Neuroblast lineage identification and Hox gene action during postembryonic development of the tritocerebrum and subesophageal ganglion in the *Drosophila* brain

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SUMMARY

In this thesis, the neuroblast lineages which give rise to the adult-specific interneurons of two *Drosophila* brain regions, the tritocerebrum and the subesophageal ganglion, were described, and an analysis of the expression and function of the developmental control genes of the Hox family in these regions was performed. In the first study (chapter 2), the Hox gene and tritocerebral maker *labial* was used together with clonal genetic labeling to identify the 4 postembryonic neuroblast lineages of the tritocerebrum. Subsequently, clonal mutation was used to show that expression of *labial* is required for timely-precise apoptosis of 2 neuroblasts, which otherwise give rise to outgrowing ectopic lineages in the tritocerebrum. In the second study (Chapter 3), the postembryonic subesophageal ganglion was investigated. First, markers for cell adhesion molecules and synapses were used to characterize the neuronanatomical structures of the subesophageal ganglion in respect to thoracic neuromeres. In this analysis, a small number of secondary axon tracts were found. Second, genetic labeling was used to identify the clonal basis for this reduction and a number of only 14 subesophageal neuroblast lineages were identified in the late larval subesophageal ganglion. Third, the subesophageal neuroblasts were traced through development, and a severe reduction in neuroblast number was found in this region to take place between middle embryonic and late larval stages. Fourth, the Hox genes *Deformed*, *Sex combs reduced* and *Antennapedia* were shown to be expressed in discrete domains in the subesophageal ganglion, and most of the subesophageal lineages in the late larva were found to be positive for one of these Hox proteins. Fifth, clonal genetic knockout was used to show that the *Dfd*, *Scr* and *Antp* genes are required for three lineage-specific functions in the subesophageal ganglion: proper axonal targeting of 2 lineages, correct cell number in 2 lineages and termination of 5 neuroblasts via programmed cell death. In chapter 4, the expression and function of Hox genes in the *Drosophila* nervous system was reviewed in the context of genetic interactions and evolutionary conservation. In summary, postembryonic generation of adult-specific neurons in the tritocerebral and subesophageal brain regions of *Drosophila* was shown to be mediated by a small number of neuroblast lineages and to involve three lineage-specific functions of Hox proteins.

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SUBESOPHAGEAL GANGLION IN THE *DROSOPHILA* CENTRAL BRAIN

1. INTRODUCTION

1.1 *DROSOPHILA* NEUROGENESIS

The central nervous system (CNS) of *Drosophila* consists of the brain (composed of the central brain and the optic lobes), as well as the ventral nerve cord (VNC; composed of the thoracic and abdominal ganglia). The central brain can be further divided into the supraesophageal ganglion (SPG) and the subesophageal ganglion (SEG). The SPG can be further subdivided into the protocerebrum (PR), deutocerebrum (DE) and tritocerebrum (TR); the SEG can be further subdivided into the mandibular (MD), maxillary (MX) and labial (LB) neuromeres. SPG and SEG, like the segmental ganglia that form the VNC have neuromeric features that reflect the overall metameric insect bauplan (Fig. 1.1). This metameric organization is most clearly manifest in the SEG with its three fused neuromeres corresponding to the mandibular, maxillary and labial segments; the metameric organization of the SPG is neuroanatomically less obvious (see Campos-Ortega and Hartenstein, 1997; Reichert and Boyan, 1997; Hartenstein et al., 2008; Ito and Awasaki, 2008; Strausfeld, 2009).

The enormous diversity of neuronal cells in the *Drosophila* CNS is generated from a special type of neural stem cell-like progenitor, the neuroblast (reviewed in Homem and Knoblich, 2012). Neuroblasts are selected by the process of lateral inhibition which involves the Delta/Notch pathway and subsequently delaminate during embryonic stages 9-11 from the ventro-lateral neuroectoderm (see Skeath and Thor, 2003; Technau et al., 2006). The SPG derives from a set of

approximately 100 bilaterally symmetrical pairs of neuroblasts that have been identified individually based on their position and molecular marker expression pattern (Urbach and Technau, 2003). Each ganglion in the ventral nerve cord derives from a set of approximately 30 bilaterally symmetrical pairs of neuroblasts which have also been identified individually based on position and marker expression as well as on the anatomical features of their neural progeny (see Technau et al., 2006). Number and identity of the neuroblasts that give rise to the SEG have only
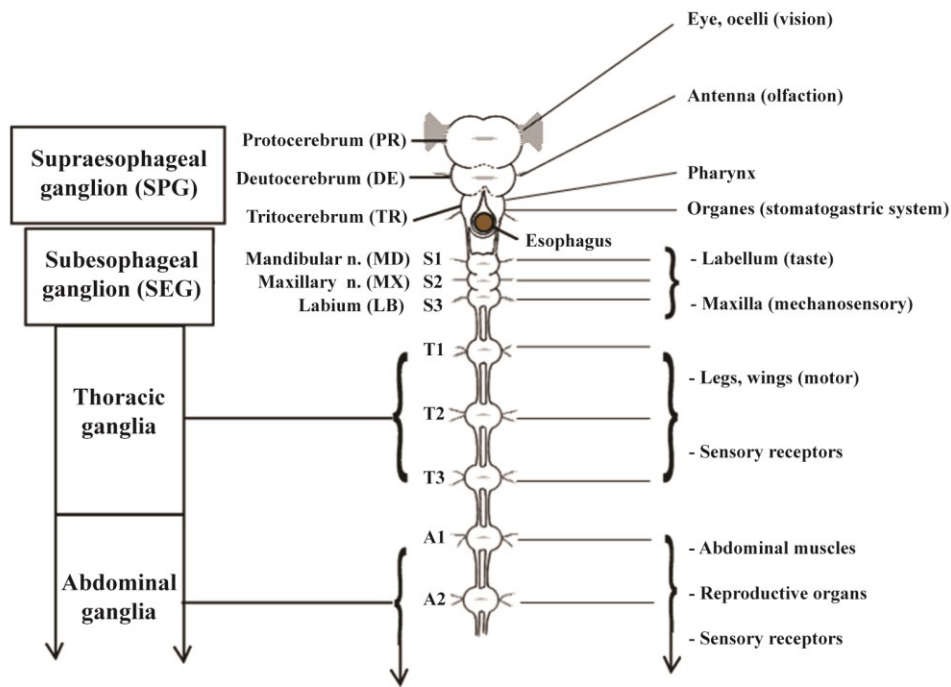
Fig. 1.1: Structural organization of the insect CNS

Scheme showing overview of the metameric structure of the insect CNS. Left side: Names of ganglia and neuromeres. Right side: Innervation and associated functions of CNS regions. The VNC consists of the thoracic and abdominal ganglia.

recently been determined; the SEG derives from approximately 80 pairs of neuroblasts (R. Urbach, personal communication). During neurogenesis, all of these neuroblasts proliferate through a series of asymmetric divisions in which they self-renew and produce secondary neural progenitor cells. In most neuroblast lineages (Type I) the secondary progenitors, called ganglion mother cells (GMCs), divide only once and give rise to two postmitotic progeny, neurons or glial cells. In a small set of different neuroblast lineages (Type II), the secondary progenitors are self-renewing intermediate progenitors which can give rise to multiple GMCs and hence serve to amplify proliferation (see Skeath and Thor, 2003; Doe, 2008; Knoblich, 2008; Reichert, 2011; Homem and Knoblich, 2012).

In *Drosophila*, neurogenesis in the central brain and VNC takes place in two phases (Fig. 1.2). In the first embryonic phase the so-called primary neurons that make up the functional central brain and VNC of the larva are generated. At the end of embryogenesis, the neuroblasts enter a period of quiescence (exceptions are 5 neuroblast pairs in the SPG which continue to proliferate). Subsequently, in early larval stages, most of the neuroblasts in the SPG of the central brain resume proliferation during a second postembryonic phase to generate the bulk of the neurons of the adult CNS (reviewed in Egger et al., 2008; Hartenstein et al., 2008). This is also the case for most of the neuroblasts in the thoracic ganglia of the ventral nerve cord, but not for the neuroblasts in the abdominal ganglia, which are eliminated by programmed cell death (see Rogulja-Ortmann and Technau, 2008). During their postembryonic phase of neurogenesis, the neuroblasts give rise to lineage-related clusters of immature adult-specific neurons, also referred to as secondary neurons, which can be identified in the brain and thoracic ganglia using neuroanatomical criteria (Pereanu and Hartenstein, 2006; Truman et al., 2004). In the subsequent pupal phase, these secondary neurons (from postembryonic neurogenesis) mature and together
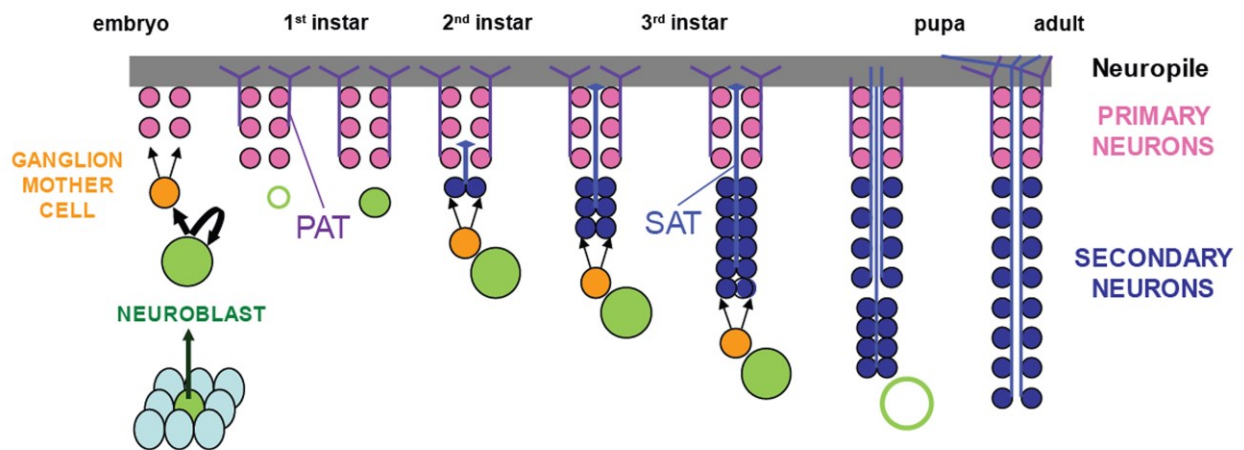
Fig. 1.2: Neurogenesis in *Drosophila*

Schematic overview showing (Type I) neurogenenesis of *Drosophila* in embryonic and postembyronic stages. Neuroblasts delaminate from the neuroectoderm and start to divide asymmetrically to produce primary neurons during embryogenesis. After a period of quiescence, neuroblasts are reactivated to produce the adult-specific secondary neurons during larval development. Primary neurons extend primary axon tracts (PATs) and secondary neurons extend secondary axon tracts (SATs). During pupal development, primary and seconadary neurons build are reconfigured to build the functional adult CNS.

with reconfigured primary neurons (from embryonic neurogenesis), they form the functional circuits of the adult (see Hartenstein et al., 2008). Adult-specific secondary lineages of the CNS have been described in detail for the SPG (Cardona et al., 2009; Ito et al., 2013; Lovick et al., 2013; Pereanu and Hartenstein, 2006; Wong et al, 2013; Yu et al, 2013) and the ventral ganglia (Truman et al., 2004).

For proper CNS neurogenesis, a time-wise precise and irreversible termination of progenitor proliferation is required (Knoblich, 2010; Reichert, 2011). Neuroblasts were shown to exit proliferation at different time points in embryonic and postembryonic stages. At the end of

embryogenesis, the majority of abdominal neuroblasts undergo apoptosis (White et al. 1994). During postembryonic stages, neuroblasts in the thorax were shown to exit by a terminal symmetric division in the pupal phase, while neuroblasts in the abdomen were shown to be terminated by apoptosis in larval stages (Bello et al., 2003; Maurange et al., 2008). The last neuroblasts to disappear are the mushroom body neuroblasts which exit in pupal stages by apoptosis and autophagy which relies on the transcription factor Foxo (Siegrist et al., 2010). Based on BrdU-incorporation experiments and due to the lack of neuroblast marker expression in the adult brain, it was believed until recently that (unlike in other insect species and vertebrates) no adult neurogenesis takes place in *Drosophila* (Ito and Hotta, 1992). However, new findings based on improved lineage-labeling methods indicate that the formation of neurons can be found indeed in the adult *Drosophila* brain (Fernández-Hernández et al., 2013).

1.2 HOX GENES AND CNS DEVELOPMENT

The field of Hoxology was initiated by William Bateson who proposed the term "homeosis" to describe the transformation of one structure of the body into the homologous structure of another body segment (Bateson, 1894). Hox genes were first identified in *Drosophila* under the name of homeotic (selector) genes which mutations lead to homeotic transformations of specific segments along the anterior-posterior axis (Lewis, 1978). In *Drosophila*, Hox genes map to two different loci on the right arm of the 3rd chromosome, referred to as the Antennapedia complex (ANTP-C) and the Bithorax complex (BX-C) (Fig. 1.3). The ANTP-C contains five Hox genes: *labial* (*lab*), *proboscipedia* (*pb*), *Deformed* (*Dfd*), *Sex combs reduced* (*Scr*) and *Antennapedia* (*Antp*). A gain-of-function mutation of the *Antennapedia* gene is also associated with one of most prominent examples for the developmental power of Hox genes, the antenna-to-leg transformation. The BX-C contains 3 Hox genes: *Ultrabithorax* (*Ubx*), *Abdominal-A* (*Abd-A*) and *Abdominal-B* (*Abd-B*). Altogether, there are 8 Hox genes in *Drosophila*, and their arrangement on the chromosome from 3` to 5` is the same as their order of expression along the anterior-posterior body axis. This phenomenon is referred to as spatial colinearity. Molecular studies have shown that Hox genes share a conserved 183 base pair DNA sequence element, the homeobox (Gehring, 1987). It encodes the homeodomain, a 61 amino acid subunit of proteins which has the ability to specifically bind DNA sequences, and therefore indicated that Hox genes encode transcription factors (Levine and Hoey, 1988). Hox genes homologous to those found in *Drosophila* were identified in all bilaterian vertebrates and invertebrates studied to date. In mammals, 39 Hox genes cluster were found which map to 4 different genomic loci, referred to as Hox-A, -B, -C and -D complexes (Favier and Dollé, 1997; McGinnis and Krumlauf, 1992;

Zeltser et al., 1996) (Fig. 1.3). Comparison of sequences from the homeobox and overall coding
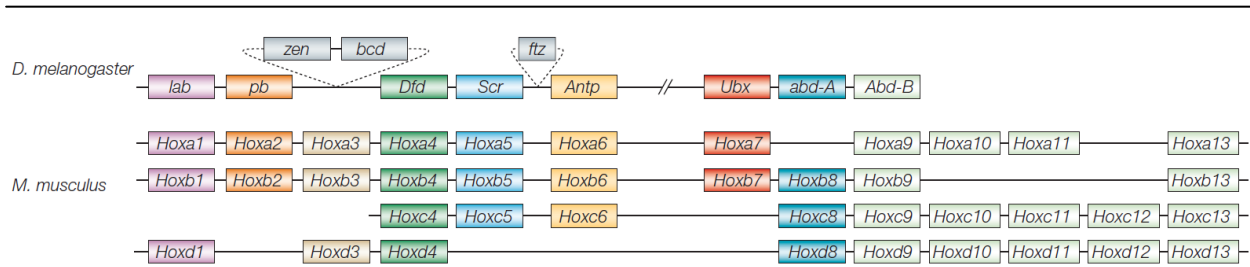
Fig. 1.3: Genetic map of the *Drosophila* and vertebrate Hox clusters

Schematic drawing of the Hox clusters in the genomes of *Drosophila melanogaster* and *Mus musculus*. Different members of the Hox family are indicated by different colors, and orthologous genes between clusters and species are labeled in the same color. Genes are shown in the order in which they are located on the chromosome. Gene abbreviations: *lab, labial*; *pb, proboscipedia*; *zen, zerknüllt*; *bcd, bicoid*; *Dfd, Deformed*; *Scr, Sex combs reduced*; *ftz, fushi tarazu*; *Antp, Antennapedia*; *Ubx, Ultrabithorax; abd-A, abdominal-A; Abd-B, Abdominal-B*. From Pearson et al., 2005.

regions showed that the mammalian Hox genes can be subdivided in 13 highly related paralogs, each of which is related to one of the *Drosophila* Hox genes (Scott, 1992). Like their *Drosophila* counterparts, mammalian Hox genes are expressed in restricted domains along anterior-posterior axis (Dollé and Duboule, 1993). This remarkable interspecies conversation of Hox gene structure and expression led to the proposal that all animals or at least all animals with bilateral symmetry may be defined by the presence of Hox genes (Slack et al., 1993; Davidson et al., 1995). For a long time it was debated how Hox proteins manage to achieve their high degree of binding specificity. It is now clear that Hox proteins use in many cases the assistance of other proteins, termed cofactors and collaborators, to fulfill this task (reviewed in Mann et al., 2009). The most

important cofactors are Extradenticle (Exd) and Homothorax (Hth) in *Drosophila* as well as Pbx and Meis in vertebrates, all of which bind DNA cooperatively with Hox proteins to increase Hox-DNA binding specificities. A big question mark in Hox gene research is still the identity of the downstream target genes (reviewed in Svingen and Tonissen, 2006). The global nature of prominent Hox gene mutant phenotypes indicates the involvement of hundreds of target genes acting in different cellular processes such as organogenesis, differentiation, cell adhesion and migration. However, to date only a very limited number of downstream targets of Hox genes have been identified.

Hox genes have been shown to play important roles in the embryonic development of the *Drosophila* central brain (reviewed in Reichert and Bello, 2010). The Hox gene *lab* is expressed in the embryonic TR of the SPG, and the Hox genes *Dfd*, *Scr* and *Antp* are expressed in the embryonic SEG (Hirth et al., 1998; Urbach and Technau, 2003) (for Hox expression in the fly CNS, see Fig. 1.4). Hox gene inactivation can cause dramatic misprojections and patterning defects in embryonic brain development. For example, if *lab* is mutated, cells of the TR neuromere (the normal *lab* expression domain) are still generated, but fail to extend axons and lack the expression of neuronal markers. This implies that *lab* is required to establish neuronal identity in its neuromeric expression domain (Fig. 1.5). In a comparable manner, the *Dfd* gene is required during embryonic neurogenesis for establishing the appropriate neuronal identity in the MD and anterior MX neuromeres. Hox genes are also required for embryonic development in the thoracic and abdominal ganglia of the VNC. The Hox genes *Antp, Ubx, abd-A* and *Abd-B* are expressed in partially overlapping domains in the embryonic VNC. Inactivation of these Hox genes does not cause gross morphological defects in embryonic ventral nerve cord development,
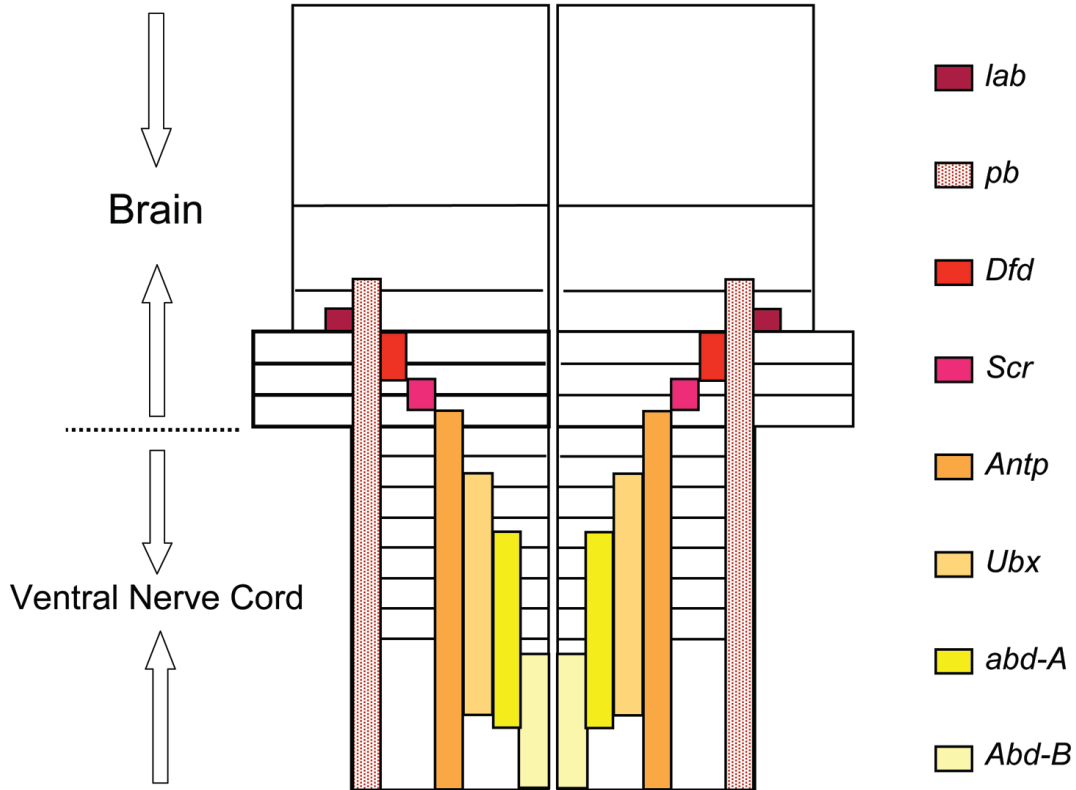
Fig. 1.4: Expression of Hox genes in the embryonic CNS of *Drosophila*

Schematic overview showing the anterior-posterior extent of Hox gene expression in the CNS of a stage 14 *Drosophila* embryo. Expression domains are indicated by color-coded bars. Horizontal lines indicate segmental neuromere boundaries. *lab*, *labial*; *pb*, *probscipedia*; *Dfd*, *Deformed*; *Scr*, *Sex combs reduced*; *Antp*, *Antennapedia*; *Ubx*, *Ultrabithorax; abd-A*, *abdominal-A*; *Abd-B*, *Abdominal-B*. Note that *pb* is only expressed in segmentally repeated groups which is indicated by dotted shading. From Reichert and Bello, 2010.

however, it does result in defective segment-specific specification, lineage identity, and cell fate as well as in defects in neuroblast-specific proliferation and programmed cell death in the thoracic and abdominal ganglia (Baumgardt et al., 2009; Berger et al., 2005; Kannan et al., 2010; Karlsson et al., 2010; Miguel-Aliaga and Thor, 2004; Prokop et al., 1998; Prokop and Technau, 1994; Rogulja-Ortmann et al., 2008; Rogulja-Ortmann and Technau, 2008; Suska et al., 2011).
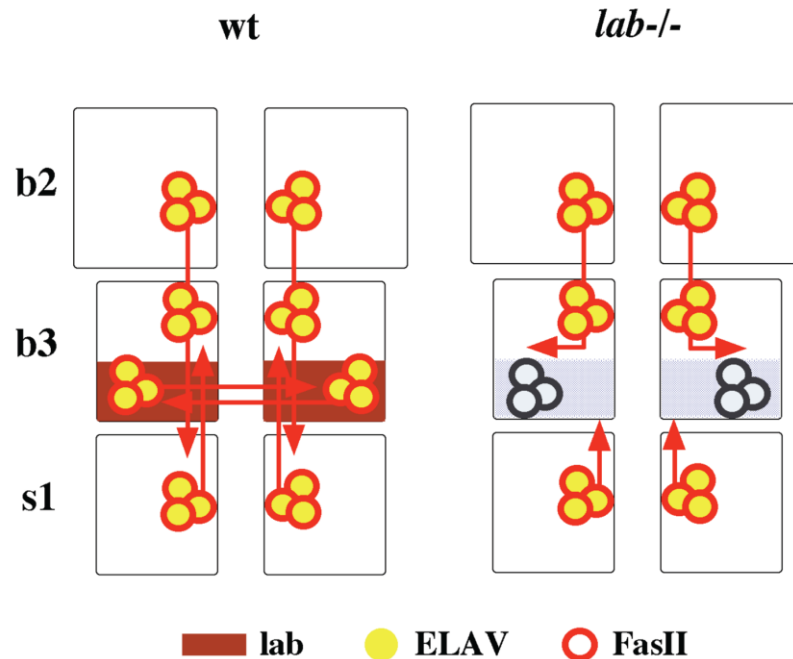
Fig. 1.5: Phenotype of *lab* mutation in the *Drosophila* brain

Simplified scheme of the deutocerebral (b2), tritocerebral (b3) and mandibular (s1) neuromeres in the embryonic brain of *Drosophila*. In the wildtype (wt), tritocerebral cells express *lab*, the neuronal marker ELAV and the cell adhesion molecule Fasciclin II (FasII). In the lab mutant, cells of the tritocerebrum do not express anymore ELAV and FasII, indicating a loss of neuronal identity. Axons (arrows) from other brain parts which project in and through the *lab* domain in the wildtype avoid the tritocerebrum in the *lab* mutant. From Reichert and Bello, 2010.

Much less is known about the expression and function of Hox genes during postembryonic development of the CNS. In the thorax, *Antp* was shown to be required as a dose-dependent determinant of motoneuron connectivity (Baek et al., 2013). *Ubx* and *abd-A* are needed to generate proper segment-specific patterns of motor activity in the larva (Dixit et al., 2008). *Ubx* has also a role in segment-specific morphology and survival of postembryonically generated neuronal cells in the thorax and abdomen (Marin et al., 2012). And in the central abdomen, a

pulse of *abd-A* expression in larval stages was shown to induce apoptosis in neuroblasts, a process which is tightly regulated by the Polycomb group of genes (Bello et al., 2003; Bello et al., 2007).

Hox genes also pattern the CNS of vertebrates (reviewed in Philippidou and Dasen, 2013). Remarkably, the anterior-posterior order of Hox gene expression domains is well conserved in the embryonic CNS of *Drosophila* and vertebrates. Hox1-Hox5 paralog group genes are expressed in the segmented hindbrain and Hox4-Hox11 genes are expressed in the spinal cord (Fig. 1.6). The embryonic hindbrain and spinal cord generate the neural circuits that build the
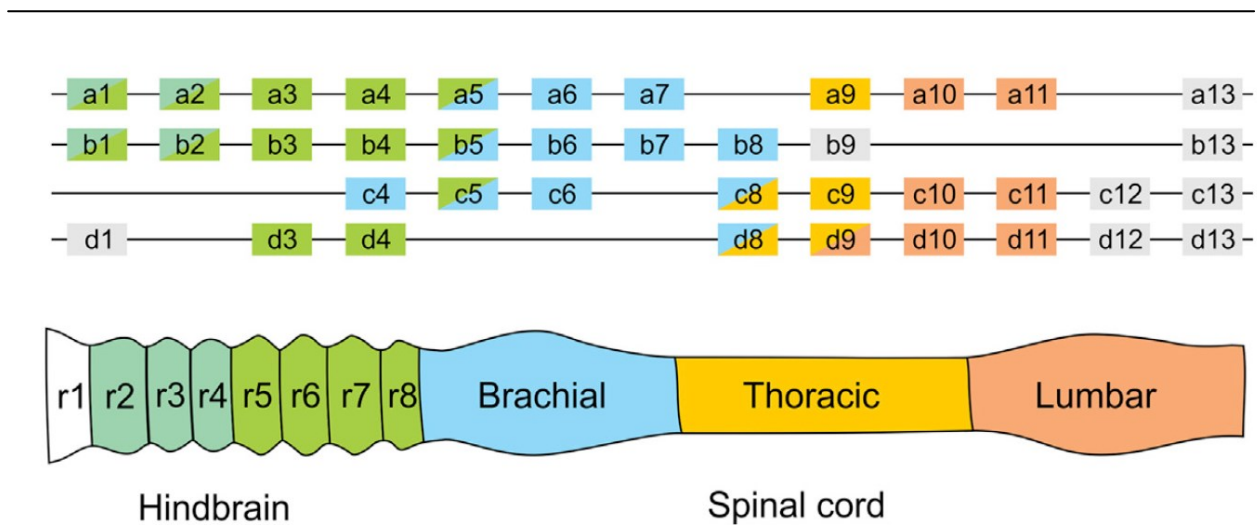
Fig. 1.6: Expression of Hox genes in the vertebrate CNS

Top: The 39 Hox genes in vertebrates are divided into 4 genomic clusters. Orthologs in *Drosophila*: *Hox1, lab*; *Hox2, pb*; *Hox3*, no ortholog; *Hox4, Dfd*; *Hox5, Scr*; *Hox6, Antp*; *Hox7, Ubx*; *Hox8, abd-A*; *Hox9-13, Abd-B*. Bottom: Color-coded anterior-posterior expression domains of vertebrate Hox genes in the rhombomeres (r1-r8) of the hindbrain and the spinal cord. From Philippidou and Dasen, 2013.

basis for important motor functions such as respiration and locomotion as well as for several sensory modalities. Most of what it is known on Hox gene action in the vertebrate CNS is based on mouse knockout experiments and manipulation of Hox activity in chick embryos. However, Hox mutant analysis in the vertebrate CNS is complex due to paralog compensations, general overlapping expression domains, genetic cross-reactions and the different time windows of Hox expression. Hox gene mutant analyses in the vertebrate CNS were performed for: the *lab*-orthologs *Hoxa1*, *Hoxb1* and *Hoxd1*; the *pb* orthologs *Hoxa2* and *Hoxb2*; *Hoxa3*, *Hoxb3* and *Hoxd3*; for the *Scr*-orthologs *Hoxa5* and *Hoxc5*; for the *Antp*-orthologs Hoxa6 and Hoxc6; the *abd-A*-orthologs *Hoxb8* and *Hoxc8*; the *Abd-B*-orthologs *Hoxc9*, *Hoxa10*, *Hoxc10*, *Hoxd10* and *Hoxb13*.

Hox1 and *Hox2* were shown to be required for compartmentalization of the mouse hindbrain, as single and double knockouts of these genes lead either the reduction or absence of specific rhombomeres and their borders (Barrow et al., 2003; Carpenter et al., 1996; Gavalas et al, 1998; Goddard et al, 1996; Mark et al., 1993; Rossel and Capecchi, 1999; Studer et al., 1998) (Fig. 1.7). Moreover, mutation of *Hoxa1* was also implicated in the hindbrain with generation of supernumerary cells that give rise to a novel functional respiratory network (Del Toro et al., 2001). Motoneurons of the hindbrain were shown to be dependent of expression of the *Hox1*, *Hox2* and *Hox3* genes for appropriate specification of identity and connectivity (Gavalas et al, 1997; Gavalas et al., 2003; Gaufo et al., 2003; Goddard et al, 1996; Guidato et al., 2003; Studer et al., 1996). *Hoxb1* and *Hoxa2* expression in neural crest was shown to be needed in a non-cell-autonomous manner for the motoneuron axon guidance (Arenkiel et al., 2004; Gendron-Maguire

et al., 1993; Prince and Lumdsen, 1994; Rijli et al., 1993). The *Hox5* and *Hox9* genes specify motoneurons in specific motor colums of the spinal cord (Jung et al., 2010; Philippidou et al., 2012). The *Hox3-Hox8* and *Hox10* genes were shown to be involved in spinal motor column and pool specification (Dasen et al., 2005; Lacombe et al., 2013; Lin and Carpenter, 2003; Shah et al., 2004; Wahba et al, 2001; Vermot et al., 2005; Wu et al., 2008). Loss of Hox genes also affects non-motoneuron populations in the vertebrate nervous system. In the hindbrain, *Hoxb1*, and *Hoxa3/b3* were shown to specify visceral sensory neurons, while *Hoxa2* mutation leads to elimination of somatic sensory neurons (Gaufo et al., 2004). Hox genes are also required for the production of serotonergic neurons and oligodendrocytes (Miguez et al., 2012; Pattyn et al., 2003). Moreover, *Hoxb8* was shown to been implicated in organization, survival and specification of neurons in the spinal cord, while *Hoxb13* acts in defining the spinal caudal boundary (Economides et al., 2003; Holstege et al., 2008; Huber et al., 2012; van der Akker et al., 1999). Thus, like in *Drosophila*, vertebrate Hox genes act in the CNS to establish regional identity, a prerequisite for correct neuronal specification, circuit formation and proper axon guidance in the nervous system.
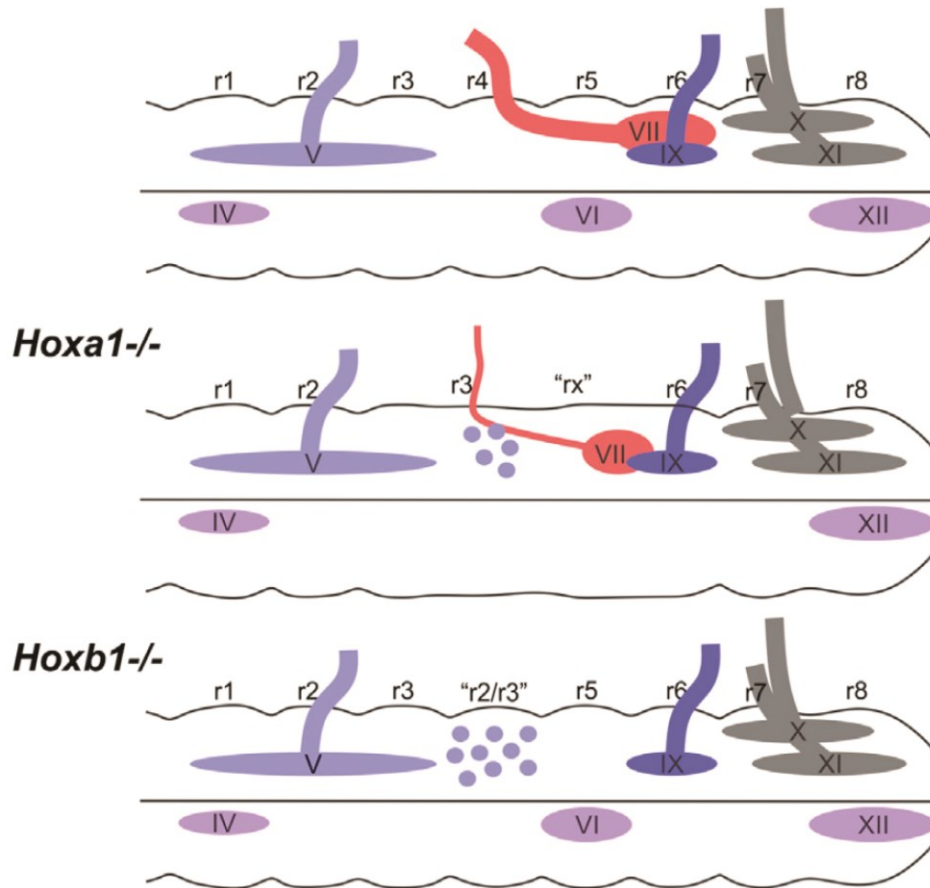
Fig. 1.7: Phenotype of Hoxa1/b1 mutation in the mammalian hindbrain

Wildype is on top. Below: Knockout of Hoxa1 and Hoxb1 leads to misspecification and disorganization of hindbrain regions. r1-r8, rhombomere1-8; rx, hybrid region with no clear rhombomeric identity. Latin numbers refer to specific nuclei: IV, trochlear; V, trigerminal; VI, abducens; VII, facial; IX, glossopharyngeal; X, vagus; XI, accessory; XII, hypglossal. From Philippidou and Dasen, 2013.

1.3 THIS THESIS

Compared to the wealth of cellular and molecular information available on postembryonic development of the neuroblasts and their lineages in the anterior supraesophageal ganglion, very little was known about the postembryonic development of the tritocerebrum and subesophageal ganglion. Thus, in the postembryonic development of the tritocerebrum and subesophageal ganglion, neither the number of proliferating neuroblasts, nor the identity of their secondary neuron lineages was known. In addition, there was complete lack on information on postembryonic expression and function of Hox genes in the tritocerebral and subesophageal brain regions.

In this thesis, the postembryonic neuroblast lineages located in the tritocerebrum and subesophageal ganglion were described, accompanied by an analysis of expression and function of Hox genes in these brain regions. In the first study (Chapter 2), the tritocerebral neuroblast lineages in the postembryonic central brain were identified by the postembryonic expression of the Hox gene *lab*, which is also required for the termination of 2 neuroblasts during early larval stages. In the second study (Chapter 3), the lineages in the postembryonic SEG were characterized, leading to the identification of a small number of 14 neuroblast lineages in the late larval brain. In addition, the Hox genes *Dfd*, *Scr* and *Antp* were shown to pattern the postembryonic SEG, in which they are also required for three lineage-specific functions. In chapter 4, Hox gene patterning in the nervous system of *Drosophila* was reviewed, and thus, findings from the previous chapters could be set in the general context of Hox gene research. Taken together, a small set of neuroblast lineages could be identified in the postembryonic

tritocerebrum and subesophageal ganglion, in both of which Hox gene action is required in a lineage-specific way for the generation of adult-specific neurons of the *Drosophila* brain.

2. THE *LABIAL* GENE IS REQUIRED TO TERMINATE PROLIFERATION OF IDENTIFIED NEUROBLASTS IN POSTEMBRYONIC DEVELOPMENT OF THE *DROSOPHILA* BRAIN

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2.1 ABSTRACT

The developing brain of *Drosophila* has become a useful model for studying the molecular genetic mechanisms that give rise to the complex neuronal arrays that characterize higher brains in other animals including mammals. Brain development in *Drosophila* begins during embryogenesis and continues during a subsequent postembryonic phase. During embryogenesis, the Hox gene *labial* is expressed in the developing tritocerebrum, and *labial* loss-of-function has been shown to be associated with a loss of regional neuronal identity and severe patterning defects in this part of the brain. However, nothing is known about the expression and function of *labial*, or any other Hox gene, during the postembryonic phase of brain development, when the majority of the neurons in the adult brain are generated. Here we report the first analysis of Hox gene action during postembryonic brain development in *Drosophila*. We show that *labial* is initially expressed in six larval brain neuroblasts, of which only four give rise to the *labial* expressing neuroblast lineages present in the late larval brain. Although MARCM-based clonal mutation of *labial* in these four neuroblast lineages does not result in an obvious phenotype, a striking and unexpected effect of clonal *labial* loss-of-function does occur during postembryonic brain development, namely the formation of two ectopic neuroblast lineages that are not present in wild-type brains. The same two ectopic neuroblast lineages are also observed following cell death blockage and, significantly, in this case the resulting ectopic lineages are Labial-positive. These findings imply that *labial* is required in two specific neuroblast lineages of the wildtype brain for the appropriate termination of proliferation through programmed cell death. Our analysis of *labial* function reveals a novel cell autonomous role of this Hox gene in shaping the lineage architecture of the brain during postembryonic development.

2.2 INTRODUCTION

The neural cells of the *Drosophila* central brain develop from a set of approximately 100 neural-stem-cell-like neuroblasts which derive from the cephalic neuroectoderm in the early embryo (reviewed in Urbach and Technau, 2004; Technau et al., 2006; Hartenstein et al., 2008). During embryogenesis, these neuroblasts divide in an asymmetric manner to self-renew and produce ganglion mother cells which generally give rise two postmitotic neural progeny (reviewed in Skeath and Thor, 2003; Doe, 2008; Knoblich, 2008). This initial phase of embryonic neurogenesis gives rise to the functional brain of the *Drosophila* larva. Towards the end of embryogenesis, most neuroblasts enter a reversible cell cycle arrest called quiescence, which separates the initial phase from the subsequent secondary phase of neurogenesis (Isshiki et al., 2001; Tsuji et al., 2008; Egger et al., 2008). In response to intrinsic and extrinsic factors involving nutritionally activated mitogens and glial cell-dependent interactions, neuroblasts resume proliferation during early larval stages (Chell and Brand, 2010; Sousa-Nunes et al., 2011). During this postembryonic phase of neurogenesis the majority of the adult-specific neurons of the brain are generated (Truman and Bate, 1988; Prokop and Technau, 1991). The adult-specific neural cells produced postembryonically by each individual neuroblast form a lineage-related cluster of immature neurons which differentiate in the pupal phase and contribute to the functional adult brain circuits (Truman et al., 2004; Pereanu and Hartenstein, 2006; Hartenstein et al., 2008).

Timely, precise and irreversible termination of postembryonic neuroblast proliferation is crucial to ensure that the correct number of neural progeny is generated and to avoid the danger of uncontrolled overgrowth (reviewed in Neumüller and Knoblich, 2009; Weng et al., 2010;

Knoblich, 2010; Reichert, 2011). This process varies in temporal and spatial respects in the developing brain, but is largely finished by the end of metamorphosis as no identifiable neuroblasts are present at adult stages (Truman and Bate, 1988; Ito and Hotta, 1992). For most of the neuroblasts of the central brain and thoracic ganglia, termination of proliferation is achieved by series of cellular adjustments, involving shrinkage, lengthening of the cell cycle, expression of nuclear *prospero* and then cell cycle exit via a symmetric final division (Maurange et al., 2008). In contrast, for neuroblasts in the abdominal ganglia, which cease dividing in larval stages, termination of proliferation involves another mechanism, namely induction of programmed cell death in neuroblasts through expression of Hox gene-encoded transcription factors (reviewed in Pearson et al., 2005; Rogulja-Ortmann and Technau, 2008; Miguel-Aliaga and Thor, 2009; Sousa-Nunes et al., 2010). More specifically, in all neuroblasts of the central abdomen, the Hox gene *abdominal-A* (*abd-A*) is expressed in a short pulse during larval development in order to trigger programmed cell death (Bello et al., 2003). This ability of Hox genes to trigger programmed cell death in the abdominal ganglia is tightly regulated by epigenetic mechanisms involving the Polycomb group of genes (Bello et al., 2007).

Hox genes have also been shown to act in the development of the central brain in *Drosophila*, and notably for the Hox gene *labial*, loss-of-function has been associated with severe pattering defects in embryonic brain development (Diederich et al., 1989; reviewed in Lichtneckert and Reichert, 2008; Reichert and Bello, 2010). During embryogenesis, *labial* is expressed throughout the tritocerebrum anlage; all thirteen neuroblasts of the tritocerebrum as well as two neuroblasts of the deutocerebrum are Labial-positive (Younossi-Hartenstein et al., 1996; Urbach and Technau, 2003). If *labial* is inactivated, postmitotic cells are generated, however, they do not extend neurites and lack the expression of neuronal markers, indicating that *labial* is required to

establish neuronal identity in the embryonic tritocerebrum (Hirth et al., 1998). Interestingly, these defects can be rescued by targeted misexpression by all Hox genes except *Abd-B* (Hirth et al., 2001). Moreover, expression of *labial* in the tritocerebrum can be subject to cross-regulatory interactions among Hox proteins during embryonic brain development (Sprecher et al., 2004).

In contrast to the extensive information on the role of the *labial* gene in embryonic brain (tritocerebrum) development, virtually nothing is known about the expression and function of *labial,* or any other Hox gene, in postembryonic brain development of *Drosophila*. Hence, it is unclear if Hox genes have any influence on the development of the adult-specific, secondary neurons that make up the bulk of the neuronal circuitry in the adult brain. Here we show that the Hox gene *labial* is expressed in late L3 larval stage brain in four neuroblasts that give rise to the identified *labial* expressing neuroblast lineages BAlp4, BAlv, TRdm and TRdl. Moreover, we demonstrate that two additional *labial* expressing neuroblasts are present in the late L2 stage – but not in the early L3 stage. Remarkably, while MARCM-based clonal mutation of *labial* in the BAlp4, BAlv, TRdm and TRdl neuroblast lineages does not result in any obvious mutant phenotype, a striking effect of clonal *labial* loss-of-function does occur, namely the formation of two ectopic neuroblast lineages that are not present in wild-type brains. These two ectopic neuroblast lineages are also observed following MARCM-based block of cell death and, significantly, these ectopic lineages are Labial-positive. Since both clonal cell death block and clonal *labial* inactivation result in the formation of the same two ectopic neuroblast lineages, these findings imply that *labial* is required in these two postembryonic brain neuroblast lineages for termination of proliferation through programmed cell death. This analysis of *labial* function reveals a novel cell autonomous role of a Hox gene in shaping the lineage architecture of the brain during postembryonic development.

2.3 RESULTS

2.3.1 The *labial* gene is expressed in four identified neuroblasts and their lineages in the late larval brain

To investigate the expression of the Hox gene *labial* in postembryonic brain development, we performed an immunocytochemical analysis of whole-mount brains of wandering third-instar (late L3 stage) larvae using a Labial-specific antibody (LAB) in combination with an anti-Bruchpilot (NC82) antibody to visualize neuropile structures. Expression of the *labial* gene was detected in two bilaterally symmetrical groups of cells located posterior to the antennal lobe and adjacent to the SEG (subesophageal ganglion) in the general region of the posterior central brain that corresponds to the developing tritocerebrum (Fig. 2.1). In confocal single optical sections, these *labial* expressing neural cells were observed in spatial association with a number of
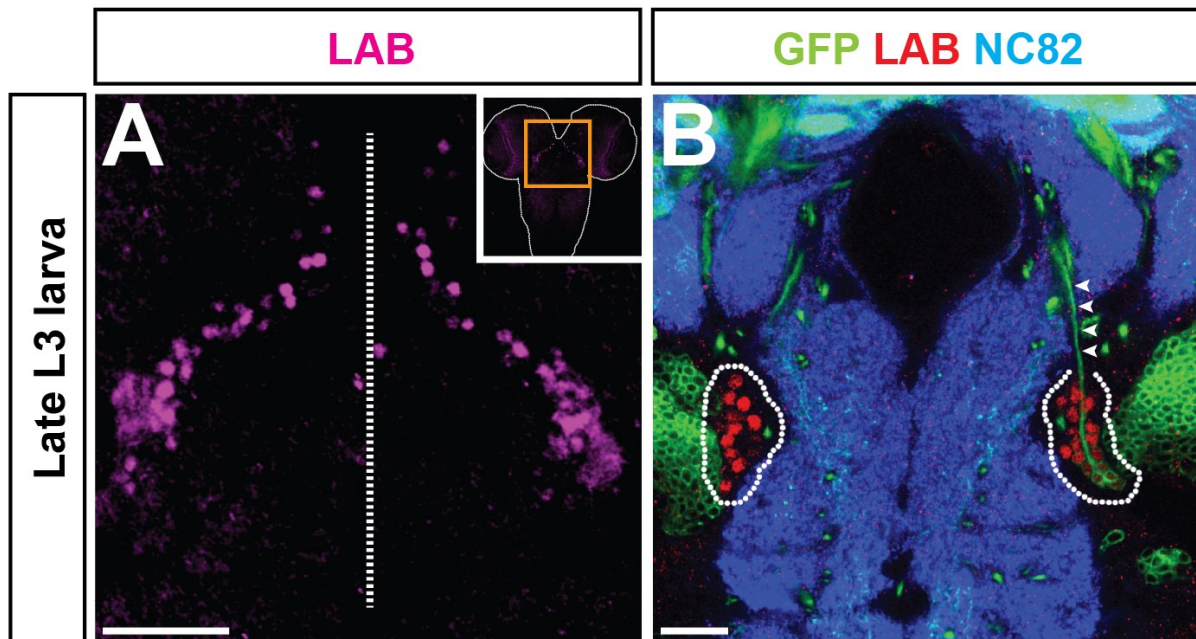
Fig. 2.1: Regionalized expression of *labial* in the posterior central brain at the late larval stage.

(A) Overview of the late L3 larval brain. Two bilaterally symmetric cell clusters express *labial*. Labeled cells are shown in a Z-projection of multiple optical sections of a whole-mount brain. Dotted line indicates midline. Inset shows total larval CNS with box indicating region of *labial* expression. **(B)** Single optical section showing *labial* expressing cells (red), nc82 immunolabeled neuropile (blue) and MZ[1407]-Gal4 driven and membrane-targeted GFP expression (green). Dotted lines indicate position of the *labial* expressing cells. Arrowheads indicate secondary axon tract of *labial* expressing cells. Scale bars: 50 μm in A; 20 μm in B.

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secondary axon tracts labeled by MZ[1407-]Gal4 suggesting that these neurons might correspond to a small set of neuroblast lineages (Luo et al. 1994; Betschinger et al., 2006). In addition to the labeled neuron groups, *labial* expression was also observed in four larger cells which co-expressed the marker *deadpan* (DPN) indicating that they were neuroblasts (San-Juán and Baonza, 2011). These four neuroblasts were also located in the same posterior central brain region and were invariably associated with the *labial*-expressing cell clusters (Fig. 2.2). Several other Deadpan-positive neuroblasts were located in the vicinity of these four *labial*-expressing neuroblasts, but none of these were Labial-positive. To identify the postembryonic lineages generated by the four *labial* expressing neuroblasts, we performed a MARCM-based clonal analysis with an ubiquitous *tub*-Gal4 driving UAS-mCD8::GFP (Lee and Luo, 1999; Lee and Luo, 2001). Clones were induced at random at 24 hours after larval hatching (ALH) and recovered at the late L3 larval stage and, therefore, only secondary (adult-specific) lineages of individual neuroblasts were labeled. MARCM-labeled clones were co-labeled with the anti-Labial antibody and with an anti-Neurotactin (NRT; BP106) antibody that is specific for secondary lineages. We recovered four neuroanatomically distinct neuroblast lineages that had Labial-immunopositive neuroblasts.
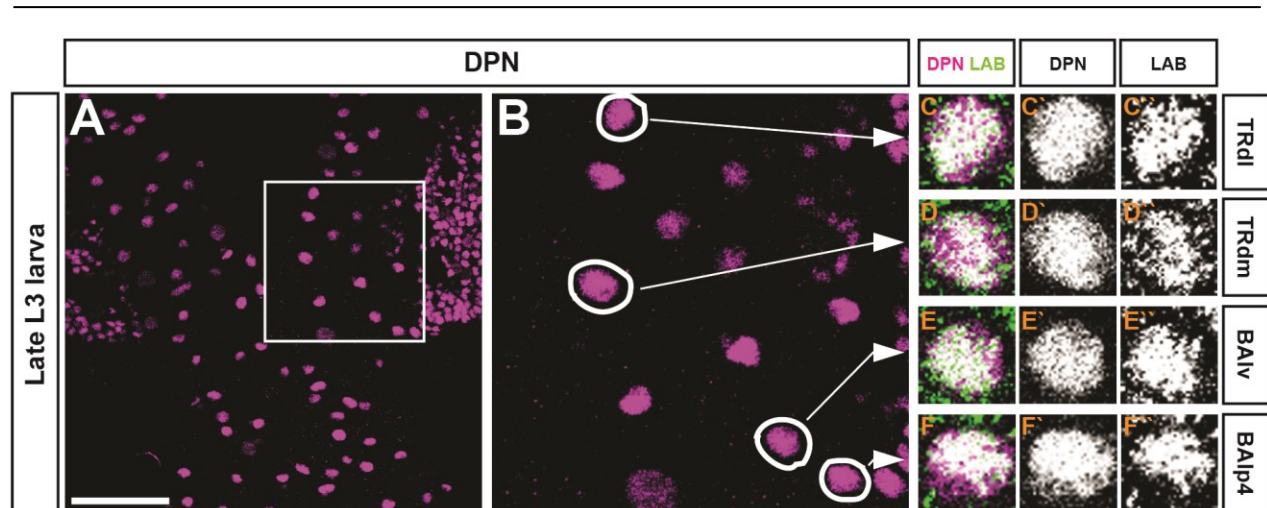
Fig. 2.2: *labial* **is expressed in four neuroblasts of the late L3 larval brain**.

Labeled cells are shown in a Z-projection of multiple optical sections. **(A)** Overview of anti-Deadpan immunolabeled cells in the L3 larval brain. Box delimits cells a region in one hemisphere of the posterior central brain. **(B)** Magnified view of the boxed region shown in A. Neuroblasts co-immunolabeled with anti-Deadpan and anti-Labial are indicated by circles. **(C-F``)** Single optical sections of each of the four Deadpan-immunolabeled neuroblasts that express *labial* at the late L3 stage. Magnified view of the circled cells shown in B. Anti-Deadpan immunolabeling is in magenta. Labial immunolabeling is in green. Based on their relative position, each of these neuroblasts can be assigned to four lineages: TRdm, TRdl, BAlv, BAlp4. Scale bar: 50 μm in A.

For further identification of theses neuroblast lineages, we determined the projection patterns of each of their secondary axon tracts relative to the ensemble of secondary axon tracts in the late larval brain based on anti-NRT immunolabeling and compared these patterns to those documented in previous lineage mapping studies (Pereanu and Hartenstein, 2006; Spindler and Hartenstein, 2010). Since all of these neuroblast lineages had an invariant and unique projection pattern of their secondary axon tracts (SAT), we were able to unambiguously assign the four *labial* expressing neuroblast lineages to four previously identified postembryonic lineages, namely BAlp4 (basoanterior lineages, posterolateral subgroup), BAlv (basoanterior lineages,

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ventrolateral subgroup), TRdm (dorsomedial tritocerebral lineage), and TRdl (dorsolateral tritocerebral lineage) (Fig. 2.3).
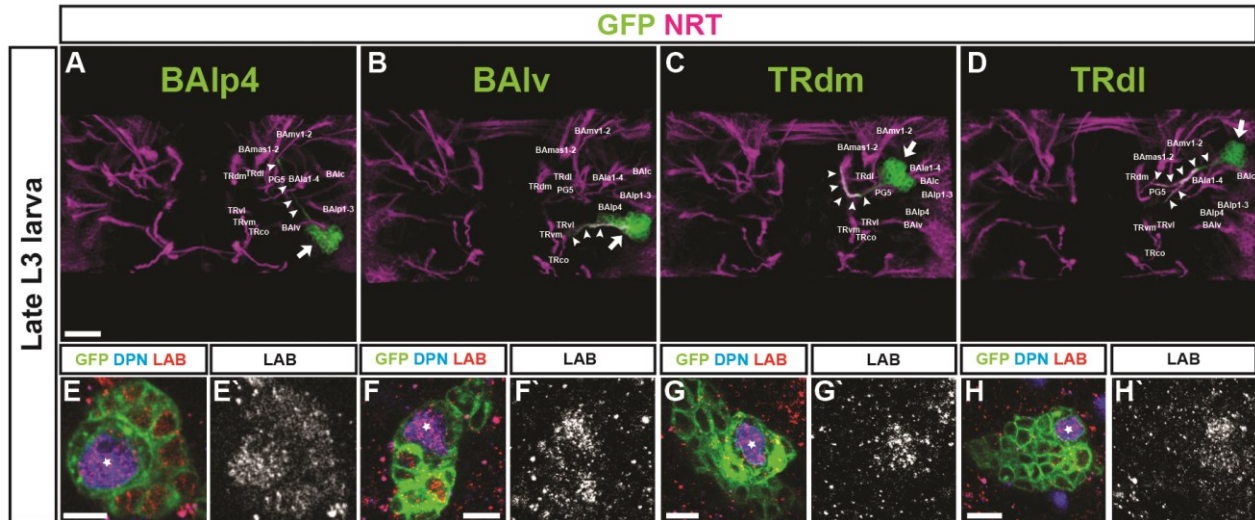
Fig. 2.3: The four *labial* expressing neuroblasts give rise to the BAlp4, BAlv, TRdm and TRdl lineage.

(A-D) Individual identified neuroblast clones are shown for each lineage together with the array of identified secondary axon tracts in the posterior central brain region of interest. GFP-labeled MARCM clones of the four neuroblast lineages are in green. Anti-Neurotactin labeling of secondary axon tracts secondary lineages is in magenta. Arrows indicate position of the cell bodies of the BAlp4, BAlv, TRdm and TRdl lineages. Arrowheads indicate secondary axon tracts of the BAlp4, BAlv, TRdm and TRdl lineages. Figures are superposition of multiple optical sections in late L3 brains. **(E-H`)** Neuroblasts in each of these four lineages express *labial*. Deadpan immunolabeling (neuroblasts) is in blue. Labial immunolabeling is in red. Single optical sections of BAlp4 (E-E`), BAlv (F-F`), TRdm (G-G`) and TRdl (H-H`). Stars indicate the neuroblast. Scale bars: 20 µm in A; 5 µm in E-H`.

2.3.2 Mutational inactivation of *labial* does not affect cell number and secondary axon tract projections in the BAlp4, BAlv, TRdm and TRdl lineages.

To investigate the role of *labial* in the development of the BAlp4, BAlv, TRdm and TRdl lineages, we compared the wildtype and *labial* mutant MARCM clones, induced at 24 hours ALH and recovered at late L3 larval stages, for each of these neuroblast lineages. Mutant GFP-labeled clones were homozygous for *lab*[14], an embryonic lethal loss-of-function allele of *labial* (Merrill at al., 1989). All of the recovered *labial* mutant clones of the BAlp4, BAlv, TRdm and TRdl lineages were similar in their general neuroanatomical features to the respective wildtype clones. They all comprised a single large cell corresponding to the neuroblast as well as an associated cluster of labeled cells corresponding to the secondary neurons, and the secondary axon tracts formed by the secondary neurons had an appropriate, wildtype-like projection pattern in all cases (Fig. S2.1). To determine if the number of cells in the *labial* mutant clones was comparable to that of the corresponding wildtype clones, we performed cells counts for each of the four lineages. For all four lineages, the total cell number was not significantly different in wildtype versus *labial* mutant clones (Fig. S2.1). Thus, average cell counts for wildtype versus mutant were 66 versus 66 (BAlp4), 70 versus 71 (BAlv), 65 versus 62 (TRdm) and 80 versus 88 (TRdl). We conclude that clonal mutation of *labial* does not alter cell number and secondary axon tract projection in the BAlp4, BAlv, TRdm and TRdl lineages.
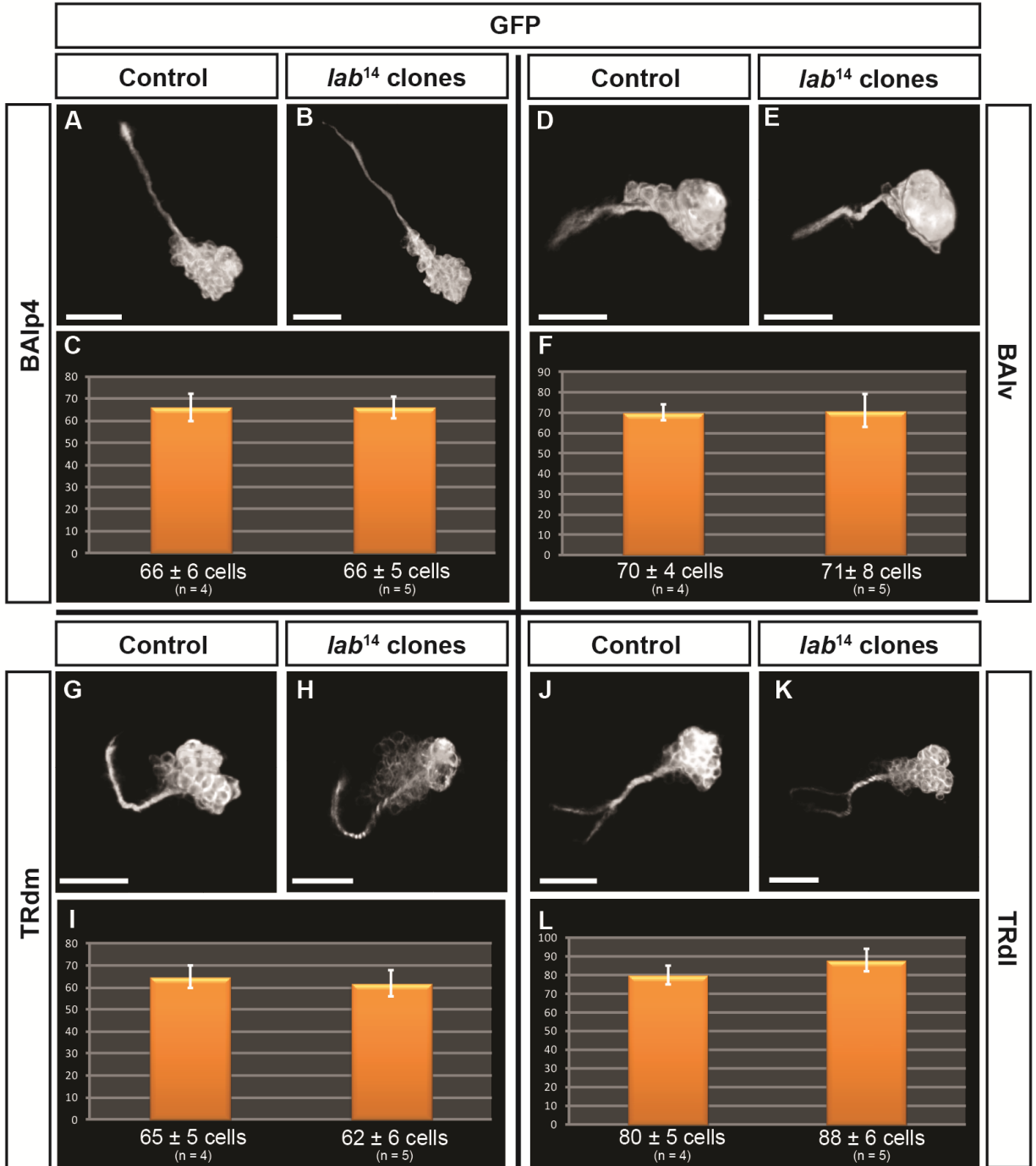
Fig. S2.1: Clonal mutation of *labial* does not affect cell number and secondary axon tract projections in the BAlp4, BAlv, TRdm and TRdl lineages.

(A, B, D, E, G, H, J, K) The projection pattern of secondary axon tracts is not significantly altered in *labial* mutant clones compared to corresponding wildtype clones. GFP labeled wildtype and *lab*[14] mutant clones of the BAlp4, BAlv, TRdm and TRdl lineages. Superposition of multiple optical sections of late L3 brains. **(C, F, I, L)** The number of cells is not significantly different in *labial* mutant clones compared to corresponding wildtype clones. Average cell number in wildtype and *lab*[14] mutant clones of the BAlp4, BAlv, TRdm and TRdl neuroblast lineages in late L3 brains. Number of clone samples indicated as n. Scale bars: 20 μm

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2.3.3 Mutational inactivation of *labial* during postembryonic development leads to the formation of identified ectopic neuroblast lineages

In contrast to the lack of overt mutant phenotype in *labial* mutant BAlp4, BAlv, TRdm and TRdl lineages, a striking and unexpected effect of clonal *labial* loss-of-function was observed in the developing L3 larval brain, namely the formation of ectopic neuroblast lineages that were not present in wildtype brains. Ectopic lineages were recovered in about 50% of all brains containing randomly induced *lab*[14] mutant clones. These ectopic neuroblast lineages could be unambiguously identified based on the projection patterns of their ectopic secondary axon tracts within the ensemble of secondary axon tracts of late larval brains (Fig. 2.4A, B). Morphologically they could be assigned to two different types, which we refer to as Ectopic1*lab* (Ect1*lab*) and Ectopic2*lab* (Ect2*lab*) lineages. Ect1*lab* was located between the BAlp4 and the BAlv lineages, had an average cell number of 107 cells (s.d.=24, n=3) and formed several secondary axon tract projections, of which one always followed an axon tract of the BAlc lineage (Fig. 2.4C-D`). Ect2*lab* was located close to the TRdm and TRdl lineage, had an average cell number of 25 cells (s.d.=1, n=3) and projected its secondary axon tract towards the midline (Fig. 2.4E-F`). These

ectopic lineages were only seen in the late larval (L3) brain. Moreover, they were never observed in MARCM-based genetic rescue experiments (clone induction: 24 hours ALH) in which a UAS-*labial* transgene under the control of the *tub*-GAL4 driver was used to express the *labial* gene in *labial* loss-of-function mutant clones (n=16). To further confirm that the formation of ectopic neuroblast lineages was indeed due to *labial* loss-of-function, we performed genetic knockdown experiments in which *worniu*-Gal4 and MZ[1407]-Gal4 were used to drive UAS-*lab*RNAi[2990] in all developing neuroblasts (Albertson et al., 2004). Ectopic neuroblast lineages comparable to those induced by *lab*[14] mutant clones resulted (Fig. 2.5A, B). These ectopic lineages were recovered in 50% of the late larval brains for the *worniu*-Gal4 driver (n=23) and in 20% of the late L3 larval brains for the MZ[1407]-Gal4 driver (n=14). In accordance with the *lab*[14] mutant clonal analysis (loss-of-function & genetic rescue), these findings indicate that the appearance of ectopic lineages is a *labial*-specific loss-of-function effect. Moreover, since the targeted knockdown of *labial* driven by *worniu*-Gal4 and MZ[1407]Gal4 is largely neuroblast-specific, these findings also suggest that the ectopic lineage phenotype was due to the absence of Labial protein in the neuroblasts themselves rather than in their neural cell progeny. This assumption is supported by the observation that both types of ectopic lineages recovered in *lab*[14] clonal MARCM experiments invariably contained a single large Deadpan-positive neuroblast (Fig. 2.5C-D`).
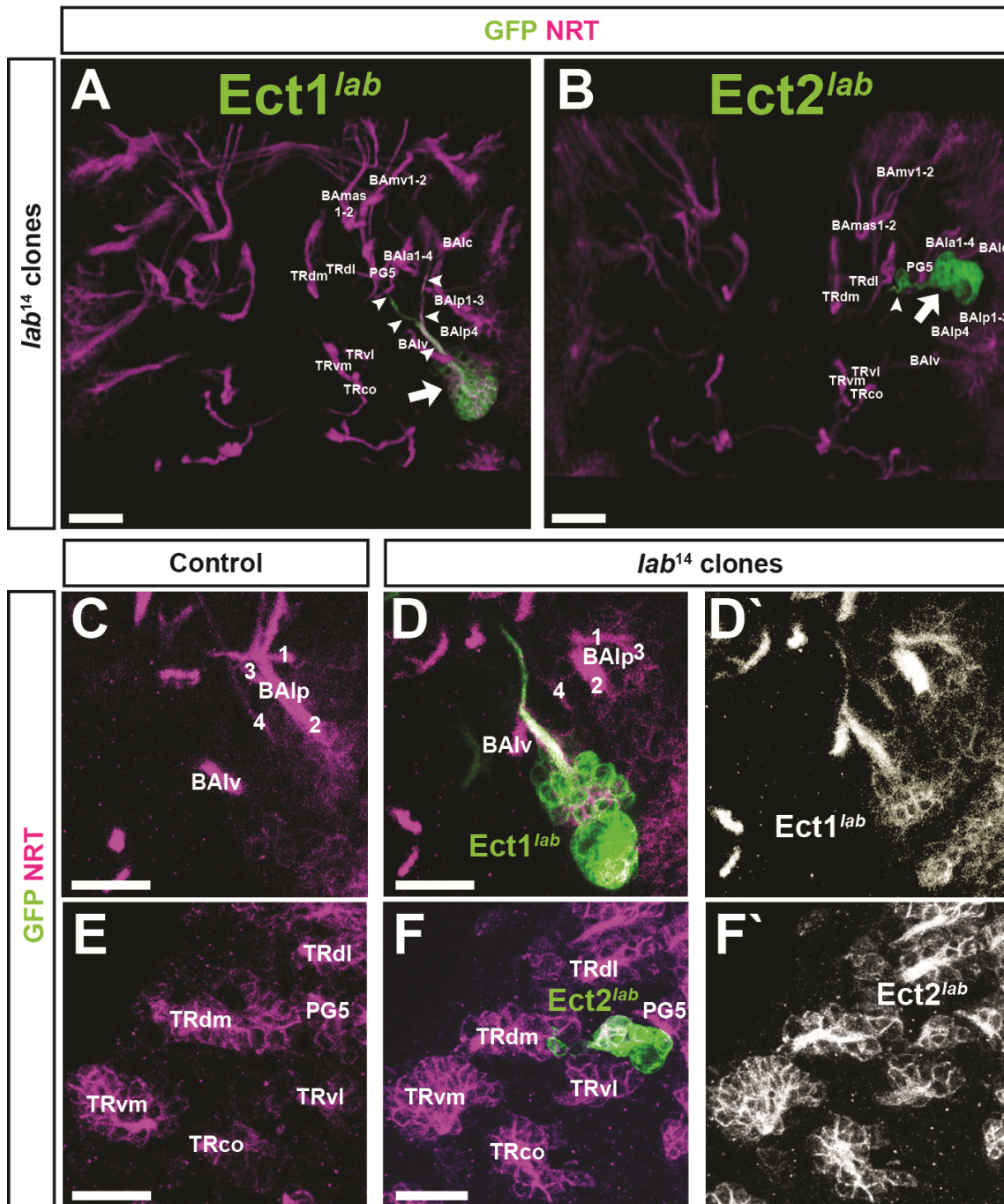
Fig. 2.4: Clonal loss-of-function of *labial* leads to the formation of ectopic neuroblast lineages. Late L3 brains; GFP-labeled mutant *lab*[14] MARCM clones are in green; secondary axon tracts labeled by anti-Neurotactin are in magenta. **(A, B)** Two identified ectopic neuroblast clones, Ect1*lab* and Ect2*lab* (arrows), are recovered in *labial* clonal loss-of-function experiments. Arrowheads indicate secondary axon tracts of Ect1*lab* and Ect2*lab*. Figures are superposition of multiple optical sections. **(C-F`)** Both of these ectopic lineages can be

identified by the projection patterns of their ectopic secondary axon tracts relative to the surrounding wildtype secondary axon tract scaffold. (C, E) Control showing corresponding wildtype axon tracts in two different optical sections. (D, D`) Ect1$^{lab}$ is present between the *labial* expressing lineages BAlp4 and BAlv and projects several axon bundles in anterior-medial direction. Single optical section. (F, F`) Ect2^{lab} is close to the *labial* expressing lineages TRdm and TRdl. Single optical section. Scale bars: 20 µm in A, B; 10 µm in C-F`.
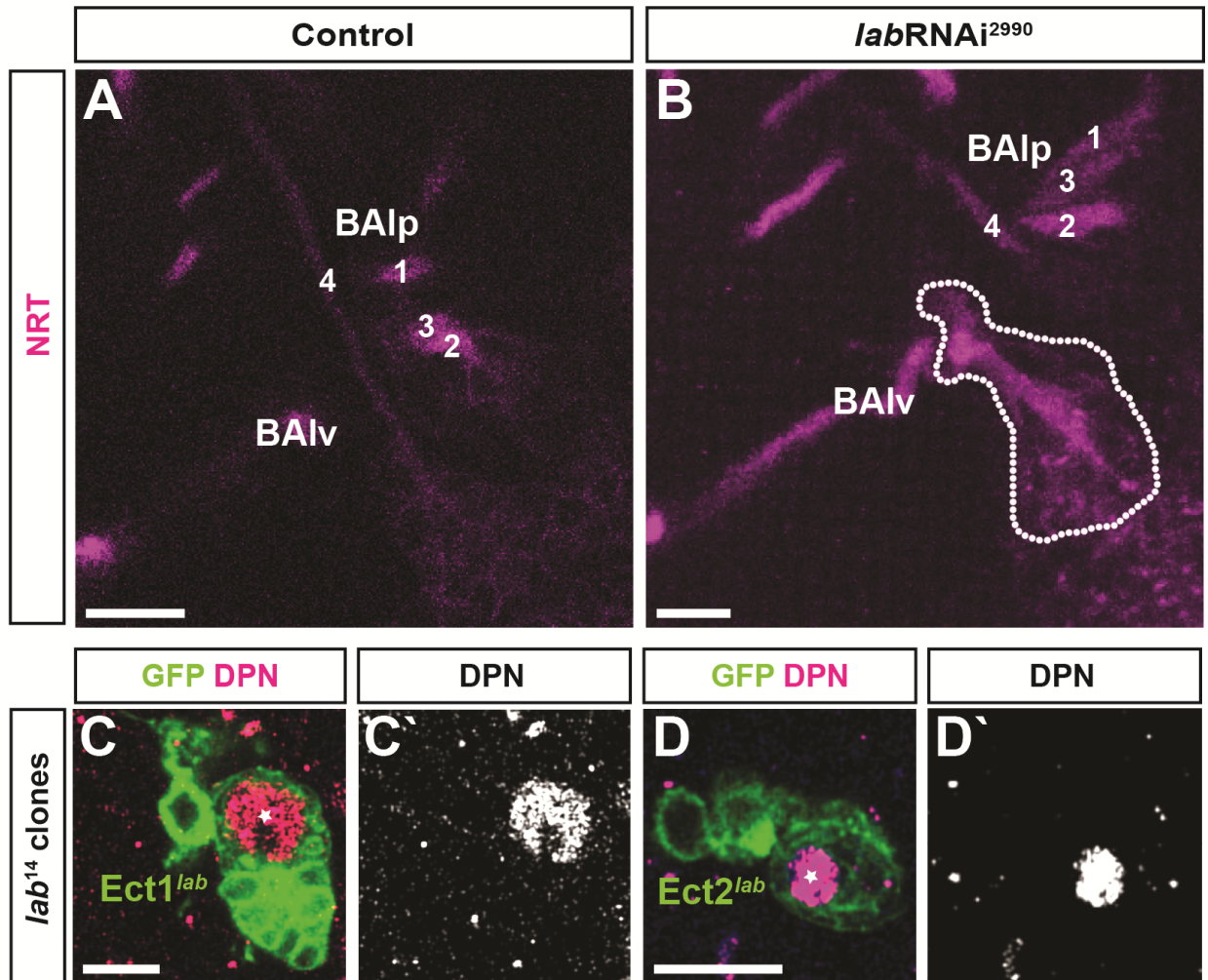
Fig. 2.5: Targeted RNAi knockdown of *labial* leads to ectopic neuroblast lineages comparable to those induced by *labial* loss-of-function mutation.

Late L3 brains. **(A)** Wildtype control showing the secondary axon tracts of the BAlp1-4 and BAlv lineages. Anti-NRT immunolabeling, Z-projection of optical sections. **(B)** UAS-*lab*RNAi^{2990} driven by MZ^{1407}-Gal4 to knockdown *labial* results in ectopic lineages. Dotted lines indicate position of Ect1^{lab} ectopic lineage relative to the secondary axon tracts of the BAlp1-4 and the BAlv lineages. Anti-NRT immunolabeling, Z-projections of optical sections. **(C, D)** Ectopic lineages contain a single Deadpan-positive neuroblast. GFP labeled *lab*^{14}

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MARCM mutant clones of Ect1^{lab} and Ect2^{lab} (green) immunostained with anti-Deadpan (magenta). Single optical sections. Stars indicate ectopic neuroblasts. Scale bars: 10 µm in A, B, C, D.

2.3.4 Additional *labial* expressing neuroblasts are present at early larval stages but are eliminated by programmed cell death at late larval stages

The cell-autonomous induction of MARCM-based mutant neuroblast clones is only possible in mitotically active progenitor cells (Lee and Luo, 1999, Lee and Luo, 2001). This implies that additional *labial*-expressing neuroblasts must be present and mitotically active during early larval brain development when the *lab*[14] mutant ectopic clones were induced. To investigate this, we determined the number of *labial*-expressing neuroblasts in the wildtype second larval instar stage (L2) by double immunolabeling with anti-Labial and anti-Deadpan. These experiments revealed the presence of six double-labeled cells indicating that six *labial*-expressing neuroblasts are present at the L2 stage (Fig. 2.6A-H``). These six neuroblasts were arranged in the L2 brain in a spatial pattern which is comparable to that of the BAlp4, BAlv, TRdm, TRdl, Ect1^{lab} and Ect2^{lab} neuroblasts in the *labial*-mutant late larval brain. Interestingly, an average of six *labial*-expressing neuroblasts were also present in the late embryonic brain implying that the number of *labial*-expressing neuroblasts does not change from the late embryonic stage to the second larval instar stage (Fig S2.2). Given that only four *labial*-expressing neuroblasts (the BAlp4, BAlv, TRdm, and TRdl neuroblasts) are present in the wild-type late L3 larval brain, these findings suggests that two of the six neuroblasts present in the L2 larval stage are missing in the L3 stage. In accordance with this assumption, double immunolabeling experiments with anti-Labial and anti-Deadpan at the early L3 stage (immediately after the L2/L3 molt) revealed only four *labial*-expressing neuroblasts, and these were arranged in a spatial pattern corresponding to the BAlp4,

BAlv, TRdm, TRdl neuroblasts characterized above in late (wandering) L3 larval stages (Fig. 2.6I-O``).
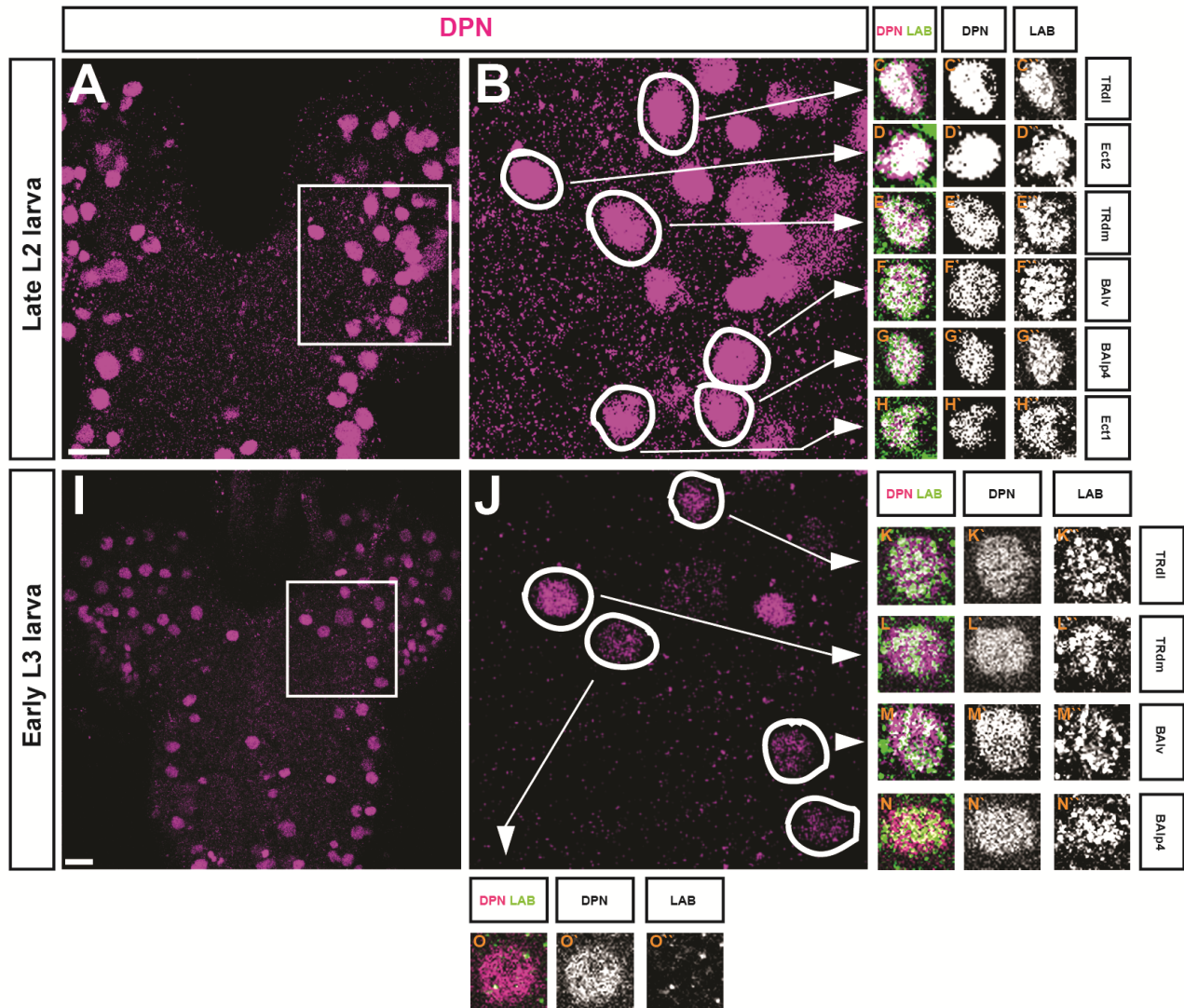
Fig. 2.6: Six *labial* expressing neuroblasts are present at the L2 larval stage.

(A) Overview of anti-Deadpan immunolabeled cells in the late L2 larval brain. Labeled cells are shown in a Z-projection of multiple optical sections. **(B)** Magnified view of the region in the box of A. Neuroblasts co-immunolabeled with anti-Deadpan and anti-Labial are indicated by circles. **(C-H``)** Single optical sections of each of the six anti-Deadpan immunolabeled neuroblasts that express *labial* at the late L2 stage. Magnification of the circled cells shown in B. Based on their relative position and appearance, each of these neuroblasts can be assigned to the BAlp4, BAlv, TRdm, TRdl, Ect1*lab*, and Ect2*lab* lineages. Deadpan immunolabeling is in

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magenta. Labial immunolabeling is in green. **(I)** Overview of anti-Deadpan immunolabeled cells in the early L3 larval brain. **(J)** Magnified view of the region in the box of I. Neuroblasts co-immunolabeled with anti-Deadpan and anti-Labial are indicated by circles. **(K-O``)** Single optical sections of each of the four anti-Deadpan-immunolabeled neuroblasts that express *labial* at the early L3 stage. Magnification of the circled cells shown in J. Based on their relative position, each of these neuroblasts can be assigned to the BAlp4, BAlv, TRdm, and TRdl lineages. Deadpan immunolabeling is in magenta. Labial immunolabeling is in green. Scale bars: 20 μm in A, I.
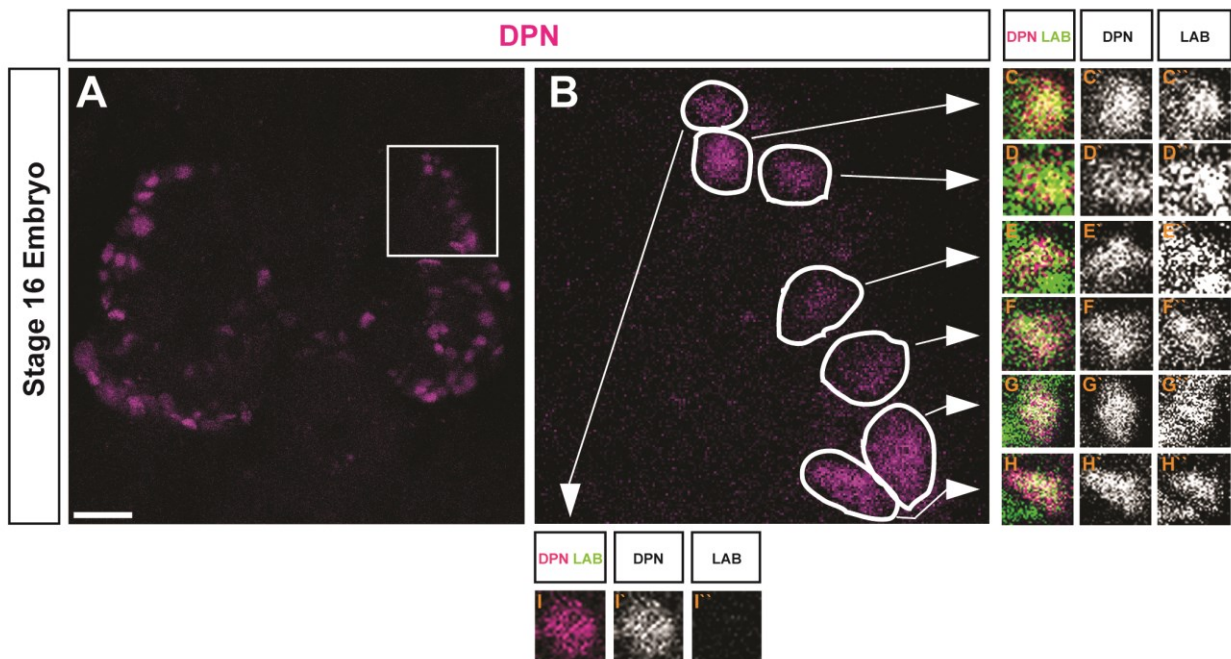
Fig. S2.2: Six *labial* expressing neuroblasts are present at embryonic stage 16.

(A) Overview of anti-Deadpan immunolabeled cells in the embryonic stage 16 brain. Z-Projection of optical sections **(B)** Magnified view of the region in the box of A. Neuroblasts co-immunolabeled with anti-Deadpan and anti-Labial are indicated by circles. **(C-I``)** Single optical sections of each of the six anti-Deadpan immunolabeled neuroblasts that express *labial* at embryonic stage 16. Scale bars: 20 μm.

What is the fate of the two *labial*-expressing neuroblasts that are present in L2 but are no longer observed in L3 wildtype larval brains? While it is conceivable that these two neuroblasts are still

present in L3 but have terminated their proliferative activity and at the same time ceased to express *labial*, a simpler explanation is that they are eliminated by programmed cell death at late larval stages. To investigate this possibility, we performed a MARCM clonal analysis of neuroblast lineages in the general region of the developing tritocerebral region using H99, a deficiency removing the proapoptotic genes *reaper*, *grim* and *head involution defective*, in an otherwise wildtype background (White et al., 1994). Homozygous H99 mutant clones were induced at 24 hours ALH and recovered in late L3 larval brains. In these experiments, a number of supernumerary ectopic lineages were observed in the corresponding region linking the central brain and the SOG. Among these, we consistently recovered two ectopic lineages that were comparable in terms of location and secondary axon tract projection pattern to the Ect1^{lab} and Ect2^{lab} lineages recovered in the clonal *lab*[14] mutant assays. We refer to these lineages as Ectopic1^{H99} (Ect1^{H99}) and Ectopic2^{H99} (Ect2^{H99}). Ect1^{H99} was located between the BAlp4 and the BAlv lineage, had several secondary axon tracts of which one always projected in a straight medial direction and manifested an average cell number of 80 cells (s.d.=11, n=9) of which an average of 70 (s.d.=12, n=9) were Labial-positive (Fig. 2.7A, C-D`). Ect2$^{H99}$ was located posterior-laterally to the TRdm and TRdl lineage, extended several secondary axon tracts that projected medially and had an average cell number of 62 cells (s.d.=8, n=12) of which an average of 31 were Labial-positive (Fig. 2.7B, E-F`). Importantly, both ectopic lineages, Ect1^{H99} and Ect2^{H99}, also consistently expressed *labial* in their neuroblast of origin (Fig. 2.7G-H`). Comparable results were obtained by targeted apoptosis block in experiments in which MZ^{1407}-Gal4 was used to drive UAS-p35^{BH2} in larval brain neuroblasts; ectopic *labial*-expressing neuroblast lineages that strongly resemble Ect1^{H99} and Ect2^{H99} in terms of location and secondary axon tract projection pattern were observed (data not shown).

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These findings indicate that two of the six *labial*-expressing neuroblasts present in early larval brain development are eliminated by programmed cell death in the late larval brain. Moreover, they indicate that blocking programmed cell death results in two (*labial*-expressing) ectopic neuroblast lineages which are comparable in neuroanatomical terms to the two ectopic neuroblast lineages recovered in *labial* loss-of-function mutant neuroblast clones. This in turn implies that *labial* is required cell autonomously in these two neuroblast lineages to terminate their proliferation through programmed cell death during late larval development.

2.3.5 Misexpression of *labial* can result in axonal misprojections but does not affect neuroblast survival

Previous studies of Hox gene action in ventral nerve cord development have shown that the Hox genes *Antp*, *Ubx* and *abd-A* are able to trigger programmed cell death in neuroblasts in which they are not normally expressed (Bello et. al., 2003). To determine if the Hox gene *labial* is also able to induce programmed cell death in central brain neuroblast lineages other than Ect1 and Ect2, we performed a clonal MARCM misexpression assay of *labial.* GFP-labeled *labial* mutant clones were induced at embryonic stage 12-15, recovered in late L3 larval brains, and were co-labeled with the neuroblast marker anti-Deadpan. No effect of *labial* misexpression on neuroblast survival was observed in the following lineages of the central brain: TRvl (n=11), BAlp2 (n=7), BAlp3 (n=4), BAmv1 (n=9), BAmv2 (n=3), BAmas1 (n=3), BAmas2 (n=2), PG5 (n=7). Similarly, no effect of *labial* misexpression on neuroblast survival was seen in the *labial* expressing lineages BAlp4 (n=9), BAlv (n=8), TRdl (n=8). Thus, the ability of *labial* to terminate neuroblast survival is likely to be restricted to the two neuroblast lineages Ect1 and Ect2.

In the TRvm and PG5 lineages, *labial* misexpression did result in aberrant secondary axon tract projection patterns. The TRvm lineage normally projects posteriorly and its secondary axon tract terminates close to where the TRco lineage SAT forms a commissure. In the *labial* misexpression assay, the secondary axon tract of the TRvm lineage projects posteriorly but then turns laterally to terminate close to the secondary axon tract of the BAlv lineage (Fig. S2.3A-B). The PG5 lineage is located medial to the BAla1-4 lineages and its secondary axon tract normally projects medially to terminate in between the bifurcating secondary axon tract of the TRdl lineage. (The PG5 lineage has not been included in previous mapping studies, see Pereanu and Hartenstein, 2006; Spindler and Hartenstein, 2010). In the *labial* misexpression assay, the PG5 lineage secondary axon tract projects medially, but then turns posteriorly to terminate close to the secondary axon tract of the BAlv lineage (Fig. S2.3C-D). Thus, while *labial* misexpression does not appear to affect neuroblast survival, it can result in aberrant secondary axon tract projection patterns in central brain lineages during postembryonic development.
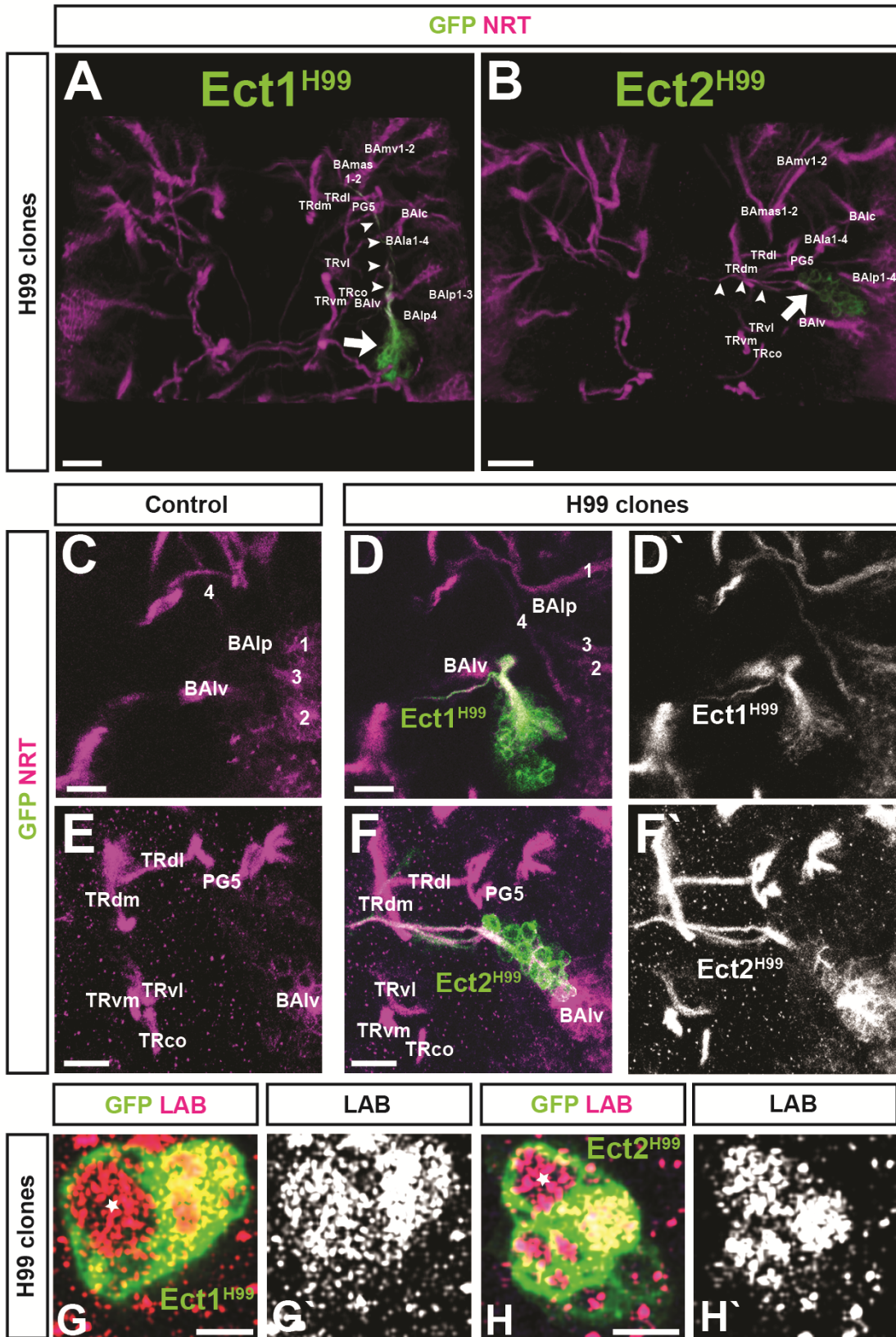
Fig. 2.7: Blocking of cell death leads to *labial* expressing ectopic neuroblast lineages.

GFP-labeled H99 MARCM mutant clones are green; secondary axon tracts labeled by anti-Neurotactin are in magenta. **(A, B)** Two identified ectopic neuroblast clones, Ect1^{H99} and Ect2^{H99} (arrows) are recovered after cell death block; both are similar in terms of position and secondary axon projection pattern (arrowheads) to Ect1^{lab} and Ect2^{lab} found in the *labial* mutant assay. Superposition of multiple optical sections. **(C, E)** Control showing corresponding wildtype axon tracts in two different optical sections. **(D-D`, F-F`)** Following clonal cell death block, both ectopic lineages can be identified by the projection patterns of their ectopic secondary axon tracts relative to the surrounding wildtype secondary axon tract scaffold. Single optical sections. (G, H) The ectopic Ect1^{H99} and Ect2^{H99} lineages express *labial* in the neuroblast (star). Labial immunolabeling is in magenta. Scale bars: 20 µm in A, B; 10 µm in C, D, E, F; 5 µm in G, H.
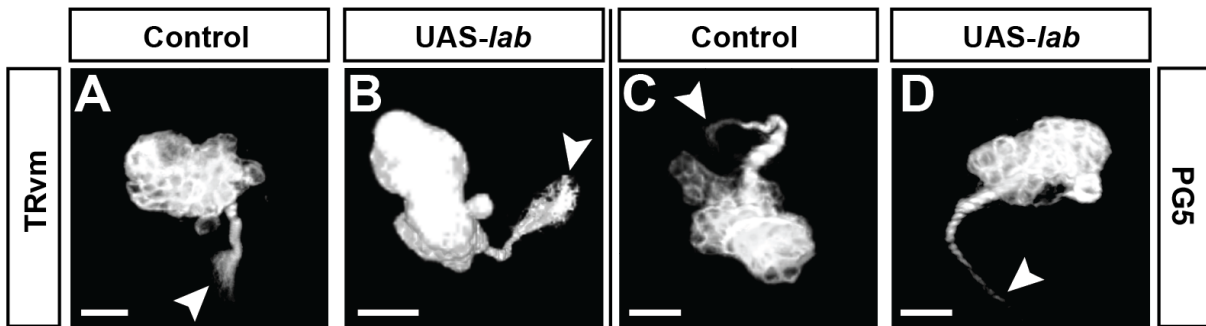
Fig. S2.3: Misexpression of *labial* can induce axonal projection defects.

Superposition of multiple optical sections of GFP-labeled clones in late L3 brains. **(A)** Wildtype clone of the TRvm lineage; its secondary axon tract projects posteriorly and terminates close to the midline (arrowhead). **(B)** Clonal misexpression of *labial* in the TRvm lineage; compared to the wild-type, the secondary axon tract terminates more laterally (arrowhead). **(C)** Wildtype clone of the PG5 lineage; its secondary axon tract projects and terminates medially (arrowhead). **(D)** Clonal misexpression of *labial* in the PG5 lineage; compared to the wildtype, the secondary axon tract projects medially but then turns to terminate more posteriorly (arrowhead). Scale bars: 10 µm.

2.4 DISCUSSION

Our findings on the role of *labial* in postembryonic brain development are in accordance with a model in which *labial* is cell autonomously required for the stage-specific programmed cell death of two of the six postembryonic neuroblasts that express *labial* during larval stages (Fig. 2.8).
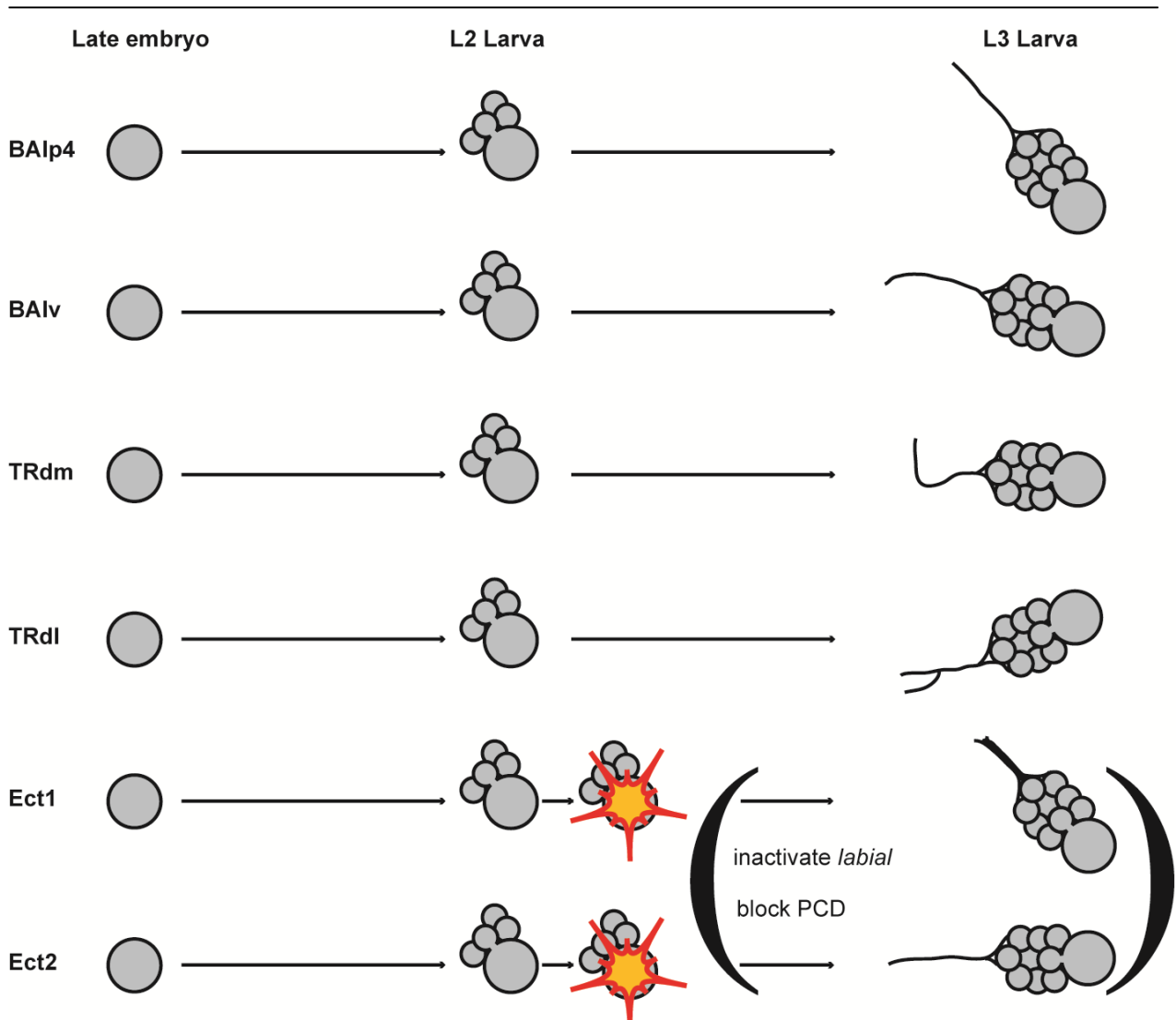
Fig. 2.8: Model for *labial*-dependent termination of specific postembryonic neuroblasts.

In this model, *labial* is cell autonomously required for the stage-specific programmed cell death of two of the six postembryonic neuroblasts that express *labial* during larval stages. The neuroblasts of the BAlp4, BAlv, TRdm and TRdl lineages express *labial* and persist to late L3 larval stages. The neuroblasts of the Ect1 and

Ect2 lineage express *labial* during early larval stages, and *labial* is required for their termination through apoptosis at the L2 to L3 transition. If *labial* is inactivated or cell death is blocked, these two neuroblasts give rise to ectopic lineages which persist to late L3 larval stages.

This model is supported by expression studies which indicate that six *labial*-expressing neuroblasts are present in the developing brain from the late embryonic stage until the end of the second larval stage (L2), while only four *labial*-expressing neuroblasts continue to be present during the third larval stage (L3). Moreover, the model is supported by functional studies which indicate that this reduction in *labial*-expressing neuroblast number is due to Labial-dependent programmed cell death of two neuroblasts during postembryonic brain development, since clonal *labial* loss-of-function leads to the recovery of two ectopic neuroblast lineages and these two ectopic neuroblast lineages are also recovered (and express *labial*) following clonal cell death block.

Given that six Labial-positive neuroblasts are present at late L2 stages, and only four Labial-positive neuroblasts are present at early L3 stages, we posit that the *labial*-dependent apoptosis of the two affected neuroblasts (Ect1, Ect2) is associated with the L2/L3 transition. Since that the L2/L3 transition involves molting that is associated with elevated levels of steroid hormones such as ecdysone, it is possible that ecdysis-triggering endocrine signals participate in the *labial*-dependent apoptotic event in Ect1 and Ect2 (reviewed in Truman, 2005). Alternatively, transiently expressed temporal transcription factors might regulate the competence of the affected neuroblasts to undergo apoptosis in a *labial*-dependent manner (Maurange et al., 2008; Chell and Brand, 2008). While the molecular nature of these signals is currently not known, they are

apparently not sufficient to elicit programmed cell death in all *labial*-expressing neuroblasts, since the *labial*-expressing BAlp4, BAlv, TRdm, and TRdl neuroblasts are not affected.

The neuroblast-specific requirement of the Hox gene *labial* in programmed cell death during postembryonic brain development reported here is novel and differs in several respects from the type of Hox-gene dependent programmed cell death that occurs in the abdominal ganglia during postembryonic development of the ventral nerve cord (Bello et al., 2003). In the larval abdominal ganglia, the Hox gene *abd-A* is expressed in a short pulse during the mid-L3 stage and results in the cell autonomous programmed cell death of all neuroblasts that express the *abd-A* pulse. In contrast, in the larval brain, the Hox gene *labial* is expressed continuously throughout early larval development in six larval neuroblasts and this only results in the cell autonomous programmed cell death in two of these neuroblasts around the L2/L3 transition. Moreover, in contrast to the general apoptotic effect of clonal misexpression of *abd-A* (as well as *Antp* or *Ubx*) in larval neuroblasts of the ventral nerve cord as reported by Bello and coworkers (2003), our studies indicate that the clonal misexpression of *labial* in larval neuroblasts of the brain does not result in apoptosis. Misexpression of *labial* does, however, result in axonal projections defects in central brain lineages. Interestingly, genetic misexpression of vertebrate Hox genes, including misexpression of the *labial* ortholog *Hoxb1*, has been shown to result in axonal projection defects of developing motoneurons (reviewed in Butler and Tear, 2007; Guthrie, 2007).

The role of *labial* in terminating proliferation in specific brain neuroblasts during postembryonic development is strikingly different from the function of this Hox gene during embryonic brain development in *Drosophila* (Hirth et al., 1998; Reichert and Bello, 2010). During embryogenesis, *labial* is expressed in all tritocerebral neuroblasts and their neural progeny, and functional

inactivation of *labial* does not terminate neuroblast proliferation since postmitotic cells are generated in the mutant domain. However, the generated cells do not express neuronal markers and do not manifest neuronal morphology implying that *labial* is required to establish regional neuronal identity in the embryonic tritocerebrum. Interestingly, there is a marked decrease in the number of neuroblasts that express *labial* during embryonic development. At embryonic stage 11, *labial* is expressed in fifteen neuroblasts of which thirteen are of tritocerebral and two of deutocerebral origin (Urbach and Technau, 2003). In contrast, at the end of embryogenesis only six *labial*-expressing neuroblasts were present in the developing brain. The fate of the remaining nine embryonic neuroblasts is not known. They may simply cease to express *labial* and remain present or they may terminate proliferation via cell cycle exit or apoptosis as it has been reported for neuroblasts in the embryonic ventral nerve cord (Abrams et al., 1993; White et al., 1994; Peterson et al., 2002)

The appearance of ectopic neuroblast lineages in the absence of *labial* during postembryonic brain development is remarkable in several respects. First, the ectopic lineages are identifiable. Only two, morphologically distinct and unique ectopic neuroblast lineages are recovered, and each for each of these, Ect1 and Ect2, neuroanatomical features such as cell number and secondary axon tract projection are reproducibly constant at the end of larval development. Other types of ectopic neuroblast lineages or lineages with variable morphologies were not observed. Second, the ectopic lineages are novel and do not represent "homeotic" transformations into any other wildtype lineages. Notably they form secondary axon tract projections that differ significantly from any other secondary axon tract projection patterns in the larval brain. Nevertheless the ectopic lineages did not distort the other surrounding neuroblast lineages; their ectopic secondary axon tracts integrated into the ensemble of secondary axon tracts of late larval

brains in an orderly manner. Third, the existence of ectopic neural lineages in the *labial*-mutant fly brain bears striking similarities to the ectopic neural assembly formation observed in a study of *Hoxa1* mutant mice (Del Toro et al., 2001). In contrast to previous analyses of mouse *Hoxa1* mutants focused on early effects on segmentation and patterning in the developing hindbrain (reviewed in Favier and Dollé, 1997; Lumsden and Krumlauf, 1996), this study shows that during later embryonic development, ectopic groups of neurons in the hindbrain of *Hoxa1* mutants derive from ectopic mutant progenitors and establish a supernumerary neuronal assembly that escapes apoptosis and even becomes functional postnatally. Thus, the *labial/Hoxa1* gene orthologs in fly and mouse appear to have remarkably similar dual roles in brain development. During early phases of brain development the *labial/Hoxa1* genes act in establishing the regional identity of neurons in specific brain neuromeres; during later phases they prevent the formation of ectopic neuronal arrays in these brain neuromeres by terminating progenitor proliferation, thus, effectively sculpting the developing brain.

The observation that brain development in flies and mammals involves not just one but two different functional roles of *labial/Hoxa1* genes, both of which appear to be evolutionarily conserved, provides additional support for the notion that comparable and conserved mechanisms operate in brain development of invertebrates and vertebrates (Reichert and Simeone, 1999; Lichtneckert and Reichert, 2005; Lichtneckert and Reichert, 2008). If this is indeed the case then a common and general strategy for generating novel functional features in brain development in bilaterian animals might be based on local changes in the regulation of *labial/Hoxa1* (and perhaps other Hox genes), which could result in the evolution of novel neuronal subsets without affecting the function of the neural circuitry already present (Brunet and Ghysen, 1999).

3. NEUROBLAST LINEAGE IDENTIFICATION AND LINEAGE-SPECIFIC HOX GENE ACTION DURING POSTEMBRYONIC DEVELOPMENT OF THE SUBESOPHAGEAL GANGLION IN THE *DROSOPHILA* CENTRAL BRAIN

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Submitted

3.1 SUMMARY

The central brain of *Drosophila* consists of the supraesophageal ganglion (SPG) and the subesophageal ganglion (SEG), both of which are generated by neural stem cell-like neuroblasts during embryonic and postembryonic development. Considerable information has been obtained on postembryonic development of the neuroblasts and their lineages in the SPG. In contrast, very little is known about neuroblasts, neural lineages, or any other aspect of the postembryonic development in the SEG. Here we characterize the neuroanatomy of the larval SEG in terms of tracts, commissures, and other landmark features as compared to a thoracic ganglion. We then use clonal MARCM labeling to identify all adult-specific neuroblast lineages in the late larval SEG and find a surprisingly small number of neuroblast lineages, 13 paired and one unpaired. The Hox genes *Dfd*, *Scr,* and *Antp* are expressed in a lineage-specific manner in these lineages during postembryonic development. Hox gene loss-of-function causes lineage-specific defects in axonal targeting, reduction in neural cell numbers as well as formation of novel ectopic neuroblast lineages. Apoptosis block also results in ectopic lineages suggesting that Hox genes are required for lineage-specific termination of proliferation through programmed cell death. Taken together, our findings show that postembryonic development in the SEG is mediated by a surprisingly small set of identified lineages and involves multiple Hox-dependent mechanisms which act in a lineage-specific manner to ensure the correct formation of adult-specific neurons in the *Drosophila* brain.

3.2 INTRODUCTION

Neurons of the *Drosophila* central brain and ventral ganglia form lineages that are generated by neural stem cell-like neuroblasts. Neuroblasts segregate from the neuroectoderm of the early embryo (Technau et al., 2006; Urbach and Technau, 2004) and undergo a series of asymmetric divisions, generating the primary neurons that differentiate into the functional CNS of the larva. After a period of quiescence, most neuroblasts resume proliferation to generate the secondary neurons which form the bulk of neurons of the adult CNS. Adult-specific secondary neurons remain immature throughout the larval phase. During metamorphosis, secondary neurons mature and together with reconfigured primary neurons, form the circuits of the adult brain (see Egger et al., 2008; Hartenstein et al., 2008).

Neuroblasts and their lineages have been described in previous work for the ventral ganglia and for the supraesophageal ganglion of the brain. Each ganglion of the ventral nerve cord derives from a set of approximately 30 neuroblast pairs which can be identified based on position, marker expression, and the neuroanatomy of their progeny (Bossing et al., 1996; Doe, 1992; Schmid et al., 1999; Schmidt et al., 1997; Technau et al., 2006). The supraesophageal ganglion (SPG), comprising three modified neuromeres (protocerebrum, deutocerebrum, tritocerebrum), derives from approximately 100 identified neuroblast pairs whose spatial organization and molecular marker expression have also been documented in detail (Urbach and Technau, 2003; Younossi-Hartenstein et al., 1996). Adult-specific secondary lineages of the CNS have been mapped for the ventral ganglia (Truman et al., 2004) and the SPG (Cardona et al., 2009; Ito et al., 2013; Lovick et al., 2013; Pereanu and Hartenstein, 2006; Wong et al, 2013; Yu et al, 2013).

Compared to the wealth of information available for the development of the SPG and ventral ganglia, much less is known about the development of the subesophageal ganglion (SEG), the part of the central brain associated with the mouthparts and responsible for the processing of gustatory information. The SEG derives from three anterior neuromeres of the ventral nerve cord which in all contain approximately 80 pairs of neuroblasts (R. Urbach, personal communication). Similar to neuroblasts of the SPG and ventral ganglia, these cells produce primary and secondary lineages, but to date no information exists regarding location and projection pattern of the SEG neuroblast lineages.

Here we focus on the neuroblasts that generate the adult-specific secondary neurons of the SEG during postembryonic development. We first characterize the general neuroanatomical features of the larval SEG compared to those of a thoracic ganglion. Based on this neuroanatomical analysis, we investigate the adult-specific neuroblast lineages in the SEG and find that a surprisingly small number of lineages, 13 paired and one unpaired lineages, are present at the late larval stage. We use clonal MARCM labeling to identify and characterize each of these lineages individually. We then show that the Hox genes *Dfd*, *Scr,* and *Antp* are expressed in the postembryonic SEG and demonstrate that most of the SEG lineages express one of these Hox genes. Subsequently, we show that Hox gene inactivation causes axonal targeting defects in two lineages, neural cell number reduction in another two lineages, and formation of five ectopic neuroblast lineage types not present in the wildtype brain. Clonal inactivation of apoptosis results in comparable ectopic lineages implying that Hox genes are required for lineage-specific proliferation termination through programmed cell death. Taken together, this report shows that postembryonic development in the SEG is mediated by a small set of neuroblast lineages and involves multiple

Hox-dependent mechanisms which act in a lineage-specific manner to ensure the correct formation of adult-specific neurons.

3.3 RESULTS

The larval SEG is composed of three neuromeres (mandibular, maxillary, and labial ganglia) each of which represents a reduced/modified version of the thoracic neuromeres described in detail in previous works (Landgraf et al., 2003; Nassif et al., 2003; Pflüger et al., 1988; Power, 1948; Truman et al., 2004; Tyrer and Gregory, 1982). To elucidate the architecture and neuroblast lineage composition of the SEG neuromeres, it is therefore helpful to first establish the relationship between landmark structures and neuroblast lineages for a "prototypical" thoracic neuromere of the larval VNC.

3.3.1 Neuroanatomical landmark structures and neuroblast lineages in thoracic neuromeres of the larval CNS

In each thoracic neuromere, connectives and commissures form a scaffold of landmark structures to which individual neuroblast lineages have been related (Truman et al., 2004). The central part of each larval thoracic neuromere is occupied by the anterior and posterior intermediate commissure (aI, pI; Fig. 3.1B, E, F). A set of three connectives, called dorso-medial tract (DMT), dorso-intermediate tract (DIT), and dorso-lateral tract (DLT), extend dorsally of the commissures; another three connectives, the ventro-medial tract (VMT), ventro-intermediate tract (VIT), and ventro-lateral tract (VLT), flank the commissures on their ventral side (Fig. 3.1A, B, E). In addition, a pair of thinner commissural bundles crosses the midline dorsally (aD, pD; Fig. 3.1E, F) and one commissure crosses ventrally (aV; Fig. 3.1A, E). Secondary neuroblast lineages of the larval thoracic ganglia are referred to by number from 0-24 (Brierley et al., 2012; Truman
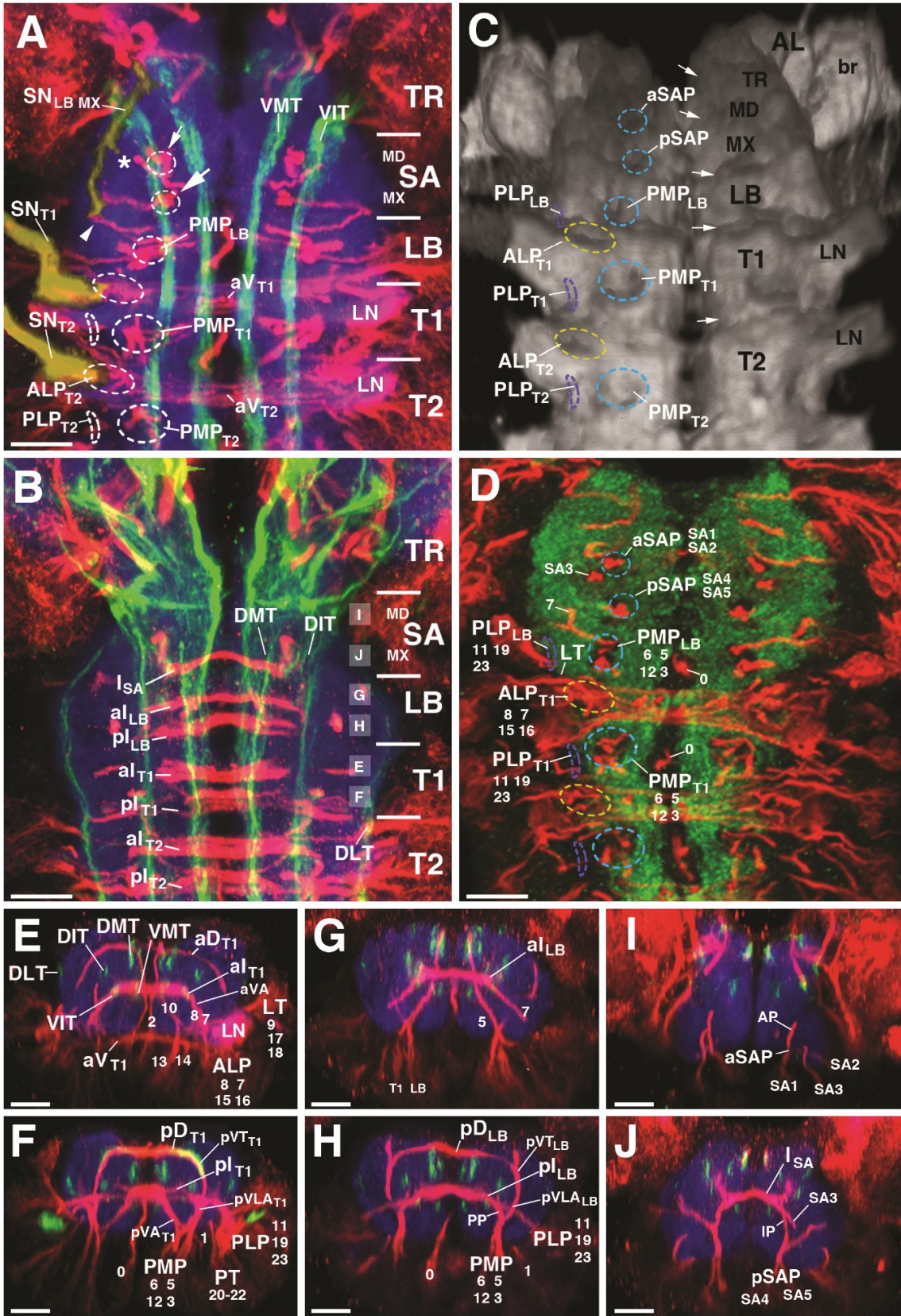
50

Fig. 3.1: Neuroanatomical landmarks of the larval ventral nerve cord.

(A, B) Z-projections of contiguous confocal sections of 3rd instar ventral nerve cord (vnc) labeled with BP106 Neurotactin (red), FasII (green), and DNcad (blue). The plane of sectioning is parallel to the length axis of the ventral nerve cord. Both z-projections represent slices of approximately 20μm thickness. (A) shows the ventral neuropile (40-60μm from the ventral surface of the vnc), (B) the central neuropile (60-80μm from the ventral surface). Only the anterior part of the ventral nerve cord and adjoining brain, including the neuromeres of the tritocerebrum (TR), subesophageal ganglion [SA anterior subesophageal ganglion, formed by two contracted neuromeres (MX maxilla, MD mandible); LB labium) and the first two thoracic neuromeres (T1, T2) are shown. Anterior is to the top. Illustrated in (A) are the ventral longitudinal connectives (FasII-positive; green; VMT ventro-medial tract; VIT ventro-intermediate tract), the ventral commissures of the thoracic neuromeres (BP106-positive; red; aV_{T1}, aV_{T2}), the primordia of the leg neuropils (LN) of the thoracic neuromeres, and the entry portals of secondary lineages (hatched lines; ALP antero-lateral portal; PLP postero-lateral portal; PMP postero-medial portal). Approximate neuromere boundaries are indicated by horizontal lines. Segmental nerves (FasII-positive) are rendered yellow ($SN_{LB \ MX}$ fused segmental nerve of the subesophageal ganglion=labio-maxillary nerve; SN_{T1} segmental nerve of T1 neuromere; SN_{T2} segmental nerve of T2 neuromere). Asterisk marks anterior (maxillary) root of the labio-maxillary nerve, arrowhead points out posterior (labial) root of the same nerve. Shown in (B) are the set of dorsal connectives (FasII-positive; green; DMT dorso-medial tract; DIT dorso-intermediate tract; DLT dorso-lateral tract), and the intermediate commissures (BP106-positive; red). Thoracic neuromeres each have a pair of intermediate commissures (aI_{T1} anterior intermediate commissure of T1; pI_{T1} posterior intermediate commissure of T1; aI_{T2} anterior intermediate commissure of T2; pI_{T2} posterior intermediate commissure of T2). Two commissures are also present in the labial neuromere (aI_{LB}, pI_{LB}). The anterior part of the subesophageal ganglion (SA) has but a single BP106-positive commissure (I_{SA}). **(C)** Volume-rendering of a series of confocal sections of larval ventral nerve cord labeled with DNcad, showing surface topography of neuropil. Small arrows point at transverse fissures in between neuromeres (LB labial neuromere; MD mandibular neuromere; MX maxillary neuromere; T1/T2 thoracic neuromeres 1/2; TR tritocerebrum). Entry portals of secondary lineages are marked by depressions in the ventral neuropil surface [blue hatched lines: postero-medial portals (PMP) and anterior/posterior portal of the anterior subesophageal ganglion (aSAP/pSAP); purple hatched lines: postero-lateral portals (PLP); yellow hatched lines: antero-lateral portals (ALP)]. **(D)** Z-projection of confocal sections of ventral nerve cord labeled with BP106 (red) and Nc82 (green), corresponding to a slice 40-50mm from the ventral surface. Hatched lines indicate entry portals of secondary lineages; BP106-positive secondary lineage tracts (red) enter into a Nc82-negative "cavity" which constitutes the portal. Individual lineages are indicated by numbers underneath or next to their corresponding entry portal. **(E-J)** Z-projections of digitally rotated confocal sections of ventral nerve cord labeled with BP106 (red), FasII (green) and DNcad (blue). Panels show slices of the nerve cord of approximately 20μm thickness.

Dorsal is to the top. Antero-posterior levels of slices is indicated by boxed capital letters ("E-J") to the right of panel B. Panels E and F correspond to an anterior and posterior level of the first thoracic segment. Note BP106-positive anterior commissures in E (aV_{T1} antero-ventral commissure; aI_{T1} antero-intermediate commissure; aD_{T1} antero-dorsal commissure) and posterior commissures in F (pI_{T1} postero-intermediate commissure; pD_{T1} postero-dorsal commissure). Note also the antero-lateral entry portal (ALP) in panel (E), and the postero-lateral and postero-medial entry portal (PLP, PMP) in (F). The FasII-positive longitudinal tracts flank the intermediate commissures dorsally (DMT, DIT, DLT) and ventrally (VMT, VIT). Secondary lineages, some of them associated with entry portals (PMP, PLP, ALP) are indicated by numbers. Panels (G) and (H) show anterior and posterior level of the labial neuromere; the arrangement of commissures, connectives and secondary lineage trajectories is similar to that in the thoracic neuromeres, excepting ventral structures that are reduced or missing in the labial neuromere; note absence of ventral commissure, leg neuropile, and secondary lineages 9, 13-14, 17-18, 20-22 (among others) associated with ventral structures. Panels (I) and (J) represent slices of the anterior subesophageal ganglion (SA) which consists of two reduced neuromeres. The anterior slice (I) illustrates the level of the anterior entry portal of the SA (aSAP) which contains the tracts of lineages SA1 and SA2; the posterior slice (J) lies at the level of the posterior entry portal of the SA (pSAP) that admits lineages SA4 and SA5. The tract of lineage SA3 forms an intermediate commissure (I_{SA}), representing the anterior-most of the three BP106-positive commissures of the SEG (see panel B). Other abbreviations: AP anterior pillar; IP intermediate pillar; LT lateral triplet (lineages 9, 17, 18); PP posterior pillar; PT posterior triplet (lineages 20-22); pVA_{T1} posterior ventral arch of T1; $pVLA_{T1}$ posterior ventro-lateral arch of T1. (A-D) ventral view; (E-J) posterior view. Scale bars: 30 μm.

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et al., 2004). The secondary axon tracts (SATs) of these lineages enter the neuropile in bundles of 3-4 at characteristic positions. As shown in Fig. 3.1C, D, the points of entry of these bundles form conspicuous, metamerically reiterated indentations ("portals") at the neuropile surface. Four lineages, 5, 6, 3, and 12, enter vertically through the posterior-medial portal (PMP; Fig. 3.1D, F). The SATs of lineages 5, 6, 12 turn medially and cross the pI commissure while a second SAT branch of lineages 6 and 12, as well as the lineage 3 SAT, turn laterally towards the dorsal neuropile, forming the posterior vertical tract (pVT; Fig. 3.1F). A dorsal branch of lineage 6 constitutes the posterior dorsal commissure (pD; Fig. 3.1F). The SATs of three lineages (11, 19,

23) enter the neuropile through the posterior lateral portal (PLP) and extend medially towards (11) or across (19, 23) the posterior intermediate commissure (Fig. 3.1D, F).

In the anterior half of each thoracic neuromere four lineages (7, 8, 15, 16) form a group of SATs that enter the neuropile through the anterior lateral portal (ALP; Fig. 3.1A, C, D, E). SATs of lineages 7 and 8 project medially and cross in the anterior intermediate commissure (aI; Fig. 3.1E). SATs of lineages 15 and 16 branch in the leg neuropile. Lineages 9, 17, and 18 form a "lateral triplet" (Truman et al., 2004), entering the anterior-lateral cortex and projecting medially in the anterior-ventral (aV) or anterior intermediate (aI) commissure (Fig. 3.1D, E). A posterior triplet of lineages (20-22) project superficially into the posterior surface of the ventrolateral neuropile, (Fig. 3.1F).

SATs of seven further lineages (0, 1, 2, 4, 10, 13, 14) enter the neuropile as individual tracts. The SAT of lineage 1 contributes to the anterior intermediate commissure (aI) of the posteriorly adjacent neuromere (Fig. 3.1F, H). SATs of lineages, 4, 13 and 14 contribute to the ventral commissure (aV); the SAT of lineage 10 contributes to the anterior intermediate commissure (aI), and the SAT of lineage 2 projects ipsilaterally along the anterior dorsal (aD) commissure (Fig. 3.1E). Lineage 0 is the only unpaired lineage; its tract extends vertically into the posterior intermediate (pI) commissure (Fig. 3.1D-F).

3.3.2 Neuroanatomical landmark structures and secondary axon tracts in the neuromeres of the larval SEG

The metameric architecture described above for the thoracic neuromeres is also apparent in the SEG, albeit in a substantially modified form (Fig. 3.1A, B). The SEG can be divided into the labium and the subesophageal anterior (SA) domain, which comprises the mandibular and maxillary neuromeres. As in the thoracic neuromeres, the paired connectives flank a set of intermediate commissures. A fully formed pI commissure (pI$_{LB}$; Fig. 3.1B) is formed in the posterior SEG, which is occupied by the labium. This neuromere comprises nine SATs which correspond to SATs in the thoracic neuromeres and are therefore numbered accordingly (Truman et al., 2004).

Two groups of paired SATs enter the posterior SEG through a postero-medial portal (PMP$_{LB}$; 3, 5, 6, 12) and postero-lateral portal (PLP$_{LB}$; 11, 19, 23), and an unpaired SAT (0) projects vertically in the midline (Fig. 3.1D, H). In front of the pI$_{LB}$ commissure, two additional commissures, aI$_{LB}$ and I$_{SA}$, are present (Fig. 3.1B). One SAT (7) forms the aI$_{LB}$ commissure (Fig. 3.1G). This characterization of the SEG indicates that a remarkably small number of individually identifiable SATs are found in the SEG neuropile. Thus, 13 different bilaterally symmetrical SAT pairs and one unpaired midline SAT are manifest in the neuromeres of the larval SEG.

To confirm that each of the SATs in the larval SEG derives from one neuroblast lineage, we performed a MARCM-based clonal analysis (Lee and Luo, 1999; Lee and Luo, 2001). Randomly labeled clones with a ubiquitous *tubulin*-Gal4 driving UAS-mCD8::GFP were induced in early larval stages and recovered in the late larval brain (100 specimens). We identified a total of 14 different neuroblast clone types in the postembryonic SEG (summarized in Fig. 3.4, Fig. 3.6O, and Table S3.3). Based on cell body position and SAT trajectory, each of these 14 different MARCM neuroblast clones corresponds unambiguously to one of the 14 SAT types that were

found to comprise the larval SEG. A total of 9 identified SEG lineages in the labial neuromere can homologized with thoracic lineages based on neuropile entry point or projection pattern within the neuropile. These are shown in horizontal and vertical projections in figure 3.2 and S3.1. Five further identified lineages located anterior to the labial neuromere cannot be homologized with thoracic lineages based on neuroanatomy; we refer to these lineages as SA1-5. They are shown in horizontal and vertical projections in Fig. 3.3. A characterization of each of these 14 neuroblast lineages is presented below. Beside these lineages, small MARCM clones of 1-5 cells without a neuroblast were also recovered in the late larval SEG. These cells may represent small clones of postembryonic neurons that have lost their neuroblast due to precocious termination of proliferation in early larval stages.

3.3.3 Complete MARCM-based identification of neuroblast lineages in the late larval SEG

Labial lineage 7 (LB7): Thoracic lineages 7 and 8 enter the neuropile together, project medially and cross in the anterior intermediate commissure. Lineage 8 also has an ipsilateral branch to the leg neuropile (Truman et al., 2004). In the SEG, one lineage resembles the 7/8 pair, projecting medially and forming a thin commissure (aI) anterior to the labial pI commissure (Fig. 3.2C). Since it has no additional ipsilateral branch we refer to it as labial lineage 7.

Labial lineage 5 (LB5): The single SAT of thoracic lineage 5 enters the neuropile lateral of 6 and projects medially following the 6c branch towards the pI commissure. Labial lineage 5 starts out at the same position as its thoracic counterparts, but does not cross the midline. Instead it projects

dorsally between the labial pI and aI commissures, turns anteriorly, and extends along the dorsal surface of the SEG towards the SPG (Fig. 3.2D).

Labial lineage 3 (LB3): In thoracic segments, the lineage 3 SAT splits into two branches. One targets the leg neuropile, the second initially follows the tracts of lineages 6 and 12 dorsally in the posterior vertical tract (pVT). The labial lineage 3 does not follow 6 or 12; its thick SAT projects straight upward and, at the intersection of the posterior intermediate (pI$_{LB}$) commissure and dorsal intermediate tract (DIT), branches into a short medial component along the pI commissure and a longer anterior component extending along the DIT (Fig. 3.2E).

Labial lineage 6 (LB6): In the thoracic neuromeres, lineage 6 bifurcates and sends one branch (6cm) through the pI commissure and another branch (6cd) towards the dorsal neuropile. The labial lineage 6 SAT is less complex. Lacking the medial 6cm branch, it continues vertically alongside lineage 12, then bends medially to form the pD commissure (Fig. 3.2F).

Labial lineage 12 (LB12): The SAT of labial lineage 12 in thoracic segments has an SAT which bifurcates into a medial branch (12c) and a lateral branch (12i) that splits into a longer dorsal branch (12id) following the posterior vertical tract and a shorter branch (12im) terminating in the neuropile flanking the DIT tract. Out of these three SAT branches, only one, 12id, is left in the labial lineage 12. It projects into the vertical tract and terminates in the dorsal neuropile (Dnp; Fig. 3.2G).

Labial lineage 0 (LB0): The cell body cluster of lineage 0 is located in the ventral midline (Fig. 3.1D). The unpaired SAT projects vertically and bifurcates at the level of the intermediate commissures. In the prothoracic neuromere, lineage 0 targets the posterior intermediate

commissure; the labial lineage 0 projects further anteriorly to the anterior commissure (Fig. 3.2H).

Labial lineages 11, 19, 23 (LB11, LB19, LB23): The SATs of thoracic lineages 19 and 23 project medially in the neuropile and cross in the pI commissure. The SATs of labial lineages 19 and 23 are identical to their thoracic counterparts, crossing in the pI (Fig. 3.2I, J). Labial lineage 11 is located near the cell bodies of 19 and 23, but is rudimentary and has no obvious SAT pattern (Fig. S3.1).

SA lineages 1-5: Lineages SA2 and SA4 enter the anterior SEG neuropile together and end near to each other in the dorsal tract neuropile (Fig. 3.3D, F). Lineage SA1 lies laterally of SA2, projects medially, and terminates ventrally in the anterior SEG (Fig. 3.3C). SA3 enters the neuropile close to SA1 and SA2, but projects posteriorly and medially, and crosses the midline in the anterior-most commissure (I_{SA}; Fig. 3.3E). Finally, SA5 enters alongside SA4, then turns laterally to terminate in the dorsolateral neuropile (Fig. 3.3G).

Taken together, these MARCM experiments identify a small set of 14 neuroblast lineages (13 paired and 1 unpaired) that comprise adult-specific neurons in the SEG. The complete set of these identified neuroblast lineages as well as their spatial relationship to adjacent lineages in the prothoracic and tritocerebral neuromeres is shown in figure 3.4.
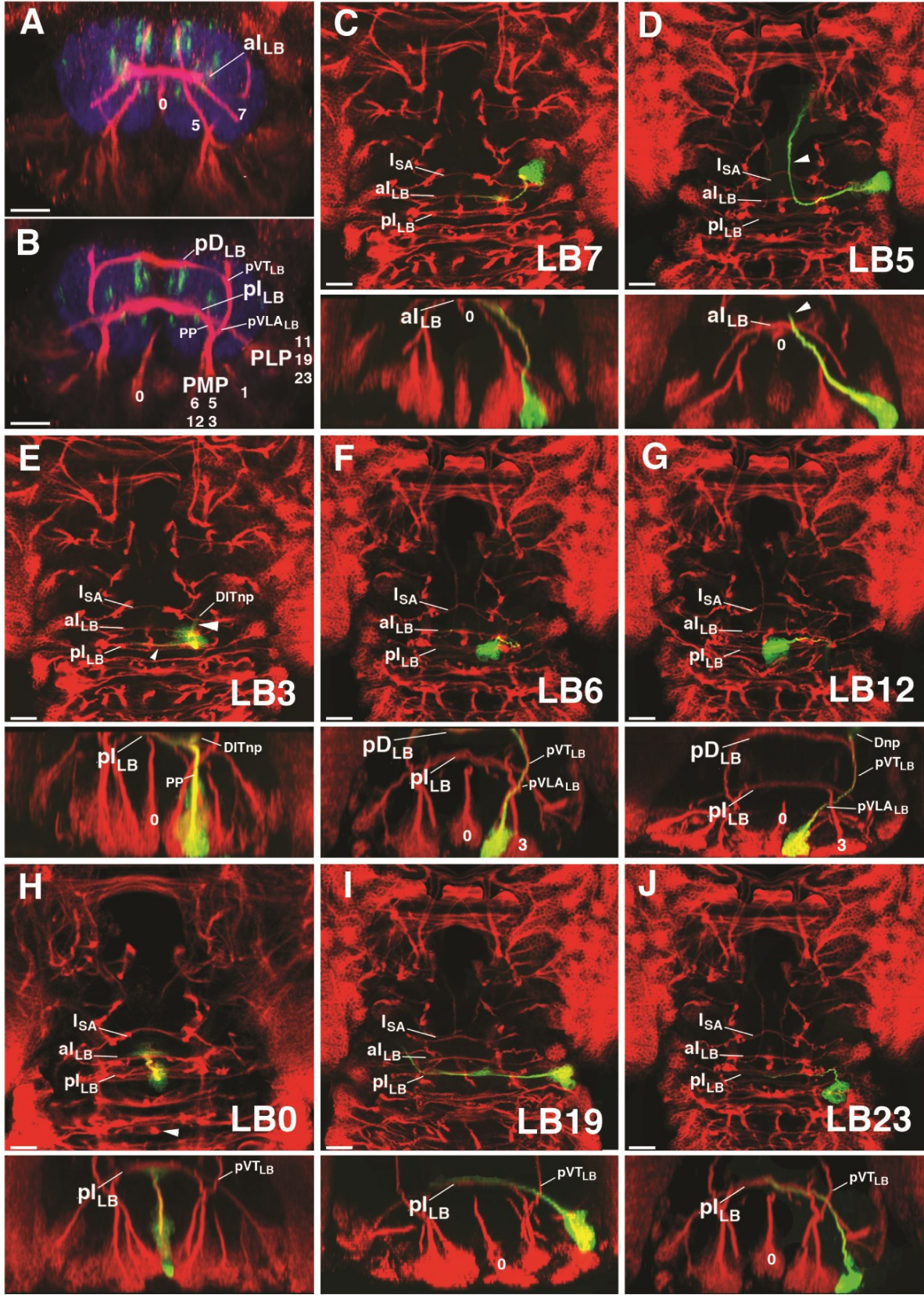
Fig. 3.2: Secondary lineages of the posterior SEG (labial neuromere).

(A, B): Transverse slices of subesophageal ganglion at the level of the anterior (A) and posterior (B) intermediate commissure of the labial neuromere (see panels G and H of Fig.1 for details and abbreviations), illustrating position of the nine lineages of the posterior SEG (annotated by numbers) relative to commissures (aI_{LB}, pI_{LB}, pD_{LB}), connectives (FasII-positive, green), and entry portals (PMP, PLP). **(C-J)**: *tubulin*-driven MARCM clones of individual SEG lineages induced at early larval stages. Each clone is shown in a ventral view (top of each panel) and posterior view (bottom of each panel). The lineage represented by a clone is identified alphanumerically at bottom right of top part of panel. Top parts of panels are z-projections of confocal sections oriented parallel to the length axis of the ventral nerve cord. BP106 labeling (red) shows intermediate level of neuropile (60-80μm from ventral surface) containing commissures (I_{SA}, aI_{LB}, pI_{LB}). GFP-labeled clones (green) are shown as z-projections through entire dorsoventral diameter of nerve cord. Note spatial relationship of clone to pattern of BP106-labeled SATs, which is characteristic of each lineage. For the bottom part of each panel, confocal stacks were digitally rotated 90deg, generating cross a sectional view of the labial neuromere corresponding to the slices shown in panels (A) and (B). Lineages LB7 (C) and LB5 (D) are located in the anterior part of labial neuromere; cross sectional views correspond to the level shown in panel (A). Note anterior intermediate commissure (aI_{LB}), formed by the SAT of lineage LB7. Arrowhead in (D) indicates conspicuous forward directed trajectory of lineage LB5. The remainder of the lineages, shown in panels (E-J), are located in the posterior part of the labial neuromere, corresponding to the level shown in panel (B). Note the posterior intermediate commissure (pI_{LB}), formed by lineages LB3, LB19, and LB23; posterior ventro-lateral arch ($pVLA_{LB}$) and posterior vertical tract (pVT_{LB}), formed by lineages LB6 and LB12, and posterior pillar (PP), formed by lineage LB3. Other abbreviations: DIT_{np} neuropile of dorso-intermediate tract. (A, B) posterior view; (C-J) ventral (upper) and posterior (lower) view. Scale bar: 30 μm in A, B; 20 μm in C-J.
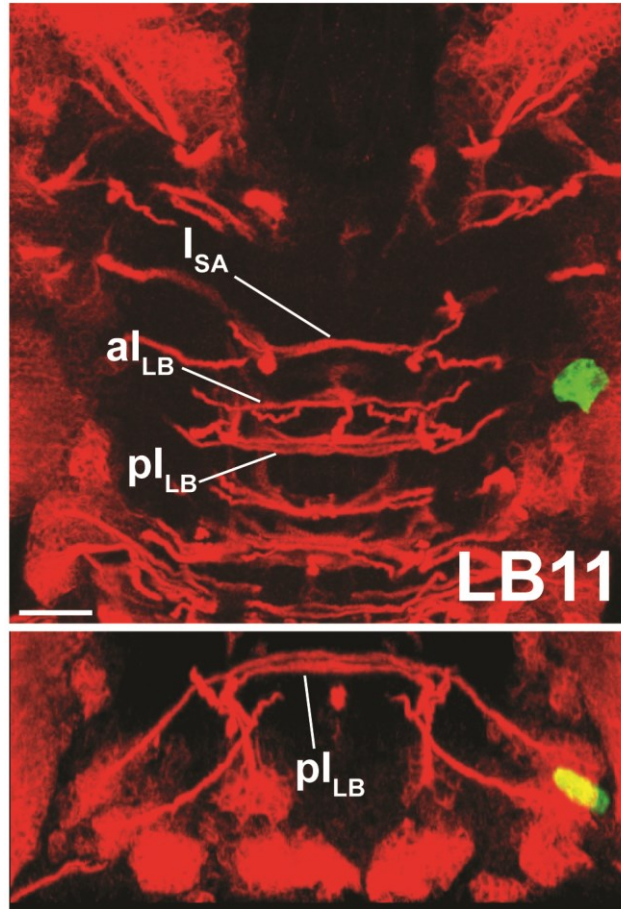
Fig. S3.1: Lineage LB11.

Z-projections of multiple optical sections of BP106 (in red) and *tubulin*-driven MARCM clones (mCD8::GFP; in green), showing a clone of lineage LB11 in the postembryonic labium. Upper panel: Ventral view, the intermediate SA (I_{SA}) as well as the anterior (aI_{LB} and posterior (pI_{LB}) intermediate commissure of the SEG are labeled. Lower panel: Posterior view, the pI_{LB} is labeled. Ventral view. Scale bar: 20 µm.
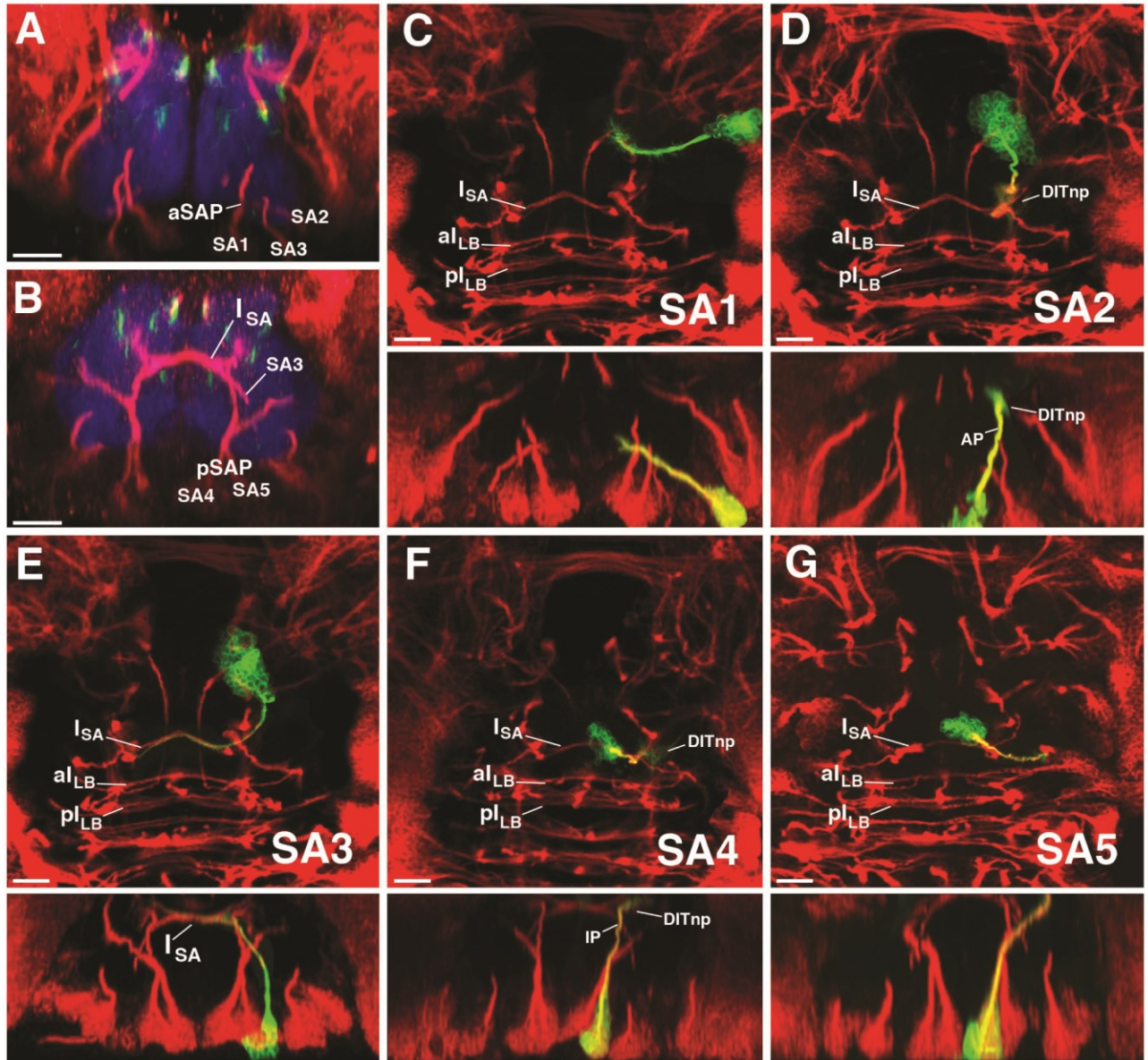
Fig. 3.3: Secondary lineages of the anterior SEG (SA domain).

(A, B): Transverse slices of subesophageal ganglion at the level of the intermediate commissure of the anterior subesophageal ganglion (I_{SA}; panel B) and the level of the aSAP portal (A) (see panels G and H of Fig.1 for details and abbreviations). **(C-G)**: The five lineages of the anterior subesophageal ganglion (SA1-5) are illustrated in the same manner as described for labial lineages in Fig. 2. SA1 and SA2 (C, D) enter through the aSAP. SA1 terminates at a ventral level; SA2 projects dorsally, forming the anterior pillar (AP) and terminates at the level of the dorso-intermediate tract neuropile (DIT_{np}). SA3 (E) enters posteriorly adjacent to the aSAP, projects dorso-posteriorly and forms the I_{SA} commissure, the anterior-most BP106-positive commissure of the SEG. SA4 (F) and SA5 (G) enter through the pSAP. SA4 projects straight dorsally, forming the intermediate pillar of the anterior subesophageal ganglion (IP), and terminates in the DIT_{np}. SA5 follows SA4 in the intermediate pillar, but then veers off laterally to terminate in the dorso-lateral neuropile. (A, B) posterior view; (C-G) ventral (upper) and posterior (lower) view. Scale bar: 30 µm in A, B; 20 µm in C-G.
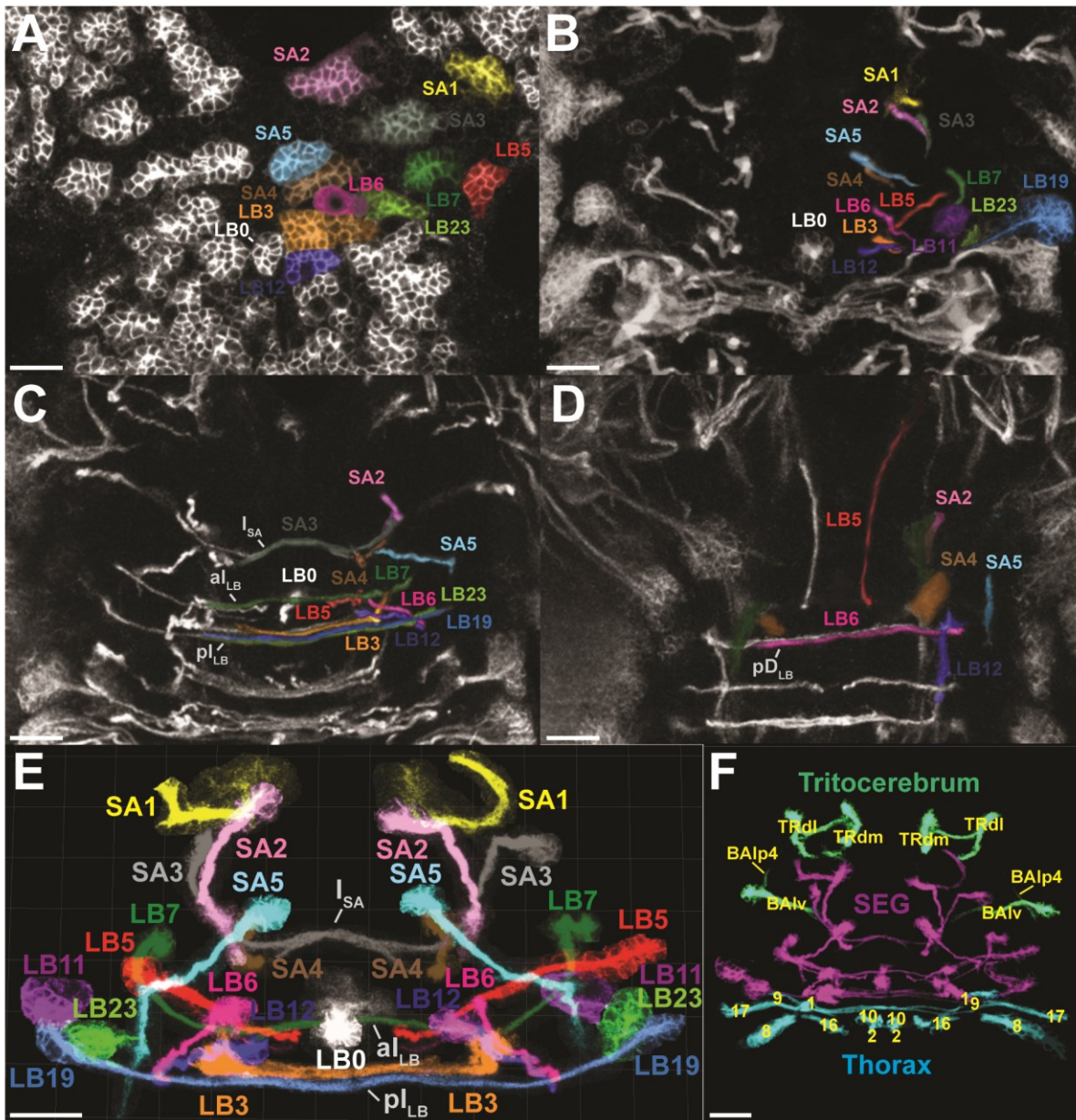
Fig. 3.4: Overview of the postembryonic SEG.

(A-E) Color-coded overview (ventral view) of the 14 lineages in the postembryonic SEG as z-projections and as a 3D-reconstruction of their SATs. (A-D) represent different levels of focal planes of the same specimen **(F)** 3D-Reconstruction of secondary axon tracts of SEG and adjacent tritocerebral and thoracic lineages (same specimen as in Fig. 4E). Ventral view. Scale bars: 20 μm.

3.3.4 Reduction of neuroblast number during embryonic and larval development

Since both the analysis of identified SATs and the clonal MARCM experiments indicate that 14 neuroblast lineages comprise adult-specific neurons in the SEG during postembryonic development, most of the approximately 80 embryonic neuroblasts in the SEG (R. Urbach, personal communication) are likely to disappear during embryonic and/or larval development. To investigate this, we first determined the number of Deadpan-positive neuroblasts during embryogenesis from stage 12 onward. (The Hox gene *labial* was used to determine the anterior boundary of the developing SEG together with *engrailed* which marks the posterior boundaries of neuromeres.) A marked decline in the number of Deadpan-positive neuroblasts was indeed observed (Fig. 3.5). Thus, while an average of 83 (s.d. ± 8, n = 10) neuroblasts was seen in the SEG at embryonic stage 12, only an average of 24 (s.d. ± 5, n = 17) neuroblasts were seen at embryonic stage 16. These data are comparable to the reduction in neuroblast number reported for abdominal neuromeres, where the bulk of neuroblasts undergo apoptosis at the end of embryogenesis (White et al. 1994). To investigate if programmed cell death also underlies the reduction of neuroblast number in the embryonic SEG, we counted neuroblasts in the SEG of embryos that were homozygous for the apoptosis-inhibiting H99 allele (White et al., 1994). In contrast to the approximately 24 neuroblasts seen in stage 16 wildtype embryos, in H99 embryos 54 neuroblasts (s.d. ± 7, n = 8) were observed in the SEG, suggesting that programmed cell death is involved in the reduction of neuroblast number seen in the late embryo (data not shown). To investigate if a further reduction in neuroblast number occurs during larval development of the

SEG, we determined the number of Deadpan-positive neuroblasts in the SEG of L1 to L3 larval stages (Fig. 3.5).
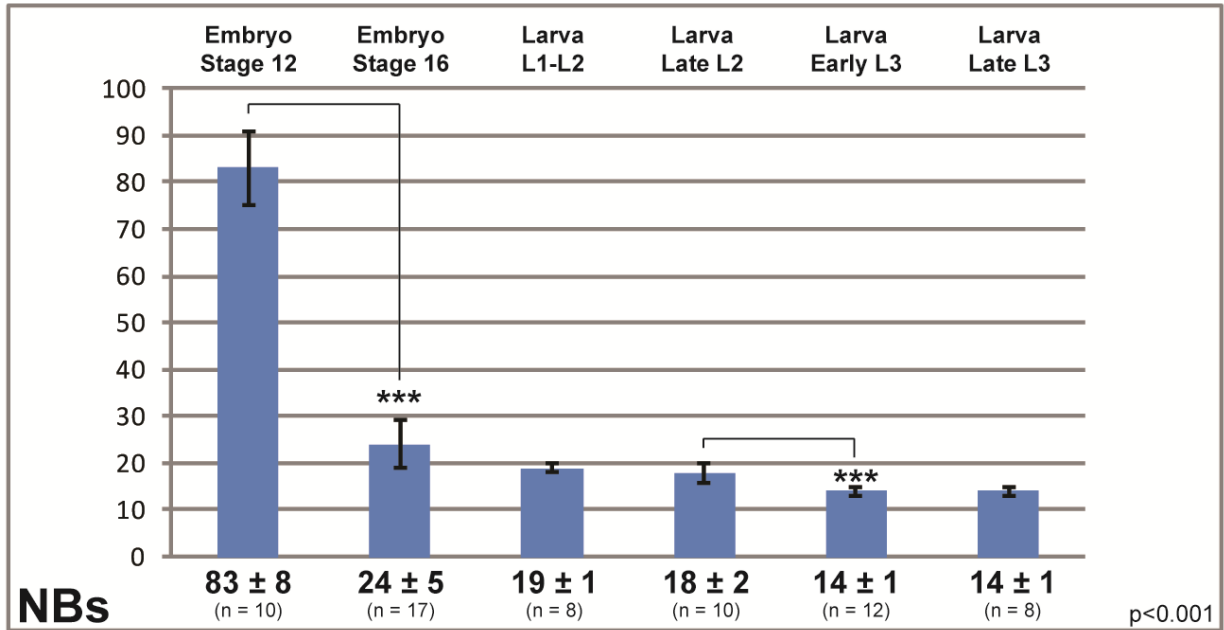
Fig. 3.5: Neuroblast numbers of the developing SEG

Counts of Deadpan-positive neuroblasts in the developing SEG region, defined by expression of *labial* and *engrailed*. Between embryonic stage 12 and 16, the average neuroblast number in the SEG significantly decreases. Between late L2 and early L3 stages, another decrease in the average number of SEG neuroblasts is evident. $p<0.001$.

During early larval stages (L1, L2), 19 neuroblasts (s.d. ± 1, n = 8) were present in the SEG. In contrast, from the early L3 stage onward until the end of larval development only 14 neuroblasts were observed in the SEG. These data imply that a significant reduction in neuroblast number occurs during postembryonic development; only 14 of the ca. 19 SEG neuroblasts seen in early larval stages remain present throughout larval development. This finding is in agreement with the results of the clonal MARCM experiments and the analysis of identifiable SATs reported above,

and supports the notion that a relatively small set of 14 neuroblasts give rise to the lineages of adult-specific SEG neurons throughout postembryonic development.

3.3.5 Lineage-specific expression of the Hox genes *Dfd, Scr,* and *Antp* in the larval SEG

During embryogenesis, the Hox genes *Deformed* (*Dfd*), *Sex combs reduced* (*Scr*), and *Antennapedia* (*Antp*) are expressed in an anteroposteriorly regionalized manner in the developing SEG (Hirth et al., 1998). To determine if these Hox genes are expressed during postembryonic SEG development, we carried out an immunohistochemical analysis of late third instar larval brains. This analysis shows that *Dfd*, *Scr,* and *Antp* are indeed expressed postembryonically in broad, non-overlapping domains in the SEG (Fig. 3.6A-C).

To characterize the expression of these Hox genes in neuroblast lineages during postembryonic SEG development we combined MARCM labeling (see previous section) with co-immunolabeling for Dfd, Scr, and Antp. This analysis showed that 11 out of the 14 neuroblast lineages in the postembryonic SEG express one of the three Hox genes. *Dfd* is expressed in three lineages: SA1, SA2 (in both in most if not all cells, weakly in NB), and (at low level; arrow) SA3 (Fig. 3.6D-F`). *Scr* is expressed in five lineages: SA4, SA5, LB5, LB6, and LB7 (in most cells, including the neuroblast; Fig. 3.6G-K`). *Antp* is expressed in three lineages: LB0, LB3, and LB19 (in a subset of cells, excluding the neuroblast; Fig. 3.6L-N`). The three remaining lineages, 11, 12, and 23, did not express any of these Hox genes. In addition, 10 out of the 14 SEG lineages

also express *engrailed* (Fig. S3.2). Hox gene and *engrailed* expression in the postembryonic SEG

lineages are summarized in Fig. 3.6O.
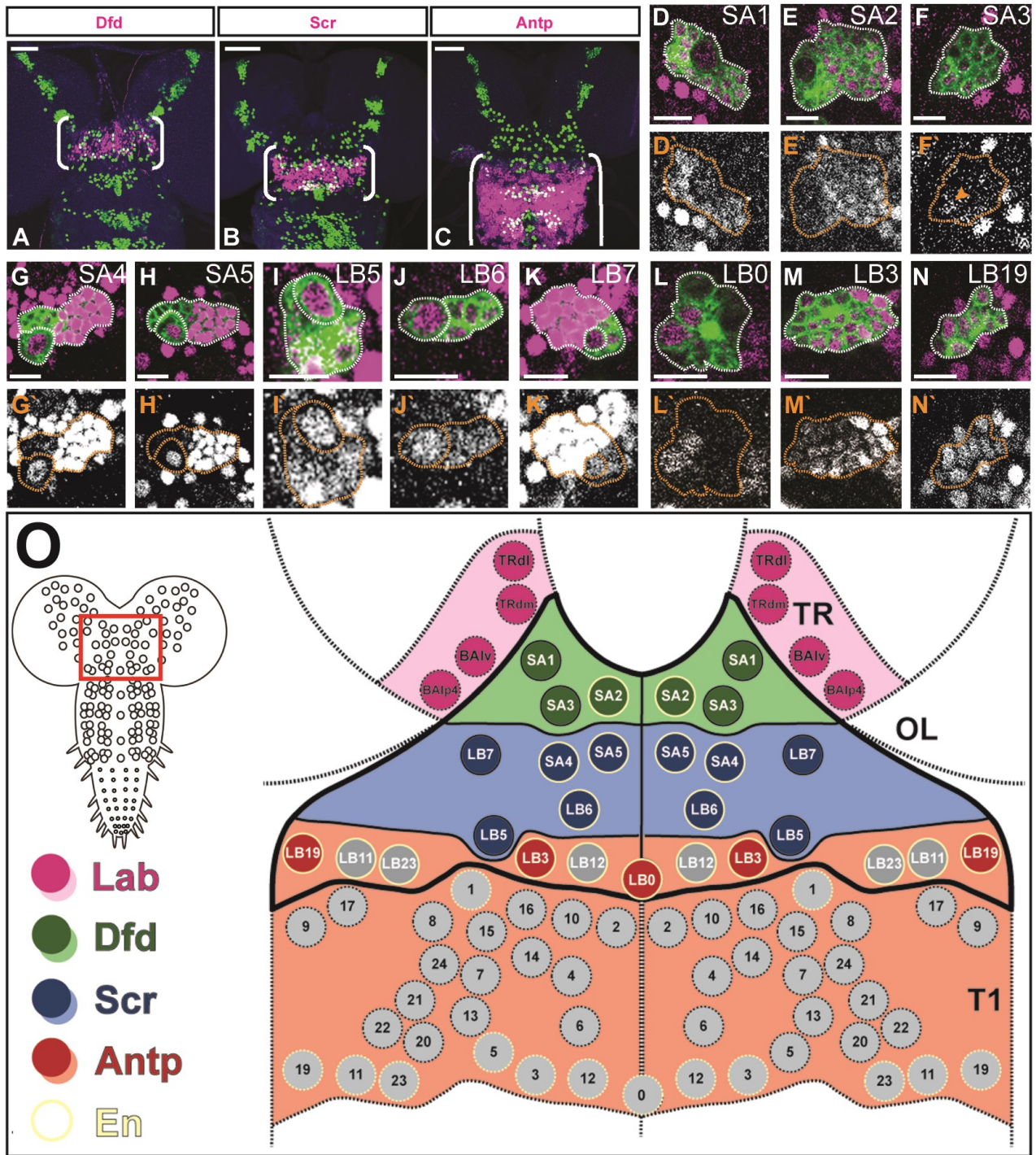
Fig. 3.6: Hox gene expression in the postembryonic SEG.

(A-C) Immunolabeling of Hox proteins in late L3 brains co-expressing *engrailed*-Gal4 driven GFP. Z-projections of multiple optical sections. **(D-N`)** MARCM clones of postembryonic SEG lineages, immunostained for Dfd (D-F`), Scr (G-K`) and Antp (L-N`). Single optical sections. **(O)** Summary scheme of Hox and *engrailed* expression in the postembryonic SEG. Lineages are represented as color-coded circles. Hatched circles indicate tritocerebral (TR) and thoracic (T1) lineages. OL, optic lobes. Ventral view. Scale bars: 30 µm in A-C; 10 µm in D-N.
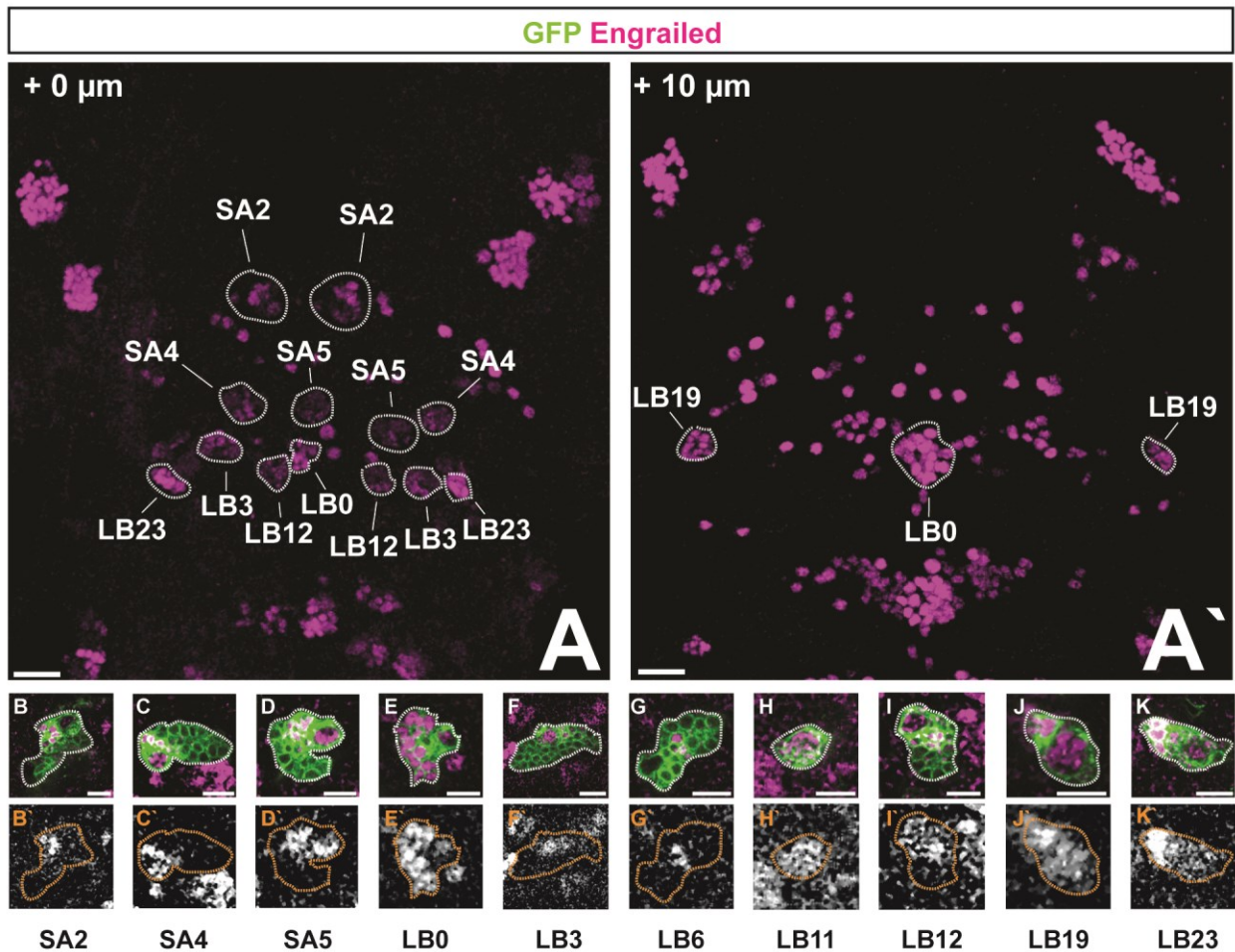
Fig. S3.2: *engrailed* expression in the postembryonic SEG.

(A, A`) Z-projections of multiple optical sections of Engrailed stainings. The SEG lineages SA2, SA4, SA5, LB0, LB3, LB12 and LB23 are detectable as *engrailed* expressing clusters on the surface, in addition to that, bigger and scattered cells are present in more dorsal regions (A`). **(B-K`)** MARCM clones

(GFP) of the postembryonic SEG lineages, immunostained with antibodies against Engrailed. Ventral view. Scale bars: 20 µm in A, A`; 10 µm in B-K.

	SA1	SA2	SA3	SA4	SA5	LB0	LB3	LB5	LB6	LB7	LB11	LB12	LB19	LB23	
Commissure			l_{SA}				pl_{LB}		pD_{LB}	al_{LB}			pl_{LB}	pl_{LB}	
Hox gene	*Dfd*	*Dfd*	*Dfd*	*Scr*	*Scr*	*Antp*	*Antp*	*Scr*	*Scr*	*Scr*			*Antp*		
wt															
Av	57	81	52	70	68	83	129	61	61	57	21	77	69	26	
S. d.	2	3	2	5	3	1	5	3	4	9	5	4	5	3	
n	3	5	3	3	5	3	5	6	3	3	3	3	3	3	
Recovered total	23	24	21	27	32	14	28	17	21	30	16	24	24	7	100 specimens
H99															
Av	83	109	75	101	93	124	129	95	78	71	N/A	94	85	N/A	
S. d.	15	20	17	15	8	10	3	11	4	11		13	12		
n	3	3	3	3	3	3	3	3	3	3		3	3		
Recovered total	23	19	15	22	31	17	10	23	19	24		16	24		184 specimens

Table S3.3: SEG summary table

Summary table showing for each SEG lineage: Commissure (which commissure their SAT joins), Hox gene (which Hox genes they express), cell numbers (average and standard deviation) for wildtype (wt) and apoptosis-blocked (H99) clones. Numbers of counted clones are indicated as n. Note that lineages LB11 and LB23 could not be identified in the H99 assay.

3.3.6 Inactivation of *Dfd*, *Scr,* and *Antp* leads to axonal misprojections, neural cell number reduction, and ectopic lineage formation

To analyze the role of Hox genes in postembryonic SEG development, Dfd^{12} or Dfd^{16} mutant (42 specimens), Scr^2 or Scr^4 mutant (49 specimens) and $Antp^{25}$ or $Antp^{NS+RC3}$ mutant (24 specimens) MARCM clones were induced 24h after larval hatching (ALH) and recovered in the late third larval stage. Three different phenotypes of lineage-specific Hox gene mutation were recovered in the late larval SEG (Fig. 3.7).
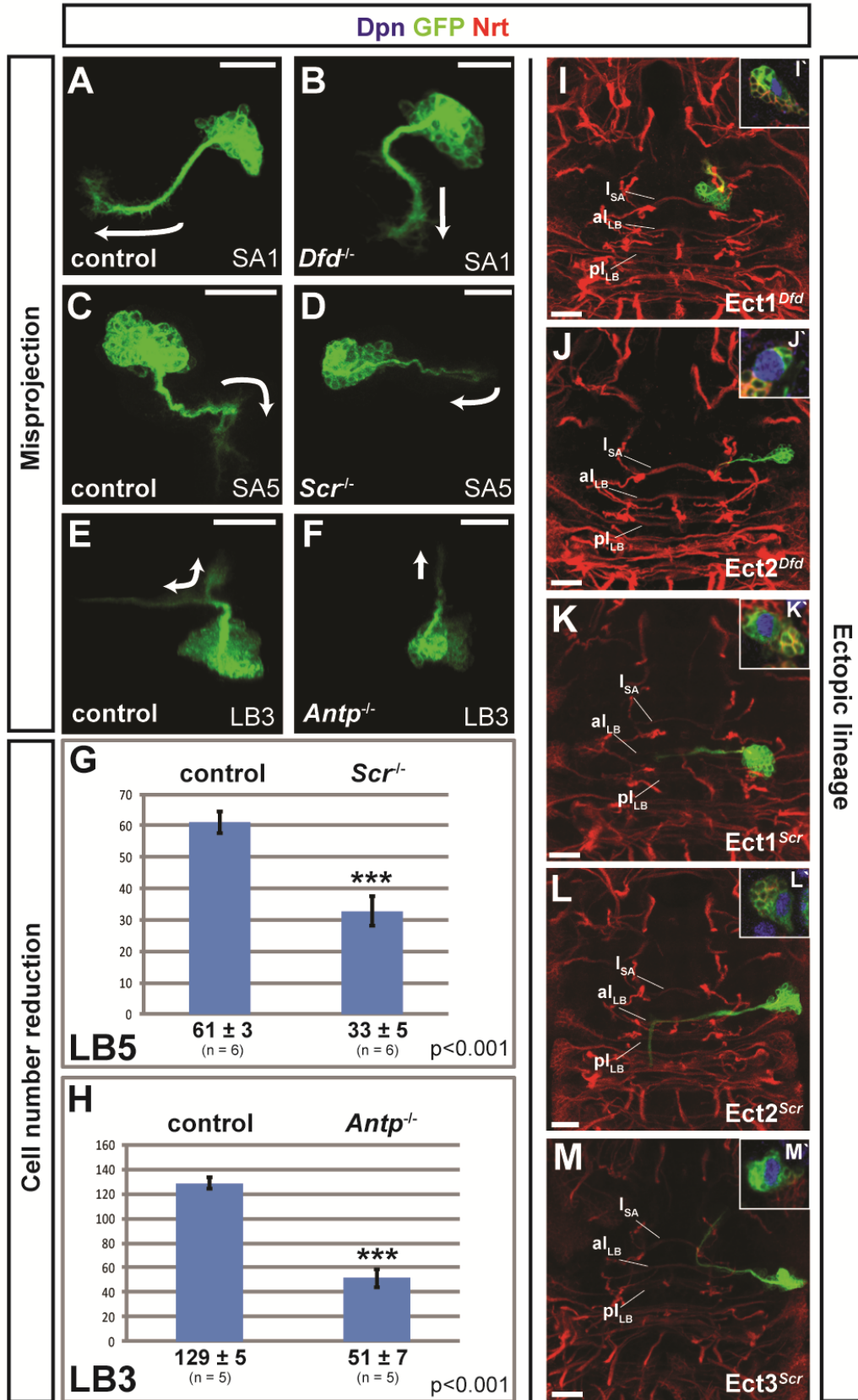
Fig. 3.7: Phenotypes of Hox gene inactivation in the postembryonic SEG

MARCM clones (GFP) in L3 brains, immunostained with anti-BP106 (Nrt) and Deadpan (Dpn). **(A-F)** Misprojection phenotypes in Hox mutant lineages. **(A)** Wildtype SA1 projects SAT medially **(B)** Dfd^{16} mutant SA1 misprojects SAT posteriorly. **(C)** Wildtype SA5 projects SAT laterally and subsequently posteriorly **(D)** Scr^2 mutant SA5 misprojects SAT medially. **(E)** Wildtype lineage LB3 has SAT that splits into anterior and medial branches. **(F)** $Antp^{25}$ mutant clone of lineage LB3 retains only anterior SAT branch. **(G-H)** Cell number reduction phenotypes of Hox mutant lineages. **(G)** The average cell number of LB5 in wildtype and *Scr* mutant clones are significantly different. p<0.001. **(H)** Average cell number of lineage LB3 in wildtype and $Antp^{25}$ mutant clones are significantly different. p<0.001. **(I-M)** Ectopic lineage formation induced by Hox mutation. **(I)** Ect1^{Dfd} with anterior-medial SAT projection **(J)** Ect2^{Dfd} with medial SAT projection **(K)** Ect1^{Scr} with medial SAT projection **(L)** Ect2^{Scr} projects SAT medially **(M)** Ect3^{Scr} with medial-anterior SAT projection. Ventral view. Scale bars: 20 μm.

First, marked axonal projection defects were manifest in three lineages. SA1: In contrast to the medially projecting SAT of the wildtype lineage, the SAT of the *Dfd* mutant SA1 lineage projected posteriorly in the SEG (Fig. 3.7A, B; arrows). This misprojection phenotype was seen in most Dfd^{12} (8 out of 9) and all Dfd^{16} (5 out of 5) mutant SA1 clones indicating that the misprojection is targeted rather than random in nature. SA5: Unlike the posteriorly projecting SAT of the wildtype lineage, the SAT of the *Scr* mutant SA5 lineage usually projected medially across the midline (Fig. 3.7C, D; arrows). Projection defects were observed in 10 out of 14 Scr^2 mutant clones and in 8 out of 10 Scr^4 mutant clones. LB3: While the wildtype SAT had an anterior as well as a medial branch, the *Antp* mutant SAT only retained the anterior branch (Fig. 3.7E, F; arrows).

Second, we observed a pronounced reduction in cell number in two SEG lineages, not associated with neuroblast loss. Whereas clones of the wildtype lineage LB5 had an average of 61 cells (s.d. ± 3, n = 6), the cell number in *Scr* mutant LB5 clones averaged 33 cells (s.d. ± 5 cells, n = 6; Fig.

3.7G). While the *Scr* mutant SAT was, in consequence, considerably thinner in the wildtype, its projection pattern appeared normal. A marked decrease in neural cell number was seen also in the *Antp* mutant LB3 lineages (Fig. 3.7H). In contrast to wildtype LB3 lineages which averaged 129 cells (s.d. ± 5 cells, n = 5), *Antp* mutant LB3 lineages had an average of 51 cells (s.d. ± 7 cells, n = 5).

A third type of Hox loss-of-function phenotype was also observed in the late larval SEG. Among the randomly induced *Dfd* and *Scr* mutant MARCM clones, we consistently found five ectopic neuroblast lineages that were never recovered in the wildtype brain (Fig. 3.7I-M). Since these ectopic neuroblast clone types could be identified based on their anatomical features, we refer to them as the Ect1^{Dfd}, Ect2^{Dfd}, Ect1^{Scr}, Ect2^{Scr}, and Ect3^{Scr} lineages. The Ect1^{Dfd} lineage was located posterior to the SA2 and SA3 lineages and projected its SAT antero-medially (Fig. 3.7I). The Ect2^{Dfd} lineage was located lateral to SA3 and projected medially (Fig. 3.7J). Quantification of cell body number showed that the Ect1^{Dfd} lineage had an average of 67 cells (s.d. ± 4, n = 10) and Ect2^{Dfd} had an average of 37 cells (s.d. ± 1, n = 2). Ect1^{Scr} was located adjacent to the SA4 and SA5 lineages, projected its SAT medially and contained an average number of 47 cells (s.d. ± 5 cells, n = 5; Fig. 3.7K). The other ectopic lineages, termed Ect2^{Scr} and Ect3^{Scr}, were located more lateral to the SA4 and SA5 lineages, projected their SATs medially (Fig. 3.7L-M). They comprised an average number of 53 (Ect2^{Scr}; s.d. ± 4 cells, n = 4) and 62 cells (Ect3^{Scr}; s.d. ± 5 cells, n = 4).

These findings indicate that Hox genes have three lineage-specific roles during postembryonic SEG development. First, *Dfd*, *Scr,* and *Antp* are necessary for the correct SAT projection of the SA1, SA5, and LB3 lineage, respectively. Second, *Scr* and *Antp* assure that the correct number of

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neurons is built in the LB5 and LB3 lineages, respectively. Third, *Dfd* and *Scr* are required to prevent the formation of five ectopic neuroblast lineages. Unlike *Dfd* and *Scr*, *Antp* does not appear to be required to prevent the formation of ectopic lineages in the SEG.

3.3.7 Clonal inactivation of apoptosis in the postembryonic SEG also results in ectopic lineages

Based on previous findings in the abdominal and tritocerebral neuromeres (Bello et al., 2003; Kuert et al., 2012), we postulated that the Hox genes *Dfd* and *Scr* might prevent the formation of ectopic neuroblast lineages in the wildtype larval SEG by inducing apoptosis of the corresponding neuroblasts. To investigate this, we performed a clonal MARCM analysis using the H99 allele (White et al., 1994). Apoptosis blocked MARCM neuroblast clones were induced at 24 hours ALH, recovered in late third larval stage brains, and co-labeled with anti-Dfd or anti-Scr antibodies (n = 184 specimens). In these experiments, we consistently recovered two ectopic neuroblast lineage types that expressed *Dfd* and three types that expressed *Scr*.

The first *Dfd* expressing ectopic lineage, $Ect1^{Dfd\text{H99}}$, was located posterior to the SA1 and SA2 lineage, projected its SAT bundle anteriorly, and had an average number of 71 cells (s.d. ± 14 cells, n = 4; Fig. 3.8A, A`). The $Ect2^{Dfd\text{H99}}$, was positioned lateral to the SA3 lineage, projected its SAT in a medial and anterior branch, and had an average number of 46 cells (s.d. ± 13 cells, n = 5; Fig. 3.8B, B`). The *Scr* expressing ectopic lineage $Ect1^{Scr\text{H99}}$ was located posterior to the SA4 and SA5 lineages, had a SAT that splits into a medial and a lateral branch, and had an average number of 83 cells (s.d. ± 9 cells, n =5; Fig. 3.8C, C`).). The other two *Scr*-expressing

lineages were located lateral to the SA5 and SA4 lineages, projected their SATs medially, and had an average cell number of 47 cells (s.d. ± 9 cells, n =5) (Fig. 3.8D, D`). Since these two lineages could be recovered together but not distinguished on a neuroanatomical basis, we refer to these ectopic lineages as Ect2/3$^{Scr\textbf{H99}}$.

The Hox expressing ectopic lineages found in the apoptosis-blocked MARCM assay were remarkably similar in neuroanatomical respects to those found in the *Dfd* or *Scr* mutant MARCM experiments. These findings imply that the Hox genes *Dfd* and *Scr* are required in these five (ectopic) neuroblast lineages for termination of proliferation through programmed cell death during early postembryonic SEG development.
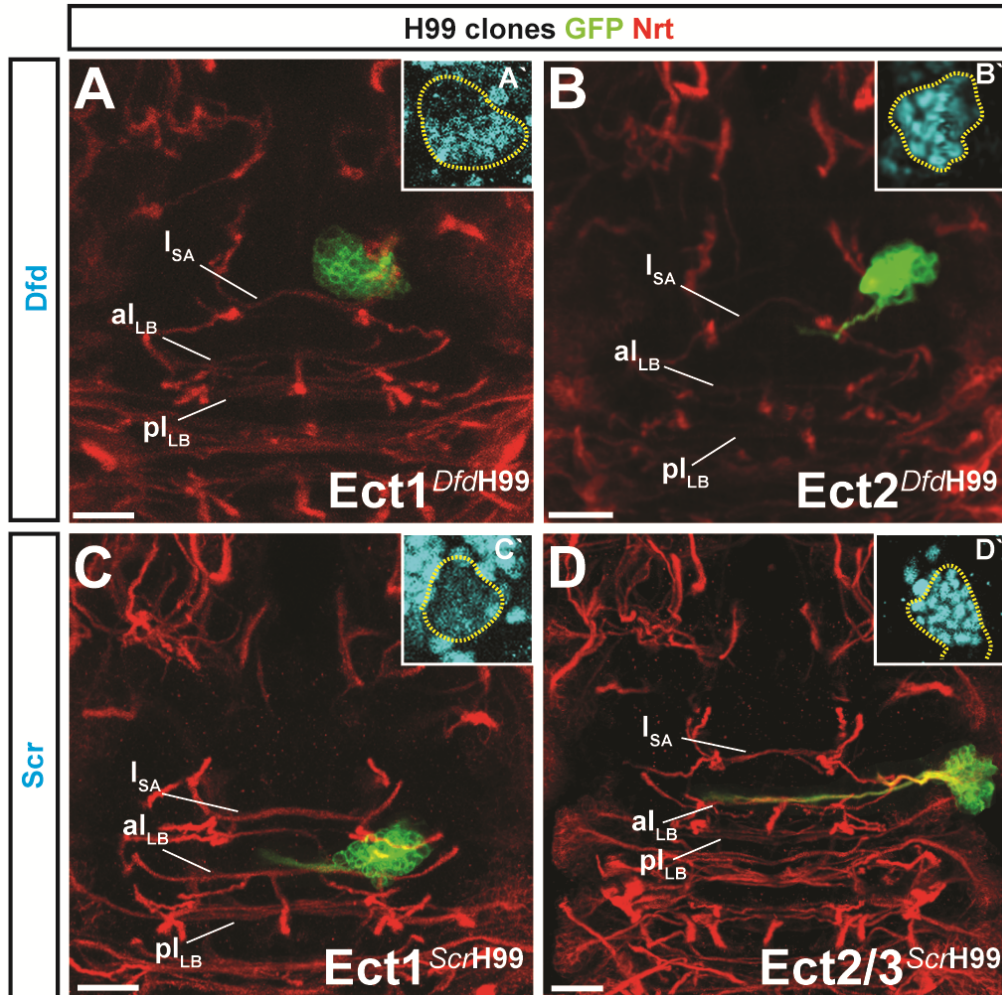
Fig. 3.8: Clonal cell death block induces Hox expressing ectopic lineages.

MARCM clones homozygous for H99 (GFP), in L3 brains immunostained with anti-BP106 (Nrt). **(A)** Ect1$^{Dfd\textbf{H99}}$ projects short SAT branch anteriorly. (A`) Expression of *Dfd* in Ect1$^{Dfd\textbf{H99}}$. **(B)** Ect2$^{Dfd\textbf{H99}}$ projects its SAT in medial direction. (B`) Expression of *Dfd* in Ect2$^{Dfd\textbf{H99}}$. **(C)** Ect1$^{Scr\textbf{H99}}$ located posterior to SA4 and SA5 projects SAT in a bifurcating bundle (C`) Expression of *Scr* in Ect1$^{Scr\textbf{H99}}$. **(D)** Ect2/3$^{Scr\textbf{H99}}$ projects SAT medially. (D`) Expression of *Scr* in Ect2/3$^{Scr\textbf{H99}}$. Ventral view. Scale bars: 20 µm.

3.4 DISCUSSION

In this report, we focus on the postembryonic neuroblast lineages that give rise to the adult-specific neurons of the SEG. We report three main findings. First, a small set of 14 uniquely identifiable postembryonic neuroblast lineages gives rise to secondary neurons in the postembryonic SEG. Second, the Hox genes *Dfd*, S*cr,* and *Antp* are expressed in a lineage-specific manner in the developing larval SEG. Third, mutation of any of these Hox genes results in severe developmental defects in specific lineages. In the following, we will discuss the major implications of these findings.

3.4.1 Reduced number of proliferating neuroblast lineages in postembryonic SEG development

A total of 14 identified postembryonic neuroblast lineages generate adult-specific secondary neurons in the late larval SEG. This is a surprisingly small number compared with the ca. 80 neuroblast lineages in the embryonic SEG (R. Urbach, personal communication). Our cell counts indicate that only approximately one fourth of these ca. 80 neuroblasts are reactivated postembryonically. This is markedly different in the SPG, where approximately 85 of the 100 embryonically active neuroblasts are reactivated and proliferate in larval stages (Dumstrei et al., 2003; Ito and Hotta, 1992; Pereanu and Hartenstein, 2006). Our experiments indicate that the fate of half of the embryonic SEG neuroblasts that are not present postembryonically is programmed cell death. The fate of the other half is unknown. They may terminate proliferation through cell cycle exit at the end of embryogenesis.

The low number of postembryonic SEG lineages has interesting consequences for the relationship between primary neurons and secondary neurons in the mature SEG. Most neuroblasts generate 10-20 neural cells embryonically and 100-150 neural cells postembryonically (see Hartenstein et al., 2008; Technau et al., 2006). Thus, the ca. 80 embryonic SEG neuroblasts should generate 800-1600 primary neural cells per hemiganglion while the 14 postembryonic neuroblasts generate approximately 900 secondary neural cells (as estimated by our cell counts) per hemiganglion. Assuming that most of the primary neurons survive metamorphosis, this suggests that a substantial fraction of the neurons in the adult SEG could be primary neurons that comprise the functional larval SEG before their integration into the adult brain.

Previous work has shown that 75 neuroblast lineages generate the secondary neurons of the three thoracic neuromeres (Ito and Hotta, 1992; Marin et al., 2012; Truman et al., 2004). This is in striking contrast to the 14 neuroblast lineages that generate secondary neurons in the three SEG neuromeres. The most evident is this reduction in the SA region (from 50 to 5 lineages), where only one commissure (I_{SA}) is present which is also built by only one lineage, SA3. The labial neuromere is also reduced but not as dramatically (from 25 to 9 lineages). The relatively small number of postembryonic neuroblast lineages in the SEG neuromeres is likely to reflect the marked reduction and fusion of segmental appendages in the three gnathal segments that are innervated by the SEG. From an evolutionary perspective, a loss/reduction of gnathal appendages in insects such as flies would eliminate or reduce the need for corresponding neural control circuitry at least in the adult. Interestingly, and in contrast to the VNC, we found no evidence for the presence of postembryonically generated motoneurons in the SEG, indicating that all secondary neurons in the SEG are interneurons.

3.4.2 Regionalized expression of Hox genes in postembryonic SEG development

During embryonic and postembryonic brain development, the Hox genes *Dfd, Scr,* and *Antp* are regionally expressed in discrete, non-overlapping domains in the neuromeres of the SEG (Hirth et al., 1998; this report). In both cases *Dfd* is expressed in an anterior domain, *Scr* is expressed in a posteriorly adjacent domain, and *Antp* expression begins in a small labial domain adjacent to the prothoracic neuromere. Moreover, while the total number of neuroblast lineages that express a given Hox gene may be different embryonically and postembryonically, most of the postembryonic neuroblast lineages do express one of these genes suggesting that Hox gene expression is a stable developmental feature of SEG lineages. Indeed, most if not all of the Hox genes that are expressed in the embryonic CNS, are re-expressed in the neuroblast lineages of the postembryonic CNS (Bello et al., 2003; Hirth et al., 1998; Kuert et al., 2012; Marin et al., 2012; this report).

3.4.3 Multiple lineage-specific functions of Hox genes in postembryonic SEG development

Our study reveals three lineage-specific requirements for Hox genes during postembryonic SEG development. First, the genes *Dfd* and *Scr* are required for correct SAT projections in the SA1 lineage (for *Dfd*) and in the SA5 lineage (for *Scr*). Interestingly, the lineage-specific loss-of-function of these Hox genes results in specific, reproducible SAT misprojections and not in

randomized axonal misprojections. The lineage-specific nature of these stereotyped misprojections suggests that Hox genes interact with other lineally acting control elements to determine the axonal projection features of the affected neurons. While the ensemble of these control elements is currently unknown, there is increasing evidence for the importance of transcription factor codes in controlling the expression of axonal guidance molecules (see Butler and Tear, 2007; Guthrie, 2007).

Second, the genes *Scr* and *Antp* are required for correct cell number in lineage LB5 (*Scr*) and in lineage LB3 (*Antp*). The fact that the *Scr* and *Antp* mutant lineages contain about half of the cells present in the corresponding wildtype lineages, suggests that these Hox genes might be required to prevent hemilineage dependent programmed cell death (e.g. Kumar et al., 2009; Lin et al., 2010; Truman et al., 2010). Further studies of Hox gene action in lineages LB3 and LB5 are necessary to confirm this hemilineage dependent cell death block and also to elucidate possible molecular interactions between Hox genes and Notch signaling in these hemilineages.

Third, the genes *Dfd* and *Scr* are required for suppressing the appearance of ectopic neuroblast lineages. When *Dfd* or *Scr* mutant neuroblast clones are induced at early larval stages and recovered at late larval stages, five distinct types of ectopic neuroblast clones are found. Each of these is identifiable based on reproducible neuroanatomical features such as position, secondary axon tract projection, and cell number. These ectopic lineages do not represent homeotic transformations of other wildtype neuroblast lineages, since all other SEG neuroblast lineages are present. Whether these ectopic lineages become functionally integrated into the adult brain of *Drosophila* is currently unknown. Evidence for an integration of ectopic neuron groups into a mature brain comes from mammalian studies, which show that *Hoxa1* mutant hindbrain

progenitors can establish supernumerary ectopic neural cell groups that escape apoptosis and give rise to a functional circuit in the postnatal brain (Del Toro et al., 2001).

Our data indicate that the appearance of ectopic neuroblast lineages is due to a lack of Hox gene dependent programmed cell death occurring in larval stages. Indeed, all Hox genes studied to date have been implicated in some aspect of programmed cell death in postembryonic neuroblasts. The *lab* gene is required for the termination of specific tritocerebral neuroblasts, *Dfd* and *Scr* are required for lineage-specific neuroblast termination in the SEG, *Antp* und *Ubx* can trigger neuroblast death if misexpressed in thoracic lineages, and *abd-A* induces programmed cell death in neuroblasts of the central abdomen (Bello et al., 2003; Kuert et al., 2012; Marin et al., 2012). We therefore conclude that a general function of Hox genes in postembryonic neural development is in the regionalized termination of progenitor proliferation as a key mechanism for neuromere-specific differentiation and specialization of the adult brain.

4. HOX GENES AND NEURAL PATTERNING IN *DROSOPHILA*

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4.1 ARTICLE SYNOPSIS

Hox genes encode conserved homeodomain transcription factors that possess significant developmental power in shaping animal morphology. Hox genes are also expressed in the nervous system of *Drosophila* and have a wide array of functions in the development of the peripheral and central nervous system. In the peripheral nervous system of *Drosophila*, Hox genes are required for sensory organ development as well as for proper formation of neuromuscular networks. During embryogenesis of the central nervous system, Hox genes are involved in the establishment of regional neuronal identity, in fate specification in different segments, in regionalized programmed cell death of postmitotic neurons and in the control of neural proliferation. During postembryonic development of the central nervous system, Hox genes are involved in limiting the proliferative potential of neural progenitors in the abdominal segments by controlling the time of their apoptosis, and this process is tightly controlled by the Polycomb group of genes. Hox genes and their cofactors are also subject to cross-regulatory interactions in neural pattering. Many features of Hox gene expression and function in the nervous system of *Drosophila* can be found in vertebrates, suggesting a high degree of evolutionary conservation for Hox gene action in neural tissues.

4.2 INTRODUCTION

"The essential phenomenon is not that there has merely been a change, but that something has changed into the likeness of something else". With his observations, William Bateson initiated homeotic gene research more than a century ago by proposing the term Homeosis to describe those natural variations in animal species, in which body appendages had acquired certain morphological features characteristic of other regions (Bateson, 1894). Homeotic genes were first characterized genetically in the fruit fly *Drosophila melanogaster* as selector genes whose mutations lead to homeotic transformations of specific segments along the anterior-posterior body axis (Lewis, 1978). In *Drosophila*, there are eight homeotic genes that map to two distinct regions of the third chromosome called the Antennapedia-Complex (ANT-C) and the Bithorax-Complex (BX-C), collectively termed the homeotic complex (Akam, 1989). (These two *Drosophila* complexes are thought to originate from the splitting of one ancestral complex.) The eight homeotic genes in *Drosophila* are named as *labial (lab)*, *proboscipedia (pb)*, *Deformed (Dfd)*, *Sex combs reduced (Scr)*, *Antennapedia (Antp)*, *Ultrabithorax (Ubx)*, *abdominal-A (abd-A)* and *Abdominal-B (Abd-B)* (Fig. 4.1).
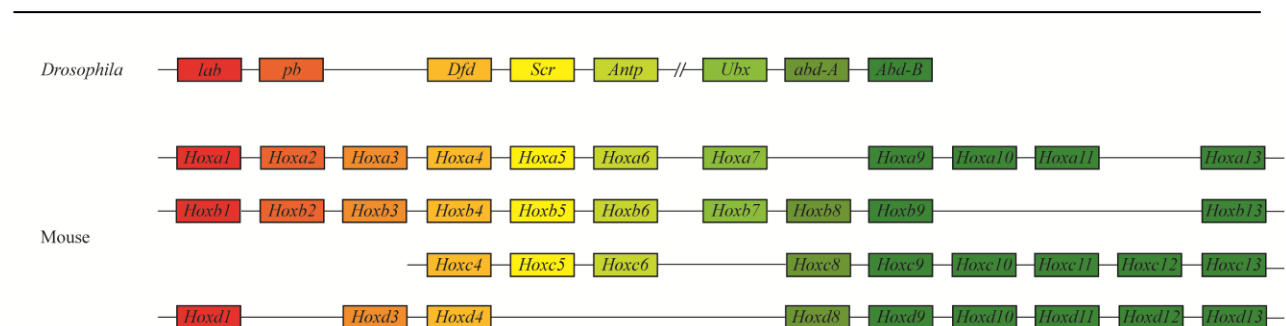

Fig. 4.1: Hox genes of *Drosophila melanogaster* and *Mus musculus*.

In this simplified schematic scheme, genes are arranged according to their physical order on the corresponding chromosomes and related genes are colored-coded. Abbreviations: *lab*, *labial*; *pb*, *proboscipedia*; *Dfd*, *Deformed*; *Scr*, *Sex combs reduced*; *Antp*, *Antennapedia*; *Ubx*, *Ultrabithorax*; *abd-A*, *abdominal-A*; *Abd-B*, *Abdominal-B*.

All of these homeotic genes (in the following referred to as Hox genes) contain a 183 base pair DNA sequence called the homeobox (Gehring, 1987). The homeobox encodes a homeodomain with sequence-specific DNA binding activities that is part of a larger protein with transcriptional regulator function, indicating that Hox genes encode transcription factors (Levine and Hoey, 1988). In *Drosophila*, Hox genes are expressed in restricted domains along the anterior-posterior axis of the embryo, and the arrangement of the expression domains of the Hox genes along the anterior-posterior axis has the same order as the arrangement of these genes on the chromosome, a phenomenon referred to as "spatial colinearity" (Kaufman, et al. 1990). Hox genes control positional identity (reviewed in Favier and Dollé, 1997; McGinnis and Krumlauf, 1992). Loss-of-function or gain-of-function mutations of Hox genes can cause homeotic transformations in the body plan of the fly. Prominent examples for homeotic mutants are the haltere-to-wing transformation in Hox mutations of the BX-C (reviewed in Lewis, 1978) and the gain-of-function mutation of *Antp*, which induces the outgrowth of a leg in place of an antenna on the head of the fruit fly (Denell, et al., 1981). Homeotic transformations of epidermally derived embryonic structures are associated with mutations in the genes *Scr*, *Antp*, *Ubx*, *abd-A* and *Abd-B*, each of which has overlapping expression domains with other Hox genes (Lewis, 1978; Sanchez-Herrero et al., 1985; Wakimoto and Kaufman, 1981). In contrast, mutation of the Hox genes *lab* and *Dfd*, whose expression domains have no "backup" Hox gene expression, result in structural deficiencies rather than transformations towards other segmental identities (Merill et al., 1987;

Merill, et al., 1989; Regulski et al., 1987). Hox genes homologous to those characterized in *Drosophila* have been found in all bilaterian vertebrates and invertebrates studied to date. The first vertebrate Hox genes were discovered in mouse and *Xenopus* by using *Drosophila* homeobox probes (Carrasco et al., 1984; McGinnis et al. 1984). In mammals such as mouse or human, a total of 39 Hox genes have been identified which can be assigned to four different genomic regions and are referred to as the Hox-A, Hox-B, Hox-C and Hox-D complexes (Favier and Dollé, 1997; McGinnis and Krumlauf, 1992; Zeltser et al., 1996). (The four mammalian complexes are thought to be the product of duplication events of a single ancestral complex.) Sequence comparisons have shown that all of these mammalian Hox genes can be divided into 13 groups of genes called paralogs, each of which is related to one of the *Drosophila* Hox genes (Scott, 1992). The Hox genes of *Drosophila* and mammals not only share structural similarities, they also exhibit comparable features of expression. Thus, murine Hox genes are expressed in restricted domains along the anterior-posterior axis of the embryo, as it is the case for their *Drosophila* Hox gene counterparts (Dollé and Duboule, 1993). The remarkable interphyletic conservation of Hox gene structure and expression patterns has led to the proposal that all animals with bilateral symmetry are defined by the presence of Hox genes (Davidson et al., 1995; Slack, 1993). A conserved role of Hox genes is also to specify positional identity along the anteroposterior body axis during early embryogenesis. During subsequent development, the positional identity values specified by Hox genes can then be interpreted differently in different tissues (and in different animals) to ensure that the cells in a given region develop into the appropriate cell type. This is exemplified by the remarkably pervasive roles of Hox genes in the regionalized patterning of the nervous system. In this review, we focus on the role of Hox genes

in patterning the developing nervous system of *Drosophila* and summarize the wide array of Hox-dependent functions that have been demonstrated in this neurogenetic model system.

4.3 ORGANIZATION AND DEVELOPMENT OF THE *DROSOPHILA* NERVOUS SYSTEM

As a basis for understanding the action of Hox genes in *Drosophila* neural patterning, we first review the overall organization and development of the nervous system in this insect (see Campos-Ortega and Hartenstein, 1997; Reichert and Boyan 1997). As in other insects, the nervous system of *Drosophila* can be subdivided in the peripheral nervous system (PNS) and the central nervous system (CNS), and both of these have a metameric organization that reflects the overall segmentation of the animal. The PNS of *Drosophila* is relatively simple and comprises the peripheral sense organs, their sensory axons, the motor axons and the stomatogastric nervous system. It contains a variety of sensory organs that are able to detect various stimuli such as light, sound, smell, taste, touch and stretch. The CNS of *Drosophila* is more complex and consists of the supraesophageal ganglion and the subesophageal ganglion as well as the segmental ganglia that form the ventral nerve cord (VNC) (Fig. 4.2).
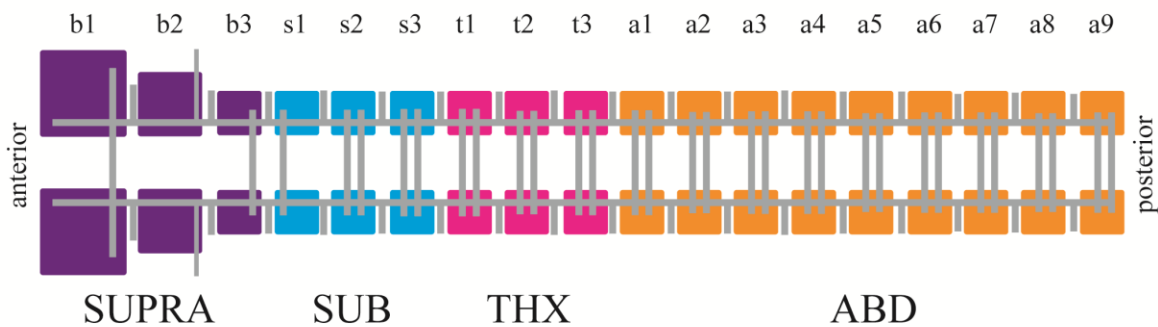

Fig. 4.2: Structural organization of the embryonic central nervous system of *Drosophila melanogaster*.

Simplified summary schemation of the neuromere compartments and their longitudinal and commissural connections. Neuromeres in different parts of the central nervous system are color-coded: supraesophageal ganglion, purple; subesophageal ganglion, cyan; thoracic ganglia, pink; abdominal ganglia, orange. Abbreviations: SUPRA, supraesophageal ganglion; b1, protocerebrum; b2, deutocerebrum; b3, tritocerebrum; SUB, subesophageal ganglion; s1, mandibular neuromere; s2, maxillar neuromere; s3, labial neuromere; THX, thorax; t1-t3, thoracic neuromeres; ABD, abdomen; a1-a9, abdominal neuromeres.

The supraesophageal ganglion and the subesophageal ganglion are both located in the head and are interconnected by paired circumsesophageal connectives. The supraesophageal ganglion, also referred to as the anterior brain, can be further subdivided into the protocerebrum, deutocerebrum and tritocerebrum. The protocerebrum is the largest neuromere of the supraesophageal ganglion and contains complex neuropile structures such as the mushroom bodies and the central complex. Laterally adjacent to the protocerebrum are the large optic lobes that receive visual input from the compound eyes. The deutocerebrum contains the antennal lobe, which receives olfactory and mechanosensory input from the antennae via the antennal nerve. The tritocerebrum is the smallest neuromere of the anterior brain and innervates the foregut via the frontal ganglion (Younossi-Hartenstein et al., 1996). The subesophageal ganglion, also referred to as the posterior brain, contains the fused mandibular, maxillar and labial neuromeres each of which have a structural organization comparable to that of the segmental ganglia of the VNC. The subesophageal ganglion innervates the mouthparts of the head. The VNC has an overt metameric organization and consists of 3 thoracic and 9 abdominal neuromeres, most of which have 2 commissures. Considerable insight into the developmental mechanisms responsible for neural patterning has been obtained for the CNS, for the peripheral mechanosensory system and for the central and peripheral visual system. Here we consider only the first two, since their development involves

Hox gene action. A stereotyped array of peripheral sensory organs that function primarily as external and internal mechanoreceptors is found in the trunk segments of the thorax and abdomen. The stereotyped spatial organization of these sensory organs and their axons in the peripheral nervous system is a result of the pattern in which the sensory organ neural precursor cells (SOPs) delaminate from the ectoderm during development (Ghysen and Dambly-Chaudière, 1989). Formation of the SOPs is best understood for the development of the external sense organs. Here, the selection of precursor cells involves two steps (Ghysen and Dambly-Chaudière, 1989; Ghysen et al., 1993): First, proneural fields are established in the developing ectoderm through expression of proneural genes of the *achaete-scute* complex providing these cells the competence of becoming a SOP (Campuzano and Modolell, 1992; Cubas et al., 1991; Romani et al., 1989; Skeath and Caroll, 1991). Subsequently, lateral inhibition guarantees that only one or a few cells adopt the fate of neural precursors while the other cells acquire the default epidermal identity. This process requires interactions between proneural genes and the neurogenic genes *Delta* and *Notch* (Artavanis-Tsakonas and Simpson, 1991; Campos-Ortega, 1988; Ghysen et al., 1993). Following its specification, each individual SOP divides and gives rise to a specific set of neurons and support cells that comprise the external sense organ. The precursors that give rise to the neural cells of the CNS are called neuroblasts. Individual neuroblasts segregate from the embryonic neuroectoderm by delamination and this process also involves proneural gene expression and lateral inhibition involving *Delta-Notch* interactions (reviewed in Skeath and Thor, 2003). The pattern of neuroblasts in the CNS is regionalized and invariant. The procephalic neuroectoderm produces the neuroblasts that form the brain and the ventral neuroectoderm produces the neuroblasts of the VNC. In the VNC, each segment gives rise to a similar set of approximately 25 neuroblast pairs. In the brain, there are about 100 neuroblast pairs present per

side (Hartenstein and Campos-Ortega, 1984; Poulson, 1950; Urbach and Technau 2003). Following delamination, the neuroblasts start to proliferate in a stem-cell like manner in which they divide asymmetrically to self-renew and produce a smaller daughter cell called a ganglion mother cell (GMC). Each GMC divides only once to generate two postmitotic daughters, neurons or glial cells. During proliferation, neuroblasts sequentially express a series of temporal identity factors, encoded by so-called temporal genes that intrinsically regulate sequential neuronal identity of their progeny (Grosskortenhaus et al., 2006; Isshiki et al., 2001; Kanai et al., 2005). Neuroblasts generally manifest two temporally distinct periods of neurogenesis, a first one during embryogenesis (primary neurogenesis) and a second one during postembryonic larval development (secondary neurogenesis). The primary neural cells generated during embryonic neurogenesis differentiate into the fully functional CNS of the larva; the secondary neural cells generated during postembryonic neurogenesis differentiate during the larval and the subsequent pupal period to generate the majority of the CNS of the adult (reviewed in Hartenstein et al., 2008). The two neurogenesis periods are separated by a period of quiescence during which most neuroblasts are mitotically inactive and reduced in size; following the quiescence period, most neuroblasts are reactivated such that by the end of larval development all persisting neuroblasts are proliferating (Hartenstein et al., 1987; Ito and Hotta, 1992; Prokop and Technau, 1991; Truman and Bate, 1988). The neuroblasts and their progeny constitute a set of stereotyped neural lineages which can be identified by position and projection pattern of their axon tracts in the CNS (Pereanu and Hartenstein, 2006; Truman et al., 2004).

4.4 HOX GENES AND PATTERNING OF THE PERIPHERAL NERVOUS SYSTEM

Hox genes have been shown to play important roles in the development of the sensory organs in the PNS. The sensory organs which are generated in the embryo are arranged in a segment-specific pattern in the trunk segments and the formation of this pattern is dependent on Hox gene action. Accordingly, mutational inactivation and ectopic overexpression of Hox genes results in major defects in the PNS such as the transformation of sensory organs and the alteration of sensory organ number and location (Heuer and Kaufman, 1992). In addition to their role in specification of sensory organ identity, Hox genes also influence the patterns of axonal projections from the sense organs into the CNS and may also contribute to the regulation of sensory cell dendrite arborization (Ghysen et al., 1983; Merritt and Whitington, 2002; Parrish et al., 2006). Furthermore, migration and terminal differentiation of specific SOPs in the trunk segments is Hox gene dependent as is the development of the mechanosensory hairs (macrochaete and microchaete sensilla) of the legs (Castelli-Gair et al., 1994; Rozowski and Akam, 2002; Shroff et al., 2007). The pervasive role of Hox genes in sense organ specification is based on molecular interactions with other developmental control genes and in several cases, some insight into these interactions has been obtained. For example, Abd-A has been shown to interact with the gene product of the proneural gene *atonal (ato)* and the zinc finger transcription factor encoded by the *senseless (sens)* gene in proprioceptive chordotonal organ development in the abdomen (Gutzwiller et al., 2010; Li-Kroeger et al., 2008). This interaction among Abd-A*,* Ato and Sens is thought to influence EGF-signalling by activation of the protease encoding *rhomboid (rho)* gene via a common cis-regulatory enhancer element (Witt et al., 2010). The EGF

signal is mediated by the ligand Spitz (Spi) which can induce epidermal cells to form SOP cells (Elstob et al., 2001; Lage et al., 1997; Okabe and Okano, 1997; Rusten et al., 2001; Shilo, 2005) (Fig. 4.3).
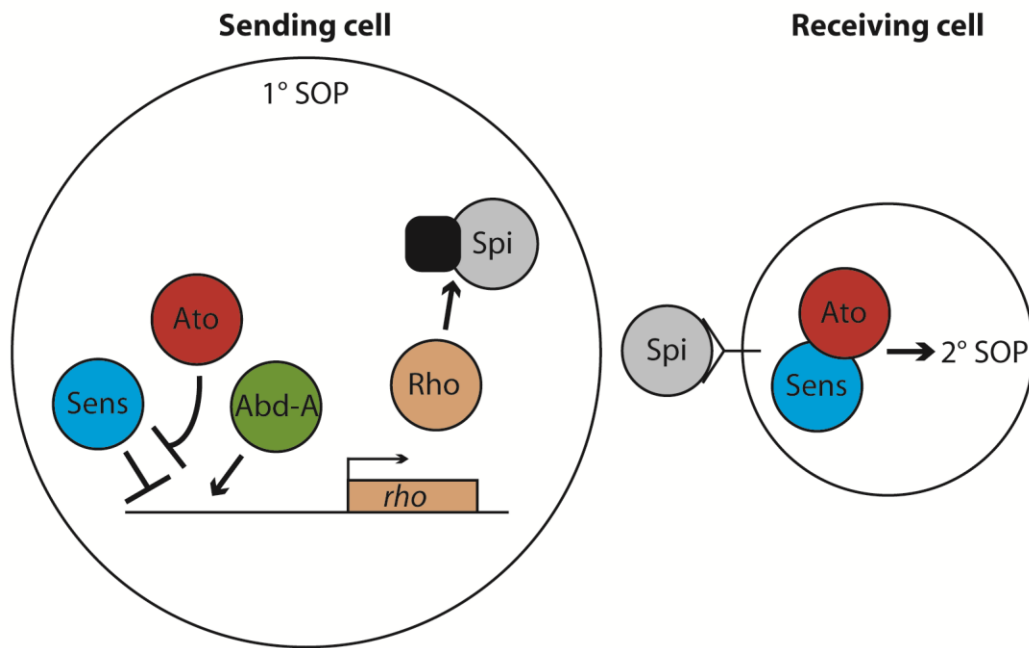
Fig. 4.3: Model of the regulation of SOP formation by Ato, Sens and the Hox protein Abd-A according to Gutzwiller et al., 2010.

In case an organ 1° SOP expresses both *ato* and *abd-A*, Sens-mediated repression of *rho* expression is abolished, thus *rho* is expressed, Spi is secreted and binds to the EGF-receptor on the receiving cell. If the receiving cell expresses the *ato* and *sens* transcription factors, a 2° SOP develops. Abbreviations: Ato, Atonal; Sens, Senseless; Abd-A, Abdominal-A; Rho, Rhomboid; Spi, Spitz; EGF, epidermal growth factor; Salm, Spalt major; SOP, sensory organ precursor.

Recently, evidence has been obtained for a role of Hox genes in the development of the peripheral neuromuscular system. Muscle development in *Drosophila* is known to depend on autonomous properties of the muscle progenitors as well as on inductive cues from neighboring cells including the innervating motoneuron axons. Hox genes may contribute to these inductive cues since targeted misexpression of *Ubx* in specific motoneurons results in dramatic alterations of morphogenesis in the muscles innervated by these motoneurons (Dutta et al., 2010). This suggests that a correct juxtaposition between the Hox-code-mediated identities of nerve and muscle might be required for correct neuromuscular development. Defects in peristaltic larval locomotor behavior that occur when Hox gene expression is perturbed during neuromuscular development support this notion (Dixit et al., 2007).

4.5 HOX GENES IN THE DEVELOPMENT OF THE EMBRYONIC CENTRAL NERVOUS SYSTEM

During embryonic development of *Drosophila*, all 8 Hox genes are expressed in the CNS (Hirth et al., 1998) (Fig. 4.4). The Hox gene *lab* has the smallest expression domain in the embryonic
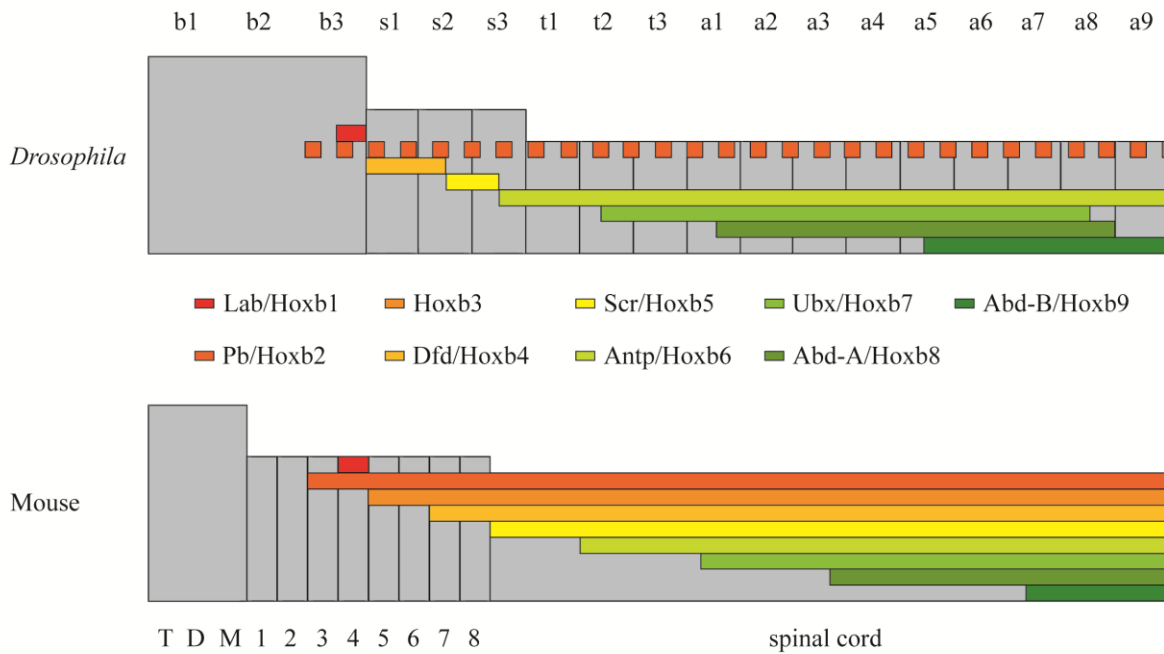
Fig. 4.4: Conserved anterior-posterior order of Hox gene expression in the embryonic central nervous system of *Drosophila melanogaster* (stage 14) and *Mus musculus* (9.5 -12.5 days).

Expression domains of the Hox genes *lab/Hoxb1*, *pb/Hoxb2*, *Hoxb3*, *Dfd/Hoxb4*, *Scr/Hoxb5*, *Antp/Hoxb6*, *Ubx/Hoxb7*, *abd-A/Hoxb8* and *Abd-B/Hoxb9* are color coded in this schematic. Anterior is to the left. Abbreviations: *Drosophila*: b1, protocerebrum; b2, deutocerebrum; b3, tritocerebrum; s1, mandibular neuromere; s2, maxillar neuromere; s3, labial neuromere; t1-t3, thoracic neuromeres; a1-a9, abdominal neuromeres; Lab, Labial; Pb, Proboscipedia; Dfd, Deformed; Scr, Sex combs reduced; Antp, Antennapedia; Ubx, Ultrabithorax; Abd-A, Abdominal-A; Abd-B, Abdominal-B. Mouse: T, telencephalon; D, diencephalon; M, mesencephalon; 1-8, rhombomeres 1-8.

CNS; it is primarily expressed in the tritocerebrum anlage. Thus, the *lab* gene is expressed in all 13 neuroblasts of the tritocerebrum and 2 neuroblasts of the deutocerebrum (Urbach and Technau, 2003). The *pb* gene has a large anterior-posterior expression domain ranging from the deutocerebral neuromere to the posterior VNC; however, atypically, *pb* expression is only detectable in small reiterated clusters of approximative 15-20 cells per neuromere. Interestingly, *pb* expressing cells are found anterior to *lab* expressing cells, which is an exception to the spatial colinearity rule (Kaufman et al., 1990). The *Dfd* gene is expressed in the mandibular neuromere and in a large anterior part of the maxillary neuromere. *Scr* is expressed in the posterior part of the maxillary neuromere and a large anterior domain of the labial (third subesophageal) neuromere. The *Antp* gene is expressed in a large domain ranging from the posterior part of the labial neuromere to the end of the VNC. In contrast to these ANT-C genes, all Hox genes of the BX-C are expressed only in the VNC. *Ubx* gene expression extends from the posterior part of the T2 neuromere to the anterior part of the A7 neuromere. The *abd-A* gene is expressed from the posterior part of the A1 neuromere to the posterior part of the A7 neuromere. The expression domain of *Abd-B* extends to the end of the VNC; its anterior border is somewhat variable and ranges from the A7 neuromere at early stages to the A5 neuromere at late stages of CNS embryogenesis. Mutational inactivation studies have been carried out for all Hox genes to determine their function in embryonic CNS development. Loss-of-function of the Hox genes *lab* and *Dfd* results in prominent defects in the embryonic brain (Hirth et al., 1998). In *lab* null mutants, marked axonal patterning defects are associated with the mutant domain in the tritocerebral neuromere. The longitudinal connectives that normally run through the tritocerebrum are missing or reduced, the tritocerebral commissure is completely absent and the connectives to the frontal ganglion project ectopically into more anterior brain neuromeres.

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Remarkably, the tritocerebral domain is not deleted in the null mutant since cells can still be detected in the mutant domain. This indicates that the tritocerebrum is not specified correctly in the *lab* mutant. Indeed, the tritocerebral mutant cells do not generate axons and axons from other parts of the brain do not project to the mutant domain. Furthermore, the tritocerebral mutant cells do not express typical neuronal markers anymore, indicating that they rather remain undifferentiated. Comparable defects are observed in the mutant domain of *Dfd* null mutants in the mandibular neuromere. Thus, the Hox proteins Lab and Dfd are required to establish regional neuronal identity in their expression domains (Fig. 4.5).
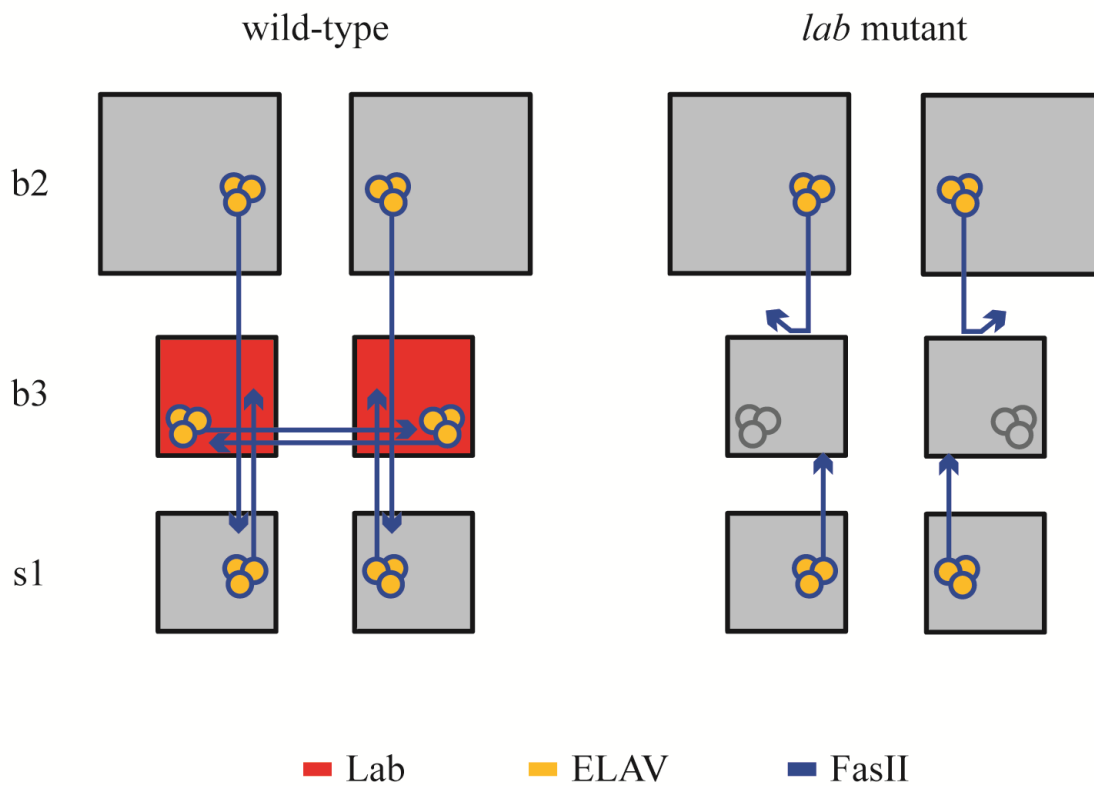

Fig. 4.5: Establishment of regional neuronal identity by the Hox protein Lab in the embryonic central nervous system of *Drosophila*.

In the wild-type, neurons (indicated by circles) of the tritocerebrum (b3) express the Hox gene *labial* (red), the neuronal-specific marker ELAV (light orange) and the cell adhesion molecule FasII (blue). In the *labial* null mutant, cells of the tritocerebrum are present but fail to extend axons and do not express ELAV and FasII due to a loss of neuronal identity, and axons from other parts of the brain avoid the *labial* mutant domain. Abbreviations: b2, deutocerebrum; b3, tritocerebrum; s1, mandibular neuromere; *lab*, *labial*; ELAV, embryonic lethality abnormal vision; FasII, fasciclin II. Anterior is to the top.

As expected, the *lab* null mutant phenotype can be rescued by targeted misexpression of the *lab* gene under the control of CNS-specific *lab* regulatory elements. Interestingly, however, all Hox proteins except Abd-B are able to replace Lab protein in this phenotypic rescue of the tritocerebral neuromere indicating that there is a significant degree of functional equivalence among the Hox proteins in the specification of the tritocerebral area (Hirth et al., 2001). This suggests that cis-regulatory elements and/or cofactors rather than Hox protein specificity are responsible for differences in Hox gene action in embryonic CNS development. In contrast to *lab* and *Dfd*, mutations in the remaining Hox genes *pb*, *Scr*, *Antp*, *Ubx, abd-A* and *Abd-B*, do not cause gross morphological defects in embryonic CNS patterning. However, analysis of individual neuroblasts in the expression domains of these Hox genes does reveal a clear role in providing identity to neuronal precursors and progeny (reviewed in Rogulja-Ortmann and Technau, 2008). For example, the thoracic and abdominal neuromeres contain serial homologs of NB1-1, and this neuroblast gives rise to segment-specific lineages that differ in the thoracic versus abdominal neuromeres (Udolph et al., 1993). Hox genes are involved in determining this segment-specific lineage identity, since Ubx and Abd-A are autonomously required to establish abdominal identity in the NB1-1 lineage. Also, the thoracic identity of the NB1-1 lineage can be overridden by Hox misexpression and Polycomb mutants (Prokop and Technau, 1994). Hox genes also play a role in

thoracic versus abdominal fate determination in the NB6-4 lineage. Thoracic and abdominal NB6-4 are different in so far as NB6-4 produces neurons and glia cells in the thoracic segments but only generates glial cells in the abdominal segments (Schmidt et al., 1997). While specification of the NB6-4 lineage in thoracic segments does not receive input from Hox genes, the abdominal NB6-4 lineage requires input from Abd-A and Abd-B for proper specification. This Hox gene input in the abdominal NB6-4 lineage downregulates the G1 cyclin, *DmCycE, (CycE)* which promotes neuronal fate in the thoracic NB6-4 lineage (Berger et al., 2005; Kannan. et al., 2010). In addition to their function of determining region-specific neural identity, Hox genes also have roles in programmed cell death (PCD) during later stages in embryonic CNS development. PCD has been shown to be involved in most of the embryonic neuroblast lineages, in some of these cases apoptosis occurs in a segment-specific manner (Rogulja-Ortmann et al., 2007). For example, the fate of the NB7-3 derived GW motoneuron, which dies in the segments T3 to A8 but survives in segments T1 and T2, is under complex Hox gene control. Thus, the Hox protein Antp is required to prevent the GW neuron from undergoing cell death while Ubx counteracts Antp and promotes apoptosis of this motoneuron (Rogulja-Ortmann et al., 2008). However, this type of Hox gene action is context- and cell-specific since other neurons in the same lineage express *Ubx* in similar patterns but do not undergo PCD. A comparable post-mitotic requirement for Hox genes in segment-specific programmed cell death has also been reported for the pioneer neurons dMP2 and MP1 (Miguel-Aliaga and Thor, 2004). Initially, these pioneer neurons are generated throughout the whole VNC but in late embryonic stages they undergo apoptosis in anterior but not posterior segments. This process is under Hox gene control, since the Hox protein Abd-B is required in posterior segments to prevent apoptosis of the dMP2 and MP1 neurons by repressing two RHG-motif cell death activators, *reaper* and *grim*. Contrasting

with these findings are very recent studies on peptidergic Va neurons in which Abd-B is playing a pro-apoptotic role (Suska et al., 2011). Besides their role in control of PCD, Hox genes are also directly involved in the control of proliferation in the embryonic CNS. The Hox genes *abd-A* and *Ubx* are involved in regulating the different number of cells generated in the thoracic versus abdominal neuromeres by controlling neural proliferation in the embryo and larva, as well as by determining the number of post-embryonic neuroblasts (Prokop et al., 1998). Interactions between Hox genes and a specific set of temporal genes have been documented using the Apterous (Ap) cluster as model system. This cluster of 4 cells expresses the LIM-HD transcription factor Ap and is produced in the thoracic neuromeres by NB5-6 at the end of its embryonic proliferative period. The fact that the Ap cluster neurons arise only in thoracic neuromeres is a result of different mechanisms which involve the Hox genes (Baumgardt et al., 2009; Karlsson et al., 2010). In abdominal segments, generation of the Ap cluster neurons is prevented in the NB5-6 lineage by premature neuroblast cell cycle exit mediated by Ubx, Abd-A, Abd-B and their cofactors. In contrast, in thoracic segments, the Ap cluster neurons of the thorax are specified by Antp, which together with the Hox cofactors Homothora*x* (Hth) and Extradenticle (Exd) interact with the gene product of the temporal gene *castor (cas)* to activate *collier (col)* and a *col*-dependent feed-forward loop that results in the differentiation of the Ap cluster neurons. Ap cluster-like cells are produced in more anterior neuromeres of the subesophageal ganglion but do not acquire the fate of Ap cluster neurons due to the absence of expression of *Antp* as well as the absent or low-level expression of the temporal factor *Grainyhead (Grh)*.

4.6 HOX GENES IN THE DEVELOPMENT OF THE POSTEMBRYONIC CENTRAL NERVOUS SYSTEM

Most *Drosophila* neuroblasts enter a quiescence period at the end of embryogenesis and resume proliferation in larval stages. However, the mechanisms that control the regulation of entry, maintenance and exit of this quiescence period are not well understood. Recently, some evidence for an involvement of Hox genes in the regulation of entry into the quiescence period has been obtained for neuroblasts in the VNC (Tsuji et al., 2008). NB3-3 in the thoracic neuromeres enters quiescence well before NB3-3 in abdominal neuromeres where this neuroblast continues to proliferate into late embryonic stages. This segment–specific difference in time of entry into quiescence is likely to be regulated by Hox genes since in *Antp* mutants, NB3-3 in thoracic neuromeres also continues to proliferate until late embryonic stages. Moreover, a comparable temporal prolongation of thoracic NB3-3 proliferation is also observed following misexpression of *abd-A*. Thus, at least for embryonic NB3-3, the Hox proteins Antp and Abd-A are required for spatial control of entry into quiescence towards the end of embryogenesis. Following their exit from quiescence during early larval stages, most neuroblasts in the brain and thoracic neuromeres resume proliferation, and their postembryonically generated progeny constitute the majority of the neurons in the adult CNS. By contrast, in the abdominal neuromeres a dramatic reduction in the number of neuroblasts takes place in late embryogenesis due to PCD such that only a small number of abdominal neuroblasts survive to generate postembryonic progeny (Abrams et al., 1993; Peterson et al., 2002; White et al., 1994). Moreover, those abdominal neuroblasts that do survive and re-initiate proliferation typically only generate a small number of progeny due to a markedly reduced proliferative period as compared to the thoracic neuroblasts or the neuroblasts

of the central brain (Truman and Bate, 1988). Remarkably, Hox genes have been shown to control this limitation of the proliferative period in many abdominal neuroblasts by controlling the timing of postembryonic neuroblast apoptosis (Bello et al., 2003) (Fig. 4.6).
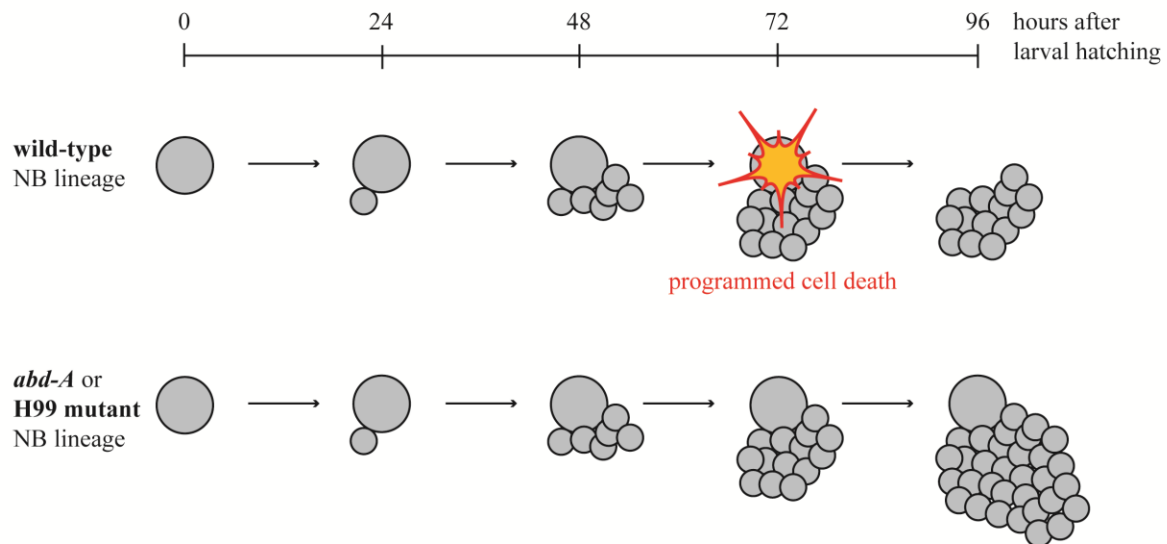
Fig. 4.6: Temporal regulation of postembryonic neuroblast proliferation by the Hox protein Abd-A. In wild-type lineages of the central abdomen, a mid third instar larval pulse (72 hours after larval hatching) of *abd-A* expression in the neuroblast triggers programmed cell death and thereby limits the final number of progeny that is produced. Blocking apoptosis or clonal mutation of *abd-A* results in the survival of the central-abdominal neuroblasts, which continue to proliferate. Abbreviations: NB, neuroblast; *abd-A*, *abdominal-A*; H99, deletion of the proapoptotic genes *reaper*, *grim* and *head involution defective*.

4.7 GENETIC INTERACTIONS BETWEEN HOX GENES IN NEURAL PATTERNING

Hox gene action during development has been shown to involve a complex set of interactions among the different Hox proteins as well as between Hox proteins and specific cofactors. Hox genes manifest complex cross-regulatory interactions in which the products of more posterior genes in the cluster are phenotypically prevalent over products of more anterior genes; this interaction process has been termed "posterior prevalence" (Duboule and Morata, 1994; Graba et al., 1997; Mann and Morata, 2000). Moreover, the transcription factors encoded by Hox genes achieve their DNA recognition specificity by binding cooperatively with cofactors. In *Drosophila*, the best studied of these cofactors are the homeodomain proteins Exd and Hth which interact with Hox proteins in a Hth/Hox/Exd combination, suggesting that Hox genes often function through such trimeric multiprotein complexes (Affolter et al., 1990; Ekker et al., 1991; Grieder et al., 1997; Hoey and Levine, 1988; Pinsonneault et al., 1997; Rieckhof et al., 1997; Ryoo et al., 1999). Hox gene interactions that manifest posterior prevalence have been analysed in the development of the *Drosophila* CNS (Sprecher et al., 2004). In these experiments, the phenotypic consequences resulting from targeted misexpression of more posterior Hox genes in the tritocerebral *lab* expression domain were studied. Remarkably, for all of these Hox genes, phenotypic defects resulted in the *lab* expression domain that are similar to those observed in *lab* null mutants (see above). These defects are due to a complete suppression of the Lab protein in the affected domain which is due to transcriptional repression of the *lab* gene. Further investigation of this type of posterior prevalence phenomenon in targeted misexpression experiments involving mutated Hox protein motifs indicate that a functional homeodomain is

crucial for executing the repressive function of the posterior Hox genes. Interestingly, the co-misexpression of a posterior Hox gene (*Ubx*) together with the two cofactors nuclear *exd* and *hth* results in a rescue of the *lab* transcriptional repression phenotype. This genetic rescue effect requires a balanced expression of both cofactors and is not attained in co-misexpression experiments involving the Hox gene and a single cofactor. In contrast to the prominent interactions among the Hox genes, there is no evidence for interactions between the Hox genes and the columnar genes, another set of homeobox-containing patterning genes involved in CNS development (Chu et al., 1998; McDonald et al., 1998; Mellerick and Modica, 2002; Skeath and Thor, 2003; Von Ohlen and Doe, 2000). This lack of interaction between the two groups of patterning genes has been documented in detail for the Hox gene *lab* and the columnar gene *ventral nervous system defective (vnd)* which act in an independent manner in the formation and specification of the tritocerebral neuromere (Sprecher et al., 2006). Mutational inactivation of the *vnd* gene results in a gap-like phenotype in the embryonic brain comparable to that observed in *lab* mutants. This phenotype is due to a marked reduction in the number of *lab*-expressing neuroblasts and to apoptosis of postmitotic neurons in the *lab* domain of the developing tritocerebrum. Nevertheless, there is no detectable crosstalk between *lab* and *vnd* genes in brain development, since in brains that are mutant for *lab* or *vnd*, the corresponding other gene is still expressed correctly in the tritocerebral domain.

4.8 EVOLUTIONARY CONSERVATION OF HOX GENE EXPRESSION AND FUNCTION IN NEURAL DEVELOPMENT

Hox genes are expressed during development of the nervous system in a wide variety of bilaterian animals, including vertebrates, hemichordates, insects, annelids and molluscs and the anterior-posterior order of their expression domains in the developing nervous system are similar in most of these organisms (Hinman et al., 2003; Hirth et al., 1998; Irvine and Martindale, 2000; Kourakis et al., 1997; Lowe et al., 2003; Wilkinson et al., 1989). This is exemplified by the almost identical order of expression of orthologous Hox genes in the embryonic CNS of fly, mouse and human (Graham et al., 1989; Hirth et al., 1998; Hunt et al., 1991; Vieille-Grosjean et al., 1997; Wilkinson et al., 1989) (Fig. 4.4). In contrast, the relative extent of the expression domains of orthologous Hox genes in the developing CNS often differs in different animals. For example, in the murine CNS, most Hox genes are expressed in broad overlapping domains, while in the fly CNS this is only true for the Hox genes *pb*, *Antp*, *Ubx*, *abd-A* and *Abd-B* and not for *lab, Dfd* and *Scr* which are expressed in discrete non-overlapping domains. Nevertheless, the degree of conservation of Hox gene expression in neural development is remarkable. Indeed, a conserved anterior-posterior order of Hox gene expression may be a common feature in the developing nervous system of all bilaterian animals. In vertebrates, as in flies, Hox proteins are required for proper development of the embryonic central nervous system, and in some cases the defects that result from inactivation of Hox gene orthologs are similar in the two animal groups (see Lichtneckert and Reichert, 2005; Lichtneckert and Reichert, 2008; Reichert and Bello, 2010; Reichert and Simeone, 2001). For example, in *Drosophila*, mutational inactivation of the Hox gene *lab* leads to loss of tritocerebral neuromere identity as well as axonal projection defects,

such as the absence of the tritocerebral commissure and defective longitudinal connectives in the tritocerebral neuromere (see above). Comparable mutant phenotypes are observed in murine mutants of the orthologous *Hoxa1/Hoxb1* genes. Thus, inactivation of *Hoxa1* causes segmentation defects as well as motor neuron axonal projection defects in mouse hindbrain neuromeres (Carpenter et al., 1993; Chisaka et al., 1992; Dollé et al., 1993; Lufkin et al., 1991; Mark et al., 1993). Moreover, *Hoxb1* is also involved in rhombomere specification since *Hoxb1* loss-of-function leads to an alteration of rhombomere 4 identity (Goddard et al., 1996; Studer et al., 1996). Cross-regulatory interactions of Hox genes are also likely to operate in murine hindbrain development. Genetic analysis of *Hoxa1* and *Hoxb1* double mutants reveals that both genes are involved in establishment and maintenance of *Hoxb1* expression in rhombomere 4 and that this involves auto- and para-regulatory interactions (Gavalas et al., 1998; Studer et al., 1998). Furthermore, as their *Drosophila* counterparts, murine Hox genes were shown to be involved in patterning the peripheral nervous system and in controlling locomotor behaviour (De la Cruz et al., 1999; Wahba et al., 2001). In view of these similar developmental genetic findings in animals as diverse as flies and mammals, it seems likely that both the expression and the function of Hox genes have been conserved in bilaterian nervous system evolution.

5. DISCUSSION

5.1 LINEAGE IDENTIFICATION IN THE POSTEMBRYONIC TRITOCEREBRUM AND SUBESOPHAGEAL GANGLION

In this thesis, the postembryonic neuroblast lineages of two brain regions of *Drosophila* were identified, the tritocerebrum (TR; see chapter 2) and the subesophageal ganglion (SEG; see Chapter 3), which account together for 4 of the 6 brain neuromeres. The neuroblasts that give rise to the lineages of the TR and SEG delaminate from the procephalic and ventral neurogenic region in the embryo (reviewed in Technau et al., 2006; Fig. 5.1). In particular, 13 tritocerebral and approximately 80 subesophageal neuroblasts neuroblasts per side delaminate in these brain regions until embryonic stage 11 (R. Urbach, personal communication; Urbach and Technau, 2003). At this stage, all tritocerebral neuroblasts express *labial* and the posterior neuroblasts of each neuromere are positive for *engrailed* (Technau et al., 2006; Urbach and Technau 2003). We used *labial* and *engrailed* expression follow the development of the tritocerebral and subesophageal neuroblasts into late embryonic stages. In this assay, we found a drastic reduction in neuroblast numbers in the TR (from 13 to 6) and SEG (from 80 to 24). Altogether, about 2/3 of neuroblasts in these regions disappear at the end of embryogenesis. This is comparable to the abdomen where the majority of neuroblast undergoes programmed cell death in late embryonic stages (White et al., 1994). Accordingly, a substantial fraction (ca. 50%) of the SEG neuroblasts terminates via cell death, as revealed by apoptosis-block experiments. The other half may be terminated by prospero-dependent cell cycle exit (Maurange et al., 2008). Only a small part of initial number of TR and SEG neuroblasts are reactivated in early larval stages to produce

postembryonic progeny. In the case of the TR, we found a number of 6 and in the case of the

SEG a number of 19 neuroblasts at the late L2 stage directly before molting. However, from the

L3 stage onwards, only 4 tritocerebral and 14 subesophageal neuroblasts were observed. The fate

of the neuroblasts that do not make it to the L3 stage is very likely programmed cell death.
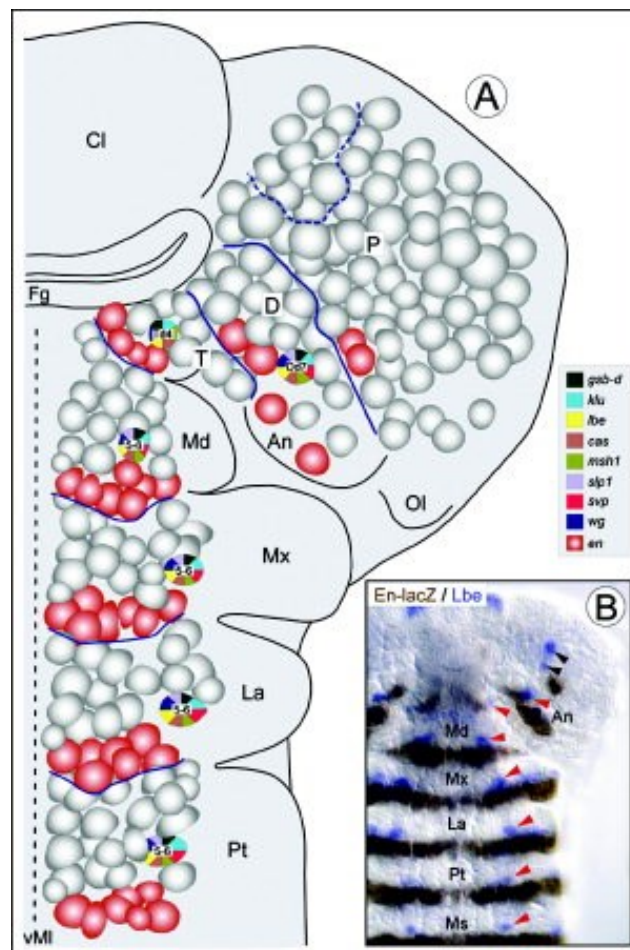
Fig. 5.1: Neuroblast map of the SPG, SEG and Prothorax at stage11 (Technau et al., 2006)

(A): Semi-schematic presentation of the right half of a flattened stage 11 *Drosophila* embryo (anterior to the top). SPG: T, Tritocerebrum; D, Deutocerebrum; P, Protocerebrum. SEG; Md, mandibular segment; Mx, maxillary segment; La, labial segment. Pt, prothoracic segment. An, antennal segment. Ol, optic lobe anlage **(B):** Stage 11 flat preparation double stained for Engrailed with the focus on the level of neuroblasts. Anterior to the top. Ms, mesothoracic segment.

This is supported by clonal apoptosis-block experiments which lead to the survival of ectopic neuroblasts corresponding with the position of the progenitor cells that disappear at the L2/L3 transition phase. The surviving neuroblasts of the TR and SEG divide until late larval stages to give rise to an invariant number of lineage clusters that account for most of the postembryonically generated cells in these brain regions (see Fig. 3.6O). In particular, the tritocerebral neuroblasts give rise to 4 lineages: BAlp4, BAlv, TRdm and TRdl. The subesophageal neuroblasts give rise to 14 newly identified lineages: SA1, SA2, SA3, SA4, SA5, LB0, LB3, LB5, LB6, LB7, LB11, LB12, LB19 and LB23. In contrast, a much higher number of postembryonic neuroblast lineages were reported to be found in the protocerebral (PR), deutocerebral (DE) neuromeres and the thorax. Of the ca. 90 embryonic neuroblasts of the PR and DE, about 80 are reactivated to produce postembryonic lineages (Urbach and Technau, 2003; Wong et al., 2013). Similarly, of the 90 thoracic neuroblasts in the embryo, about 75 are reactivated and give rise to postembryonic lineages (Skeath and Thor, 2003; Truman et al., 2004). Thus, a dramatic reduction of neuroblast lineages takes place in the postembryonic TR and SEG regions.

Especially evident is this reduction in the anterior SEG. As described in chapter 3.3.1 and 3.3.2, late larval landmark features defined by Truman et al. (2004) for the thoracic neuromeres are present only partially in the SEG. Prominent features of the larval thoracic neuromeres are the ventral, intermediate and dorsal commissures (aV, aI, pI, aD, pD; Truman et al., 2004). In contrast, only the posterior region of the larval SEG (the labium) has aI, pI and pD commissures – aV and aD are missing. In the anterior SA region, only the aI commissure is present, while no other commissures were found. Moreover, 9 lineages in the posterior SEG could be assigned to

thoracic counterparts due to the neuroanatomical similarity, but no lineages in the SA region resembled any thoracic lineages.

What could be the reason for this reduction in neural cell generation in the TR and SEG in postembryonic stages? To address this question, we have to know more about the functions of the neurons in these regions. Interestingly, both regions, the TR and the SEG, are associated with gustation. Sensory neurons of the internal mouthparts project along the pharyngeal nerve to the TR and the SEG is the fly`s primary gustatory center that receives input from incoming gustatory sensory fibers from the labellum via the labial nerve (Miyazaki and Ito, 2010; Rajashekhar and Singh, 1994; Fig. 5.2). If a common function of these regions is indeed the processing of taste information, a reduction would likely reflect a reduced need for this kind of processing in the adult. Given the higher number of embryonic neuroblasts in the TR and SEG, this indicates that embryonic born neuronal lineages play an important role in the functions of these brain regions. However, none of the primary lineages of the TR and SEG are known to date. Only single gustatory neurons that might origin from the embryonic subesophageal neuroblasts were described (Mann et al., 2013; Marella et al., 2012). In contrast, the secondary tritocerebral and subesophageal lineages in the adult were identified recently (Volker Hartenstein, personal communication; Wong et al., 2013). Future experiments are going to hopefully connect embryonic with postembryonic neurogenesis in the TR and SEG, which would help to elucidate the mechanisms behind gustatory processes in the *Drosophila* nervous system.
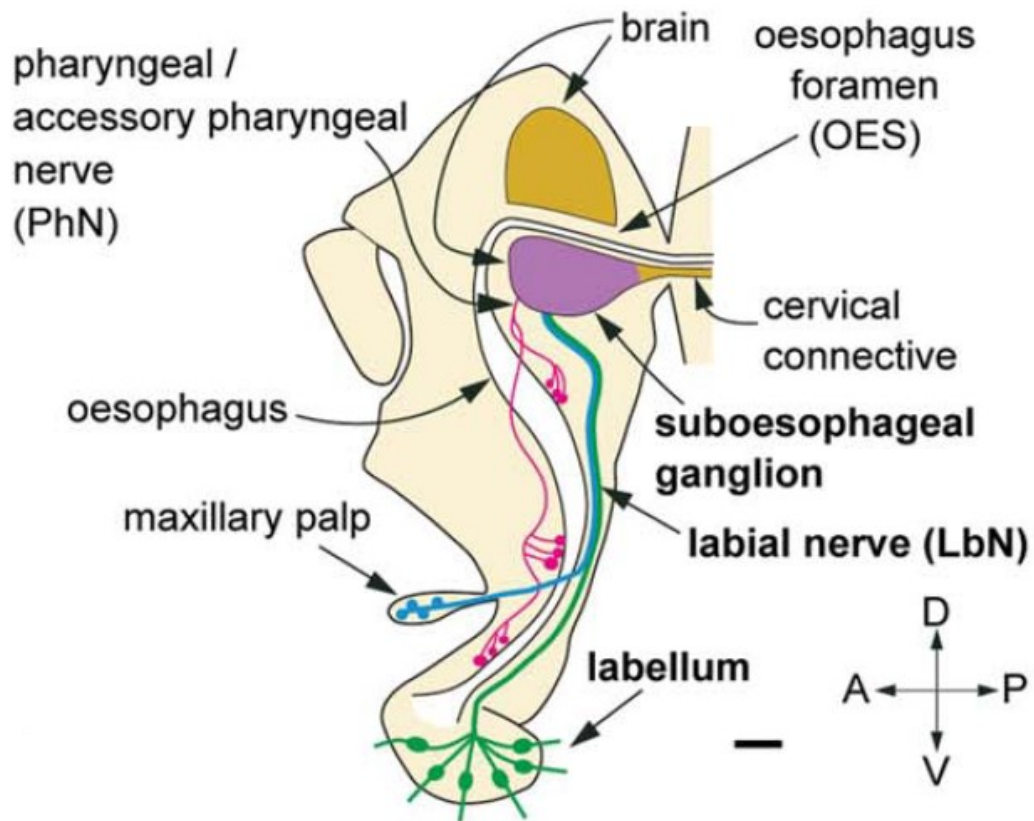
Fig. 5.2: Schematical sagittal section of a fly head

Gustatory receptor neurons (GRNs) send their axons directly via the labial nerve to the subesophageal ganglion. Gustatory neurons along the esophagus project via the pharyngeal and accessory pharyngeal nerves, which enter a more anterior region than the labial nerve. Dorsal up, anterior to the left. From Myazaki and Ito, 2010.

5.2 GENERAL FEATURES OF HOX GENE ACTION IN BRAIN DEVELOPMENT

In this work, the expression and function of the Hox genes *lab*, *Dfd*, *Scr* and *Antp* in postembryonic brain development of *Drosophila* was analyzed. Thus, in this study, all Hox genes of the ANTP-C were covered except *proboscipedia*. Hox gene expression can be detected already in the embryonic brain. *lab* starts to be expressed at embryonic stage 9 in the ectoderm of the intercalary segment, and all neuroblasts which arise from Lab-positive neuroectoderm also express *lab*. Altogether, 15 lab-expressing neuroblasts delaminate from this region, of which 13 are belonging to the TR and 2 to the DE. (Urbach and Technau, 2003). In the SEG, 44 neuroblasts express *Dfd*, 25 neuroblasts express *Scr,* and 7 neuroblasts express *Antp* during embryonic development (R. Urbach, personal communication). *Dfd* expression covers the MD and the anterior MX neuromere, *Scr* is expressed in the posterior MX and most of the LB neuromere and *Antp* expression is present in the posterior LB neuromere. At stage 11, all of these neuroblasts have delaminated. At Stage 14, Hox genes are expressed in a similar parasegmental pattern as described for the neuroblast maps above, indicating that Hox expression is inherited to the postmitotic progeny (Hirth et al., 1998). In subsequent larval stages, Hox genes continue to be expressed in discrete, non-overlapping domains (Fig. 5.3). Thus, Hox gene expression is likely to be a stable feature of *Drosophila* brain development. The lineage analysis in chapter 2 and 3 revealed a small number of postembryonic neuroblast lineages in in the late larval TR and SEG (altogether 18 neuroblast lineages). Most of these lineages in these regions express Hox genes. In the late larva, we found 4 Lab-positive lineages in the TR, as well as 3 Dfd-positive, 5 Scr-positive and 3 Antp-positive lineages in the SEG. Only 3 lineages in these brain regions are Hox-negative. Due to parasegmental Hox gene expression in the larva, it is not possible to assign the
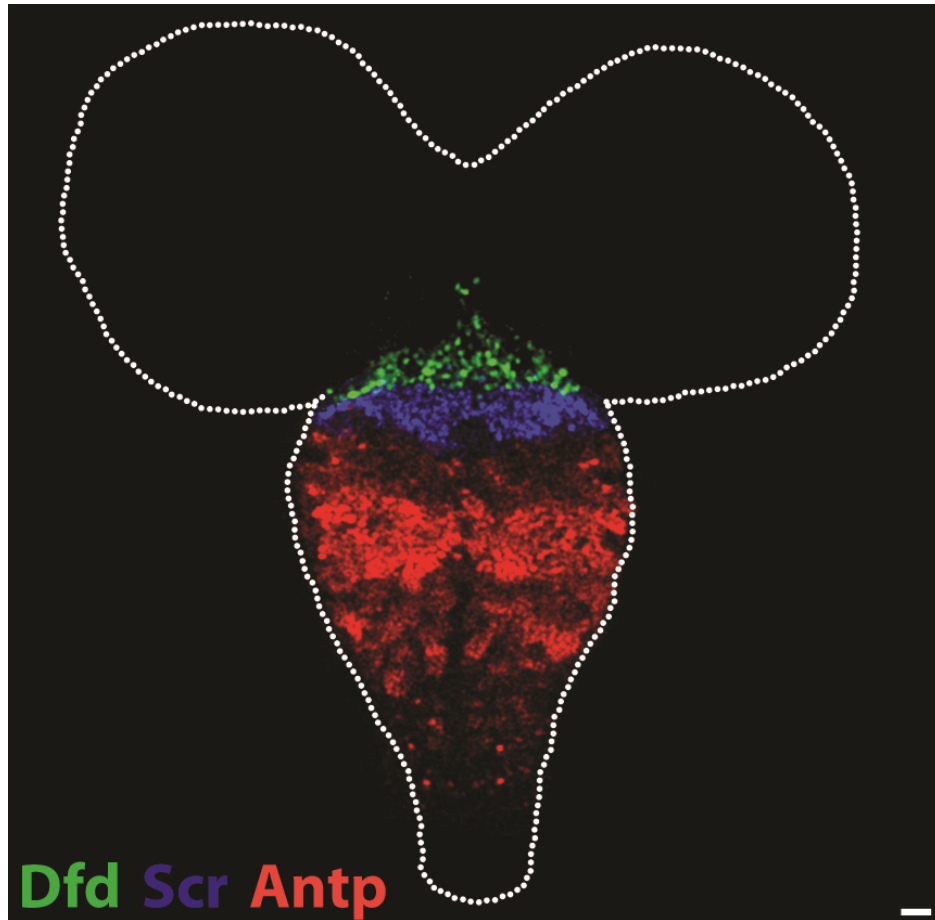
Fig. 5.3: Expression of *Dfd*, *Scr* and *Antp* in the postembryonic *Drosophila* brain and VNC

Immunolabeling against Dfd (green), Scr (blue) and Antp (red) proteins in the late L3 larval brain and VNC. Z-projections of multiple optical sections. Anterior is to the top. Scale bar: 20 µm.

postembryonic SEG lineages to the MD or MX neuromere according to Hox expression patterns. As in the embryo, however, *Antp* is expressed in the larval brain in the posterior region of the LB neuromere. *lab*, *Dfd*, *Scr* and *Antp* are also expressed in a similar pattern in the adult brain (P. A. Kuert, unpublished).

What are the functions of Hox genes in *Drosophila* brain development? Generally, the role of Hox proteins is to determine regional identity along the anterior-posterior axis. The global nature of their mutant phenotypes in appendages (like the antenna-to-leg transformation) indicates that Hox genes must act on many different levels of biological hierarchies. This is indeed reflected in *Drosophila* brain development. In the embryonic brain, *lab* and *Dfd* are expressed in the TR and MD/MX neuromeres of the SEG, and mutational inactivation of these genes leads to a loss-of-neuronal markers in these regions (Hirth et al., 1998). Thus, in the embryonic brain, Hox genes act on the level of neuromeres and are required for proper neuronal identity in the TR and anterior SEG domains. The role of Hox genes in postembryonic brain development of *Drosophila*, however, is different from their role in the embryo.

First, *lab*, *Dfd* and *Scr* act on the level of progenitor cells in the larval TR and SEG. These genes are expressed in postembryonic neuroblasts during L3, but at the same time are required at early larval stages to terminate the proliferation of neuroblasts, which otherwise would give rise to ectopic lineage clusters. As *Antp* and *Ubx* can trigger neuroblast cell death in the thorax and *Abd-A* is required to induce neuroblast apoptosis in the abdomen, the timely precise termination of neuroblasts is likely to be a common postembryonic function of Hox proteins in *Drosophila* nervous system development (Bello et al., 2003). Interestingly, the action of these Hox genes seems to be different from anterior to posterior. In the postembryonic *Drosophila* CNS, in the TR and SEG, Hox genes (*lab*, *Dfd*, *Scr*) are expressed mostly in the neuroblasts, in the posterior SEG and thorax Hox genes (*Antp*, *Ubx*) only partially in neuroblasts and in the central abdomen Hox genes (*Abd-A*) are only expressed in short pulse to kill the progenitor cells (Bello et al., 2003; Marin et al., 2013; P. A. Kuert, unpublished). This suggests that the ability of Hox proteins to terminate postembryonic neuroblasts in the *Drosophila* CNS increases from anterior to posterior

regions. Another difference in Hox gene-dependent neuroblast termination between the (more anterior) postembryonic brain and (more posterior) VNC is the timing: In the central abdomen, neuroblasts are terminated by a pulse of *Abd-A* expression occurring at the mid-L3 stage (Bello et al., 2003). In contrast, supernumerary neuroblasts in the brain (TR and SEG) are terminated at the L2/L3 molting stage. Also, there seems to be no pulse of Hox expression involved, as the additional Hox expressing neuroblasts are present from early larval stages on in the SEG (P. A. Kuert, unpublished). These results indicate the action of additional factors which are responsible for the timing of the Hox-positive neuroblast termination in the postembryonic brain. As the termination event occurs at a molting stage, ecdysone signaling would be a likely candidate for such a factor (reviewed in Ou and King-Jones, 2013). However, MARCM experiments inducing misexpression of a dominant-negative allele of the ecdysone receptor did not phenocopy the Hox mutant phenotype of ectopic lineages (induced in the early larva; recovered in late L3; n = 15 specimens). This indicates that ecdysone is not the timer which controls the Hox-dependent neuroblast apoptosis in the brain. Another timing mechanism in *Drosophila* neurogenesis is the family of so-called temporal genes which are subsequently expressed in neuroblasts and their progeny to specify different temporal identities (reviewed in Li et al., 2013). Furthermore, a model was proposed in which the successful execution of the temporal cascade is required for the termination of neuroblasts in the ventral nerve cord (Maurange et al., 2008). In this model, neuroblasts would either terminate their proliferation via prospero-dependent cell cycle exit in the thorax at the pupal stage (Type I) or undergo Hox-dependent programmed cell death in the abdomen at larval stages (Type II). Is it reasonable that the Hox-dependent termination of neuroblasts in the TR and SEG is a Type II event involving the temporal cascade? To address this question, we performed a MARCM analysis with a mutant allele of the larval-specific temporal

gene *seven-up* (*svp*; induced in the early larva; recovered in late L3; n = 62 specimens). Indeed, in these experiments ectopic lineages in the TR and SEG were found which are were neuroanatomically similar to the ectopic lineages recovered in Hox and cell death mutant assays (Fig. 5.4). We conclude from these experiments that, in the central brain, Hox and temporal proteins are required together for neuroblast termination. However, ectopic lineages similar to the *Dfd*-dependent ectopic lineages were not found in the *svp* mutant assay, implicating the existence of other additional mechanisms of neuroblasts termination in the central brain.
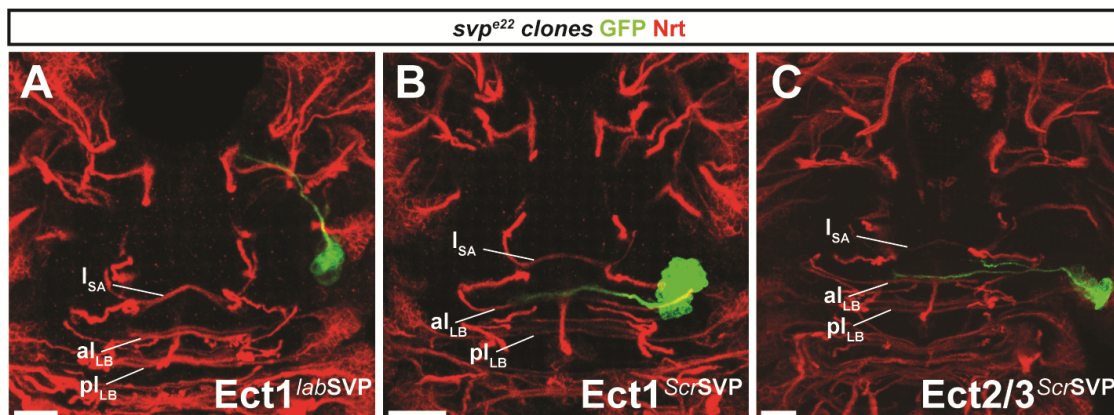
Fig. 5.4: Clonal mutation of the temporal transcription factor *seven-up*.

MARCM clones homozygous for *svp^{e22}* (GFP), in L3 brains immunostained with anti-BP106 (Nrt). Intermediate commissure of SA region (I_{SA}); Anterior and posterior intermediate commissure of LB neuromere (aI$_{LB}$; pI$_{LB}$) **(A)** Ect1^{labSVP} in the tritocerebral region. **(B, C)** Ect1^{labSVP} and Ect2/3^{labSVP} in the posterior SEG region. Ventral view. Scale bars: 20 μm.

Second, Hox proteins in the postembryonic TR and SEG act on the level of progeny cells. Expression of Hox genes in progeny cells of all TR neuroblast lineages and all but three SEG neuroblast lineages were found in the late L3 brain. Expression patterns vary and ranged from

very low expression in a few cells in SA3 to strong expression in all cells of the BAlp4 lineage. We could identify two different requirements for Hox proteins in the postembryonic central brain that act on the progeny level. Unlike the embryonic brain, loss of Hox genes is not associated with loss of neuronal identity, as Hox mutant neurons in the postembyronic TR and SEG did still extend axons (Hirth et al., 1998). However, neurons of the SA1, SA5 and LB3 lineages exhibited reproducible axonal misprojections when mutated for *Dfd*, *Scr* or *Antp*, respectively. In addition, misexpression of *lab* could induce similar defects in the PG5 lineage which is located close to the TR. This data indicates that all Hox genes in the central brain (with the exception of the *proboscipedia* gene, for which there is no data) are able to influence the guidance of SATs. A second type of Hox mutation phenotype found in the postembryonic brain was a reduction in cell number of about 50 % in the LB3 and LB5 lineage. Two main reasons could explain this result. On one hand the neuroblast could divide slower and thus give rise to fewer neurons until the late L3 stage. However, the neuroblast of LB3 is clearly Hox-negative in the late larva which makes a neuroblast-dependent action to be unlikely. On the other hand, cell number reduction could come from programmed cell death in the progeny cell themselves. This explanation is more favorable since the percentage of cell number reduction (around 50%) is likely to involve hemilineage-dependent programmed cell death involving Notch signaling (e.g. Kumar et al., 2009; Lin et al., 2010; Truman et al., 2010). We conclude from our experiments that Hox gene expression is required for neuronal survival in the LB3 and LB5 lineages. Moreover, this function of Hox genes seems to be involved in other part s of the *Drosophila* nervous system, as *Antp* was shown to be required for survival for motoneurons in the postembryonic thorax (Baek et al., 2013).

Altogether, we could identify three different functions of Hox genes in the postembryonic TR and SEG (Fig. 5.5). First, Hox genes are required for the timely-precise termination of specific

neuroblasts during early larval stages, second, they are required and sufficient in specific lineages for targeting of secondary axon tracts and third, they are needed for neuronal survival in specific lineages. However, in the majority (TR: 4 out of 4; SEG: 7 out 11) of Hox-expressing neuroblast lineages in the postembryonic central brain, we could not identify a reproducible Hox gene mutation phenotype.
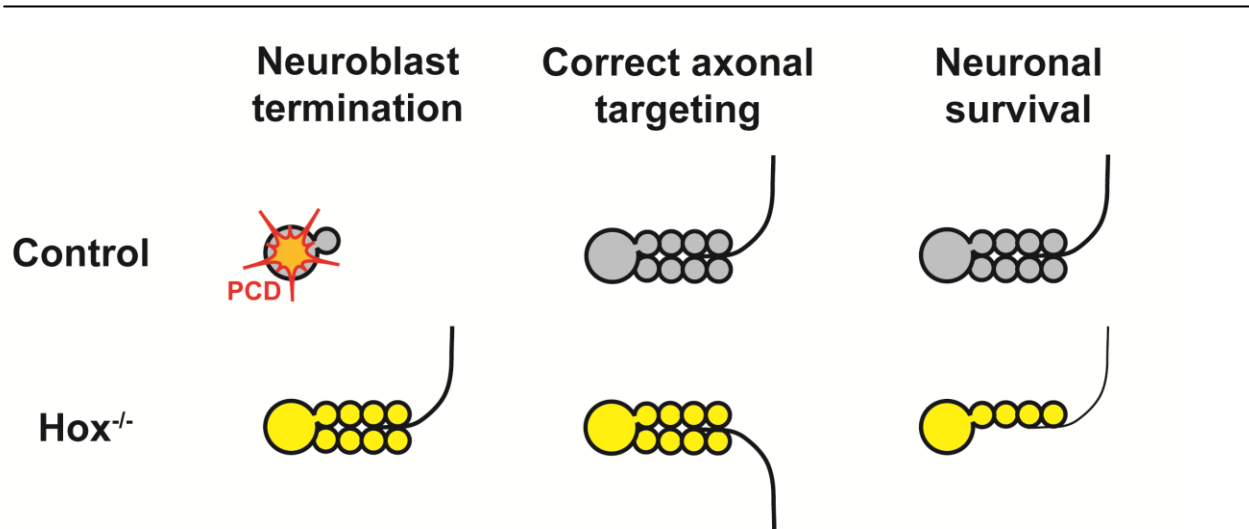
Fig. 5.5: Summary of Hox functions in the postembryonic brain of *Drosophila*

In this model, Hox genes are cell autonomously required for three different, lineage-specific functions in postembryonic development of the *Drosophila* brain. First, Hox genes act on the level of progenitor cells to induce programmed cell death in neuroblasts that would otherwise give rise to ectopic lineages. Second, Hox genes are required for lineage-specific correct targeting of secondary axon tracts. Third, Hox genes are required for neuronal survival in specific lineages, most likely in a hemilineage-dependent manner.

What might be the function of Hox genes in those postembryonic lineages that did not show a phenotype of Hox gene mutation in the late larval brain? As stated above, Hox genes are also expressed in the adult central brain in a similar pattern as in the larva. This suggests that Hox proteins are required not only for the development, but also for the functioning of differentiated

Drosophila neurons. Indeed, it was recently shown that Hox genes are required in neurons for activation and maintenance of a neuropeptide, Leucokinin (Estacio-Gómez et al., 2013). In particular, *Scr* was shown to be sufficient to activate the expression of Leucokinin in thoracic and abdominal segments. Thus, it might be true that Hox genes are needed for the control of neuropeptide expression in the central brain too.

How does this study help us to understand the brain development in animals other that *Drosophila*? Vertebrates, for instance, do not have a tritocerebrum or subesophageal ganglion. However, Hox genes are expressed during CNS development of diverse bilaterian animal groups, including vertebrates, hemichordates, insects, and annelids, and in all of these animal groups the order of Hox gene expression domains in the developing CNS appears to be conserved (see Reichert and Bello, 2010). In the mammalian mouse model, *Hoxb* orthologs of the *Drosophila* genes *lab*, *Dfd*, *Scr* and *Antp* are expressed in the rhombomeric hindbrain and spinal cord in an order similar to the fly Hox genes (see Fig. 4.1). In addition, not only expression but also functions of Hox genes seem to be conserved in insects and mammals (see Philippidou and Dasen, 2013). Similar to the embryonic phenotype of the *lab* mutation in the TR and the *Dfd* mutation in the SEG , knockout of the *lab*-ortholog *Hoxb1* leads to a loss of identity in the rhombomere4 region, as rhombomere4-specific markers are not expressed any more in this region (Hirth et al., 1998; Goddard et al., 1996; Studer et al., 1996). Another *lab*-ortholog – *Hoxa1* – is required to prevent the formation of cells that otherwise give rise to an ectopic neuronal circuit in the mouse hindbrain (Del Toro et al., 2001). This Hox function also strikingly reminds on the function of *lab*, *Dfd* and *Scr* in the postembryonic *Drosophila* brain, where they are required to terminate neuroblasts before they produce ectopic lineages. Moreover, the functions of Hox genes in axonal targeting and neuronal survival in the *Drosophila*

postembryonic nervous system seem to be conserved as well. Deletion experiments in mice showed that the *Scr*-orthologs *Hoxa5* and *Hoxc5* are required for branching and survival of neurons in the phrenic motor column which controls the rhythmic neuronal firing needed for respiration in mammals (Philippidou et al., 2012). The requirement of Hox genes to promote neuronal survival in the larval SEG is also conserved. Similar to the cell number reduction in the *Antp*-mutant lineage LB3, mutation of the Antp-orthologs *Hoxa6* and *Hoxc6* leads to a cell number reduction of ca. 50% in the mammalian lateral motor column (Lacombe et al., 2013). Taken together, these findings suggest that Hox gene expression patterns and functions, which were found in the postembryonic tritocerebrum and subesophageal ganglion of *Drosophila*, are remarkably well conserved in the development of the mammalian nervous system.

6. METHODS

6.1 GENETICS

Unless otherwise stated fly stocks were obtained from the Bloomington Stock center. Gal4 lines that were used: *engrailed*-Gal4, UAS-H2B::YFP (A. Brand, P. Fichelson, S. Sprecher) \ *fasciclin*-GFP (Siebert et al., 2009) \ *inscuteable*-Gal4>UAS-GFP (L. Jan, Y. Jan) \ GAL4^{Mz1407}, UAS-mCD8::GFP^{LL5} (Luo et al. 1994; Betschinger et al., 2006; B. Bello) \ w; *wor*-GAL4, UAS-mCD8::GFP^{LL5} (Albertson et al, 2004). Additional lines: Df(3L)H99, kni^{ri-1} p^p \ w^{1118}. For early larval heatshock MARCM experiments (Lee and Luo, 1999; Lee and Luo, 2001), embryos were collected on standard medium over a 4-8 hour time window, raised at 25°C for 24 or 48 hours and then heat-shocked for 1 hour at 37°C. For embryonic heatshock MARCM experiments, embryos were collected for an 8 hour time window on standard medium at 18°C, raised at 18°C for 15 hours and afterwards heatshocked for 1 hour at 37°C. MARCM drivers: hsFLP^{122}; *tub*P-GAL4, UASmCD8::GFP^{LL5}; FRT2A, *tub*PGAL80^{LL9} (B. C. Bello) \ hsFLP^{122}; *tub*P-GAL4, UAS-mCD8::GFP^{LL5}; FRT82B, *tub*P-GAL80^{LL5} (B. C. Bello). MARCM responders: FRT2A, Df(3L)H99, *kni*$^{ri-1}$ (B. C. Bello) \ FRT82B \ FRT82B, *Antp*$^{NS+RC3}$, *e* (B. C. Bello) \ FRT82B, *Ki*^{1}, *Dfd*^{12}, *p*^{p} (B. C. Bello) \ FRT82B, *Ki*^{1}, *pb*^{5}, *Dfd*^{16}, *e*^{1} (B. C. Bello) \ FRT82B, *lab*^{14}, *p*^{p} (P. A. Kuert) \ FRT82B, *pb*^{27}, *Scr*^{2}, *p*^{p}, *cu*, P{*w*$^+$, *ry*$^+$}90E (Percival-Smith et al., 1997) \ FRT82B, *Scr*^{4}, *Antp*^{25}, *p*^{p} (B. C. Bello) \ FRT82B, *svp*^{e22} (B. C. Bello) \ UAS-EcR^{DN}; FRT82B \ UAS-*labial*; FRT82B \ UAS-*labial*; FRT82B, *lab*^{14}, *p*^{p} (P. A. Kuert). For RNAi experiments, UAS-*lab*RNAi^{2990} (obtained from VDRC) was crossed to GAL4^{Mz1407}, UAS-mCD8::GFP^{LL5} and *wor*-

GAL4, UAS-mCD8::GFP^{LL5}, *act*GFP^{JMR1}. For p35 cell death block experiments, UAS-p35^{BH2} was crossed to GAL4^{Mz1407}, UAS-mCD8::GFP^{LL5}. For p35 cell death block experiments, UAS-p35^{BH2} was crossed to GAL4^{Mz1407}, UAS-mCD8::GFP^{LL5}.

6.2 IMMUNOHISTOCHEMISTRY

Embryos and larval brains were fixed and labeled as previously described (Bello et al., 2007; Patel, 1994; Pereanu and Hartenstein, 2006). Primary antibodies: guineapig-anti-Deadpan (1:500 or 1:1000; J. Skeath), guineapig-anti-Deformed (1:200; W. McGinnis), mouse-anti-Antennapedia (1:100; Condie et al., 1991), mouse-anti-BP106 Neurotactin (1:10 or 1:20; DSHB), mouse-anti-Engrailed (1:1 or 1:5; DHSB), mouse-anti-nc82 (1:20; DSHB), rabbit-anti-Deformed (1:100; T. Kaufman), rabbit-anti-Labial (1:200; F. Hirth, H. Reichert), rabbit-anti-Sex combs reduced (1:400; LeMotte et al., 1989), rat-anti-Labial (1:200; F. Hirth, H. Reichert), rat-anti-N-Cadherin (1:25; DSHB). Alexa (Molecular Probes) and Dylight (KPL) secondary antibodies were used between 1:200 and 1:500.

6.1 MICROSCOPY AND IMAGE PROCESSING

Images were recorded using a Leica SP or a Zeiss LSM 700 Meta confocal microscope. Optical sections ranged from 1 to 2 µm. Resolution of images were either 512x512 or 1024x1024 pixels. Collected images were processed and arranged using ImageJ, Fiji, Adobe Photoshop and Adobe Illustrator. For highlighting specific MARCM clones, cell bodies and neurites from other lineages in the vicinity were removed in every single optical section. In Fig. 5.3, background signal and staining in the optic lobes were removed. 3D models were generated with Fiji 3D viewer and Bitplane Imaris.

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