividing NBs sequentially express the TFs **Hb → Kr → Pdm-1 → Cas** that link birth order with neuronal fate (Kambadur et al., 1998; Isshiki et al., 2001; Pearson and Doe, 2003)(reviewed in Harris, 2001; Livesey and Cepko, 2001b). Neurons that are generated from progenitors that haven't undergone many rounds of cell division (Hb or Kr positive) take on early fates, while those that have been produced by NBs that have divided for longer (Pdm or Cas positive) take on late determination fates. Progression of the cell cycle is required to allow the proper readout of this molecular timer as termination of the cell cycle in *string* mutants prevents NBs from switching TF expression (Isshiki et al., 2001). The release of the cell-cycle brake at later times results in the normal sequence of factors being resumed without any TF skipping. This indicates that the underlying switching mechanism is based on counting cell-cycles rather than elapsed time. An ultimate TF, Grh, has been proposed to take part in the series, **Hb → Kr→ Pdm-1 → Cas ⇨ Grh** (Brody and Odenwald, 2000). Grh is expressed in late embryonic and larval NBs, however, it is not known yet whether Cas directly regulates Grh. Moreover, this last TF differs from the others in the sequence because its expression appears to be exclusive to NBs (and possibly GMCs) and it is maintained during the numerous divisions that accompany post-embryonic neurogenesis (Uv et al., 1997; Bello et al., 2003).

In this thesis, I asked whether the TF sequence is involved in the generation of segment-specific neural features. First, I addressed the possibility that the

Hb → Kr→ Pdm-1 → Cas series might not be invariant from segment-to–segment. For this purpose, I studied the THB cells, a group of embryonic neurons that are specific to the thorax and express Hb. By analysing the mechanism of THB patterning, I found thorax-specific modulation of the TFs and possible gene skipping in the canonical series (Section 6.1). I next explored the functions of the candidate TF series factor Grh during larval neurogenesis and identified a segment-specific role in the control of neural proliferation. I find that, in the thorax, Grh is required for proliferation by promoting pNB divisions and survival, whereas, in the abdomen, it is required for AbdA-mediated cell death and thus limits proliferation (Sections 6.2 and 6.3).

6.1 Segment-specific sublineage switching patterns

For the first time, to my knowledge, a thorax-specific exception to the usual sequence of NB sublineage factors has been identified. In the thorax, the THB progenitor NBs produce more Hb-expressing cells at the apparent expense of the other TFs in the series. Less Kr and Cas-expressing progeny are generated and, importantly, the expression of Pdm-1 appears to be skipped. Evidence for this last point was provided by showing that no Pdm-1 positive neurons are contained within the THB lineages. However, I cannot rule out the possibility that Pdm-1 was transiently expressed in the THB NBs but not subsequently maintained in the neurons. THB lineage-labelling experiments indicated that the late THB progenitors also produce Cas-negative cells. Although it has not yet been addressed by immunostaining, I favour the possibility that these late-born neurons were produced from a Grh-positive NB.

The THB pattern requires Hth (but not thoracic Hox genes) and is suppressed in the abdomen by the activities of Ubx and AbdA. This suggests a role for *Ubx/abdA* either in repressing the THB pattern by modulating TF switching or by inhibiting division and/or survival of THB progenitors. One possibility is that Hth is required, directly or indirectly, for altering the timing of Hb expression in the THB lineages. In the nervous system of *C. elegans*, heterochronic genes have been implicated in the temporal control of gene expression (Slack and Ruvkun, 1997).

These genes may be sensitive to different cell-cycle phases and act by orchestrating the developmental timing of cell divisions and cell fates. Many heterochronic genes encode microRNAs and RNA binding proteins. Interestingly, in nematodes these genes are required for regulating *hbl-1* and *lin-29*, homologues of Drosophila *hb* and *Kr* (Pasquinelli and Ruvkun, 2002; Abrahante et al., 2003). Thus, the very speculative possibility exists that the expression of fly microRNAs might regulate sublineage switching and that they could be regulated in a segment-specific manner by *hth*.

The linkage of birth order to neuronal fate is of wide interest because it appears to be conserved during the development of the vertebrate nervous system, where it is generally referred to as "Histogenesis" (Caviness and Sidman, 1973). For example, in certain regions of the vertebrate nervous system, including the mammalian retina and the cerebral cortex, cells are generated in a highly stereotyped temporal sequence. In the cortex, neural precursors and their progeny pass through a sequence of different TFs which appear to determine which of several laminae become their final destination (Frantz et al., 1994a; Frantz et al., 1994b; McConnell, 1995). The intrinsic and/or extrinsic elements taking part in this temporal mechanism in the vertebrate have not been fully characterized yet, but, similar to the fly NBs, the neural progenitor cells undergo temporal changes in their competence to generate specific progeny types and possibly also undergo a progressive restriction of cell fate with age (Doe et al., 1998; Livesey and Cepko, 2001b; Ohnuma and Harris, 2003; Pearson and Doe, 2003). Very interestingly, a mammalian *Pdm* homolog, *SCIP/Oct-6* is both temporally and spatially regulated during cerebral cortical development (Frantz et al., 1994a). It would be interesting to know whether, similar to what I see in the fly CNS, there is an involvement of Hox genes or Meis factors (mammalian homologues of Hth) in shaping this process.

6.2 Regulation of NB divisions by the Hox genes, PcG genes and Grh

In addition to their well-known role in the specification of cell fates along the AP axis, it has long been argued that Hox genes can play a role in the regulation of cell proliferation (Garcia-Bellido, 1975; Duboule, 1995). In principle, this can be

achieved in two different ways, regulation of the cell-cycle or regulation of precursor survival. Considering the first mechanism, the vertebrate Hox gene, *Hoxb4*, has been shown to induce cell proliferation in the haematopoietic lineages (Antonchuk et al., 2002; Owens and Hawley, 2002; Bjornsson, 2003). Regarding regulation of cell survival, during the development of the fly embryonic CNS, *abdA* has been implicated in reducing NB number in the abdomen (Prokop et al., 1998). Direct evidence for *abdA*-dependent NB death has so far only been described in the larval CNS (Bello et al., 2003). Hox genes have also been found to limit cell survival in other developmental contexts. For example, *Deformed* and *Abominal-B* have been shown to reduce cell number by inducing *reaper*-mediated apoptosis during head morphogenesis and posterior segment boundary formation respectively (Lohmann et al., 2002). Similarly, during vertebrate limb development, *Hoxa13* is required for normal interdigital cell death (Stadler et al., 2001). Interestingly, at least one vertebrate Hox gene, *Hoxb13,* appears to regulate negatively neuronal cell number by a mechanism involving cell death. Mice lacking *Hoxb13* function show overgrowth of caudal spinal cord and tail vertebrae and this phenotype is associated with a great reduction in neural cell death (Economides et al., 2003). However, it is not yet clear whether *Hoxb13* is required in neural progenitors or some other cell type. In this thesis, I have primarily focused on the negative role of Hox genes in neural proliferation. More specifically, I have addressed how they are regulated in NBs and how their expression leads to cell death.

The PcG genes have been extensively implicated in the maintenance of the Hox repressed status (Paro, 1993; Pirrotta, 1995). However, I have shown that *3Pc* genes (*Psc*, *Asx* and *Pcl*) do not appear to be required for Hox repression in the fly postembryonic NB lineages of the thorax; suggesting that Hox expression within a given NB lineage is kept off by a mechanism other than 3Pc. Similar to this idea, Hox gene de-repression is not causal for the haematopoietic abnormalities in PcG mutant mice (Jacobs and van Lohuizen, 2002). Thus, segment-specific and lineage-specific mechanisms of Hox repression may be distinct. Alternatively, lack of Hox expression in pNBs may reflect a lack of positive regulators in this cell-type, rather than an active repression.

Like *3Pc*, *grh* also does not appear to be required for controlling Hox expression, neither in the thoracic nor in the abdominal NB lineages. I find that grh^{B37} MARCM clones in PS6 do not mis-express Ubx and that, at mid-L3, grh^{370} abdominal pNBs undergo the normal burst of AbdA.

In conclusion, the positive and negative regulators of Hox expression within the fly NB are still unknown and a genetic screen, based on MARCM, is currently been carried out in the laboratory to find this class of genes. More specifically, it would be exciting to test whether the timing of the AbdA burst of expression at mid-L3 is dependent on the number of divisions the NB has undergone. This could be explored by analysing the AbdA pattern of expression in animals where *string* function has been altered (Edgar and O'Farrell, 1989), so that cell cycle speed is changed and thus uncoupled from developmental time. The identification of Hox regulatory mechanisms other than the known ones (segmentation genes and the PcG/trxG genes) would be interesting and would provide a possible way of investigating Hox-dependent cell proliferation in vertebrates.

By using MARCM, I find that genes belonging to the PcG complex are required for normal thoracic NB divisions. In the absence of PcG genes, clone size is dramatically reduced and the NB disappears. As they appear to act via a Hox-independent mechanism, they presumably regulate cell cycle or cell death factors (Gould, 1997; Muyrers-Chen and Paro, 2001; Jacobs and van Lohuizen, 2002). Similarly, I showed that Grh controls the speed of pNB divisions as well as precursor survival in the thorax. Therefore, PcG genes and *grh* are both essential for normal thoracic NB divisions but whether they genetically interact is not known. One possibility could be that *3Pc* genes are required for maintaining Grh expression. Alternatively, it is also possible that *3Pc* and *grh* act in parallel pathways. Analysis of Grh expression and of the effects of its overexpression in *3Pc* clones need to be carried out to address this point. I favour the idea of cooperation between PcG proteins and Grh and suggest that these factors may interact to repress unknown target genes involved in the cell cycle and/or apoptosis. This hypothesis is based on the observation made by Tuckfield *et al.* that the human PcG protein DinG directly binds CP2, a mammalian member of the *grh*-like family of TFs (Tuckfield et al.,

2002). This interaction is necessary for repressing the transcription of downstream target genes. Importantly, the formation of PcG-Grh-like complexes seems to be conserved in evolution, as the authors also demonstrated that Grh binds to the DinG fly homologue, Dring, in electro-mobility shift experiments. The model in which PcG and Grh cooperate to promote thoracic NBs divisions is very speculative and needs to be rigorously tested. This would require obtaining evidence, by GST pull-down or immunoprecipitation from protein extracts, that Grh binds to a Pc complex containing at least one of the 3Pc factors. The identification of relevant promoter regions from cell cycle and/or cell death target genes, might then allow the roles of this protein-complex to be determined *in vivo*.

6.3 Grh defines competence for AbdA-dependent NB death

It is evident that NBs respond differently to Hox expression depending on their age. Ubx and AbdA are expressed in some of the early (stage 8-12) embryonic NB lineages (Prokop et al., 1998), yet many of these NBs keep dividing and produce AbdA-positive progeny. Conversely, AbdA expression in the NB at mid-L3 leads to cell death. Prokop *et al.* proposed a "cell-memory" mechanism whereby early embryonic expression of Hox factors in NBs is what influences segment-specific NB numbers and divisions at later stages (Prokop et al., 1998). However, Bello *et al* found larval *abdA* expression in a burst and showed that an overproliferation phenotype is found when abdA mutations are induced after larval hatching, arguing against the "cell memory" model (Bello et al., 2003). I have shown that providing strong and stable expression of AbdA in grh^{370} NBs does not result in their death, indicating that precursor apoptosis is dependent on Grh expression. Thus, in the vm, vl and dl NBs of the abdomen, Grh is required for AbdA to trigger the correct output of cell death. For this reason, I defined Grh as a late competence factor for AbdA apoptotic activity. The molecular mechanism underlying this role of Grh remains unknown, however, one possibility that could be tested is that Grh acts as a novel Hox cofactor, perhaps directly binding AbdA to activate the cell death pathway. Although, as described earlier, Grh can bind to Dring *in vitro* (Tuckfield et al., 2002), so far evidence is lacking of any other proteins that bind Grh. It is interesting

to notice that the human Grh-related protein CP2 is found to be involved in temporally controlling the expression of globin in fetal erythrocytes. This is achieved through the formation of stage-specific heteromeric complexes with NF-E4, a novel tissue and development-specific protein (Zhou W, 2000). In the fly CNS, Grh may work in a similar way, as a part of a later stage complex that acts differently on cell proliferation according to the presence or absence of segment-specific factors such as Hox genes. Another possibility is that Grh may be differentially modified, for example phosphorylated (Liaw et al., 1995), in the thorax or abdomen and in this way exert segment-specific functions. A Grh-context mechanism may explain how Hox sensitivity is temporally regulated to make old, Grh-positive pNBs different from young, Grh-negative NBs and thus competent to die upon receiving the appropriate AbdA pulse (Figure 6.1). The late embryonic phase of Grh expression (from stage 14, Bray et al., 1989) might also account for the reduction in the number of abdominal NBs at the end of embryogenesis (Prokop et al., 1998) and, in this regard, the possibility of a late embryonic pulse of AbdA expression needs to be addressed. The observation of ectopic pNBs in the abdomen of grh^{370} larvae is consistent with this model. Continued Grh expression in larval pNBs would then permit the remaining abdominal pNBs to be culled by a second AbdA pulse at the mid-L3 stage. This raises the question of how vm, vl and dl normally escape cell-death in the late embryo. Do they fail to express Grh or AbdA or are they protected by an as yet unknown factor?

In addition to different ages of NBs displaying different sensitivities to Hox expression, different stages of neuronal differentiation do too. Imaginal neurons express Ubx or AbdA for long periods but this does not compromise their survival. Once again, this might be explained by the fact that Grh expression is excluded from these post-mitotic cells. In this regard, experiments are underway to test whether misexpressing Grh in neurons leads to these cells dying.

Whether Grh is acting in a CNS-specific manner to induce Hox-mediated cell death or whether it plays the same role in other tissues is also not known. In this regard, the possible requirement of Grh in the apoptotic events triggered by *Deformed* and *Abdominal-B* during epidermal segment formation needs to be

assessed (Lohmann et al., 2002). It would also be interesting to determine whether, during vertebrate neurogenesis, *Hoxb13* is inducing cell death (Economides et al., 2003) through cooperation with CP2 or other Grh-like competence factors. However, the functions of mammalian *grh*-related genes (Wilanowski, 2002) within the vertebrate CNS have not yet been identified.

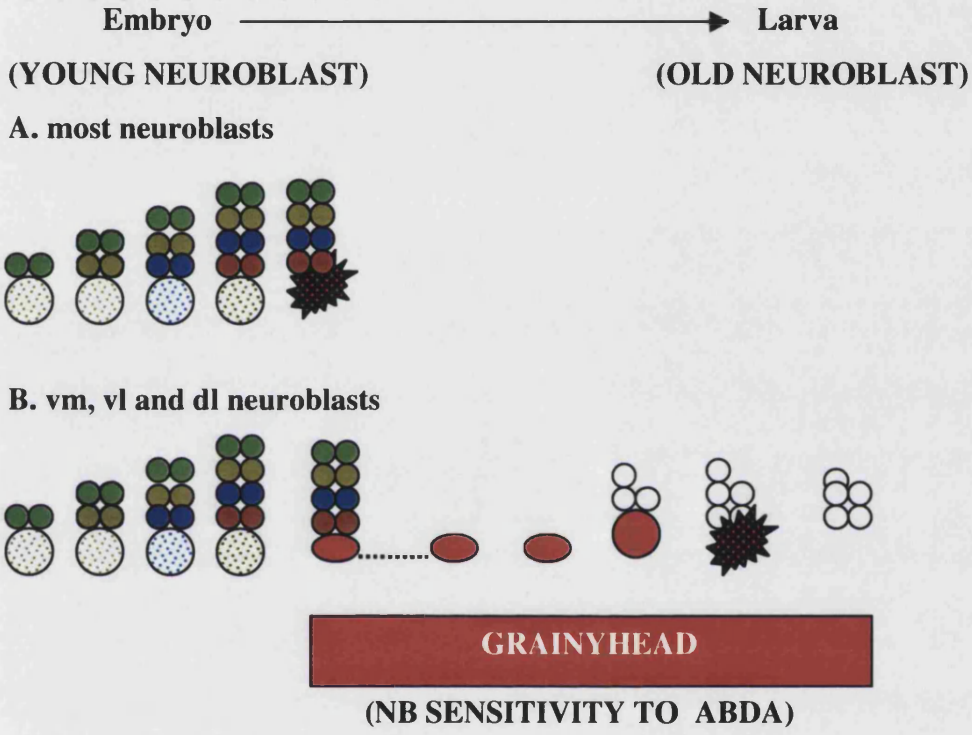


FIGURE 6.1 GRH DEFINES THE NB WINDOW OF SENSITIVITY TO ABDA.
Time line of two idealised abdominal NB lineages. Large circles and intermediate ovals represent dividing and quiescent NBs respectively. Small circles represent the progeny neurons. Early embryonic (stage 8-12) NBs sequentially express Hb (light green), Kr (dark green), Pdm-1 (blue) and Cas (brown). At these stages the abdominal NBs divide despite AbdA expression. The ultimate factor in the series is Grh (red), expressed from stage 14. (**A**) From stage 14, 27/30 NBs in the abdomen are lost via apoptosis (starburst). This may be triggered by not yet identified AbdA pulses. (**B**) Three abdominal lineages do not undergo apoptosis and reiterate divisions during larval life as vm, vl and dl. At mid-L3 a second wave of apoptosis is triggered by a burst of AbdA expression. Note that while the progeny neurons retain the expression of Hb → Kr → Pdm → Cas and form a layered CNS, Grh is not expressed in the post-mitotic cells.

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