Analysis of the *engrailed*-expressing neuroblast lineages in *Drosophila* brain development

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to Sachin, Ma and Papa

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“You can know the name of a bird in all the languages of the world, but when you're finished, you'll know absolutely nothing whatever about the bird... So let's look at the bird and see what it's doing - that's what counts. I learned very early the difference between knowing the name of something and knowing something”- Richard Feynman.

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Summary

The segment polarity gene *engrailed* (*en*) encodes a homeodomain transcription factor which is expressed in metamerically reiterated stripes in the embryonic neuroectoderm, in some primary neuroblasts and their progeny; usually located at the posterior boundary of each embryonic CNS neuromere. In this thesis, we have analyzed the expression of *en* in the larval and adult brains. In the late larval brain, four secondary neuroblast lineages (three protocerebral lineages and one deutocerebral lineage) express *en* in specific subsets. However, in the adult brain, only three of the four lineages express *en*.

In a first study (Chapter 2), we have characterized the neuropile innervation pattern of *en*-expressing central brain neuroblast lineages in embryonic, larval and adult stages. Firstly, based on *en* expression data and anatomical criteria, we are able to link primary lineages in the larva to secondary, adult-specific lineages. Secondly, the neurons of the *en*-expressing lineages form most arborizations, particularly their proximal branches, in the same brain neuropile compartments throughout development. Thirdly, the *en*-positive lineages of differing neuromeric origin and therefore, from different brain neuromeres innervate a non-overlapping set of neuropile compartments. Thus, the lineages appear to respect boundaries between neuromere-specific compartments in the brain and our findings support a model for neuromere-specific brain neuropile. Moreover, using genetic labeling techniques, this attempt is the first of its kind that links larval and adult brain anatomy at higher resolution.

In our second study (Chapter 3), we have analysed the total number and pattern of *en*-expressing, adult-specific cells in each of the four, identified larval neuroblast lineages mentioned above. Firstly, there are lineage-specific differences in number as well as expression of *en* in the four lineages examined. Secondly, this difference is established due to programmed cell death, which has a pronounced effect on the number of cells; approximately half of the immature adult-specific neurons in three of the four lineages are eliminated by cell death during development. Furthermore, programmed cell death selectively affects *en*-positive versus *en*-negative cells in a

lineage-specific manner and, thus, controls the relative number of *en*-expressing neurons in each lineage. Our data provide evidence for a hemilineage-specific cell death model; i.e, either half of a lineage is targeted by programmed cell death. Further testing of this model by analysis of single and two cell clones in one of the four lineages also supports the proposed model. Finally, Notch signalling is involved in the regulation of *en* expression and consequently, is implicated to play a role in generation of the hemilineages. This study is the first of its kind to demonstrate the prominent role of lineage-specific programmed cell death in the generation of neuronal number and lineage diversity in the *Drosophila* postembryonic, central brain.

In conclusion, the use of *en* as a molecular marker has helped us characterise brain anatomy in greater detail. In addition, the analysis of the *en*-expressing neurons in the central brain has revealed a surprisingly predominant and lineage-specific role of programmed cell death in the control of neuronal number.

Index

1 Introduction

1.1 *Drosophila* NEUROGENESIS

In insects, the embryonic brain consists of a supraoesophageal ganglion that can be subdivided into the protocerebral, deutocerebral, and tritocerebral neuromeres and a suboesophageal ganglion that is subdivided into the mandibular, maxillary, and labial neuromeres. The developing ventral nerve cord extends posteriorly from the suboesophageal ganglion into the body trunk (Campos-Ortega and Hartenstein, 1997). In this work, we will use the term 'brain' equivalent to the supraoesophageal ganglion.

In *Drosophila*, most neuroblasts (Nbs) have two proliferative periods: an initial brief period during embryogenesis that generates the primary neurons of the functional larval central nervous system (CNS), and a second prolonged period during larval and early pupal stage that generates the secondary or adult-specific neurons. The secondary neurons make up 90% of the adult CNS. These two neurogenic periods are separated by a time window lasting from late embryogenesis to approximately the second half of first instar stage where most brain Nbs persist in a cell-cycle arrested state (Prokop and Technau, 1991; Truman and Bate, 1988).

Proliferating Nbs undergo sequential cycles of self-renewing divisions, dividing asymmetrically to produce ganglion mother cells (GMCs) that in turn divide once to produce two post-mitotic daughter cells (Truman and Bate, 1988). Thus, during larval life the adult-specific progeny of each Nb accumulates in a growing cluster of immature neurons that extend fasciculated neurites (or secondary lineage axon tracts, SATs) close to the neuropile but wait until metamorphosis to complete their extension to adult specific synaptic targets (Dumstrei et al., 2003; Truman et al., 2004; Zheng et al., 2006). Whereas the primary, larval-functional progeny of each Nb show a high degree of phenotypic diversity (Brody and Odenwald, 2000; Kambadur et al., 1998), the adult-specific cells in a given lineage are

remarkably similar and typically project to only one or two initial targets in the larva (Pereanu and Hartenstein, 2006; Truman et al., 2004; Zheng et al., 2006). During metamorphosis the adult brain forms by neuronal remodelling of larval functional neurons and final morphogenesis of adult-specific neurons (Lee et al., 2000; Marin et al., 2005; Zheng et al., 2006).

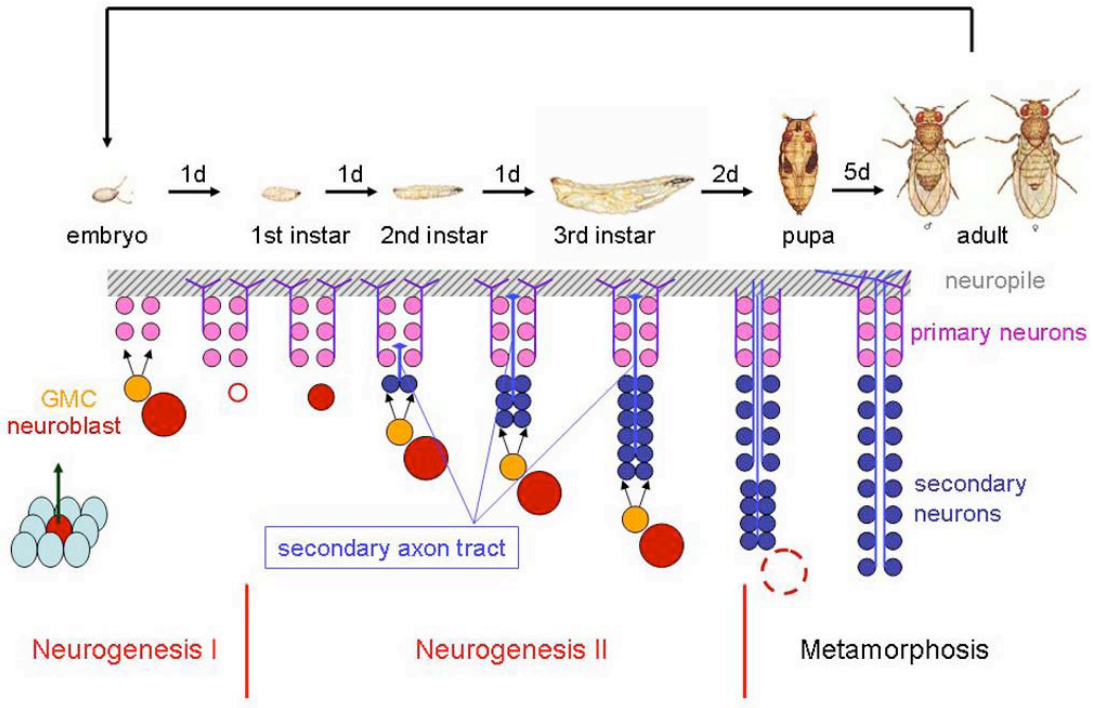

Figure 1-1. Lifecycle and neurogenesis in *Drosophila*. Two phases of neurogenesis, separated by a quiescent state of the neuroblast, produce primary and secondary neurons of the same lineage. Whereas the primary progeny of the neuroblast quickly differentiates into functional neurons of the larva, cell of the secondary lineage wait until metamorphosis to extend their projections (see text for more detail).

The neural-stem cells like neuroblasts, for the most part, generate the vast arrays of different neural cell types that characterize the complex circuits of the brain. It is thus, one of the most challenging problems in biology as to what are the molecular mechanisms underlying this process. In the past two decades, significant progress has been made in understanding the mechanisms underlying specification and division control in neural stem cells in *Drosophila.*

In the early embryo, approximately 100 bilaterally symmetrical Nbs segregate from the neuroepithelium in a stereotyped array, each of which can be uniquely identified and can generate lineage-specific subunits of the brain. A detailed analysis of the expression of over 30 developmental control genes in the embryonic brain has shown that specific combinations of gene expression uniquely identifies all embryonic brain Nbs (Urbach and Technau, 2003a, b). Over the past decade, molecular genetic analysis utilizing mainly the Gal4\UAS system (Perrimon and Brand, 1993) in early embryonic development has resulted in the identification of developmental control genes that are involved in generating the larval functional brain. For a number of these developmental control genes, loss of function analyses have revealed severe defects in neurogenesis, patterning, and circuit formation during embryonic brain development (Hirth et al., 1995, 1998, 2003; Kammermeier et al., 2001; Noveen et al., 2000; Urbach and Technau, 2003a, b, c). However, it is still largely unclear how these genes, and the embryonic process that they control, relate to the anatomical and functional diversities as well as specify the clonal sub-units of the adult brain.

Classical neuroanatomical studies describe the architectural subunits of the adult *Drosophila* brain in terms of their spatial coordinates as they appear in the adult (Strausfeld, 1976). A number of recent publications have addressed the analysis of the developmental origin of adult brain units taking advantage of the MARCM (Mosaic Analysis with a Repressible Cell Marker) system (Jefferis et al., 2001; Jefferis et al., 2004; Kimura et al., 2005; Komiyama et al., 2003; Lee and Luo, 1999; Marin et al., 2005; Zheng et al., 2006). Upon heat-shock induced mitotic recombination (by induction of yeast Flippase) in the Nb all clonally related cells are labeled with a membrane-bound marker, and therefore, projection patterns of neurons can be studied in the context of overall brain architecture. Furthermore, clonal mutant analysis enables us to study homozygous mutant clones in a heterozygous background. In

addition, fine neuronal morphology or the timing of developmental processes can be studied at single-cell clone resolution with MARCM (reviewed in Lee and Luo, 2001).

Moreover, a recently published Nb lineage atlas of developing adult brain in the late larva subdivides each brain hemisphere into approximately 100 clonal lineages, each represented by a fasciculated neurite bundle that forms an invariant pattern in the neuropile (Pereanu and Hartenstein, 2006). Furthermore, to date, only a few early developmental control genes have been analysed in postembryonic brain development (Callaerts et al., 2001; Hassan et al., 2000; Hitier et al., 2001; Kurusu et al., 2000; Lichtneckert et al., 2007, 2008; Pereanu and Hartenstein, 2006). Therefore, the question arises whether classical developmental control genes implicated in early embryonic neurogenesis and neural patterning, are re-used at later developmental stages in the brain. Observations of that kind have been recently made in vertebrates (Zapala et al., 2005). Another question that arises is how this limited number of neuroblasts can generate the enormous number of neural cell types that make up adult brain circuitry. The underlying molecular developmental mechanisms of these processes are currently poorly understood.

The overall goal of the research study done here was to analyze some of the developmental mechanisms by which neuroblasts generate the lineage-specific units of the adult brain and specify the number and diversity of cell types in each of these units through a lineage-based molecular genetic dissection.

1.2 THE *engrailed* GENE IN INSECT NEURAL DEVELOPMENT

The *engrailed* (*en*) gene is a homeodomain-containing transcription factor with numerous, overlapping roles in embryonic development. It was first characterized as a *Drosophila* mutation that fails to form a correct wing segment with anterior and posterior boundaries (Garcia-Bellido and Santamaria, 1972). The fly embryo, during its development, is divided into fourteen segments. This process is controlled by gap genes and pair rule genes (Nusslein-Volhard et al., 1987). Within each segment, the segment polarity gene *en* is sufficient to specify the anterior- posterior polarity. Expression of *en* imparts posterior identity to the compartment within the segment and its absence is sufficient to give the compartment an anterior identity (Kornberg, 1981).

en is expressed in metamerically reiterated stripes in the embryonic epidermis and deriving neuroectoderm (Bossing et al., 1996; Younossi-Hartenstein et al., 1996). This striking periodic expression of *en* also extends more anteriorly into regions of the embryonic head that are not obviously segmented.

en expression in the embryonic head is seen in five discrete clusters of cells (Diederich et al., 1991), though some discrepancy in the number has been reported (DiNardo et al., 1985). The five clusters of *en* expression appear in the following sequence from stage 8 onwards during embryogenesis (stages after Campos Ortega and Hartenstein, 1997): the “en antennal stripe”, the “en headspot”, the “en intercalary spot”, the “en expression in the anterior dorsal hemispheres” and the “en expression in the clypeolabrum”. Subsequently, the en antennal stripe and the en head spot split in two groups of cells, thus generating in total 7 en spots. This notable pattern argues for the presence of seven head segments (4 pregnathal and 3 gnathal) in *Drosophila* (Schmidt-Ott and Technau, 1992). The development of the *Drosophila*

central nervous system (CNS) begins after gastrulation between embryonic stages 9-11, as single cells within the ventral neuroectoderm enlarge and delaminate into the embryo to form a stereotyped array of about 100 neuroblasts (NBs) (Doe, 1992). In the embryonic CNS, *en* expression is seen in the neuroectoderm and in some neuroblasts (and GMCs) that derive from these neuroectodermal domains, and in well defined groups of postmitotic primary and secondary neurons, usually located at the posterior boundary of each CNS neuromere (Bossing et al., 1996; Schmid et al., 1999; Urbach and Technau, 2003a, c; Younossi-Hartenstein et al., 1996).

en expression in the putative embryonic brain has been reported in 9 NBs as follows: from stage 8 in the antennal segment(*en* antennal stripe as), four deutocerebral NBs (Dv8, Dd5, Dd9, Dd13) delaminate; from stage 9 in the ocular segment, the *en* head spot (hs), from which two protocerebral NBs (Ppd5, Ppd8) evolve; and from stage 10 in the posterior intercalary segment (*en* intercalary stripe; en is), which gives rise to three to four tritocerebral NBs (Tv4, Tv5, Td3). Therefore, all four pregnathal head segments contribute to the early embryonic brain (Urbach and Technau, 2003a, c). The spatial distribution of the en-positive NBs closely corresponds to the *en* domains of their origin in the ectoderm. This suggests they demarcate the posterior borders of the respective brain neuromeres Also, *en* expression domains in the ventral nerve cord (VNC) define the posterior segmental compartments (DiNardo et al., 1985; Poole et al., 1985), from which NBs of row 6 and 7 and NB1-2 derive (Broadus et al., 1995).

Besides serving as a marker of neuromeric boundaries in the embryonic CNS, *en* also plays a role in neural development. For instance, *en* is expressed in subsets of fly embryo (stage 16) interneurons of the central as well as peripheral nervous systems (PNS). These neurons do not express cell adhesion molecules Connectin or Neuroglian, whereas other neurons that are *en*-negative strongly express these adhesion molecules (Siegler and Jia, 1999). Moreover,

Connectin and Neuroglian expression is eliminated in interneurons when *en* expression is driven ubiquitously in neurons, and greatly increased when *en* genes are lacking in mutant embryos, suggesting that *en* is a negative regulator of *connectin* and *neuroglian*. Differential *en* expression is thus, crucial in determining the pattern of expression of cell adhesion molecules and thus constitutes an important determinant of neuronal shape and perhaps connectivity. Another role for *en* has been demonstrated in the fly embryonic VNC, where *en* interacts with genes like *frazzled* to guide axons and aid in formation of the posterior commissures in the VNC (Joly et al., 2007). Thus, *en* seems to not only impart neuroblast identity, but also has early roles in organizing the architecture of the embryonic CNS.

Most studies addressing the expression and function of *en* have so far been done in the embryonic CNS. In contrast, information on the above in the fly postembryonic brain is lacking. In a recent atlas of adult-specific, secondary lineages, Pereanu and Hartenstein have reported *en* expression in the the late larval central brain as follows: a cluster of primary neurons projecting to the tritocerebrum, three adult-specific lineages in the protocerebrum (DPLam, DALv2/3), and one in the deutocerebrum (BAla) (Pereanu and Hartenstein, 2006). This compares well to the 4 pregnathal segments seen in the embryonic head. In addition, *en*-positive, secondary lineages have also been identified in the VNC (Truman et al., 2004).

Homologues of *en* are present in numerous animal groups including annelids (Prud'homme et al., 2003; Seaver and Kaneshige, 2006; Seaver et al., 2001; Shain et al., 2000; Wedeen and Weisblat, 1991), mollusks (Jacobs et al., 2000; Moshel et al., 1998; Nederbragt et al., 2002; Iijima et al., 2008; Wanninger and Haszprunar, 2001), insects (Boyan and Williams, 2002; Diederich et al., 1991; Peterson et al., 1998; Rogers and Kaufman, 1996; Schmidt-Ott and Technau, 1992; Younossi-Hartenstein et al., 1993;), echinoderms (Sintoni et al., 2007) and vertebrates (Joyner, 1996; Simon et al., 2004; 2005). These homologues have a high degree of

functional rather than sequence conservation. The expression pattern of this segment polarity gene is similar in most of the species except in vertebrates, where the expression pattern is regionalized rather than segmentally reiterated. A few studies have also been reported on the role of *en* in other insect nervous systems, such as cockroach and grasshopper.

In the cockroach cercal system, the large 6m medial sensory neuron requires persistent *en* expression post mitotically, to establish its correct axonal arborization and trajectory (Marie et al., 2002). Knockout of En using dsRNA at different time points in development results in incorrect development of the neuron's arbors, trajectory and synaptic connections. In the grasshopper, each GMC from the medial neuroblast lineage (MNB) produces two neurons of asymmetric type: one is *en*-positive (of interneuronal fate); and one is *en*-negative (of efferent fate). The mature neuronal population results from differential neuronal death among the *en*-negative efferent neurons during the course of embryogenesis (Jia and Siegler, 2002). The continued production of two different types of neurons within a lineage, followed by selective death of some neurons may be a normal mechanism across insect neuronal lineages to generate more interneurons versus relatively few efferent neurons. This provides a flexible and responsive strategy whereby neuronal populations can be tailored to match segmental diversity across the insect body plan, without reconfiguring individual neuroblast lineages. Thus, the homeodomain transcription factor *en* plays diverse roles in a variety of insect nervous systems studied.

1.3 THE *engrailed* GENE IN THE VERTEBRATE NERVOUS SYSTEM

The expression and function of the two *en* homologues (*En*-1 and *En*-2) in the vertebrate mouse model system has been well studied. During mouse development, the homeobox gene *En-1* is specifically expressed across the mid-hindbrain junction, the ventral ectoderm of the limb buds, and in regions of the hindbrain, spinal cord, somites and somite derived tissues (Wurst et al., 1994). Mice homozygous for a targeted deletion of the *En-1* homeobox die shortly after birth and exhibit multiple developmental defects. *En1* is required for midbrain and cerebellum development and postnatally for dorsal/ventral patterning of the limbs. Interestingly, when the coding sequences of *En1* were replaced with Drosophila *en*, mice expressing this transgene had a near complete rescue of the lethal *En1* mutant brain defect and most skeletal abnormalities but not postnatal limb defects (Wurst et al., 1994). These studies demonstrate that the biochemical activity utilized in mouse to mediate brain development has been retained by En proteins across the phyla, and indicate that during evolution vertebrate En proteins have acquired two unique functions during embryonic and postnatal limb development and that only En1 can function postnatally (Hanks et al., 1998). In addition, *En-2* is also required postnatally, dividing the cerebellum into anterior and posterior regions (Millen and Joyner, 1995). Strikingly, four of the *Wnt-7B* expression domains that are adjacent to the *En-2* domains are lost in *En-2* mutant embryonic cerebella. There is some evidence of a potential network of regulatory genes that establish spatial cues in the developing cerebellum by dividing it into a grid of positional information required for patterning foliation and afferents. Similar gene regulatory networks may also exist in *Drosophila* and participate in early patterning of the developing nervous system; arguing for the existence of evolutionarily conserved mechanisms across the phyla.

En-1 and *En-2*, in addition, control the developmental fate of midbrain dopaminergic neurons. *En-1* is highly expressed by essentially all dopaminergic neurons in the substantia nigra and ventral tegmentum, whereas *En-2* is highly expressed by a subset of them (Simon et al., 2001). These neurons are generated and differentiate into their dopaminergic phenotype in *En-1*/*En-2* double null mutants, but are lost soon thereafter. The *en* genes are thus required for the maintenance, rather than the differentiation of the midbrain dopaminergic neurons.

In the developing midbrain of *Xenopus*, *En-2* is expressed in a caudal-to-rostral gradient where it patterns the optic tectum, which is the target of retinal input (Brunet et al., 2005). It has been demonstrated that an external gradient of *En-2* protein strongly repels growth cones of axons originating from the temporal retina and, conversely, attracts nasal axons. *En-2* thus, also participates directly in axonal turning and therby, topographic map formation in the vertebrate visual system.

En-1 expressing cells are expressed in a heterogenous population of interneurons in the chick embryonic spinal cord. Here it directs synaptic connections with motoneurons indicating that it may be involved in interneuron-motoneuron connectivity (Wenner et al., 2000). Furthermore, homologues of the murine *en* genes have been identified in zebrafish (*eng-1* and *eng-2*). Their expression pattern has been characterized in relation to the regionalization of the CNS and generation of morphological boundaries, suggesting a biochemically conserved function for *en* (Fjose et al., 1992).

1.4 PROGRAMMED CELL DEATH IN THE NERVOUS SYSTEM OF *Drosophila*

Programmed cell death is an orchestrated form of cell death in which cells are actively involved in their own death. Cell death occurs during development to provide separation of tissue layers, to sculpt structures and to control cell numbers in both vertebrates and invertebrates. Several examples of the same are well known. For instance, programmed cell death acts to separate the vertebrate digits (Chen and Zhao, 1998), the vertebrate trachea, and the esophagus (Zhou et al., 1999).

Cell death has been recognized as an important event in the normal development of the mammalian nervous system, where it appears to be fundamental for the control of the final number of neurons and glia cells. Cell death is also required for morphogenetic processes involved in development, such as neural tube closure (Homma et al., 1994). During early embryogenesis, approximately 50% of the differentiated neurons die through programmed cell death; this is critical for the correct establishment of neuronal connectivity (Oppenheim, 1989). Between E12 and E16, proliferating neural precursors and newly postmitotic neuroblasts in the ventricular and intermediate zones of the cerebral cortex undergo extensive cell death (Blaschke et al., 1996, 1998; Thomaidou et al., 1997). Also, the neurons, which have failed to establish synaptic connections with target fields or have produced worthless synapses, are eliminated by programmed cell death; a process called neurotrophic cell death which is associated with axon guidance and the limitation of trophic factors (de la Rosa and de Pablo, 2000; Kuan et al., 2000). Therefore, the delicate balance between proliferation and death of neural cells ultimately determines the size and shape of the nervous system.

In *Drosophila* neural development, many examples of the same can be cited as well. Excess cells are eliminated by cell death and whole structures are deleted due to the occurrence of

programmed cell death in the fly, for example during insect metamorphosis (Baehrecke, 2002). Early appearance of cell death is observed in the dorsal cephalic region, within the gnathal segments and in the clypeolabrum as the germ band begins to retract (stage 11). Thereafter, as germ band retraction proceeds (stages 12 and 13), cell death becomes widespread throughout the embryo, particularly in the ventrolateral portions and around the procephalic lobes during neuroblast segregation and mitosis. Eventually, prominent cell death appears throughout the CNS as the ventral nerve cord condenses (stage 16, Abrams et al., 1993). During this stage, the neuroblasts of the abdominal neuromeres die through a *reaper* dependent mechanism (Peterson et al., 2002). A recent systematic analysis of all neuroblasts lineages in the embryonic VNC shows that there might be a strict spatio-temporal regulation in the cell death pattern (Rogulja-Ortmann et al., 2007). In particular, Hox genes have been shown to be involved in regulation of programmed cell death in the VNC. For instance, *Abdominal B* (*AbdB*) expression has been shown to be essential for survival of differentiated neurons in the posterior segments of the embryonic VNC (Miguel-Aliaga and Thor, 2004). In addition, *Ultrabithorax* (*Ubx*) and *Antennapaedia* (*Antp*) act antagonistically in differentiated motoneurons of the NB7-3 and NB 2-4t lineages to regulate apoptosis (Rogulja-Ortmann et al., 2008). In the larval VNC, neuroblasts of the abdominal segments undergo apoptosis due to a pulse of *Abdominal* A (*AbdA*) expression, thus ensuring appropriate neuronal numbers (Bello et al., 2003). The NB7-3 lineage in the embryonic VNC has been extensively studied and identified neurons of this lineage have been shown to undergo cell death (Karcavich and Doe, 2005; Lundell et al., 2003; Novotny et al., 2002). Moreover, midline glial cells are also known to undergo cell death during embryonic life (Sonnenfeld and Jacobs, 1995) as well as during metamorphosis, under the influence of ecdysteroids (Awad and Truman, 1997).

The mechanism of neuronal cell death at the molecular level has been extensively well studied. From the initial cloning of molecules that genetically regulate programmed cell death

in *C. elegans*, the three main apoptotic genes *hid, grim* and *reaper* have beeen cloned in *Drosophila* (White et al., 1994) as well as their vertebrate homologs in mice. Apoptosis can be a kind of cell fate which can be inherited within a cell lineage like any other cell fate determinant (reviewed in Hidalgo and Constant, 2003). Among the molecular genetic mechanisms that cell autonomously regulate the apoptotic cell fate in a neuronal lineage is the asymmetric inheritance of cell-fate determinants, Notch and Numb. The membrane protein Numb inhibits the activation of Notch signaling, thus resulting in reciprocal cell fate duplication in the two daughter cells (reviewed by Cayouette and Raff, 2002; Knoblich, 2008; Lu et al., 2000; Skeath and Thor, 2003). Cell death is determined by the asymmetric segregation of Numb at mitosis, which prevents the expression of pro-apoptotic genes in the cell inheriting Numb; consequently activating Notch signaling in the other sibling cell. This binary cell death decision has been demonstrated in the multidendritic lineage of the PNS that gives rise to the mechanosensory vmd1 neuron (Orgogozo et al., 2002). In the embryonic CNS, the NB 7-3 lineage of the VNC has been well studied with respect to cell death decisions in the postmitotic neurons of this lineage. Here, the daughter cell of GMC-2 in which Numb inhibits Notch signaling survives, whereas its sibling in which Notch signaling is active is programmed to die (Karcavich and Doe, 2005; Lundell et al., 2003; Novotny et al., 2002). Whether the asymmetric inheritance of Notch and Numb is a general mechanism regulating programmed cell death in postembryonic, adult-specific neuroblasts lineages has not yet been demonstrated.

1.5 THIS THESIS

The analysis of *en* expression and function has been widely reported in the embryonic CNS. In the embryonic brain, *en* is used as a neuromere boundary marker. In addition, *en*-expressing lineages have also been characterised and studied in other insect nervous systems like cockroach and grasshopper. In contrast, very little is known about the expression of *en* in the postembryonic central brain of *Drosophila*. In this thesis, we have analyzed the morphology and characteristics of *en*-expressing cells in the postembryonic central brain. In a first study, we have traced *en*-expressing lineages from embryo to adult and analyzed their projection pattern and the corresponding, innervated brain neuropiles at high resolution. The difference in neuronal number and expression pattern of the four *en*-expressing lineages caught our interest. We have revealed a surprisingly predominant role of lineage-specific cell death that gives rise to this difference in neuronal number. This is the first study in the postembryonic central brain that demonstrates a major role of programmed cell death in generating lineage diversity. Together, these data open new questions about the general principles and mechanisms governing overall organization of the brain as well as regulation of neuronal numbers during neural development. The resulting data sets are presented in chapters 2 and 3.

2. The arborization pattern of *engrailed*-positive neural lineages reveal neuromere boundaries in the *Drosophila* brain neuropile

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2.1 SUMMARY

The *Drosophila* brain is a highly complex structure composed of thousands of neurons that are interconnected in numerous exquisitely organized neuropile structures such as the mushroom bodies, central complex, antennal lobes, and other specialized neuropiles. While the neurons of the insect brain are known to derive in a lineage-specific fashion from a stereotyped set of segmentally organized neuroblasts, the developmental origin and neuromeric organization of the neuropile formed by these neurons is still unclear. In this report, we use genetic labeling techniques to characterize the neuropile innervation pattern of *engrailed* (*en*)-expressing brain lineages of known neuromeric origin. We show that the neurons of these lineages project to and form most arborizations, in particular all of their proximal branches, in the same brain neuropile compartments in embryonic, larval and adult stages. Moreover, we show that *en*-positive neurons of differing neuromeric origin respect boundaries between neuromere-specific compartments in the brain. This is confirmed by an analysis of the arborization pattern of *empty spiracles* (*ems*)-expressing lineages. These findings indicate that arborizations of lineages deriving from different brain neuromeres innervate a non-overlapping set of neuropile compartments. This supports a model for neuromere-specific brain neuropile, in which a given lineage forms its proximal arborizations predominantly in the compartments that correspond to its neuromere of origin.

2.2 INTRODUCTION

The insect CNS is composed of two spatially separated parts, the ventral nerve cord (VNC) which is located in the trunk region, and the brain which is located within the head. The overall composition of the VNC consists of a chain of relatively uniform segmental units referred to as neuromeres; each neuromere corresponding to one body segment of the trunk (thorax and abdomen. In the head, segments have fused and become strongly modified, and as a result, neuromere boundaries are difficult to define in the brain. The insect brain can be divided into two parts, namely a supraesophageal ganglion and a subesophageal ganglion (Bullock and Horridge, 1965; Hanström, 1928; Holmgren, 1928). The subesophageal ganglion can be further subdivided into three fused neuromeres called the mandibular, maxillary and labial neuromeres, which correspond to the neuromeres of the three fused gnathal segments of the head posterior to the esophagus. The supraesophageal ganglion is also classically subdivided into three parts, namely the protocerebrum, deutocerebrum and tritocerebrum. Tritocerebrum and deutocerebrum are generally considered to be the segmental neuromeres of two strongly modified segments, the intercalary segment and antennal segment respectively. The neuromeric nature of the protocerebrum, by far the largest part of the insect brain, is unclear; it may be correspond to a single, unsegmented acron (called ocular segment in some recent papers), or have multiple segmental (ocular, labral) and non-segmental (acron) components (Diederich and Kaufman, 1991; Schmidt-Ott and Technau, 1992; Urbach and Technau, 2003a, c).

This structural complexity of the supraesophageal ganglion contrasts with the relative simplicity of the thoracic and abdominal neuromeres. Each of these manifests the same, basic bilaterally symmetric structure consisting of a cortex of cell bodies surrounding a series of longitudinal tracts, transverse commissures and regionalized domains of interspersed

neuropile areas (Tyrer and Gregory, 1982; Burrows, 1996). Many aspects of this structural organization are clearly serially reiterated throughout the VNC such that segmentally homologous tracts, neuropile regions and even individual neurons can be identified in each of the neuromeric units. Due to their relative structural simplicity, the ganglionic neuromeres of the VNC have been intensively studied, both anatomically and physiologically. In consequence, much is known about the organization of these neuromeres as well as the structure of a large number of their component neurons. One general neuroanatomical feature of neurons in the VNC that has emerged from these studies is the fact that most of the neurons in a given neuromere restrict their proximal (often dendritic) arborizations to the neuropile of that neuromere. This is obviously the case for the numerous local neurons, which by definition have no aborizations outside of the neuromere. Over half of the neurons in a thoracic or abdominal ganglion are local neurons of this type (see Burrows, 1996). However, it is also true for the vast majority of the motoneurons and intersegmental interneurons in the VNC. Although the processes of large intersegmental interneurons by definition spread over several ganglia, most of these interneurons have their proximal arborizations located in the same neuromere as their cell body, while their distal processes project to other neuromeres and ganglia. Most sensory neurons of a given segment project their axons into the neuromere of that segment and also form their terminal arborizations there, and motoneurons typically form dendrites in the neuromere that contains their somata (Landgraf et al., 2003; Merritt and Whitington, 1995; Schrader and Merritt, 2000; Zlatic et al., 2003).

To a significant extent, the internal structure and neuronal organization of the three neuromeres of the subesophageal ganglion in terms of tracts commissures and neuropiles is similar to that of the VNC neuromeres. In contrast, the larger supraesophageal ganglion manifests highly organized neuropile structures such as the mushroom bodies, central complex and antennal lobes which have no obvious equivalents in other neuromeres of the

CNS. Moreover, due to its complex and hidden segmental organization, it is difficult to determine the neuromere boundaries within the neuropile of the supraesophageal ganglion. The antennal and intercalary segments possess afferent and efferent axons, and the neuropile compartments that contain arborizations of these fibers (in particular the afferent, sensory axons) are usually assigned "with some confidence" to the corresponding neuromeres. Thus, the morphologically well delineated antennal lobe and antenno-mechanosensory-motor center (AMMC), which receive input from the antenna, are considered part of the deutocerebrum (Homberg et al., 1989). Similarly, the domain of arborization of the pharyngeal nerve, belonging to the intercalary segment, delineates a small neuropile domain usually referred to as tritocerebrum (Rajashekhar and Singh, 1994). However, these sensory compartments most likely represent only parts of the deutocerebrum and tritocerebrum, given that in a prototypical trunk segment, the arborizations of sensory afferents fill only part of the volume of the corresponding segmental neuromere. Thus, the lack of information about neuromeric boundaries together with the highly fused nature of the brain neuropile confound attempts to understand the neuroanatomical construction principles of the supraesophageal ganglion. It also makes it difficult to relate the serially homologous organization of the prototypical ganglionic pattern elements in the VNC to the structural organization of the brain.

The serially homologous organization of the insect CNS arises during development. From an early stage onward, the insect embryo (germ band) becomes subdivided into a series of reiterated metameres. Each segmental metamere contains a relatively stereotyped set of progenitor cells, called neuroblasts, which generate the neurons and glia of the mature nervous system. These neuroblasts as well as the cellular and molecular mechanisms by which they generate the neurons of the CNS have been most throughly studied in *Drosophila* (Hartenstein et al., 2008a; Technau et al., 2006). All of the neuroblasts in the developing brain and VNC of *Drosophila* have been individually identified based on their position in the

neuroectoderm and on their specific combination of marker gene expression. The supraesophageal ganglion derives from approximately 100 bilaterally symmetrical neuroblast pairs (Urbach and Technau, 2003a; Younossi-Hartenstein et al., 1996), each of which generates a characteristic lineage of neural progeny (neurons and glia). Based on the expression of segment polarity genes, it is possible to assign a defined neuromere of origin to each of the embryonic brain neuroblasts (Urbach and Technau, 2003a). Moreover, through clonal labeling techniques, it is also possible to follow the development of the neurons generated by individual brain neuroblasts through embyronic, larval and pupal stages and into the adult brain. Thus, the neuropile domains of the brain, in which neurons of a known neuromeric origin form their arborizations; can now in principle be determined. This, in turn, should make it possible to delineate the specific brain neuropile domains that correspond to specific brain neuromeres.

In this report, we reconstruct the projection pattern of the *Drosophila* neuroblast lineages expressing the segment polarity gene *en* from embryonic to adult stages in order to contribute to our understanding of neuromere boundaries in the brain. The *en* gene is expressed in neuroblasts located at the posterior boundary of each CNS neuromere (Bossing et al., 1996; Schmid et al., 1999; Urbach and Technau, 2003a; Younossi-Hartenstein et al., 1996) and, hence, the neurons that derive from these neuroblasts are of known neuromeric origin. We identify *en*-expressing neural lineages in protocerebrum, deuterocereberum and tritocerebrum, and determine the trajectory of the axon tracts as well as the innervated brain neuropile compartments for these lineages in protocerebrum and deutocerebrum. Moreover, we show that the neurons of the *en*-expressing lineages project to and innervate the same brain neuropile compartments in embryonic, larval and adult stages. An analysis of the arborization domains of *en*-expressing neurons in these stages reveals boundaries between neuromere-specific compartments in the brain that are respected by neurons of differing neuromeric

origin. This is confirmed by analysis of the arborizations made by deutocerebral *ems*-expressing neurons, which in part also restrict their arborizations to the neuropile region delimited by deutocerebral *en*-expressing neurons.

2.3 RESULTS

2.3.1 The *engrailed*-expressing primary neurons in embryonic brain development

The *engrailed* (*en*) gene is expressed in metamerically reiterated stripes in the embryonic neuroectoderm and the neuroblasts that delaminate from these neuroectodermal domains. The stripes of *en* expression define the posterior segmental compartments and, correspondingly, the *en*-expressing neuroblasts define the posterior boundary of each neuromere (Bossing et al., 1996; Urbach and Technau, 2003b; Younossi-Hartenstein et al., 1996). In the preoral procephalic neuroectoderm that gives rise to the supraesophageal ganglion of the brain, we can distinguish, in all, nine *en*-positive neuroblasts; three belong to the intercalary segment, four to the antennal segment, and two to the more anterior ocular segment. As is the case for the neuromeres of the ventral nerve cord, these neuroblasts define the posterior boundaries of the preoral neuromeres; the *en*-expressing neuroblasts of the intercalary segment define the posterior boundary of the tritocerebrum, those of the antennal segment the boundary of the deutocerebrum, and those of the ocular segment the boundary of the protocerebrum (Fig. 1A).

Following their delamination from the neuroectoderm at embryonic stages 9-11, procephalic neuroblasts begin to proliferate and generate the primary neurons of the larval brain. During this phase, *en* expression, as assayed by anti-En immunoreactivity, is maintained in the neuroblasts and the neurons generated by several of these neuroblasts. Subsequently, at embryonic stages 13-15 (Campos-Ortega and Hartenstein, 1997), neurons initiate neuronal differentiation and axonal outgrowth. At these stages, the *en*-positive neurons in the supraesophageal (procephalic) neuromeres are observed in three clusters grouped along the neuraxis anterior and dorsal to the three clusters of the *en*-positive neurons of the

subesophageal (gnathal) neuromeres (Fig.1C). The protocerebral cluster, which appears to be composed of 2-3 closely apposed neuroblast lineages, emits a single, short primary axon tract

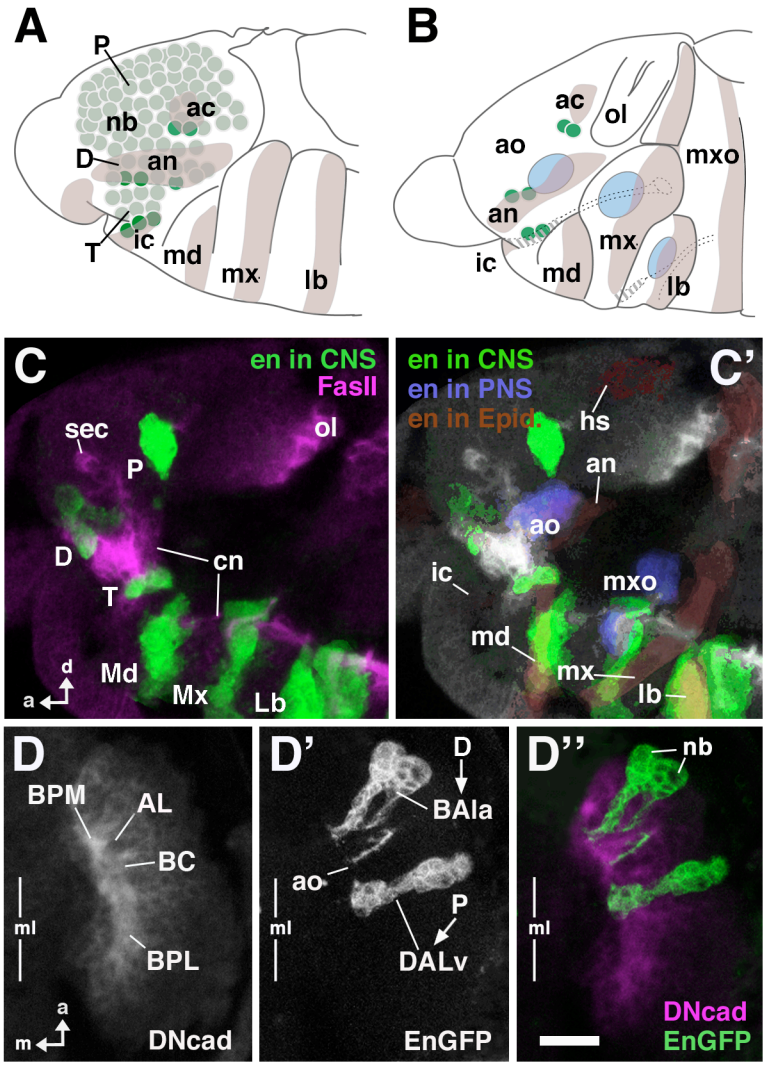

Figure 2-1. Metameric *engrailed* expression in the embryonic head. A: Schematic of early (stage 11) embryonic head, lateral view. Ectodermal *engrailed* (*en*) stripes corresponding to posterior domains of head segments are shaded [lb labium; mx maxilla; md mandible; ic intercalary segment; an antennal segment; ac acron ("head spot")]. Brain neuroblasts (nb) are shaded green. Dark green indicates sets of *en*-positive neuroblasts. It is notable that these neuroblasts spatially overlap with *en* expression domains. B: Schematic of mid-stage (stage 13) embryonic head, lateral view. Spatial relationship of *en*-positive neuroblasts and ectodermal *en* strips is maintained. Blue areas indicate primordia of head sensory complexes (labium, maxilla, antenna) which partially overlap with *en* stripes. C: Z-projections of confocal sections of stage 14 embryonic head labeled with antibody against En (green) and Fasciclin II (magenta); lateral view. Note metamerical clusters of neurons (lineages) derived from *en*-positive neuroblasts. The three posterior clusters (Md, Mx, Lb) demarcate

the three neuromeres of the gnathal segments, which will later form the subesophageal ganglion. The anterior clusters correspond to the neuromeres of the supraesophageal ganglion (P protocerebrum, derived from acron; D deutocerebrum, derived from antennal segment; T tritocerebrum, derived from intercalary segment). C': Same Z-projection as in C. The different tissues contributing to *en* expression domains are shown in different colors. *en*-positive clusters of neurons are in green. *en* in the ectoderm (by that stage: epidermal primordium) is shaded brown; parts of sensory primordia expressing *en* (ao antennal organ; mxo maxillary organ) are in blue. Note that the *en*-positive clusters and the corresponding *en* stripes from which they derive are still in close proximity, except for the protocerebral cluster of neurons that, due to morphogenetic movements in the head, has moved away from the epidermal head spot (hs). D, D', D'': Z-projection of confocal sections of stage 15 embryonic brain labeled with antibodies against DN-cadherin (D; magenta in D'') and GFP expressed by an *en*-Gal4 driver (D'; green in D''); dorsal view; only right brain hemisphere is shown; vertical line indicates midline (ml). Note pattern of *en*-positive lineages (deutocerebral BAla3; protocerebral DALv) and their relationship to the primordia of brain compartments (AL Antennal lobe; BC basocentral; BPL baso-posterior lateral; BPM baso-posterior medial). Each lineage consists of the superficial neuroblast (white line and arrowhead indication, D') and a chain of primary neurons. Other abbreviations: cn cervical connective; ol optic lobe; sec supraesophageal commissure.
Bar: 10µm (for C-D)

that extends straight medially towards the center of the nascent brain neuropile. This position marks the point where the baso-central (BC; nomenclature of larval brain compartments according to Younossi-Hartenstein et al., 2003; 2006) neuropile compartment will appear several hours later (see Fig. 2). The deutocerebral cluster of *en*-expressing neurons, which may comprise 1 or 2 neuroblast lineages, emits axon tracts that converge posteriorly and defines the position where the baso-posterior medial compartment (BPM) will appear (see Fig. 2). (In addition, *en*-positive sensory neurons of the antennal organ, the larval equivalent of the antenna, project towards the central neuropile laterally adjacent to the deutocerebral cluster, a position that defines the nascent antennal lobe). The tritocerebral cluster of *en*-positive cells encircles the base of the embryonic tritocerebral primordium in the shape of a horizontal crescent. No axons can be discerned emanating from this tritocerebral cluster, and at late embryonic stages, and most of the *en*-expressing cells in the cluster have adopted the shape and position of glial cells; these cells were not characterized further in this study.

2.3.2 Compartment-specific arborizations of *engrailed*-expressing primary neurons

In the late embryo (stage 16 to hatching), the primary axon tracts of the neurons in all primary lineages, including those that are *en*-positive, begin to form extensive arborizations, and as a result the developing brain neuropile, subdivided into several compartments, emerges (Pereanu and Hartenstein, 2006; Younossi-Hartenstein et al., 2006). These neuropile compartments can be individually identified and followed throughout larval and pupal development into the adult brain (see Hartenstein et al., 2008b). The entire morphology of the developing En-immunoreactive neurons, including their cell bodies, neurites, and arborizations, can be revealed by an *en*-Gal4 driver coupled to a UAS-mCD8::GFP reporter and then related to nascent brain neuropile structures revealed by anti-DNcadherin immunolabeling (Fig. 1D). Moreover, by relating the compartments innervated by *en*-expressing neuronal clusters to the compartments innervated by identified neuroblast lineages (see Pereanu and Hartenstein, 2006), one can assign the neurons of a given *en* cluster to identified neuroblast lineages.

In the protocerebrum of late embryonic stages, two clusters of *en*-expressing neurons become apparent (Fig. 2A). The protocerebral cluster of neurons that had already been *en*-positive at earlier stages is located next to the nascent BC compartment (Fig. 2B, H). The primary axon tract that derives from these neurons projects to the BC compartment. The neurites from this tract form arborizations throughout the compartment and also continue medially and anteriorly and form arborizations in the CPI, CPL and CA compartments, where they intermi-

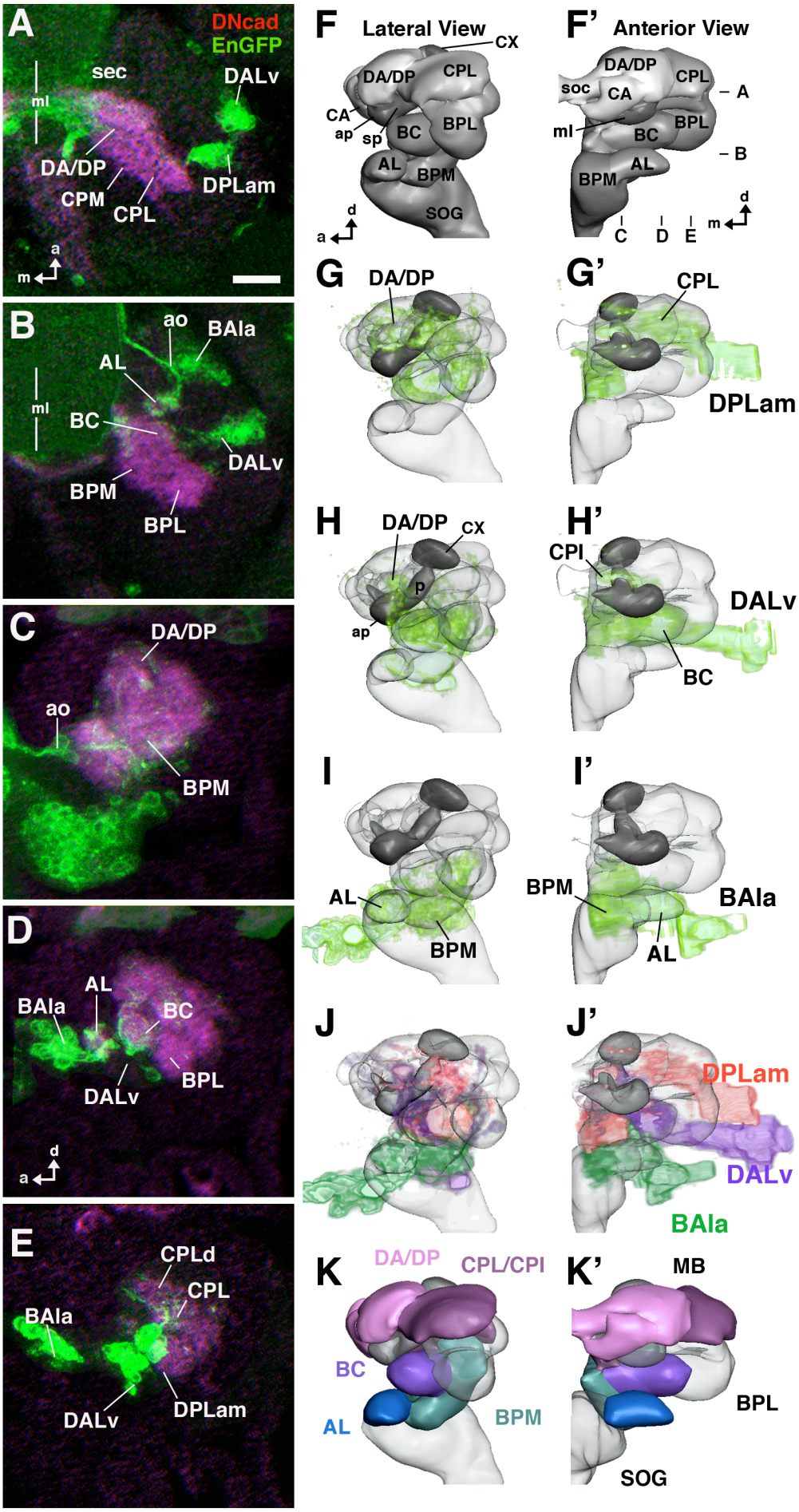
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Figure 2-2. *engrailed* lineages in the late embryonic brain. A, B: Z-projection of confocal sections of stage 17 embryonic brain labeled with antibodies against DN-cadherin (magenta) and GFP expressed by an *en*-Gal4 driver (green); dorsal view; only right brain hemisphere is shown; vertical line indicates midline (ml). Dorso-ventral focal plane of A and B is indicated in panel F' to the right. C-E: Z-projections of parasagittal confocal sections of stage 17 embryonic brain prepared as the one shown in A/B. Anterior is to the left, dorsal up. Medio-lateral focal planes of C-E are indicated in panel F'. F, F': 3D digital models of stage 17 embryonic brain hemisphere in lateral view (F) and anterior view (F'), showing neuropile compartments (AL larval antennal lobe; ap anterior appendix of larval mushroom body; BC baso-central; BPL baso-posterior lateral; BPM baso-posterior medial; CA centro-anterior; CPL centro-posterior lateral; CX calyx; DA dorso-anterior; DP dorso-posterior; sec supraesophageal commissure; ml medial lobe of mushroom body; SOG subesophageal ganglion (=anterior ventral nerve cord); sp spur of mushroom body). G-I, G'-I': Digital brain models as in F/F', with volume renderings of the *en* lineages visible in stage 17 embryo (DPLam, DALv, BAla3; shaded green). J, J': Montage of the *en* lineages in one model (DPLam red; DALv purple; BAla3 green). K, K': Digital brain models as in F/F', with neuropile compartments innervated by *en* lineages shown in different colors. En expression in late embryo appears in three main clusters. The dorsal-most cluster corresponds to the DPLam lineage (A, E, G/G'), which arborizes in CPL, CPI, DP and DA (A, C-E, G/G'). The medial cluster (DALv; A, B, E, H/H') consists of two adjacent lineages, DALv2 and DALv3, with indistinguishable projections in the embryo and early larva. Proximal projections are in the BC compartment (B, D, H/H'); more distally, projections overlap with those of DPLam in CPI (see G' and H') and DA and CA (see G, H, F). In the deutocerebrum, one lineage (BAla3) is distinguished (B, E, I/I'). It projects to the BPM compartment (C, I/I'). The larval AL compartment (antennal lobe) is labeled by sensory afferents of *en*-positive antennal organ (ao; B-D, I/I'). Bar: 10µm

-ngle with branches from other procephalic lineages, as well as with *en*-positive fibers that ascend from the ventral nerve cord (Fig. 2). Based on their close association with the BC compartment we can identify the *en*-expressing neurons in this protocerebral cluster as members of the two DALv2/3 neuroblast lineages of the larva (see below). The second *en*-positive protocerebral cluster is located anterior and dorsal to the DALv2/3 cluster (Fig.2A, E, G). (At the earlier embryonic stages described above, this group of neurons might have been nested in the observed protocerebral *en*-cluster; alternatively, *en*-expression may appear de novo in this cell cluster between embryonic stage 15 and late16.) The primary axon tract from

this second cluster enters the neuropile more dorsally than the DALv2/3 axons, and arborizes in the CPL, posterior CPI, and DA compartments. Based on this arborization pattern, one can identify the neurons in this second cluster as members of a third protocerebral *en*-positive lineage namely the DPLam lineage of the larva (see below). The deutocerebral cluster of *en*-positive neurons projects its axon tracts from anterior into the BPM compartment (Fig. 2 D, E, I). There they form arborizations which are intermingled with unidentified *en*-positive ascending fibers. The BPM compartment (like the antennal lobe) is also filled with *en*-positive sensory endings from the antennal organ. Their compartment-specific arborization pattern identifies the neurons in the deutocerebral cluster as members of the BAla3 lineages as defined for the larva (see below).

This general pattern of neurite projections and arborizations of the three groups of *en*-positive primary neurons in the late embryo is maintained throughout the larval period (Fig. 3B, C). Based on this, one can assign distinct larval neuropile compartments to at least part of the arborizations made by the neurons of any of these given lineages. Thus, the proximal arborization of the larval DPLam lineage, as described above for the embryo, is found in the CPL compartment of the larval brain, close to where the cell bodies are located. Similarly, the proximal arborizations of the larval DALv lineages, as described above for the embryo, are in the BC compartment of the larval brain that is adjacent to the DALv cell bodies. The more distal branches of DPLam and DALv are found in the larval DA, DP, CPI and CPM compartments, where they appear to intermingle (Fig. 3B, C, D). Finally, the *en*-positive neurons of the larval BAla3 lineage, as described above for the embryo, form a long tract that passes the antennal lobe and reaches the more posteriorly located larval BPM compartment, where it arborizes.

In summary, most of the neuropile compartments of the late embryonic as well as the larval

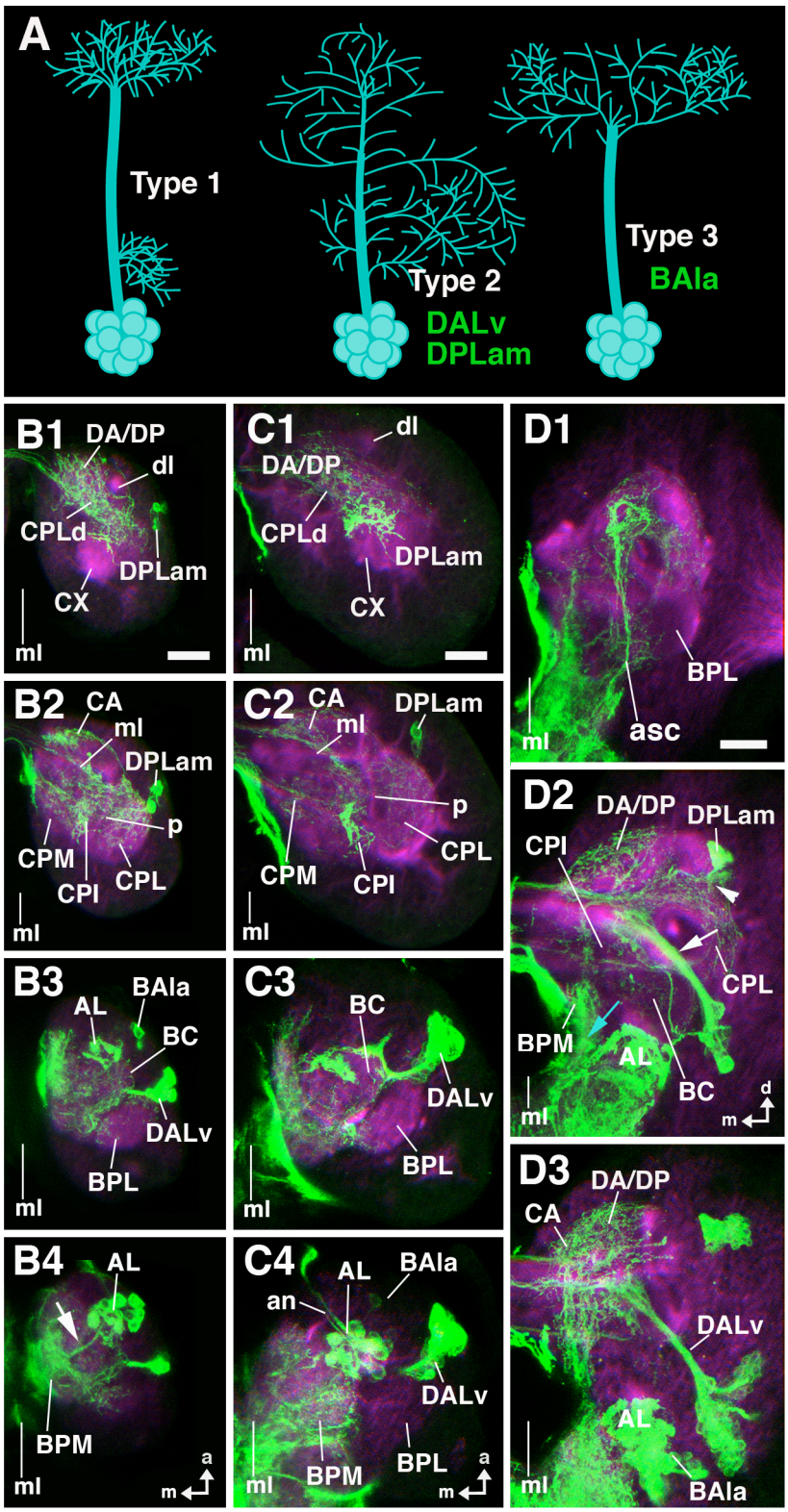

Figure 2-3. *engrailed* lineages during larval development. A: Schematic representation of different types of lineages encountered in brain (type 1: separate proximal and distal arborization; type 2: continuous arborization; type 3: distal arborization; Larsen et al., personal communication). B1-B4,

C1-C4, D1-D3: Z-projection of confocal sections of larval brains labeled with antibodies against DN-cadherin (magenta) and GFP expressed by an *en*-Gal4 driver (green); only right brain hemisphere is shown; vertical line indicates midline (ml). B1- B4: first larval instar, dorsal view, focal plane goes from dorsal (B1) to ventral (B4). C1-C4: second larval instar, dorsal view, focal plane goes from dorsal (C1) to ventral (C4). D1-D3: late third larval instar; anterior view; focal plane goes from anterior (D1) to posterior (D3). The arborization pattern of *en* lineages is similar as shown for late embryo in Fig.2. The protocerebral DPLam lineage (cell bodies shown in B2, C2, D2) arborizes widely in CPL, CPI, DP/DA, and CA. DALv (comprising two neighboring lineages; cell bodies in B3, C3, D3) has arborizations in BC, as well as CPI and DP/DA where they overlap with fibers of DPLam. BAla3 (cell bodies in B3, C4, D3) arborizes in BPM. *en*-Gal4 driven GFP labeling in larval antennal lobe (AL; B4, C4, D3) is due to *en*-positive afferents from antennal organ. At third instar, secondary neurons have been added to *en* lineages. They form distinctive secondary axon tracts (SATs) that project into territory innervated by primary neurons (white arrow head in D2: SAT of DPLam; arrow in D2: SAT of DALv2/3; blue arrow in D2: SAT of BAla3). Note that there are *en*–positive fibers ascending from the ventral cord (asc), as well as *en*-positive surface glia (sg). Other abbreviations: BPL baso-posterior lateral compartment; CPM centro-posterior medial compartment; CX calyx; dl dorsal lobe; ml medial lobe; p peduncle). Bars: 10µm (B1-B4; C1-C4); 25µm (D1-D3)

brain can be assigned to at least one of the *en*-lineages; CPL and BC to the proximal arborizations of the DPLam lineage and the DALv lineages, respectively; CPI, CPM, DP and DA to the distal arborizations of the DPLam lineage and the DALv lineages; BPM to arborizations of the BAla3 lineage (Fig. 2 F, J, K). The only major brain compartments that appear to lack arborizations from neurons of *en*-expressing lineages are the mushroom body, and the BPL.

2.3.3 Secondary neurons in *engrailed* lineages innervate the same brain compartments as their primary neuron siblings

After a period of mitotic quiescence during the early larval period, neuroblasts reactivate proliferation and produce adult-specific secondary neurons. Secondary neurons belonging to one and the same neuroblast lineage form axons that fasciculate in a coherent bundle referred

to as a secondary axon tract (Dumstrei et al., 2003). To investigate the lineages of secondary *en*-expressing neurons in more detail, we carried out a MARCM-based clonal analysis with a faithful *en*-Gal4 driving UAS-mCD8::GFP and therefore, we recovered positively labeled clones for analysis that comprised *en*-expressing neurons. (Clones were induced at larval hatching and therefore only adult-specific secondary cells were labeled.) In this analysis, we consistently recovered, in the late third instar larval brain (L3), *en*-positive clones in the supraesophageal ganglion that correspond to 4 different neuroblast lineages, and all *en*-expressing secondary neurons in the supraesophageal ganglion belong to one of these four lineages. (However, not all of the secondary neurons in these lineages are *en*-expressing.) Location of cell bodies, projection of secondary axon tract, and the innervated set of compartments positively identify these four lineages as the DPLam, DALv2, DALv3, and BAla3 lineages (Fig. 4).

Secondary axon tracts of most, if not all, neuroblast lineages in the brain are thought to grow along the neuronal processes of primary neurons, possibly using them as guidance structures (Larsen et al., personal communication). This suggests that the secondary neurons of the four *en*-expressing lineages might innervate the same brain compartments as do their lineage-related *en*-expressing primary neurons. MARCM clonal analysis indicates that this is indeed the case (Fig. 4I, J). The protocerebral DPLam lineage projects its secondary axon tract postero-medially into the CPL compartment, thus innervating the same neuropile domain densely innervated by the primary DPLam neurons (Fig.4A, E). Approaching the peduncle of the mushroom body from an antero-dorsal direction, this secondary axon tract branches and sends one branch medially across the peduncle, the other one ventrally. The secondary axon tracts of the two protocerebral DALv2/3 lineages form one fascicle that grows along the BC compartment, passes underneath the medial lobe of the mushroom body, and reaches the midline. The DALv3 lineage has a secondary axon tract that is split, forming a dorsal and a

ventral branch (Fig. 4B, F). These represent the larval forerunners of the two commissures that flank the ellipsoid body in the adult brain (see below).

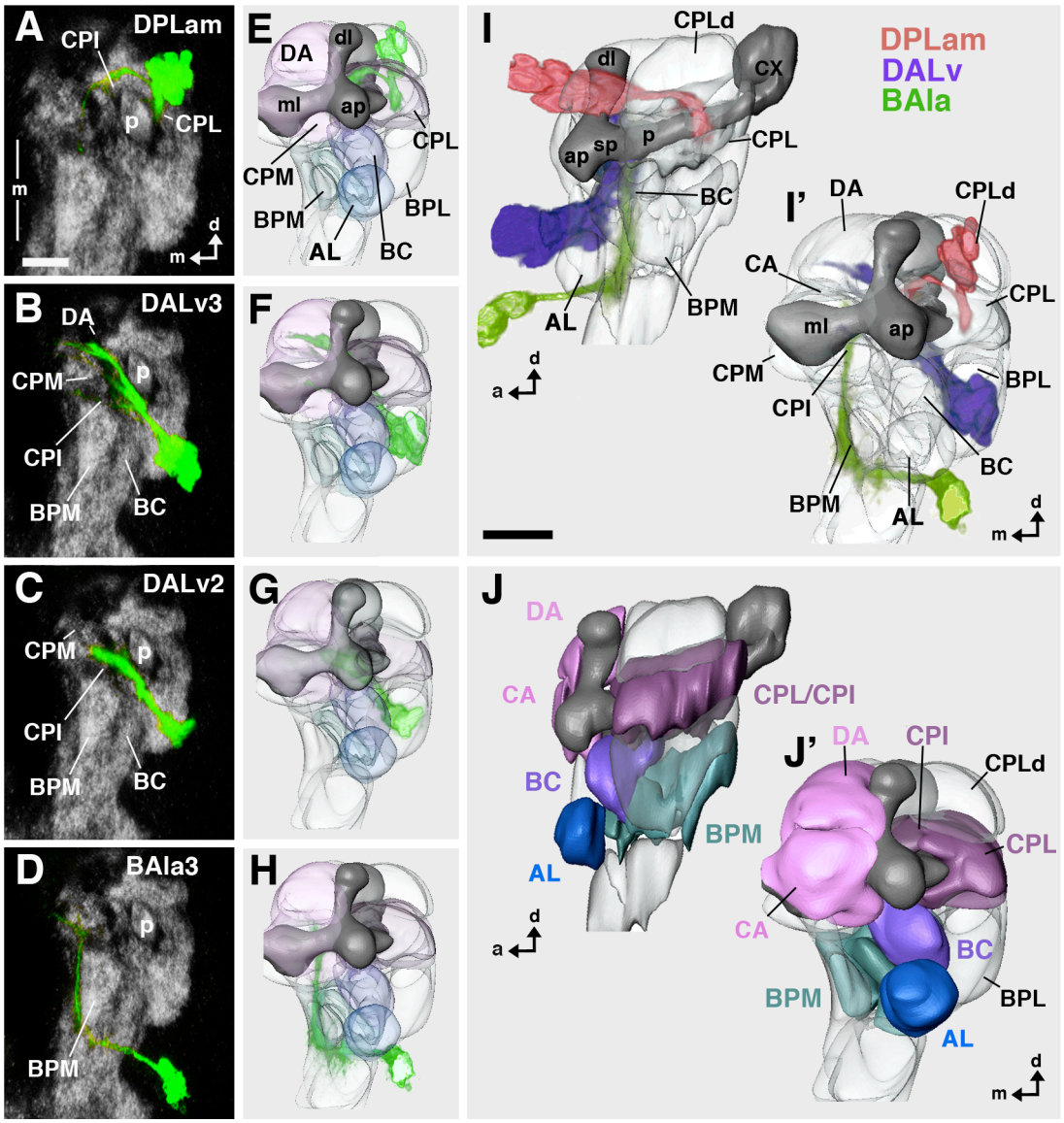

Figure 2-4. Secondary neurons of *engrailed* lineages visualized in late third instar larva by *en*-Gal4 driven MARCM clones. Heat shock to induce Flippase mediated recombination was given shortly after larval hatching, leading to labeling of secondary neurons. A-D: Z-projection of confocal sections of late larval brains labeled with antibodies against Nc82 (grey) and GFP expressed by an *en*-Gal4 driver (green); anterior view; only right brain hemisphere is shown; vertical line indicates midline (ml). E-H: Digital 3D models of third instar brains (anterior view) with volume renderings of secondary *en* lineages (green). The lineages correspond to that shown in A-D. In models, mushroom body is shown in dark gray for better orientation. Compartments are rendered transparent. I, I': Digital 3D model of third instar brain with montage of all four *en* lineages; lateral view (I) and anterior view (I'). J, J': Digital models showing neuropile compartments in lateral view (J) and anterior view (J').

Lineages depicted in I/I' are shown in different colors: DPLam red, DALv2/3 purple; BAla3 green. Compartments in E-J' are shaded in colors that reflect the lineages they are innervated by (dark blue: antennal organ; light blue: BAla3; purple: DALv2/3; burgundy/pink: DPLam plus DALv. Note that axon tracts of secondary lineages are associated with the same compartments that were innervated by primary neurons of corresponding lineages (see Fig.2/3). For abbreviations of compartments see legend of Fig.2. Bar: 25µm

The DALv2 has a single unbranched secondary axon tract that stops short of the midline (Figs. 4C, G). The deutocerebral BAla3 secondary axon tract, similar to the primary BAla3 neurons, passes ventro-medially of the antennal lobe and grows backward into the BPM compartment (Fig. 4D, H). (Although one or two deutocerebral BAla3 neuroblast lineages may express *en* in the late embryo, only a single clone of secondary neurons can be recovered in larval stages suggesting that there is indeed only one lineage or, alternatively, that one of the two neuroblasts does not generate *en*-expressing secondary neurons since anti-En antibody immunostaining reveals only a single deutocerebral cluster of secondary neurons; data not shown). No *en*-positive secondary clones were recovered for the *en*-positive tritocerebral neuroblasts implying that they do not generate *en*-positive secondary cells.

In a recent clonal analysis of *Drosophila* neuronal lineages, three main morphological classes of lineages were distinguished (Larsen et al., personal communication; Fig.3A). Lineages belonging to the first class (type 1) elaborate distinct, spatially separate proximal and distal arborizations. For example, the lineages of olfactory projection neurons between antennal lobe and protocerebrum form proximal, dendritic arborizations in one or more glomeruli of the antennal lobe, and have their terminal, axonal arborizations in the calyx or lateral horn of the protocerebrum (Rodrigues and Hummel, 2008). The second class of lineages (type 2) forms branches that are distributed more or less evenly along the entire length of the axon tracts. One cannot distinguish, in these lineages, between a defined proximal input and a distal output domain. Type 3 lineages form a relatively long tract without proximal arborizations

(i.e., branches that are formed in the neuropile adjacent to the cell bodies); arborizations are restricted to the distal part of fibers. Our MARCM-based clonal analysis shows that all three of the protocerebral *engrailed*-positive lineages (DPLam, DALv2/3) are type 2 lineages. In contrast, the deutocerebral BAla3 neurons show characteristics of type 3 lineages.

2.3.4 The arborization pattern of secondary *engrailed*-lineages may delimit boundaries between adult brain neuropile compartments

Secondary neurons remain undifferentiated during the larval stage; aside from the single undivided fibers that are assembled into the secondary axon tracts, no terminal branches or synaptic connections are formed (see Hartenstein et al., 2008a). During the subsequent pupal phase, secondary neurons undergo substantial maturation processes involving generation of terminal and proximal arborizations as well as formation of synaptic interconnections and, thus, attain the mature neuron morphology that characterizes the adult brain. To determine the neuroanatomical features of the *en*-expressing neuroblast lineages in the mature brain (supraesophageal ganglion), we generated MARCM clones at larval hatching and recovered labeled, *en*-positive clones in the adult. In order to reveal the spatial relationship between the neuroblast clones and the mature neuropile compartments of the adult brain, these preparations were also labeled with the Nc82 antibody, which stains synaptic neuropile.

Figure 5 shows the MARCM-labeled arborizations of the BAla3, DALv3 and DPLam lineages in different frontal sections of the adult brain as well as 3D digital models of the lineages relative to the neuropile compartments. It is notable that the DALv2 lineage can no longer be recovered in the adult brain, suggesting that the neuroblast or the neurons of this lineage no longer express *en*. In all cases, the adult brain compartments occupied by the

arborizations of the secondary lineages largely conform to the larval compartments innervated by the primary lineages. Moreover, in most cases the arborizations of the different *en*-positive

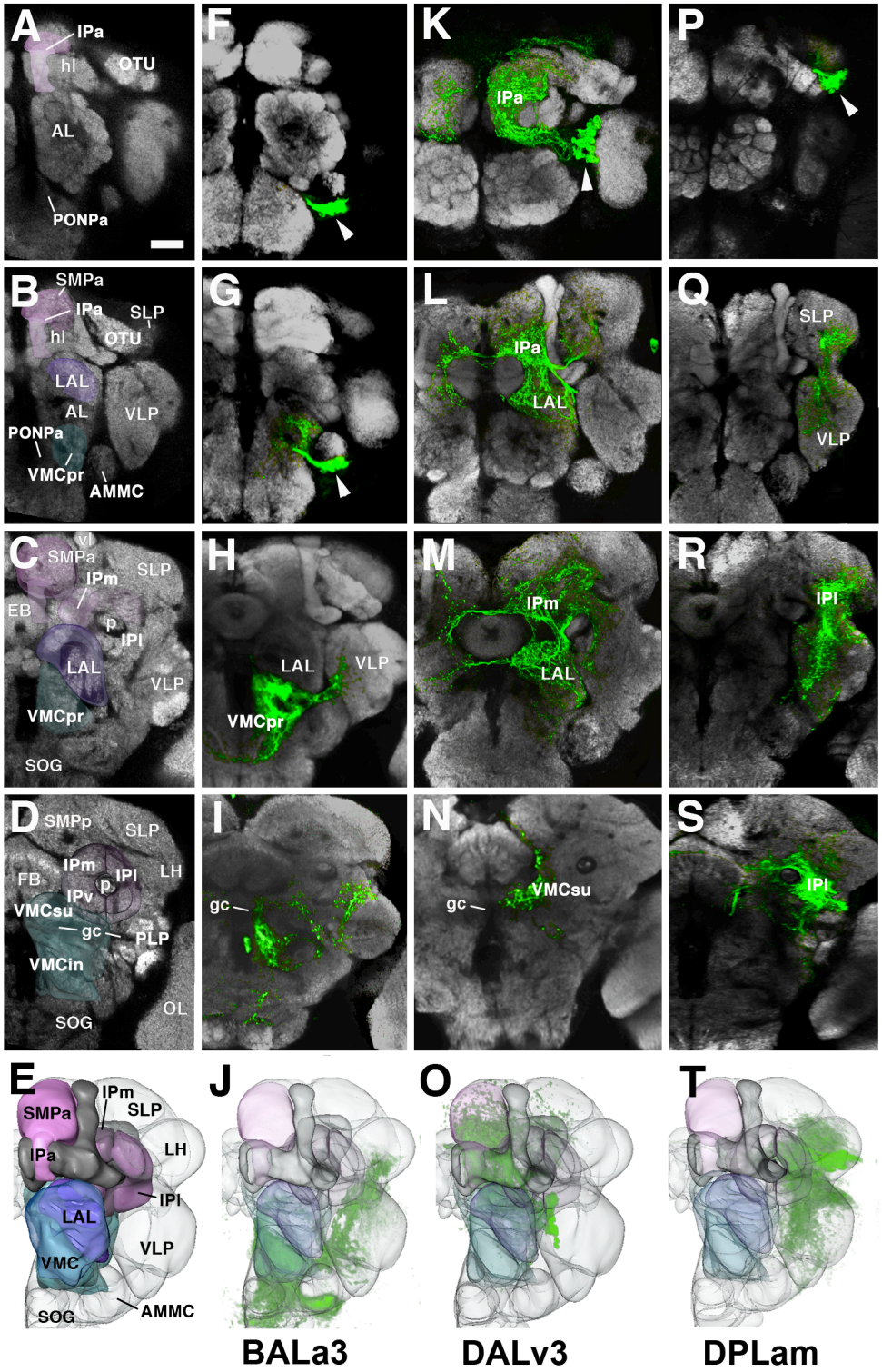

Figure 2-5. Arborization of secondary *engrailed* lineages in adult brain. A-S: Upper four panels of each column show Z-projections of frontal confocal sections of adult brains labeled with Nc82 antibody (synapses, grey) which delineates neuropile compartment. Each panel represents a Z projection of optical sections ("brain slice") of approximately 20 micron thickness. Rows of panels

start anteriorly in the brain (top) and move posteriorly (second to bottom). Panels of the first row (A, F, K, P) show the neuropile right in front of the horizontal lobe of the mushroom body (hl); the second row (B, G, L, Q) right behind the horizontal lobe; third row (C, H, M, R) at the level of the ellipsoid body (EB); fourth row (D, I, N, S) at the level of the fan-shaped body. E-T: The bottom row presents 3D digital models of adult brain neuropile in anterior view. The first column identifies the compartments (AL antennal lobe; AMMC antennomechanosensory and motor center; CCX central complex; CX calyx of mushroom body; EB ellipsoid body; FB fan-shaped body; gc great commissure; hl horizontal lobes of mushroom body; IP inferior protocerebrum; IPa anterior domain of IP; IPl lateral domain of IP; IPm medial domain of IP; IPv ventral domain of IP; LAL lateral accessory lobe; LH lateral horn; OL optic lobe; OTU optic tubercle; p peduncle of mushroom body; PLP postero-lateral protocerebrum; PONPa anterior perioesophageal neuropile; PSi inferior posterior slope (VMCpo); PSs superior domain of posterior slope (=VMCpo); SLP superior lateral protocerebrum; SMP superior medial protocerebrum; SMPa anterior domain of SMP; SMPp posterior domain of SMP; SOG suboesophageal ganglion; vl vertical lobes of mushroom body; sp spur of mushroom body; VLP ventrolateral protocerebrum; VMC ventromedial cerebrum; VMCin infracommissural domain of VMC; VMCpo postcommissural domain of VMC; VMCpr precommissural domain of VMC; VMCsu supracommissural domain of VMC) . Color scheme corresponds to that one of Figure 4: the VMC, whose larval forerunner (BPM) is innervated by *en* lineage BAla3, is shaded blue; LAL (larval precursor: BC), innervated by DALv2/3, is purple; IPa (larval precursor: CA), SMPa (larval precursor: DA) and IPm (larval precursor: CPI), all innervated by DALv and DPLam, are pink; IPl (larval precursor: CPL), innervated mostly by DPLam, is burgundy. The second, third and fourth column each shows the arborization pattern of a single *en* lineage (BAla3 (F-J), DALv3 (K-O), and DPLam (P-T), respectively). Clusters of cell bodies of the lineages are identified by arrowheads. Note that the compartments that contain arborizations of the secondary neurons largely correspond to those that had input from primary neurons of the corresponding lineage. An exception is DPLam, where secondary neurons contribute substantially to the VLP, which had no primary arborizations; moreover, secondary DPLam neurons do not project to the SMP and IPa, both of which did receive primary neuronal input. Bar: 25µm

lineages largely respect each others' territories and do not intermingle (Fig. 6A-C). In doing so, these lineages appear to delimit boundaries that may correspond to the borders of the protocerebrum, deutocerebrum and tritocerebrum in the adult brain neuropile.

The BAla3 lineage forms dense terminal arborizations in the anterior part of the ventro-medial cerebrum (VMC), the compartment that develops from the larval BPM (Pereanu et al.,

personal communication; Fig. 5H). Some longer branches are directed laterally into the ventro-lateral protocerebrum (VLP), the descendant of the larval BPL, as well as ventrally into the subesophageal ganglion (Fig. 5H, I). The BAla3 arborization defines a relatively sharp boundary medially and anteriorly towards the anterior perioesophageal neuropile (PONPa) (Fig. 7A, B). This BAla3 arborization-negative domain corresponds to the anterior perioesophageal domain that harbors the sensory terminals of pharyngeal nerve axons, which originate in the intercalary segment (Fig. 7D). Furthermore, overlapping with this sensory neuropile, but continuing further posteriorly, is the compartment called flange (Rajashekhar and Singh, 1994; Strausfeld, 1976), which receives input from the pars intercerebralis (PI) (Fig. 7C). Both pharyngeal sensory afferents and PI input define the tritocerebrum in *Drosophila* (Rajashekhar and Singh, 1994) and other insects (Bullock and Horridge, 1965; Zaretsky and Loher, 1983). Based on this, we suggest that this anterior perioesophageal domain, which is not innervated by the *en*-expressing BAla3 lineage, corresponds to the tritocerebrum (Fig. 6D, E).

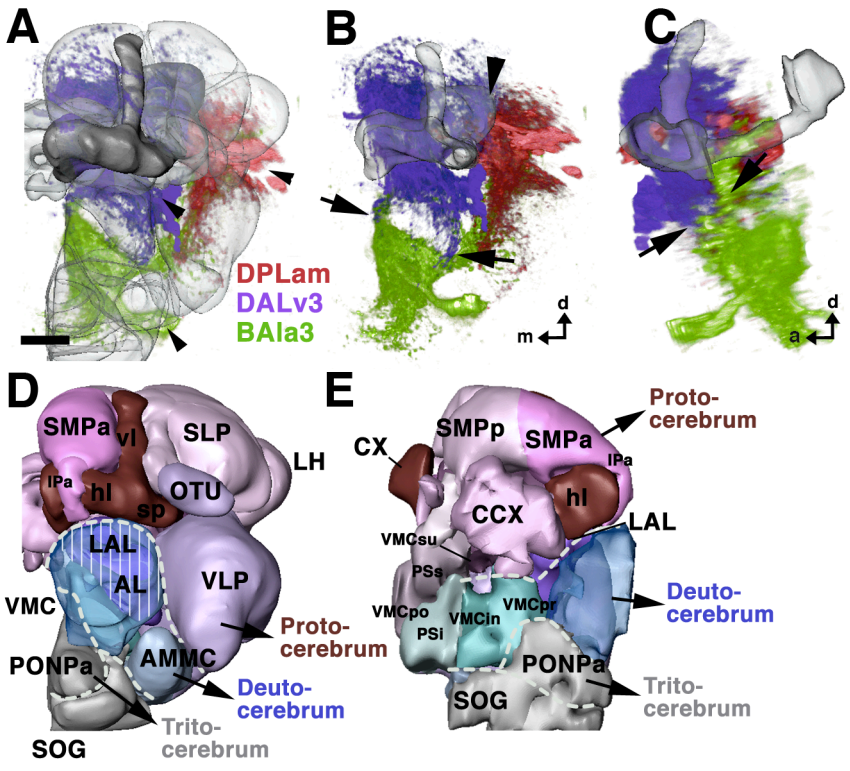

Figure 2-6. Secondary *engrailed* lineages in the adult brain and suggested neuromere boundaries. A-C: montages of volume renderings of the three *en* lineages shown in different colors (DPLam: red; DALv3: purple; BAla3: green). A and B show anterior views; in A, neuropile compartments are shaded. C presents a lateral view. Arrowheads in A point at the cell body clusters of the three lineages. Arrows in B and C outline the sharp boundary between arborizations of DALv3 (above) and BAla3 (below). This boundary corresponds to the structurally distinct boundary between LAL compartment (above) and VMC (below). Large arrowhead in B demarcates another, less sharply demarcated boundary between the IPm and IPl (figure 5); DPLam neurites are largely confined to the IPl, DALv3 neurites to the IPm. D, E: 3D digital models of the neuropile compartments of the adult brain, presenting suggested neuromere boundaries as white hatched lines. D shows anterior view, E medial view. Color coding is similar to that used in Figures 4 and 5. Compartments of the protocerebrum are shown in shades of red and purple, deutocerebral compartments in blue, and the tritocerebrum in grey. Note that in D, the hatched line indicating the proto-deutocerebral boundary arches dorsally over the deutocerebral antennal compartment (AL), which is rendered transparent to let one see through to the VMC and LAL located behind. The LAL (vertical lines) belongs to the protocerebrum, while the VMC (no vertical lines) to the deutocerebrum. Also, note that the volume renderings in A-C do not reveal the fact that BAla3 terminal arbors are excluded from the PONPa; this is visible in Z-Projections shown in Fig.5F, G. For abbreviations of neuropile compartments, see Fig.5. Bar: 25µm

The DALv3 lineage fills the lateral accessory lobe (LAL, descendant of the BC compartment) and the medial inferior protocerebrum (IPm, descendant of the larval CPI; Fig. 5L, M). In addition, the anterior part of the inferior protocerebrum (IPa) that wraps around the horizontal lobes of the mushroom body like a cuff contains dense terminal arborizations of DALv3. Commissural fibers that cross dorsal and ventral of the ellipsoid body carry input of DALv3 to the contralateral IPm and LAL (Fig.5M). Noteworthy is the sharp boundary between DALv3 and BAla3 that coincides with the morphologically distinct boundary of LAL (DALv3) and anterior (precommissural) VMC (BAla3). More posteriorly, at the level of the great commissure (gc), the boundary coincides with this commissure; BAla3 fibers are restricted to the territory below the commissure (the infracommissural VMC; Fig.5I), and DALv3 to the one above the commissure (supracommissural VMC; Fig.5N). We propose that

this boundary can be used to delineate the protocerebral-deutocerebral borderline within the neuropile (Fig. 6D, E).

The domain of arborization of DPLam is in the inferior lateral protocerebrum (IPl; Fig.5R) which develops from the larval CPL, and, more posteriorly, reaches into the inferior medial protocerebrum (Fig. 5S). In addition, branches extend into the ventrolateral protocerebrum (VPL) and superior lateral protocerebrum (SLP) adjacent to the IPl (Fig. 5Q, R). Projecting both DPLam and DALv3 into the same 3D model makes it clear that the two lineages divide the inferior protocerebrum in such a way that DALv3 innervates mostly medial and anterior domains while DPLam innervates mostly posterior and lateral domains (Fig. 6D, E).

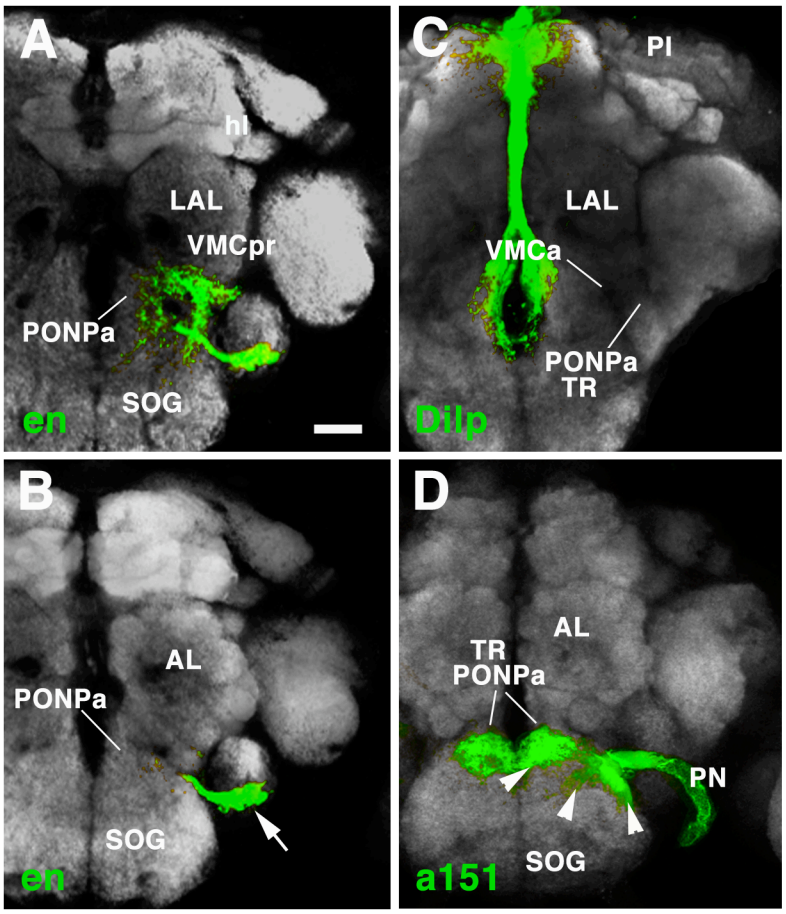

Figure 2-7. Tentative delineation of the tritocerebrum. A, B: Z-projections of frontal confocal sections of adult brain labeled with Nc82 antibody (grey; labels neuropile compartments). The *en*-positive BAla3 lineage is shown in green; arrow in B points at cell bodies. Panel A represents

neuropile at level of mushroom body horizontal lobe (hl) and lateral accessory lobe (LAL); focal plane shown in B represents neuropile more anteriorly, at the level of the antennal lobe (AL). C, D: Z-projections of confocal sections at levels corresponding to those shown in A and B, respectively. In C, projection of Dilp-positive neurons of the Pars intercerebralis (PI) to the anterior perioesophageal neuropile (PONPa) is visualized by Dilp-Gal4, UAS-mcd8GFP (Rulifson et al., 2002). In D, the sensory afferents of the pharyngeal nerve (PN, labeled by Gal4-driver line a151; kindly provided by Dr.Julie Simpson, JFRC, USA), which carries axons of the sensory neurons that originate in the intercalary segment, are shown in the anterior-most tip of the perioesophageal compartment. Note that the anterior perioesophageal neuropile is devoid of arborizations of the deutocerebral BAla3 lineage (A, B). We take this finding to support the notion that the PONPa represents the tritocerebrum (TR). Bar: 25µm

2.3.5 Secondary lineages expressing the *empty spiracles* gene arborize in neuromere-specific neuropile compartments

To what extent do other neuroblast lineages respect the proposed boundaries between neuromere-specific compartments in the brain? To address this, we focused on the secondary neuron lineages in the larval brain that comprise *ems*-positive neurons (Fig. 8B); the development of these lineages was analysed by Lichtneckert et al. 2007; 2008. In the embryo, two lineages are known to derive from protocerebral neuroblasts that co-express *ems* and *en*, and are thus identfied as the DALv2/3 neuroblasts (Fig.8A). However, in the post embryonic larval brain, only one of the two neuroblast lineages, DALv3, co- expresses *ems* and *en*. (We show above that the dendritic arbors of the DALv2/3 secondary neuron lineages are restricted to protocerebral-specific compartments, hence they will not be considered further here.)

Among the remaining lineages that express *ems* in the larval brain, five are deutocerebral (Urbach and Technau, 2003a; Younossi-Hartenstein et al., 1997). Analysis of the secondary axon tracts of these lineages relative to major brain neuropile structures using clonal MARCM

labeling combined with anti-Ems and anti-Neurotactin co-immunostaining identifies them as BAlc1/2, BAmv2/3 and BAlp3 (Fig. 8C-E). Moreover, characterization of their domains of

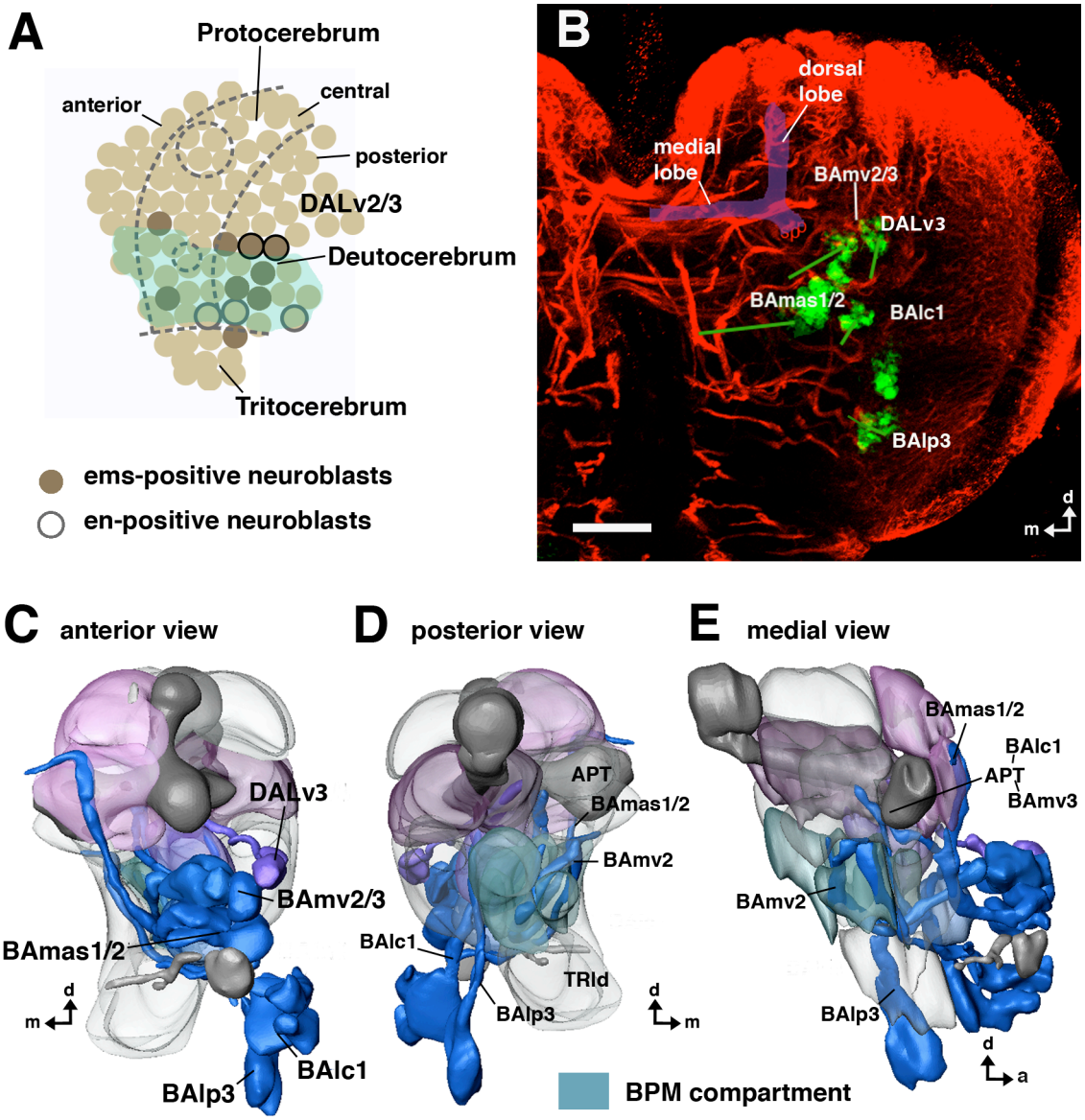

Figure 2-8. Projection of ems-positive lineages in the late larval brain. A: Schematic map of embryonic brain neuroblasts (lateral view; after Urbach and Technau, 2003a, b; Sprecher et al., 2007). Neuroblasts that were classified as deutocerebral based on their spatial relationship to *en* and other markers are shaded green. *ems* (shaded in brown) is expressed in one tritocerebral neuroblast, four deutocerebral neuroblasts, and four neuroblasts along the posterior border of the protocerebrum. Two of these are the DALv2/3 neuroblasts that also turn on *en* (shown as black-frame neuroblasts). B: Z-projection of confocal section of a late larval brain labeled with anti-Neurotactin (red; visualizes secondary lineages), and anti-Ems (green). The Ems-positive lineages of interest are pointed out by labels; they include the protocerebral DALv3, and the deutocerebral BAmv2/3, BAlc1/2 and BAlp3. Green lines connect the clusters of somata to the belonging, BP106-positive axon tracts. C-E: Digital

3D models of late larval brains with neuropile compartments rendered semi-transparent and *ems*-positive lineages rendered blue (deutocerebral) and purple (protocerebral). Three deutocerebral lineages, BAmv3, BAlc1, and BAlp3 connect the deutocerebral antennal lobe with the protocerebrum; two deutocerebral lineages, BAmv2/3, project to the BPM compartment (D, E). BAmas1/2 are most likely tritocerebral, since they have proximal arbors in this compartment (Lichtneckert et al., 2007). Bar: 25µm

arborization in the adult brain indicates that arbors are indeed found in deutocerebral compartments as proposed above. Thus, BAlc1/2 and BAmv3 form projection neurons which have dendritic arbors in the antennal lobe (of the deutocerebrum) and which connect the antennal lobe with the protocerebrum (Lichtneckert et al., 2008; Pereanu and Hartenstein, 2006; VH, unpublished). Also, the BAlp3 and BAmv2 lineages project neurites to the VMC and form arbors there in the adult brain, providing further support for the idea that this compartment forms part of the deutocerebrum.

The remaining *ems*-positive lineage can be identified as BAmas2 based on its neuroanatomical features (Fig. 8B-E). The somata of the secondary neurons in this lineage are located most ventrally of all the *ems*-positive lineages in the larval brain; these neurons most likely represent the progeny of the unique tritocerebral neuroblast that expresses *ems* (Urbach and Technau, 2003a). The characteristic axon tract of BAmas2 projects up in the median bundle towards the protocerebrum (Lichtneckert et al., 2007; Pereanu and Hartenstein, 2006). In the adult, BAmas2 produces dendritic arborizations in the small neuropile domain that receives sensory afferents from the pharyngeal nerve, and thereby probably represents part of the tritocerebrum (see Fig.7).

Taken together, these findings indicate that the adult-specific neurons of both the *en*-expressing lineages and the *ems*-expressing lineages form their domain of arborization in discrete sets of compartments in the brain neuropile. Moreover, they suggest that lineages

deriving from different brain neuromeres innervate a non-overlapping set of neuropile compartments. These observations support a model for neuromere-specific brain neuropile, in which a given lineage forms its arborizations predominantly in the compartments that correspond to its neuromere of origin.

2.4 DISCUSSION

2.4.1 *engrailed* expression and neuromere boundaries in the *Drosophila* brain

In this paper we have analyzed the pattern of neurite projections and arborizations of the *en*-positive lineages in the brain of *Drosophila* embryos, larvae and adults. Our data demonstrate that arborizations of individual lineages are restricted to discrete neuropile compartments, or parts thereof. Furthermore, they show that the arborization pattern seen at the larval stage (formed by primary neurons) is remarkably similar to the pattern of the adult (formed by secondary neurons).

Based on the characteristic trajectory of axon tracts and their terminal arborizations, we can follow the *en*-positive lineages unambiguously from late embryo to adult. But the question arises whether the *en*-positive neuroblasts, which demarcate the posterior neuromere boundary within the early embryonic (stage 8-11) neural primordium, are the same cells that we recognize at later embryonic stages. It could be argued that the expression pattern of transcription factors, generally speaking, is dynamic; in the neural primordium, neuroblasts expressing *en* at stage 11 could lose expression during stage 12/13, and at the same time, other neuroblasts gain expression, so that the neuroblasts visible at stage 15 are different ones from those at stage 11. We argue that this scenario is very unlikely, and even if correct, the neuroblasts gaining *en*-expression would have to be the immediate neighbors of the neuroblasts that lose expression. The basis for this argument is the fact that the time interval between stage 11 and stage 15 (when tracts are clearly distinguishable) lasts only about 5 hours, and observations of embryos at very close increments (15min) can be, and were, made. In such a sequence of observations it is evident that initially, small groups of *en*-positive neuroblasts demarcate the posterior neuromere boundary. Some of these neuroblasts generate

en-positive clusters of GMCs and neurons, which stay in contact with their parent neuroblasts. As time passes, the clusters grow larger, and finally start producing axon tracts. Given this continuity of observations we can be quite certain that the metameric groups of lineages we define in the late (stage 15) embryo stem from within the metameric groups of neuroblasts defined at stage 11.

The arborization patterns of *en*-expressing lineages provide support for the existence of neuromere boundaries that coincide with morphologically defined neuropile compartment boundaries. According to our data, the boundary between protocerebrum and deutocerebrum corresponds to the interface between LAL/CC and ventromedial cerebrum (VMC). In view of the domains of the fly brain neuropile that are classically referred to as protocerebral (see, for example, Strausfeld, 1976), the findings reported here are entirely confirmatory; these domains include the superior and inferior protocerebrum, lateral horn, mushroom body, central and accessory complex, ventrolateral protocerebrum, and optic lobe. In regard to deutocerebral domains, we add the VMC to the two traditionally accepted sensory domains of the deutocerebrum, namely the antennal lobe and AMMC. Specifically, the precommissural VMC and the infracommissural VMC that flank the great commissure receive dense innervation from the *en*-positive BAla3 lineage, as well as from other deutocerebrally derived lineages expressing *ems* (e.g., BAmv2). For the tritocerebral neuropile, we tentatively assign the anterior perioesophageal neuropile domain, which lies medial and anterior of the VMC and is excluded from the BAla3 arborization, to the tritocerebrum. This coincides well with previous anatomical studies (Rajashekhar and Singh, 1994) that designated the region receiving input from the pars intercerebralis (via median bundle) and from the pharyngeal nerves as tritocerebral.

Our argument for assigning the VMC to the deuterocerebrum is based on the unity of neuronal cell bodies and their (proximal) arborizations. Thus, in the clearly metameric segmental ganglia of the ventral nerve cord the majority of neurons have most of their terminal arbors (i.e., arbors of all local neurons, dendrites of motorneurons, dendrites of many intersegmental interneurons, axon terminals of sensory neurons) in the neuropile of the same neuromere that produces their cell bodies. In other words, neurons whose cell bodies derive from segment A have neurites that largely arborize in the segmental neuropile of segment A. If this situation also applies to the neuromeres of the brain, then we would predict, for example, that the VMC should be considered part of the deutocerebral neuropile. This is because the BAla3 neurons clearly derive from the deutocerebrum and the large majority (more than 90%) of their terminal arborizations fall within the realm of the VMCpr and VMCi. To test this notion, future analyses of the arborization pattern of other lineages, as well as of connectivity patterns within the VMC, will be needed. Some insight may also be gained by identifying the serial homologs of the brain lineages in the thoracic ganglia of the ventral nerve cord, and then comparing key neuroanatomical as well as other features between homologous lineages in brain and ventral ganglion neuromeres. We are optimistic that the numerous genetic markers (e.g. Pfeiffer et al., 2008) that are becoming available at an increasing rate will soon provide more tools to study the development of anatomical modules such as compartments and neuromeres in the *Drosophila* brain.

2.4.2 VMC: features of a novel deutocerebral neuropile compartment

Currently, very little is known about the structure and function of the VMC. Classical neuroanatomical accounts dealing with the deutocerebrum in arthropods make no mention of this region (Bullock and Horridge, 1965; Homberg et al., 1989; Strausfeld, 1976). In Strausfeld's atlas of the Musca brain (1976), arguably the most detailed account of insect

brain anatomy, the only named compartment posterior to the lateral accessory complex (= ventral body) is the posterior slope. An anterior boundary of the slope is not indicated, leaving open the question whether or not a separate, distinct compartment is located between the slope and the LAL. Thus, based on published data it is not currently possible to relate the posterior slope to parts of the VMC; the posterior slope could correspond to only the posterior-most VMC layer or even to the entire VMC compartment. Irrespective of this uncertainty regarding boundaries, it has been found that the posterior slope of Musca and other insects receives ascending fibers from the subesophageal and thoracic ganglia (Strausfeld, 1976). Similarly, collateral axons of descending neurons from the ventrolateral protocerebrum are known to branch in the posterior slope. In a recent study of neurons connecting the brain and ventral nerve cord of *Drosophila* (Hartenstein et al., 2008a) we found that the larval BPM compartment, which corresponds to the adult VMC, also receives a large amount of ascending axons from the ventral nerve cord; furthermore, one of the groups of descending neurons (BP-DN) has cell bodies located in the posterior cortex adjacent to the BPM. These findings suggest that the BPM/VMC, similar to other basal neuropile compartments like the LAL (Homberg, 1994; Homberg et al., 2004) or ventrolateral protocerebrum (Strausfeld and Gronenberg, 1990), may represent an important output center where multimodal sensory input, integrated upstream or within the VMC neuropile, is used to drive descending "command neurons" that influence motor behavior.

Some of its neuroanatomical features suggest that the VMC might correspond to the dorsal "motor neuropile" of the deutocerebrum. Given the 90 degree upward turn of the neuraxis that occurs in the head between subesophageal ganglion and brain, positions that are ventral in the subesophageal (or thoracic) ganglia are anterior in the (basal) brain, and positions that are dorsal in the gnathal and ventral ganglia (like the motor neuropiles of the thoracic segments) are posterior in the brain. Are there indications that the VMC, a posterior (deutocerebral)

compartment, receives dendrites of motorneurons, like the dorsal neuropile in the thoracic segments? Although strongly modified from early developmental stages onward, the antennal segment, to which the deutocerebrum belongs, has all the hallmarks of a segment, including a myomere that gives rise to a number of mesodermal tissues, including somatic muscles and part of the vascular system (DeVelasco et al., 2006; Ullman, 1964). In the antennal segment, the myomere-derived muscles are the extrinsic muscles that move the antenna. These muscles are innervated by deutocerebral motoneurons. The central and peripheral projection of these antennal motor neurons has been studied in several insect taxa (ant: Ehmer and Gronenberg, 1997; moth: Kloppenburg et al., 1997; cockroach: Baba and Comer, 2008), but not in *Drosophila*. Dendrites of the antennal motorneurons are reported to branch in the AMMC, which is called the "dorsal lobe" in certain species by some authors (e.g., Ehmer and Gronenberg, 1997). From the description given and markers shown, it is not clear whether the AMMC/dorsal lobe of other insects corresponds to what is defined (strictly by input from the antennal nerve) as the AMMC in *Drosophila*, or whether it might also include a more posterior domain corresponding to the VMC in *Drosophila* (Peterson et al., 1998). Careful anatomical studies of the antennal motor neurons in *Drosophila* should clarify this issue.

2.4.3 Expression and function of *engrailed* at neuromere boundaries in other animals

Detailed studies of early *en* expression in the head neurectoderm have been performed for a variety of arthropods, including many insects (Boyan and Williams, 2002; Diederich et al., 1991; Peterson et al., 1998; Rogers and Kaufman, 1996; Schmidt-Ott and Technau, 1992; Younossi-Hartenstein et al., 1993), as well as a few crustaceans (Sintoni et al., 2007). Outside the arthropods, we know of *en* expression in several annelids (Prud'homme et al., 2003; Seaver and Kaneshige, 2006; Seaver et al., 2001; Shain et al., 2000; Wedeen and Weisblat, 1991), mollusks (Iijima et al., 2008; Jacobs et al., 2000; Moshel et al., 1998; Nederbragt et al.,

2002; Wanninger and Haszprunar, 2001), ascidians (Canestro et al., 2005; Imai et al., 2002), Cephalochordates (Beaster-Jones et al., 2008; Holland et al., 1997) and, of course, vertebrates (Joyner, 1996; Simon et al., 2004; 2005). In arthropods and most annelids, *en* expression is seen in the early embryo in a metameric striped pattern that coincides with segment boundaries. In addition, as shown for *Drosophila*, *en* is typically expressed in the progeny (or derivatives) of cells in the early *en* stripes (e.g. segmental clusters of neurons). Some annelids seem to have lost the early phase of *en* expression and maintain the gene only during a later phase in neurons and other metamerically organized cell types (Seaver et al., 2001). Metameric expression of *en* is not seen in vertebrates and invertebrate deuterostomians, except Amphioxus (Holland et al., 1997). In vertebrates, *en* appears in the neurectoderm in a single broad stripe that demarcates the mid-hindbrain boundary. However, as for invertebrates, some of the derivatives of the *en*-positive neurectoderm (e.g. parts of the cerebellum) maintain *en* expression during subsequent developmental phases.

In view of the fact that *en* is a transcription factor, its function very much depends on the type of genes activated or repressed by it. It is sometimes assumed that these downstream factors (in case of *en* as well as many other early expressed patterning genes) form part of a defined molecular developmental pathway. This assumption is often misleading, or at least unproductive. All one can reliably generalize about the role of *en* is that is required for both early (morphogenetic) and late (differentiative) aspects of the cells it is expressed in. With respect to its early function, *en* appears to be involved in setting up segmental boundaries, such that adhesion molecules or other structural proteins that form part of the cellular mechanism for generating or maintaining segmental boundaries (invagination of cells to form furrow; differential adhesion of cells to stabilize boundaries) are under the control of *en* (Dahmann and Basler, 2000). As far as the late function of *en* is concerned, loss of function studies of *en* in *Drosophila* and vertebrates show that cell types expressing *en*, such as the

adrenergic and serotonergic neurons of the vertebrate brainstem, are often absent or reduced in *en* mutants (Simon et al., 2004; 2005), or show pathfinding defects (Joly et al., 2007). In most of these studies, antibodies raised against the En protein were used to study *en* expression, and, since En is a nuclear protein, the morphology of *en*-expressing neurons (i.e., neurite projections, terminal arborizations) as well as the neuropile compartments they contribute to could not be analyzed in detail. It will therefore be important to develop and extend the type of genetic labeling techniques available in the *Drosophila* model system and as utilized in this report, to investigate the precise anatomical features of wildtype and mutant *en* neurons and neuronal compartments in the developing brains of other vertebrate and invertebrate animals.

3. Lineage-specific programmed cell death in the postembryonic brain development of *Drosophila*

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3.1 SUMMARY

The Drosophila central brain is composed of thousands of neurons that derive from approximately 100 neuroblasts per hemisphere. Functional circuits in the brain require precise neuronal wiring and tight control of neuronal numbers. How this accurate control of neuronal numbers is achieved during neural development is largely unclear. Specifically, the role of programmed cell death in control of cell numbers has not been studied in the central brain neuroblast lineages. Here, we focus on four postembryonic neuroblast lineages in the central brain identified on the basis that they express the homeobox gene *engrailed* (*en*). For each lineage, we determine the total number of adult-specific neurons generated as well as number and pattern of *en*-expressing cells. We then demonstrate that programmed cell death has a pronounced effect on the number of cells in the four lineages; approximately half of the immature adult-specific neurons in three of the four lineages are eliminated by cell death during postembryonic development. Moreover, we show that programmed cell death selectively affects *en*-positive versus *en*-negative cells in a lineage-specific manner and, thus, controls the relative number of *en*-expressing neurons in each lineage. Furthermore, we provide evidence that Notch signaling is involved in the regulation of *en* expression. Based on our findings, we conclude that lineage-specific programmed cell death plays a prominent role in the generation of neuronal number and lineage diversity in the Drosophila brain.

3.2 INTRODUCTION

The Drosophila brain is generated by approximately 100 neural stem cell-like neuroblasts that derive from the cephalic neuroectoderm in the early embryo (Urbach and Technau, 2003b). In the embryo, neuroblasts divide repeatedly in an asymmetric mode whereby they self renew and generate an intermediate progenitor cell called ganglion mother cell (GMC). The GMC usually divides once to produce two post mitotic progeny (Knoblich, 2008; Pearson and Doe, 2004; Skeath and Thor, 2003). Following a brief period of quiescence, during postembryonic larval development, neuroblasts re-enter the cell cycle and continue to divide in this asymmetric mode, giving rise to adult specific neurons which make up more than 90% of the adult CNS (Prokop and Technau, 1991; Truman and Bate, 1988). The adult specific neurons generated during larval life from each neuroblast form a lineage-related cluster of immature neurons, which wait until metamorphosis to differentiate and form functional circuits in the adult (Dumstrei et al., 2003; Pereanu and Hartenstein, 2006; Truman et al., 2004; Zheng et al., 2006).

It is generally believed that each postembryonic neuroblast generates between 100 to 150 neurons (for exceptions, see Bello et al., 2008; Boone and Doe, 2008; Bowman et al., 2008). It is thought that an autonomous program in the neuroblast determines its proliferative capacity and thereby, number of cells that constitute its lineage (reviewed by Hidalgo and Constant, 2003). Studies addressing this issue have pointed to two possible mechanisms; one being regulation of neuroblast proliferation and the second being elimination of a precursor neuroblast or its progeny, the postmitotic neurons, through programmed cell death.

Programmed cell death has been reported to occur widely in the fly CNS. In the late embryo, prominent cell death appears throughout the CNS as the ventral nerve cord (VNC) condenses

(Abrams et al., 1993). Among the cells in the VNC are neuroblasts of abdominal neuromeres that die via a *reaper* dependent mechanism (Peterson et al., 2002). Moreover, recent systematic analysis of the number and identity of dying cells in the embryonic VNC implies that there might be a strict spatio-temporal regulation in cell death pattern (Rogulja-Ortmann et al., 2007).

Studies conducted on identified neuroblast lineages have pointed to possible mechanisms involved in elimination of neuroblasts or postmitotic neuronal/glial cells. For instance, Hox genes are shown to be involved in regulation of programmed cell death in the developing CNS. In the embryonic VNC, *Abdominal B* (*AbdB*) expression is essential for survival of differentiated neurons in the posterior segments (Miguel-Aliaga and Thor, 2004). In the larval VNC, neuroblasts of the abdominal segments undergo apoptosis following a pulse of *Abdominal A* (*AbdA*) expression, thus regulating neuronal numbers (Bello et al., 2003). In addition, *Ultrabithorax* (*Ubx*) and *Antennapaedia* (*Antp*) act antagonistically in differentiated motoneurons of the NB7-3 and NB 2-4t lineages to regulate apoptosis (Rogulja-Ortmann et al., 2008). The NB7-3 lineage in the embryonic VNC has been extensively studied and identified neurons of this lineage have been shown to undergo cell death (Karcavich and Doe, 2005; Lundell et al., 2003; Novotny et al., 2002). Programmed cell death has also been reported for midline glial cells during embryonic life (Sonnenfeld and Jacobs, 1995) and during metamorphosis, where ecdysteroids play a key role in inducing cell death (Awad and Truman, 1997).

Although the intrinsic and extrinsic determinants controlling lineage and neuron-specific programmed cell death in the developing CNS are poorly understood, there is some evidence that cell fate determinants Notch and Numb are involved (Karcavich, 2005). During GMC division, the Numb protein is segregated asymmetrically from the GMC to one of its two

sibling daughter cells (reviewed by Cayouette and Raff, 2002; Lu et al., 2000; Skeath and Thor, 2003). In consequence, Numb blocks Notch signaling in this GMC daughter and allows the cell to assume a "B-fate". In contrast, in the other GMC daughter that does not receive Numb, Notch signaling occurs and hence, that cell assumes a different "A-fate". Importantly, as demonstrated for the lineage of neuroblast 7-3, this interaction between Numb and Notch can be responsible for controlling programmed cell death; the daughter cell of GMC-2 in which Numb inhibits Notch signaling survives, whereas its sibling in which Notch signaling is active is programmed to die (reviewed in Karcavich, 2005). A similar binary cell death decision has been demonstrated in the lineage giving rise to multidendritic neuron vmd1a in the peripheral nervous system (Orgogozo et al., 2002). Broadly speaking, Notch/Numb signaling maybe acting generically as an important mechanism, enabling the two siblings of each GMC to acquire different fates and hence, give rise to neuroblast lineages that comprise two sublineages ("B-hemilineage" and "A-hemilineage"), each with different cell fate potential.

Until now, most of the studies addressing the issue of cell death and differential cell fate have been done in the VNC. In contrast, very little is known about these processes in the central brain. In this study, we identify four postembryonic neuroblast lineages in the central brain based on the criterion that they express the homeobox gene, *en*. For each lineage, we show that they have a characteristic, relatively invariant lineage size as well as a lineage-specific number of *en*-expressing cells. We then demonstrate that programmed cell death dramatically influences number of cells in three of the four lineages. In these three, approximately half the neurons in each lineage are eliminated by programmed cell death. Furthermore, we show that programmed cell death specifically targets *en*-negative cells in two of the three lineages, whereas in the third lineage, *en*-positive cells are selected to die. In this manner, differential cell death controls the relative number of *en*-expressing neurons in each lineage. Finally, we

provide evidence that Notch signaling is involved in regulation of *en* in each of the four lineages. In conclusion, our data indicate that lineage-specific programmed cell death plays a significant role in controlling neuronal number and in generating lineage diversity in the Drosophila brain.

3.3 RESULTS

3.3.1 Four neuroblast lineages produce the *engrailed*-expressing neurons in the postembryonic central brain

The *en* gene is expressed in metamerically reiterated stripes in the embryonic neuroectoderm, in some neuroblasts (and GMCs) that derive from these neuroectodermal domains, and in well defined groups of postmitotic primary and secondary neurons, usually located at the posterior boundary of each CNS neuromere (Bossing et al., 1996; Schmid et al., 1999; Urbach and Technau, 2003a, c; Younossi-Hartenstein et al., 1996). In our analysis of mechanisms involved in the control of neuronal cell number, we focused specifically on a set of *en*-expressing neural cells in the Drosophila central brain (supraesophageal ganglion without optic lobes).

Analysis of the adult central brain using anti-En immunocytochemistry indicated that characteristic numbers of *en*-expressing cells were present and arranged in fixed spatial patterns. In particular, three spatially separated clusters of *en*-positive cell bodies were manifest (Fig. 1A, B). We refer to these clusters as the anterior cluster (AC), medial cluster (MC) and posterior cluster (PC). (Numerous *en*-expressing cells were also seen in the subesophageal ganglion; these cells were not characterized further in this study). Analysis of late L3 brain showed that the general arrangement of the *en*-expressing cell clusters in the supraesophageal ganglion was already established by end of larval development (Fig. 1C).

The clustered arrangement of these *en*-positive cells suggests that they might belong to several neuroblast lineages. (In the lineages of the central brain, the neuroblast and the secondary post-mitotic neurons generated by this neuroblast form a tight cluster at least until

metamorphosis; see Dumstrei et al., 2003). To investigate the lineage-dependent origin of *en*-expressing cells in more detail, we carried out a MARCM-based clonal analysis with a ubiqui--tous tubulin-Gal4 driving UAS-mCD8::GFP and selected labeled, secondary clones for anal-

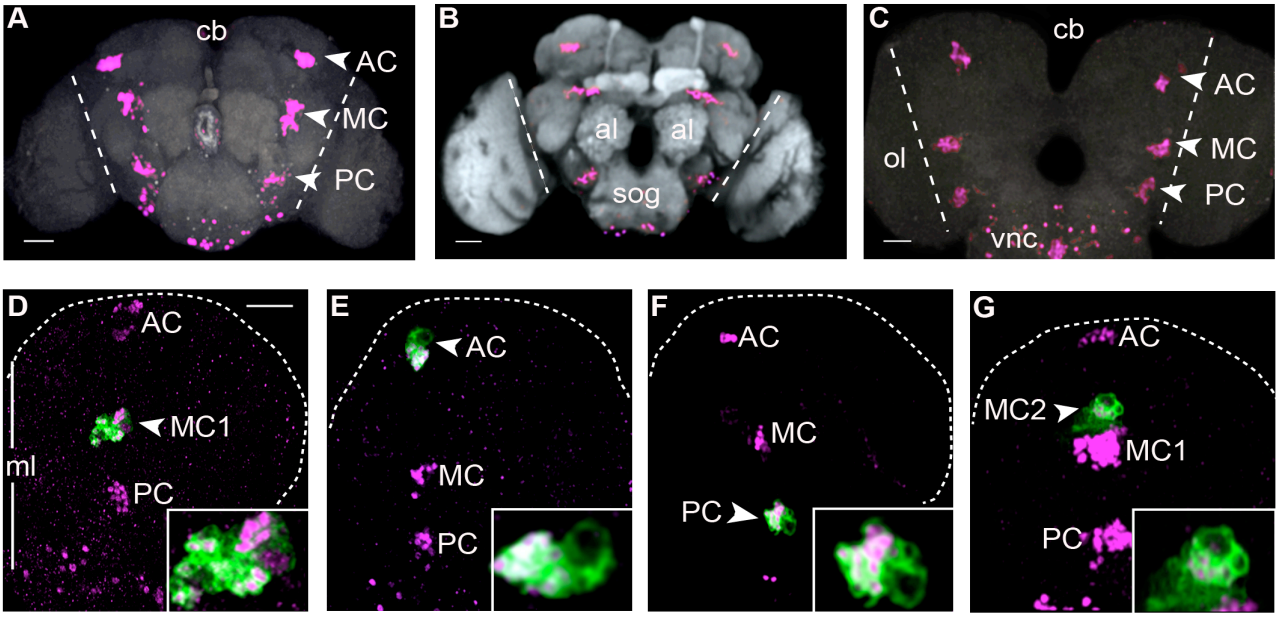

Figure 3-1. *engrailed* is expressed in three clusters in the *Drosophila* postembryonic central brain. Anti-En in magenta. A, C: Frontal view, maximal projections of wildtype (Oregon R) adult and L3 brain respectively. Arrowheads: *en*-expressing clusters- Anterior Cluster (AC), Medial Cluster (MC) and Posterior Cluster (PC). B: Frontal view, maximal projection of adult brain (y,w; rbo^2; rbo-eGFP, Huang et al., 2004). Anti-GFP (grey) highlights neuropile. D-G: Frontal view, L3 brains. Right hemisphere shown. The three *en* clusters correspond to four neuroblast lineages in L3. Z projections of selected panels of wildtype, GFP-labeled MARCM clones expressing *en* (inset- higher magnification). D: MC1, E: AC, F: PC and G: MC2 lineage. Abbreviations: cb central brain, sog subesophageal ganglion, ol optic lobes, al antennal lobes, vnc ventral nerve cord, ml midline. Scale bars: A-C: 50µm, D-G: 30 µm.

-yses that contained immunolabeled *en*-positive cells. In this analysis, we consistently recovered neuroblast clones containing *en*-positive cells in the central brain that corresponded to four different neuroblast lineages (Fig. 1D-G). While cells of AC cluster belonged to one lineage and cells of PC cluster belonged to another lineage, the cells of MC cluster belonged to two different lineages that were closely apposed in the brain. We refer to the two lineages

that make up MC cluster as MC1 and MC2 lineages. Based on location of cell bodies, projection of secondary axon tracts, and the innervated set of compartments we identified these four lineages, according to criteria and terminology established by Pereanu and Hartenstein (2006), as DPLam (AC), DALv2 (MC1), DALv3 (MC2), and BAla3 (PC); the AC, MC1 and MC2 lineages are thus protocerebral and PC lineage is deutocerebral (Kumar et al., 2009, in press).

3.3.2 Number and spatial pattern of *engrailed*-expressing neuronal cells are lineage-specific

Although our clonal analysis indicated that the *en*-expressing cells in the central brain belong to four lineages, not all cells in these four lineages were *en*-expressing. The overall lineage size (all GFP positive cells in a clone, excluding the neuroblast) and the number of *en*-positive cells (GMCs and differentiated neurons) were found to be different for the four lineages (Fig. 2). In terms of overall cell number, MC1 lineage was strikingly larger than the other three lineages. MC1 neuroblast clones contained approximately 130 cells, while AC, PC and MC2 neuroblast clones comprised 60-80 cells. MC1 neuroblast clones also had the largest number of *en*-expressing cells (average of 87 $\pm$ 7.2). AC and PC neuroblast clones had somewhat less *en*-positive cells (averages of 63 $\pm$ 5.9, for AC, 55 $\pm$ 4.7 for PC) and, remarkably, the MC2 neuroblast clone had less than twenty (17 $\pm$ 3.4) *en*-positive cells.

To characterize the lineage-specific differences in more detail, we combined MARCM-based clonal analysis with anti-En and neuron-specific marker labeling (anti-Elav) and analyzed the spatial arrangement of *en*-positive versus *en*-negative cells in all four lineages at late L3.

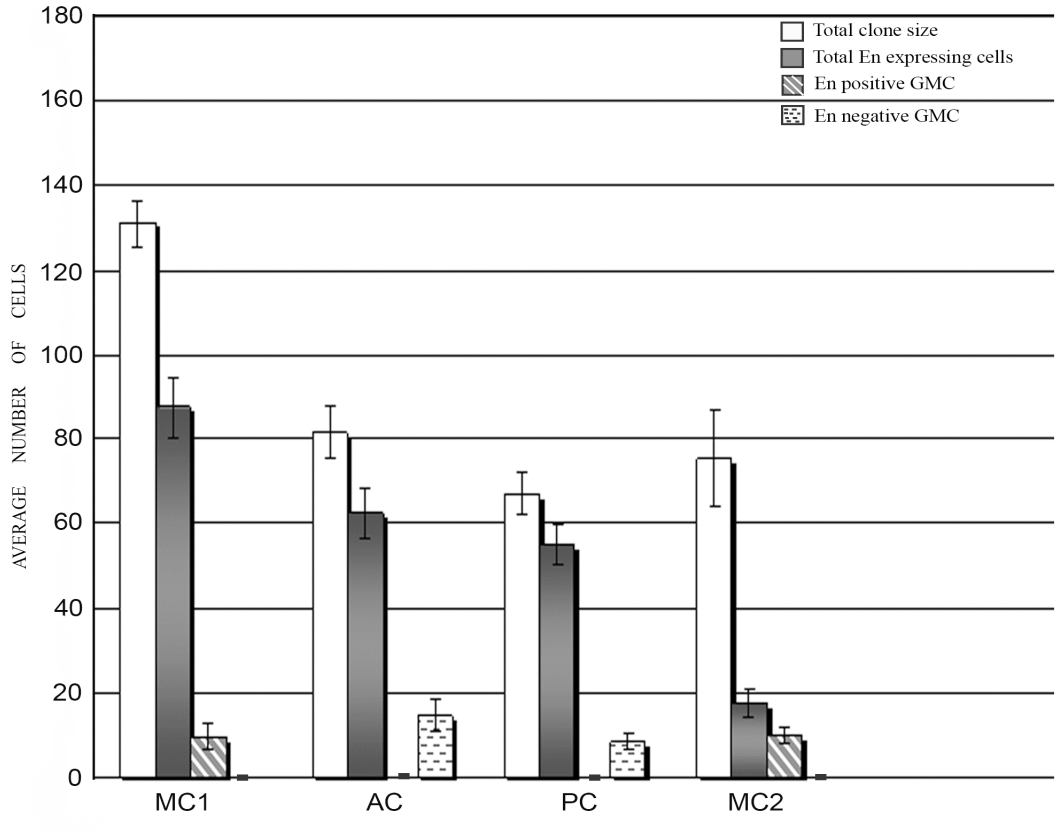

Figure 3-2. Clone size difference between *engrailed*-expressing lineages. Histogram: total clone size (all GFP-positive cells, excluding neuroblast), number of *en*-expressing cells (Elav-negative GMCs and Elav-positive neurons) and number of *en*-positive and *en*-negative GMCs of the four, wildtype MARCM clones. Average number of cells plotted against type of lineage. Sample size (n): MC1, AC, PC n=12 each and MC2 n=7. Error bars indicate standard deviation.

In the MC1 lineage, the large cortically located neuroblast and an average of 10 $\pm$ 3.1 immediately adjacent GMCs and undifferentiated neurons consistently expressed *en* (Fig. 2, 3A). In addition to the neuroblast and GMCs, the clone contained a large number of Elav-expressing cells that represent secondary neurons of the lineage. Approximately two thirds of these neurons were *en*-positive and one third were *en*-negative (Figs. 3A, A', 5A'). When labeled clones were examined at different levels that extended from the outer cortical layer towards the central neuropile, there was no obvious segregation into clonal subregions

containing preferentially *en*-positive or *en*-negative neurons. Rather, both types of neurons appeared to be spatially intermixed throughout the MARCM clone (summarized in Fig. 3A’’).

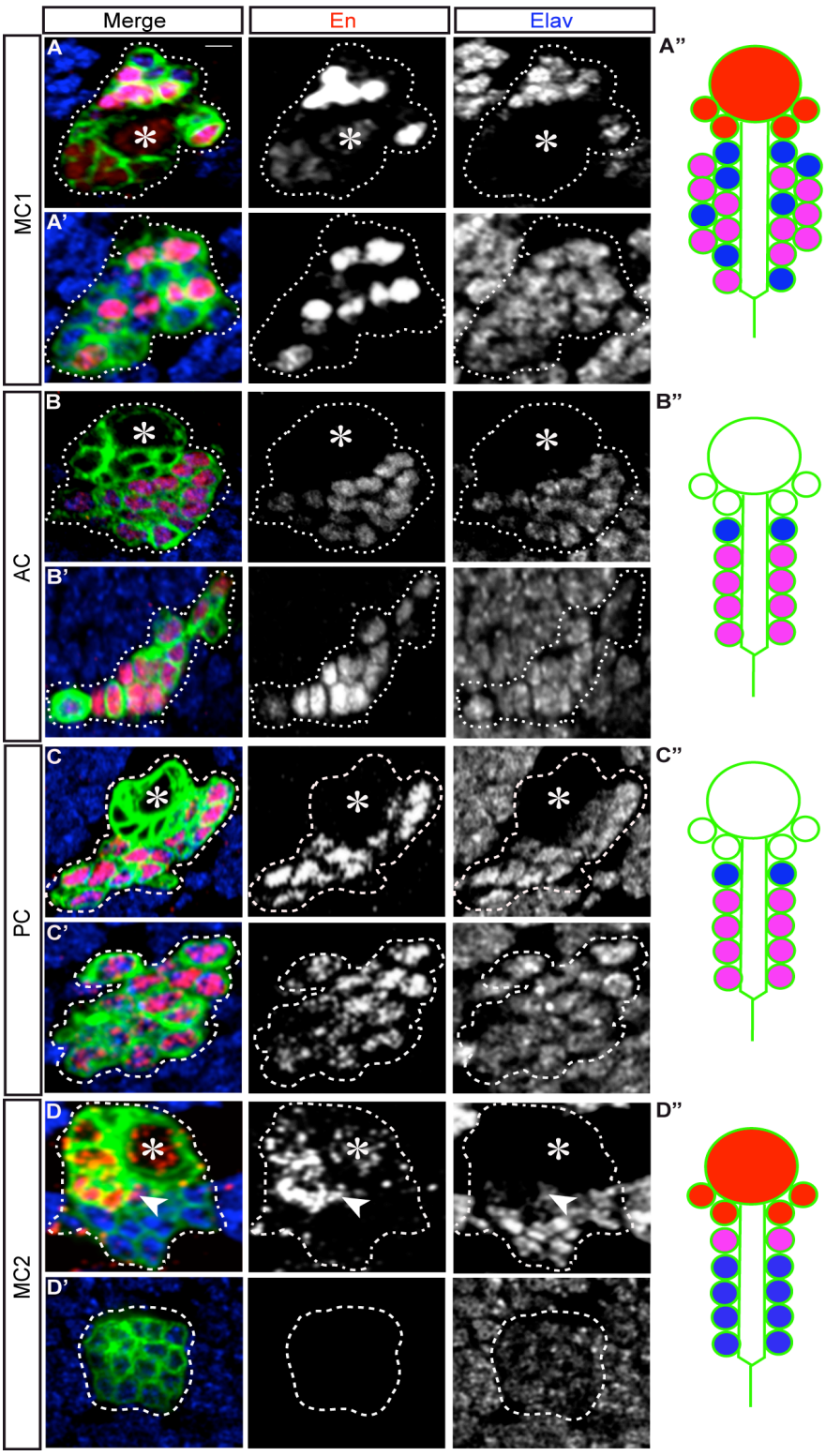

Figure 3-3. Lineage-specific expression of *engrailed*. Single sections of wildtype MARCM clones. Anti-GFP (green), Anti-En (red), Anti-Elav (blue). White dotted lines outline clones. Star indicates Neuroblast. Lineages as follows: MC1 (A-A'), AC (B-B'), PC (C-C') and MC2 (D-D'). A-D: Optical section at a superficial focal plane. A, D: Neuroblast and new born progeny express *en*. D: White arrowhead indicates *en*-positive neuron. A'-D': Optical section at a deeper focal plane. A"-D": Schematics represent MC1, AC, PC and MC2 respectively. Color code according to immunostaining; i.e., schematic *en*-positive and negative neurons in pink and blue respectively. Scale bar A-D': 10μm.

In the AC and PC lineages, the large neuroblast and the adjacent small group of GMC-like cells (averages of 15 $\pm$ 3.8 and 9 $\pm$ 1.8 respectively) did not express *en* (Fig. 2, 3B, C). In addition to the neuroblast and GMC-like cells, the clones contained a large number of Elav-expressing cells representing secondary neurons of the lineage. Almost all of these neurons were *en*-positive; only a very small number (< 5) were *en*-negative (Figs. 3B, B', C, C', 5B, B', C, C'). Thus, in both AC and PC lineages, virtually all the neurons throughout all clonal subregions extending from the outer cortical layer towards the central neuropile expressed *en* (summarized in Fig. 3B'', C'').

Finally, in MC2 lineage, as in MC1, the neuroblast and the small group of (average 10 $\pm$ 1.8) adjacent GMC-like cells expressed *en* (Fig. 2, 3D, D'). Expression of *en* was also observed in a small number of Elav-expressing secondary neurons (<10) located near the neuroblast. Based on their position relative to the neuroblast, these neurons may be recently born. However, the remaining secondary neurons in all clonal subregions of MC2 lineage were consistently *en*-negative (Figs. 3D, D', 5D'). Thus, in contrast to their neuroblast of origin, most neurons in MC2 did not express *en* (summarized in Fig. 3D'').

In summary, we observed a striking neuronal heterogeneity among the lineages that contain *en*-expressing adult-specific neurons in the brain. The MC1 lineage is the largest in terms of

its cell number as compared to the other three and the secondary neurons of MC1 are a mixture of both *en*-positive and *en*-negative cells. The AC and PC lineages are comparable in size and have a similar pattern of *en* expression; almost all the neurons in these two lineages express *en*. The MC2 lineage, although comparable in size to the AC and PC lineages, is drastically different in terms of its *en* expression in that almost all neurons in the MC2 lineage do not express *en*.

3.3.3 Programmed cell death shapes neuroblast lineage size in postembryonic brain development

To investigate if lineage-specific programmed cell death is involved in determining the total number of cells in these lineages, we blocked apoptosis by generating H99 mutant MARCM neuroblast clones in which the three pro-apoptosis genes, *head involution defective* (*hid*)*, grim* and *reaper* (*rpr*) were eliminated in the genomic region at 75C, due to a homozygous deficiency Df3(3L)H99 (Chen et al., 1996; Grether et al., 1995; White et al., 1994). We then quantified the number of cells in wildtype versus H99 clones for each *en* lineage. Both types of clones were co-immunolabeled with anti-En and anti-Elav. Due to lack of programmed cell death, the total number of cells in all four lineages increased. However, the magnitude of these increases was markedly different for the MC1 lineage as compared to the AC, PC, and MC2 lineages (Fig. 4).

In the H99 MC1 lineage, a relatively small increase in total cell number from an average of 131 $\pm$ 5.5 cells to an average of 154 $\pm$ 7.7 cells was observed. In contrast, in the H99 AC lineage, a striking increase from an average of 81$\pm$ 6.3 cells to an average of 151 $\pm$ 7.6 cells was seen. This value, which corresponded to an approximate doubling in cell number in H99 AC lineages, was similar to the total number of cells observed in the H99 MC1 lineage.

Comparable, albeit slightly less pronounced increases were observed in the PC lineage (increase in average cell number from 67 ± 5 to 118 ± 10) and the MC2 lineage (increase in average cell number from 75 ± 11.4 to 125 ± 9.6) following apoptosis block. The marked increase in total cell number observed in H99 clones of AC, PC, and MC2 lineages indicates that programmed cell death plays a major role in determining the size of these lineages, whereas it appears to have only a minor role in size control of MC1 lineage.

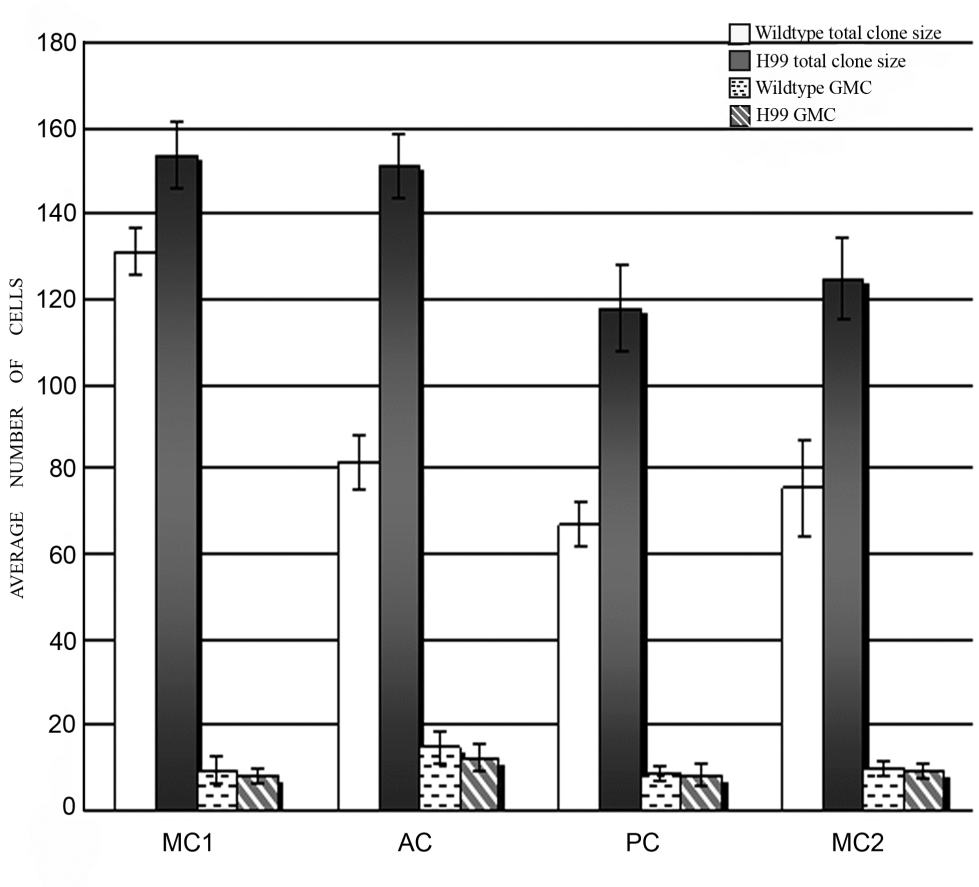

Figure 3-4. Effect of blocking cell death on total clone size of *engrailed*-expressing lineages. Histogram shows difference in total clone size (all GFP-positive cells, excluding neuroblast) and GMCs (all Elav negative, but *en*-positive or *en*-negative cells) between wildtype and H99 *en* lineages. Sample size (n) for H99 lineages: MC1, AC, PC n=12 each and MC2 n=9. Error bars indicate standard deviation.

3.3.4 Programmed cell death controls the number of *engrailed*-expressing neurons in a lineage-specific manner

Given the lineage-specific role of programmed cell death in determining the total clonal cell number in wildtype and H99 clones (Fig.4), we wondered if cell death determines the number of neurons in a lineage-specific manner. To investigate this, we quantified the number of *en*-positive and *en*-negative neurons in wildtype versus H99 MARCM clones for each lineage.

In MC1 neuroblast clones, the number of *en*-positive neurons was similar in H99 and wildtype; however, the number of *en*-negative neurons increased from approximately 44 $\pm$ 6.3 in wildtype to 71 $\pm$ 6 in H99 clones (Fig 5A, A'). This implies that the increase in overall cell number observed in H99 MC1 clones (Fig. 4) is due primarily to an increase in number of surviving *en*-negative neurons. This results in an approximately equal number of *en*-negative and *en*-positive secondary neurons in H99 MC1 clones (summarized in Fig. 5A'').

In H99 AC and PC neuroblast clones, the number of *en*-positive neurons was comparable to that in wildtype. However, the number of *en*-negative neurons increased dramatically from 4 $\pm$ 2.5 cells in wildtype to 72 $\pm$ 5.6 cells for AC clones and from 3 $\pm$ 1.7 cells in wildtype to 55 $\pm$ 6.1 cells for PC clones following apoptosis block (Fig. 5B, B', C, C'). Indeed, the marked increase in overall cell number observed in H99 AC and PC clones (Fig. 4) appears to be almost exclusively due to an increase in the number of surviving *en*-negative neurons. Thus, after apoptosis block, these neuroblast clones also contained approximately equal numbers of *en*-negative and *en*-positive secondary neurons (summarized in Fig. 5 B'', C'').

In MC2 neuroblast clones, the number of *en*-positive neurons was clearly affected by apoptosis block and increased markedly from 8 $\pm$ 3.6 in wildtype to approximately 39 $\pm$ 9.8 cells in apoptosis-blocked clones (Fig. 5D, D'). In contrast, the number of *en*-negative neurons showed a much smaller increase in wildtype versus H99 clones. Thus, in contrast to the other three lineages, the striking increase in cell number observed in H99 MC2 clones

(Fig. 4) appears to be due primarily to an increase in number of surviving *en*-positive neurons (summarized in Fig. 5D’’).

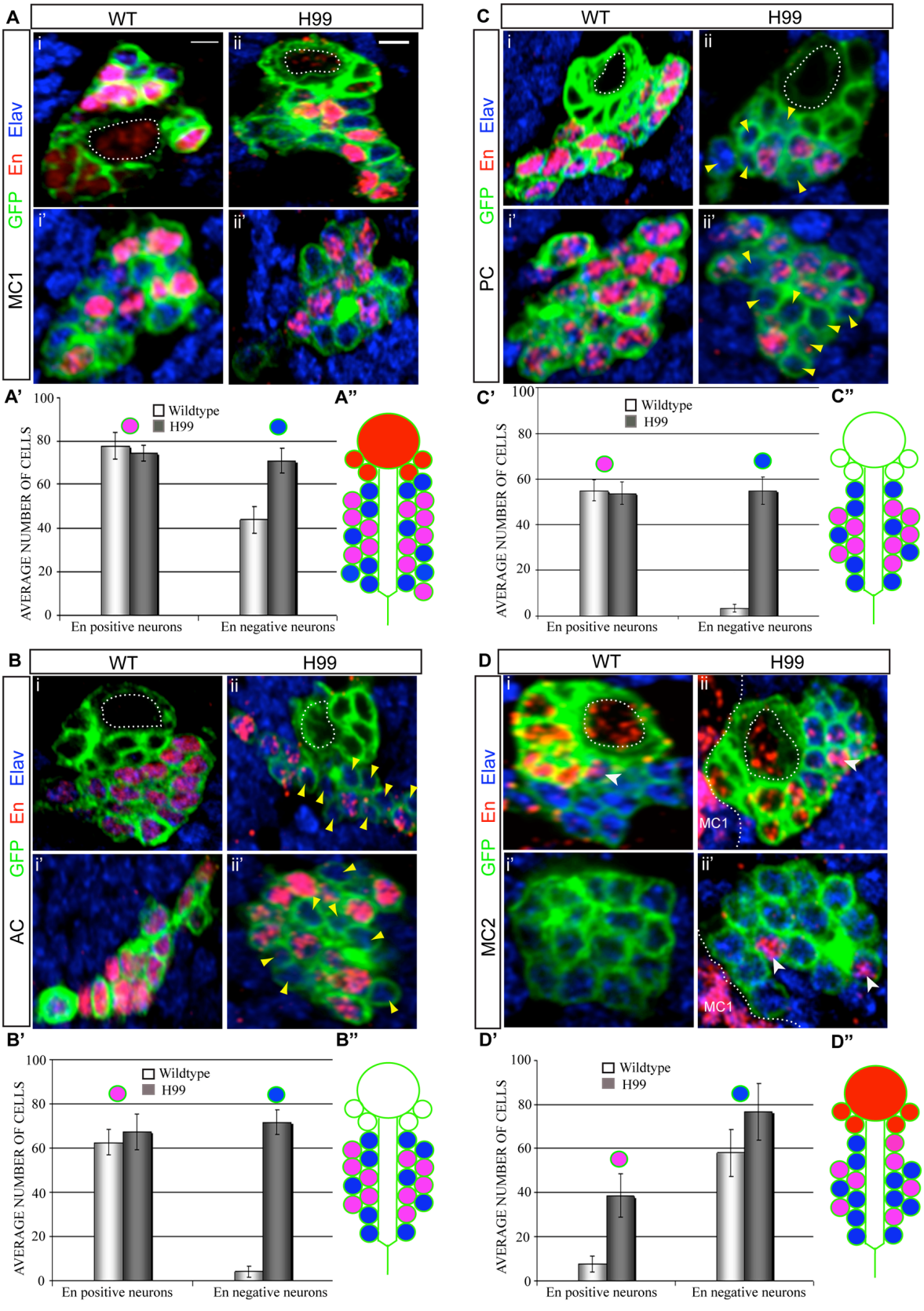

Figure 3-5. Lineage-specific effect of blocking cell death on number of *engrailed*- expressing neurons. A-D: Z projections of WT (i-i') and H99 (ii-ii') MARCM clones (MC1, AC, PC and MC2 respectively). Anti-GFP (green), Anti-En (red) and Anti-Elav (blue). White dotted lines outline Neuroblast. Top row (A-D i, ii): Superficial optical section. Bottom row (A-D i', ii'): Deeper optical section. Yellow arrowheads indicate *en*-negative neurons; white arrowheads indicate *en*-positive neurons. Histograms A'-D': Effect of blocking cell death (H99) on number of *en*-positive and *en*-negative neurons, compared to WT. White and grey bars: WT and H99 *en*-lineages respectively. Left Bars: number of *en*-positive neurons; right hand bars: number of *en*-negative neurons. A"-D": Schematics of H99 MC1, AC, PC and MC2 MARCM clones respectively. Color codes according to immunostaining. Error bars indicate standard deviation. Scale bars A-D: 10μm.

Taken together, these findings indicate that programmed cell death has lineage-specific effects on *en*-positive and *en*-negative neurons in the postembryonic brain. In the MC1 lineage, programmed cell death eliminates some *en*-negative neurons but does not affect *en*-positive neurons. In the AC and PC lineages, programmed cell death eliminates virtually all *en*-negative neurons, but does not affect *en*-positive neurons. By contrast, in the MC2 lineage, programmed cell death eliminates majority (approximately three fourths) of the *en*-positive neurons but affects only few *en*-negative neurons.

3.3.5 Evidence for a hemilineage-specific effect of programmed cell death on *engrailed* expression

The observed lineage-specific effects of programmed cell death on neuronal number and *en* expression support a model in which each of the four neuroblasts generates two distinct hemilineages. In this model, each GMC division gives rise to one *en*-positive and one *en*-negative neuron which are then selectively targeted by programmed cell death.

According to this model, programmed cell death would target primarily (i) the *en*-negative hemilineage of AC and PC neuroblasts, (ii) the *en*-positive hemilineage of MC2 neuroblast,

and (iii) neither hemilineage of the MC1 neuroblast (Fig. 8). This model makes the following predictions. First, in the AC and PC lineages, most of the surviving neurons should be *en*-positive. Second, in the MC2 lineage, most of the surviving neurons should be *en*-negative. Third, in the MC1 lineage, both *en*-positive and *en*-negative neurons should mostly survive. Fourth, following apoptosis block, all four lineages should be of approximately the same size. Fifth, following apoptosis block, all four lineages should contain comparable numbers of *en*-positive and *en*-negative neurons.

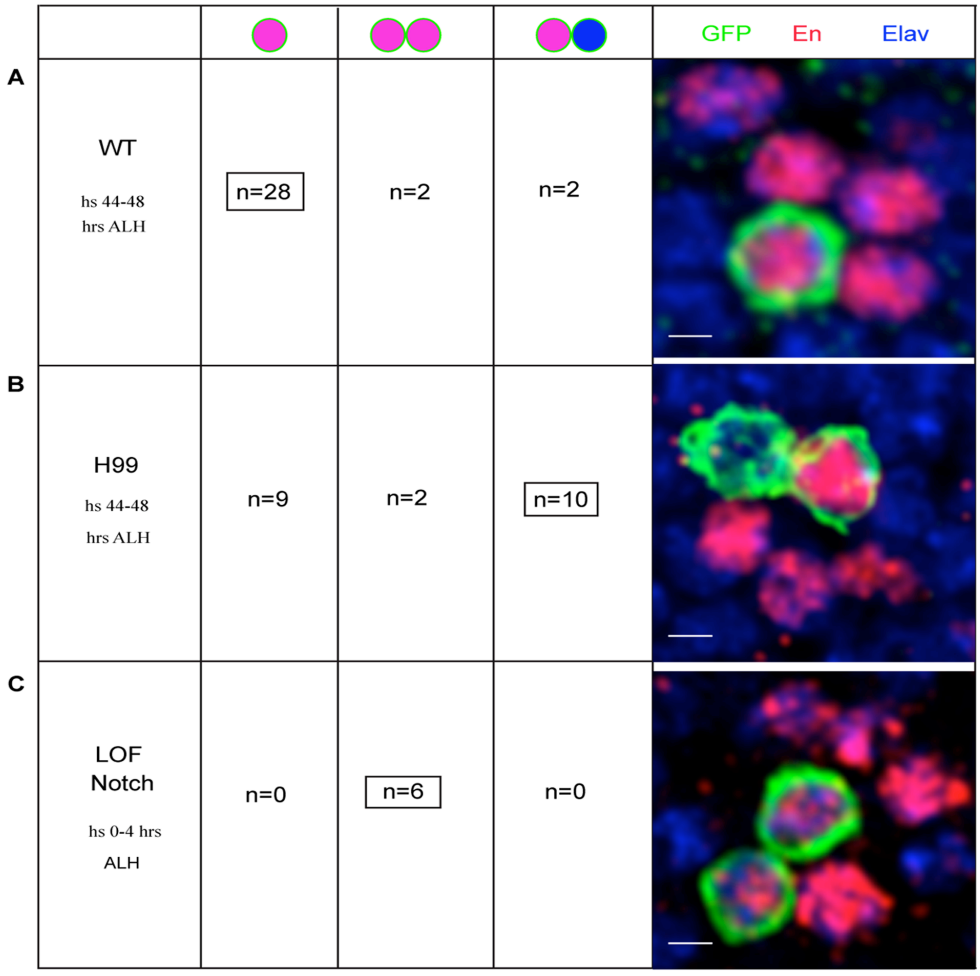

Figure 3-6. Evidence for hemilineage-specific cell death in PC lineage. Figure shoes GFP-labeled single and two-cell MARCM clones (green) in PC lineage. Anti-En (red) and Anti-Elav (blue). Time of heat shock treatment indicated. Top row: Schematic of clone types examined; pink and blue circles represent *en*-positive and *en*-negative neurons respectively. n shows number of times a clone type was

recovered; most frequent clone type boxed and represented in corresponding figure panel. A: WT PC clones. Majority single *en*-positive neurons (n=28 from 286 examined brain hemispheres). Very few two-cell clones recovered (n=4). B: H99 PC clones. Majority two-cell clones, having an *en*-positive and *en*-negative neuron (n=10 from 280 examined brain hemispheres). Single *en*-positive neurons also obtained (n=9). C: Notch mutant PC MARCM clones. All two cell clones with *en*-positive neurons (n=6 from 544 examined brain hemispheres). Scale bar A-C: 10μm.

While the hemilineage model explains majority of our findings, there are several results that are not in full quantitative accord with it. First, a small number (<5) of *en*-negative neurons are found in AC and PC lineages; the model predicts that all neurons should be *en*-positive. Second, a small number (<10) of *en*-positive neurons are found in the MC2 lineage; the model predicts that all neurons should be *en*-negative. Third, a low level of cell death (involving approximately 20 *en*-negative neurons each) does occur in the MC1 and MC2 lineages; the model predicts that no cell death should occur in the MC1 lineage, while only *en*-positive neurons should die in MC2 lineage.

To test the validity of this model in more detail, we focused on the PC neuroblast lineage and recovered both wildtype and H99 single-cell and double-cell MARCM clones, co-immunolabeled with anti-En and anti-Elav. In MARCM experiments, double-cell clones are recovered when both daughter cells of a GMC are labeled following the recombination event, whereas single-cell clones are recovered when only one daughter cell of a GMC is labeled following recombination (see Lee and Luo, 2001). However, a single-cell clone will also be also recovered in place of a double-cell clone if one of the two daughter cells of the GMC undergoes apoptosis between the recombination event and the time of MARCM clone recovery.

According to the model, the *en*-negative hemilineage is primarily targeted by programmed cell death in the wildtype PC lineage. Hence, double-cell clones which comprise both GMC

daughter cells should be rare and single-cell clones should be predominant in wildtype brains. This was indeed the case (Fig. 6A). In wildtype, 87% non-neuroblast clones recovered were single-cell neuronal clones. Also according to the model, following apoptosis block, both *en*-positive and *en*-negative hemilineages survive. Hence, both double-cell clones comprising one *en*-positive and one *en*-negative neuron, and single-cell clones should be recovered in apoptosis blocked clones of the PC lineage. This was also the case (Fig. 6B). Following apoptosis-block, double-cell clones and single-cell clones were recovered in approximately equal numbers. Moreover, over 80% of double-cell clones consisted of one *en*-positive and one *en*-negative neuron.

Finally, since Notch-signaling is known to be an important factor in assigning differential cell fate to the two daughter cells of a GMC (see Karcavich, 2005), we blocked Notch signaling in the PC lineage by generating Notch loss-of-function clones. Given that loss of Notch signaling should cause both GMC daughters to adopt the same fate, the model predicts that recovered two-cell clones would consist of two surviving *en*-positive neurons. The experimental results confirm this prediction; all recovered two-cell clones comprised two *en*-positive neurons (Fig. 6C).

3.3.6 Notch signaling influences number of *engrailed*-expressing neurons in a lineage-specific manner

The analysis of Notch mutant two-cell clones in the PC lineage suggests that loss of Notch signaling results in a neuronal cell fate transformation such that both daughters of GMCs in this lineage become *en*-positive. To determine if loss of Notch signaling might have comparable effects on *en*-expression in neurons of other *en* lineages, we recovered Notch

loss-of-function neuroblast clones for all four lineages. Neuroblast MARCM clones were co-immunolabeled with anti-En and anti-Elav. We then quantified number of *en*-positive and *en*-negative neurons in wildtype versus Notch loss-of-function neuroblast clones for each lineage.

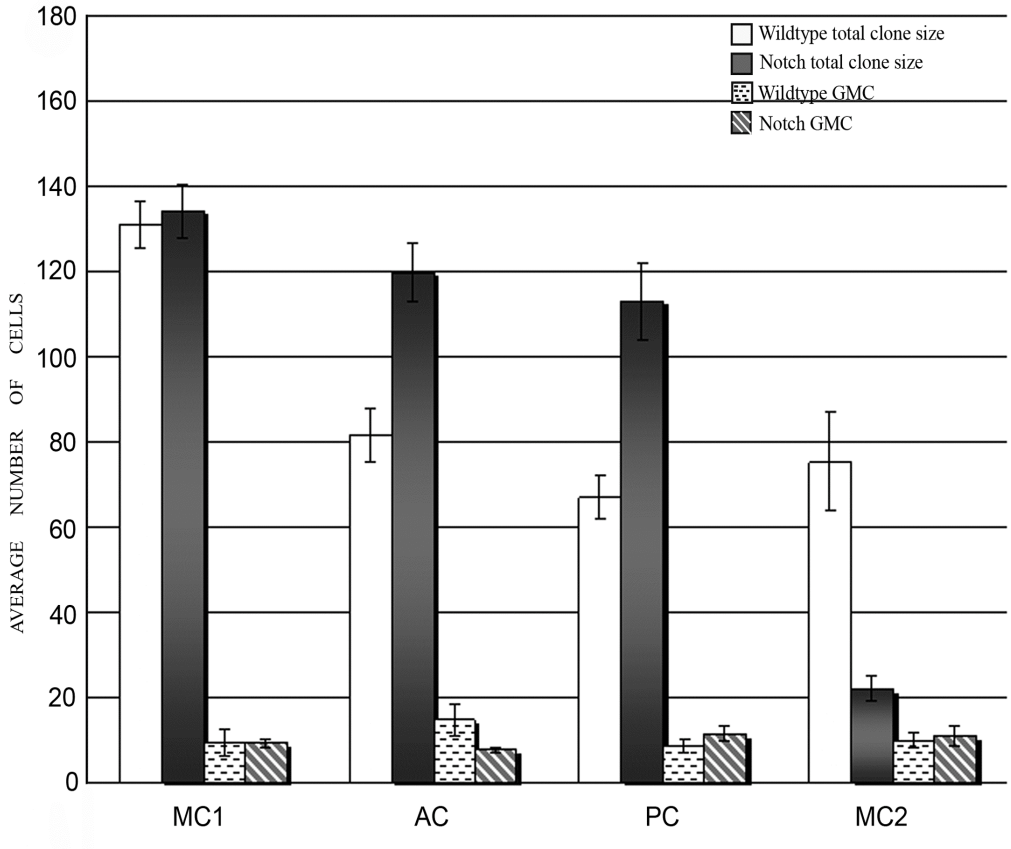

Figure 3-7. Effect of loss-of-Notch function on total clone size of *engrailed*-expressing lineages. Histogram shows difference in total clone size (all GFP-positive cells, excluding the neuroblast) and GMCs (all Elav-negative, but *en*-positive or *en*-negative cells) between wildtype and Notch mutant, *en*-expressing clones. Error bars indicate standard deviation.

In Notch loss-of-function MC1 neuroblast clones, all neurons were *en*-positive; *en*-negative neurons were no longer seen (Fig. 8A, A'). Moreover, as expected, given the low level of programmed cell death in the MC1 lineage, there was no obvious size decrease or increase in mutant versus wildtype clones (Fig. 7). This suggests that most neurons fated to become *en*-

negative in wildtype adopted an *en*-positive phenotype in absence of Notch signaling (resulting phenotype schematized in Fig. 8A'').

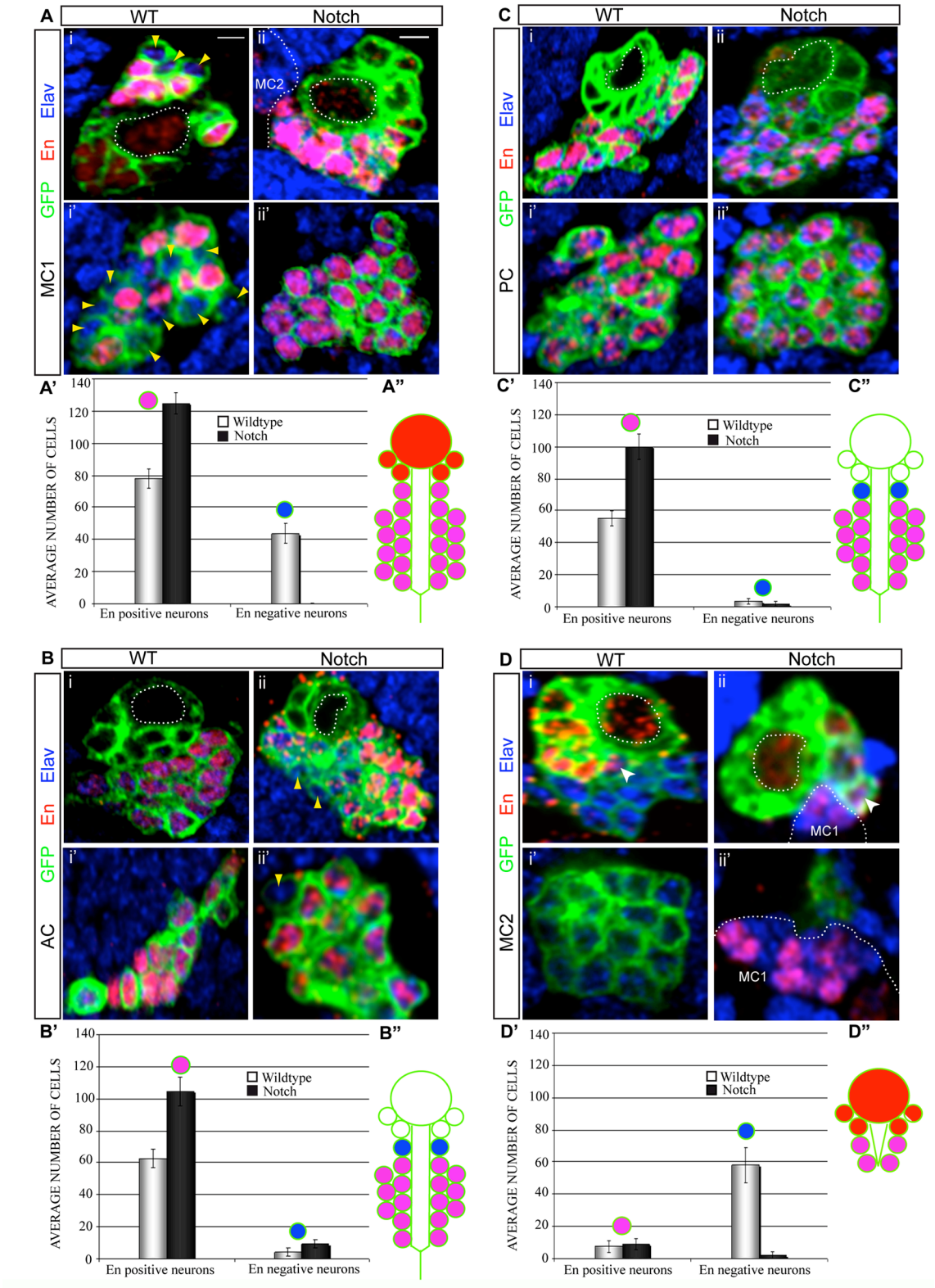

Figure 3-8. Effect of loss-of-Notch function on *engrailed* lineages. A-D: Z projections of WT (i-i') and Notch mutant (ii-ii') MARCM clones (MC1, AC, PC and MC2 respectively). Anti-GFP (green), Anti-En (red), Anti-Elav (blue). All top and bottom row panels (A-D) similar to Figure5. Yellow arrowheads indicate *en*-negative neurons; white arrowheads indicate *en*-positive neurons. A'-D': Histograms show effect of loss of Notch function on number of *en*-positive and negative neurons, compared to WT. A"-D": Schematics of Notch mutant MC1, AC, PC and MC2 clones respectively. Color codes according to immunostaining. Sample size (n) for Notch mutant lineages: MC1 n=6, AC n=4, PC n=9 and MC2 n=7. Error bars indicate standard deviation. Scale bars A-D: 10μm.

In Notch loss-of-function AC and PC neuroblast clones, the number of surviving *en*-positive neurons increased markedly (105 $\pm$ 9 for AC; 100 $\pm$ 7.9 for PC) as compared to wildtype (63 $\pm$ 5.9 for AC and 55 $\pm$ 4.7 for PC), while the small number (<10) of *en*-negative neurons did not change significantly (Fig. 8B, B'', C, C'). Resulting mutant clones were almost twice as large as wildtype clones (Fig. 7). This is what would be expected if most neurons fated to become *en*-negative in wildtype, and thus programmed to die in AC and PC lineages (see Fig. 9), adopted an *en*-positive phenotype and hence survived in absence of Notch signaling (resulting phenotype schematized in Fig. 8B'', C'').

In contrast, in Notch loss-of-function MC2 neuroblast clones, the number of surviving *en*-negative neurons decreased dramatically (2 $\pm$ 2.3) as compared to wildtype (58 $\pm$ 10.8), while the small number (< 10) of *en*-positive neurons was not affected (Fig 8D, D'). The resulting mutant clones were very small compared to wildtype clones and did not have a discernable projection pattern, probably due to very few neurons (Fig. 8, 9). This is what would be expected if most neurons fated to become *en*-negative in wildtype, and thus programmed to survive in MC2 lineage (see Fig. 10), adopted an *en*-positive phenotype and hence died in absence of Notch signaling (resulting phenotype schematized in Fig. 8D'').

Taken together, these findings indicate that Notch signaling has lineage-specific effects on number of *en*-expressing neurons in the four brain neuroblast lineages. Loss of Notch function caused a marked increase in number of surviving *en*-positive neurons in AC and PC lineages, a dramatic decrease in surviving *en*-negative neurons in MC2 lineage and an increase in *en*-positive neurons as well as a corresponding decrease in *en*-negative neurons in MC1 lineage. These results support the hypothesis that loss of Notch signaling causes all neurons in the four lineages to acquire an *en*-positive cell fate and the cells survive or die depending on lineage-specific context.

3.4 DISCUSSION

3.4.1 Lineage-specific cell death in the postembryonic brain

Programmed cell death has been extensively investigated in the embryonic insect CNS. In VNC neuromeres, programmed cell death has important roles in generating dramatic differences in neuronal numbers in abdominal versus thoracic ganglia. Thus, although both abdominal and thoracic neuromeres begin proliferation via a nearly identical number of embryonic neuroblasts, mature thoracic ganglia comprise approximately 3000 neural cells, whereas mature abdominal ganglia comprise approximately 500 neurons. Programmed cell death together with different neuroblast lifespans and proliferation rates are responsible for this difference (reviewed in Buss et al., 2006). Segment-specific programmed cell death has also been shown to occur during CNS embryogenesis (Miguel-Aliaga and Thor, 2004; Rogulja-Ortmann et al., 2007).

In postembryonic CNS development of holometabolous insects such as flies, a combination of programmed cell death and neuronal process reinnervation allows the larval nervous system to reorganize and innervate new body structures (Truman, 1983; Truman et al., 1992; Weeks, 2003; Weeks and Truman, 1985). During metamorphosis many adult-specific neurons in the ventral ganglia are targeted by programmed cell death, particularly in abdominal segments (Bello et al., 2003; Booker and Truman, 1987; Booker et al., 1996). Furthermore, extensive cell death occurs during postembryonic development in the insect visual system where cells are overproduced and those that do not make the appropriate targets are eliminated by apoptosis (Bonini and Fortini, 1999). In contrast, very little is currently known about the prevalence and functional roles of programmed cell death in development of the insect adult central brain.

In this report, we identify four neuroblast lineages in the postembryonic central brain and find that programmed cell death occurs in all four lineages, albeit to different extents. Whereas cell death plays only a minor role in the MC1 lineage, it has dramatic effects in AC, PC, and MC2 lineages, in which nearly half of the adult-specific neuronal progeny are programmed to die during larval development. It is noteworthy that the adult-specific neurons targeted by cell death are generated during larval development and are eliminated before their respective neuroblasts stop proliferating (12-24 hours after pupal formation). Because the cell death reported here occurs before neuronal differentiation, it is probably not involved in events of brain reorganization that take place during metamorphosis.

Another central feature of the cell death events demonstrated here is that none of the four lineages are completely eliminated by cell death; all four neuroblasts and a significant number of their neuronal progeny survive at the end of larval development, and these neuronal progeny are largely present in the adult. In this sense, the programmed cell death reported here is likely to be functionally different from the cell death observed in the ventral ganglia, where the neuroblast itself undergoes apoptosis, regulating neuronal numbers in the abdominal segments (Bello et al., 2003; Truman and Bate, 1988).

Our experiments indicate that programmed cell death plays a prominent role in determining lineage-specific features; if cell death is blocked the total neuronal number increases in all four lineages and the number of *en*-expressing neurons increases in AC, PC and MC2. Furthermore, we examined the axonal projection pattern of H99 and Notch mutant *en*-expressing lineages, comparing them to wildtype (Fig. 9). Both H99 and Notch mutant PC lineages showed an additional projection which was not present in the wildtype, while the other three H99 lineages did not appear to change drastically in their projection patterns, as

compared to wildtype. In conclusion, programmed cell death appears to contribute to the cellular diversity of neuronal lineages in the central brain.

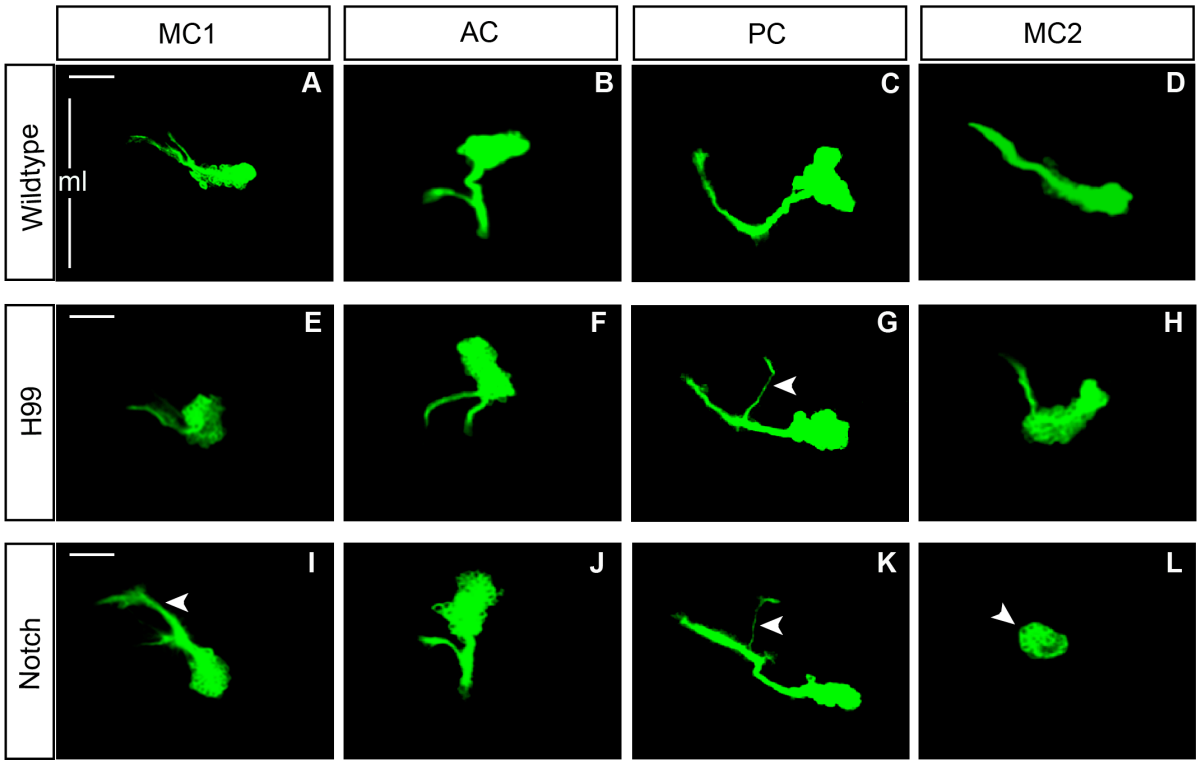

Figure 3-9. Axonal projection patterns of wildtype, H99 and Notch mutant, *en*-expressing lineages. Each panel represents Z projections of GFP-expressing, MARCM clones in L3 brain. Axon tracts are shown, extending from cell body clusters of respective clones. A-D: Wildtype, E-H: H99, I-L: Notch mutant MC1, AC, PC and MC2 lineages respectively. White arrowheads point to additional (G, K) or altered (I) projections, as compared to wildtype. L: Due to small clone size and consequently, very few neurons, Notch mutant MC2 lineage does not appear to have an axonal projection. ml midline. Scale: 50 µm.

3.4.2 Differential cell fate, Notch signaling and generation of hemilineages in the brain

Studies on neuroblast lineages in the developing ventral ganglia indicate that proliferating neuroblasts generate a largely invariant clone of neural cells. In general, each neuroblast division produces a distinctly fated GMC, and each GMC division produces two sibling

progeny of different fates (Egger et al., 2007; reviewed in Technau et al., 2006). There is some evidence that the fate of these progeny is controlled by the parental GMC; the two siblings are restricted to a pair of different cell fates with one sibling adopting an "A" fate and the other adopting a "B" fate (JW Truman, personal communication; Skeath and Doe, 1998). This, in turn, has led to a model in which a neuroblast lineage can be thought to be composed of two hemilineages, with one hemilineage comprising "A" fate cells and the other hemilineage comprising "B" fate cells. It is thought that an interaction between Notch and Numb is responsible for generating distinct neural fates of the two GMC daughter cells, with a loss of Notch or Numb resulting in reciprocal cell fate duplication (Karcavich, and Doe, 2005; Lundell et al., 2003; Novotny et al., 2002). However, Notch signaling does not appear to confer a particular fate, rather it acts generically as a mechanism to enable two siblings to acquire different fates, and other developmental control genes which are inherited from the specific parental GMC are thought to be instrumental in determining the final identity of each progeny (Karcavich, 2005).

Our findings on lineage-specific cell death support a comparable model in which all four brain neuroblasts can generate one *en*-positive hemilineage and one *en*-negative hemilineage (Fig.10). In this model, programmed cell death is then targeted in a lineage-specific manner to either the *en*-negative hemilineage (AC, PC), or the *en*-positive hemilineage (MC2) or neither hemilineage (MC1). Alternatively, *en*-positive and *en*-negative neurons in the lineages could be generated in a temporal fashion and subsequently, *en*-positive or *en*-negative neurons could be eliminated in a lineage-specific manner. However, our results point that this is unlikely. In particular, in the PC lineage, more than 80% of the two-cell clones examined were composed of one *en*-positive and one *en*-negative cell. If the above did occur, a significant number of two cell *en*-positive clones should have been obtained along with two-cell clones comprising one *en*-positive and one *en*-negative neuron. Similar analysis of single and two cell clones in

the other three *en* lineages is further required to confirm the occurrence of hemilineage-specific programmed cell death.

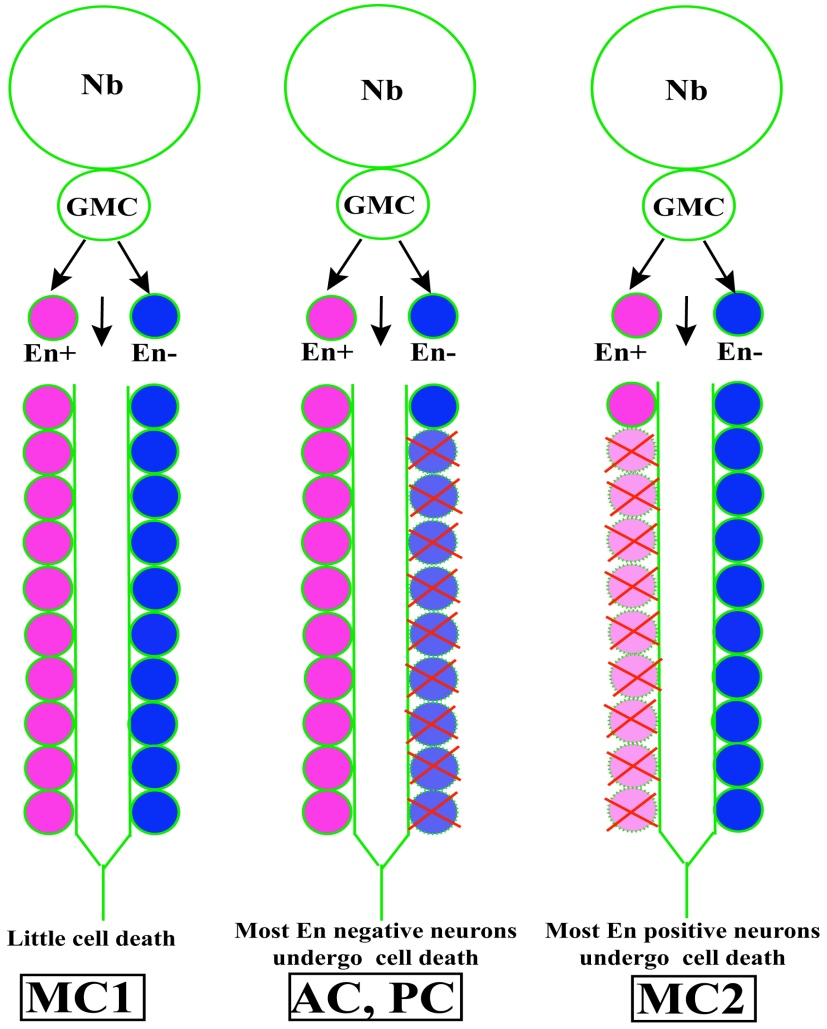

Figure 3-10. Model for hemilineage-specific cell death and generation of lineage diversity. Abbreviations: Nb Neuroblast, GMC Ganglion Mother Cell, En+ *en*-positive neuron, En- *en*-negative neuron. Neurons in dotted lines and red crosses undergo cell death.

Based on our experimental results, we postulate that Notch signaling is an important generic mechanism underlying generation of the two different hemilineages; since in absence of Notch signaling, cell fate duplication of GMC siblings occurs (Fig. 6). Indeed, our analysis of Notch loss-of-function neuroblast clones suggests that in absence of Notch signaling most of

the neurons in the four lineages acquire an *en*-positive cell fate (Fig. 8). Alternatively, in the four lineages examined, being *en*-positive maybe the default state of the cells, and Notch induces secondary fate by repressing *en* in subsets of cells in each lineage. These *en*-positive neurons then appear to survive or undergo programmed cell death depending on lineage-specific context. However, it remains to be seen whether Notch itself acts on the apoptotic machinery, independent of *en*.

In this study, we used *en* as a molecular marker to identify four lineages in the postembryonic central brain. Might *en* itself be functionally involved in regulating programmed cell death in these lineages? For the PC lineage, there is some indication that the total clone size is reduced by approximately half in *en* loss-of-function mutants, as compared to wildtype (A. Kumar, unpublished). Although, this suggests that *en* may be involved in promoting survival of *en*-positive neurons in PC (and probably AC), it does not explain the role of *en* in the MC2 lineage; where it would have to play an opposing role, since *en*-positive neurons die in this lineage. Thus, *en* could act either as a pro-apoptotic or an anti-apoptotic factor, depending on lineage-specific context. Moreover, a direct genetic interaction between *en* and the apoptotic machinery remains to be investigated. Since *en* is known to have multiple interactions with other proteins (Alexandre and Vincent, 2003; Chanas and Maschat, 2005; Joly et al., 2007; Kobayashi et al., 2003), a complex regulatory network involving target proteins of *en* maybe responsible for regulating apoptosis in a lineage-specific manner. Further analysis of interactions with such target proteins is necessary to reveal the full regulatory network in more detail.

3.4.3 Serial homology of brain neuroblast lineages?

The lineage-specific effects of cell death and of Notch signaling in AC and PC lineages are distinctly different from those observed in MC1 or MC2 lineage. However, when compared with each other, many aspects of AC and PC lineages are similar. In wildtype, both lineages consist of similar numbers of adult-specific neurons, and majority (approximately 80%) of these neurons are *en*-positive whereas neuroblasts and GMCs are *en*-negative in both lineages. Blocking cell death results in a substantial (approximately double) increase in total cell number in both lineages and this increase is almost exclusively due to an increase in number of surviving *en*-negative neurons in both lineages. Moreover, loss of Notch function causes a marked increase in number of surviving *en*-positive neurons without affecting number of *en*-negative neurons in both lineages. The only significant difference between AC and PC lineages observed in this study is that the AC lineage is located in the protocerebrum whereas the PC lineage is located in the deutocerebrum.

What might be responsible for these similarities of the AC and PC neuroblast lineages? There is some evidence for existence of serially homologous neuroblasts in the fly brain and VNC (Urbach and Technau, 2003b). In the VNC, serially homologous neuroblasts, defined by comparable time of formation, similar positions in the neuromeric progenitor array and similar expression of developmental control genes such as segment polarity genes, dorsoventral patterning genes and other molecular markers, can give rise to almost identical cell lineages (Bhat, 1999; Bossing et al., 1996; Broadus and Doe, 1995; Doe, 1992; Schmidt et al., 1997). This suggests that similar regulatory interactions take place during development of serially homologous neuroblasts and their neural cell lineages. A comparison of molecular expression patterns in neuroblasts of different neuromeres of the brain and ventral ganglia suggests that several of them might be serial homologs of each other (Urbach and Technau, 2003b). For example, neuroblasts NB5-6 in the abdominal, thoracic and subesophageal

ganglia have been proposed to be homologous to NBDd7 in the deutocerebrum and NBTd4 in the tritocerebrum.

Given the remarkable similarities in AC and PC neuroblast lineages, it is possible that the protocerebral AC lineage and the deutocerebral PC lineages represent serial homologs. If this is the case, then investigations of the cellular and molecular mechanisms that control their lineage-specific development should be useful for our understanding of how regionalized neural diversity in the brain evolves from a basic metameric ground state. However, since neither the combination of developmental control genes expressed in AC and PC neuroblasts nor the position of the two brain neuroblasts in their neuromeres of origin are currently known in sufficient detail, further experiments are needed before the issue of serial homology can be resolved for these brain neuroblast lineages.

4 Discussion

The datasets presented in chapters 2 and 3 provide insights into the expression of the homeodomain containing transcription factor *en.* The analysis of *en*-expressing lineages in the postembryonic brain has revealed insights into the organizing principle of the brain neuropile as well as provided surprising information about the predominant role of programmed cell death in regulating neuronal numbers and shaping lineage characteristics. Several aspects of the results obtained are discussed in detail below.

4.1 *engrailed* EXPRESSION THROUGH BRAIN DEVELOPMENT

In the early embryo approximately 100 bilaterally symmetrical brain neuroblasts segregate from the neuroepithelium in a stereotyped array. Out of these, 9 identified neuroblasts express *en*. 2 of these are protocerebral, four are deutocerebral and the rest of the neuroblasts are tritocerebral (Urbach and Technau, 2003a). It is notable that *en*-positive neuroblasts are located at the posterior boundary of their respective brain neuromeres, similar to expression in the ectoderm and VNC. During late embryonic stages, *en* expression is restricted to 2-3 closely apposed neuroblast lineages in the protocerebral neuromere, 1-2 neuroblast lineages in the deutocerebral neuromere, and a single cluster of *en*-expressing cells in the tritocerebral neuromere. However, postmitotic *en*-expressing progeny in the tritocerebral neuromere adopt glia-like morphology at later embryonic stages (stage 15 onwards) and were not characterized further in our study.

This expression data at primary lineage resolution was studied in embryonic stages and was followed into the larval brain and subsequently at secondary lineage resolution in the late larval and adult brain. Approximately 100 secondary lineages have been identified in each hemisphere in the late larval brain (Pereanu and Hartenstein, 2006). Here we have found that *en* is exclusively expressed in lineage-specific subsets in 4 secondary lineages per hemisphere. Out of these, only 2 lineages express *en* in the neuroblasts; the other 2 do not. 3 of the *en*-expressing secondary lineages have been attributed to the protocerebrum and one to the deutocerebrum, based on arborizations of the lineages from known neuromeric origin in the embryo. Only 3 of the 4 lineages continue to express *en* in the adult brain- two in the protocerebrum and one in the deutocerebrum. A general image of the *en* expression pattern in the developing *Drosophila* brain thus, emerges.

4.2 TRACING *engrailed*-EXPRESSING LINEAGES DURING DEVELOPMENT

At the developmental stages studied, *en* expression appears persistent in the posterior boundaries of neuromeres, even though expression is successively restricted to only 3 lineages in the adult out of 9 neuroblasts in the embryo. Our data links groups of *en*-expressing cells in the late embryo to lineages of a set of identified *en*-positive neuroblasts. Though it is likely that *en*-expression is maintained in some progeny cells of some of these neuroblasts, it could be argued that expression patterns are highly dynamic and, as shown previously for several other genes, expression of a particular gene can be switched on at later stages in progeny cells produced by adjacent neuroblasts that did not express this gene. In addition, our results do indicate that dynamic expression of *en* does occur; *en*-expression is switched off in some progeny of *en*-positive neuroblasts (some of these neuroblasts do not produce any *en*-positive progeny). Conversely, only one of the two clusters of *en*-positive neurons in the late embryonic protocerebrum had already been *en*-positive at earlier stages (chapter 2). Linking gene expression to a specific neuroblast and its lineage requires clonal analysis. We have generated positively labelled MARCM clones during postembryonic stages. Since this technique does not work well for the embryo, we traced primary neurons in the embryonic brain by anti-En immunoreactivity.

Even though based on the characteristic trajectory of axon tracts and their terminal arborizations, we can follow the *en*-positive lineages unambiguously from late embryo to adult; the question still arises whether the *en*-positive neuroblasts, which demarcate the posterior neuromere boundary within the early embryonic (stage 8-11) neural primordium, are the same cells that we recognize at later embryonic stages. In the neural primordium, neuroblasts expressing *en* at stage 11 could lose expression during stage 12/13, and at the same time, other neuroblasts gain expression, so that the neuroblasts visible at stage 15 are

different ones from those at stage 11. We argue that this scenario is very unlikely, and even if correct, the neuroblasts gaining *en*-expression would have to be the immediate neighbors of the neuroblasts that lose expression. The basis for this argument is the fact that the time interval between stage 11 and stage 15 (when tracts are clearly distinguishable) lasts only about 5 hours, and observations of embryos at very close increments (15min) can be, and were, observed. In such a sequence of observations it is evident that initially, small groups of *en*-positive neuroblasts demarcate the posterior neuromere boundary. Some of these neuroblasts generate *en*-positive clusters of GMCs and neurons, which stay in contact with their parent neuroblasts. As time passes, the clusters grow larger, and finally start producing axon tracts. Even when it is clear that transcription factors are expressed dynamically, and are turned off in tissue A and then on in B, one always sees "remnants" of expression in A, if one observes the embryo 15min later. (Example: genes like *snail*, very strongly and "cleanly" on in mesoderm until stage 8, and neuroblasts after 9 (Ip et al., 1994; Kosman et al., 1991; Leptin, 1991). There is a clear transitional stage of 30min or so where one can observe the expression wane in the mesoderm, and wax in the neuroblasts). Hence, given this continuity of our observations on *en*-expressing cells, we can be quite certain that the metameric groups of lineages we define in the late (stage 15; 11-12h) embryo stem from within the metameric groups of neuroblasts defined at stage 11.

The equivalence of single *en*-positive neuroblasts in the early embryo to *en*-expressing secondary lineages could be further analyzed. The link is mainly established based on *en* expression data and anatomical criteria and this is one of the first attempts that have done so. A different promising approach for fate mapping of primary lineages into later developmental stages had been demonstrated for *atonal*-positive cells using an *atonal*-specific GAL4 line in combination with a special self-perpetuating UAS-GAL4 construct (Hassan et al., 2000). Additional markers, which were used for the characterization of single delaminating

neuroblasts in the early embryo (Urbach and Technau, 2003b, c) could probably be also be used, in terms of a combinatorial code, to further identify distinct neuroblasts and their lineages during embryonic and larval development. Also, future clonal characterization of additional gene expression patterns during postembryonic brain development will be very informative on the question if successive spatial restriction reflects a general trend for transcription factors that act as patterning genes during early development.

4.3 EVIDENCE FOR A NEUROMERE-SPECIFIC BRAIN NEUROPILE

A general neuroanatomical feature of neurons in the VNC is that most of the neurons in a given neuromere restrict their proximal (dendritic) arborizations to the neuropile of that neuromere. This is the case for the numerous local neurons, which do not have aborizations outside of the neuromere to which they belong (Burrows, 1996). This general scheme is also true for the proximal arborizations of a majority of the motoneurons and intersegmental interneurons in the VNC. However, their distal processes project to other neuromeres and ganglia. Most sensory neurons of a given segment project their axons into the neuromere of that segment and also form their terminal arborizations there, and motoneurons typically form dendrites in the neuromere that contains their cell bodies (Landgraf et al., 2003; Merritt and Whitington, 1995; Schrader and Merritt, 2000; Zlatic et al., 2003).

In contrast, the internal structure and organization of the supraesophageal ganglion (brain) manifests highly organized neuropile structures such as the mushroom bodies, central complex and antennal lobes which have no obvious equivalents in other neuromeres of the CNS. Moreover, due to its complex and hidden segmental organization, it has been difficult to determine the neuromere boundaries within the neuropile of the brain. Thus, the lack of information about neuromeric boundaries together with the highly fused nature of the brain neuropile have so far confounded attempts to understand the neuroanatomical construction principles of the brain.

The results presented in chapter 2 support the presence of a brain neuropile organization in which a given lineage forms its arborizations predominantly in the neuropile corresponding to its neuromere of origin; thus organizing the brain architecture into neuromere-specific compartments. We have traced *en*-expressing lineages in the embryo, coming from known neuromeric origin; to the larval and the adult brains. Based on their arborizations and general principles valid in the VNC and subesophageal ganglion, we could identify the neuropile

compartments innervated by *en*-expressing lineages; and found them to be the same throughout development. Moreover, we observe that the arborizations (in particular, proximal) of lineages that derive from different neuromeres do not overlap and respect boundaries; thus organizing the brain neuropile into neuromere-specific compartments. Not only is this true for *en*-expressing lineages, but analysis of *ems*-expressing deutocerebral lineages also confirms this finding. Further analysis of the arborizations of other brain lineages through development may perhaps reveal whether this is a general organizing principle in the brain. These data have also described previously less known brain neuropile regions at high resolution and opened further questions about their functional role.

4.4 ROLE OF CELL DEATH IN SHAPING *engrailed*-EXPESSING BRAIN LINEAGES

Cell death is a prominent feature in CNS development, functioning in the removal of redundant or mis-specified cells as well as in the spatial patterning and synaptic matching of neuronal circuit elements (Truman et al., 1992; Buss et al., 2006). In these processes precise spatio-temporal regulation of cell death is required to ensure that the correct numbers of neural cells and the appropriate cell types are eliminated. How this regulation is achieved and how cell death is integrated with neural development and differentiation processes are important questions that remain largely unanswered. However, it is clear that cell death can be extensive in brain development. Indeed, in *Drosophila* and mammalian nervous systems, upto 50% of the neurons generated in specific areas are removed by cell death (Raff et al., 1993; White et al., 1994).

In *Drosophila* neural development, cell death has been widely studied in the embryonic VNC (Abrams et al., 1993; Miguel-Aliaga and Thor, 2004; Rogulja-Ortmann et al., 2007; Rogulja-Ortmann et al., 2008). There are also few reports about the occurrence of cell death in the abdominal segments of the larval VNC (Booker and Truman, 1987; Booker et al., 1996; Bello et al., 2003) and during metamorphosis to organize and innervate new structures in the CNS (Truman, 1983; Weeks and Truman, 1985; Truman et al., 1992; Weeks, 2003). However, very little is known about the occurrence, extent or functional roles of cell death in the development of adult-specific neurons of the *Drosophila* central brain.

The results presented in chapter 3 demonstrate the occurrence of programmed cell death in postembryonic brain development. We have identified three neuroblast lineages in which roughly half of the cells appear to be eliminated by cell death. In three of the four

postembryonic lineages generated by *en*-expressing neuroblasts (AC, PC and MC2), cell death differentially affects sublineages and selectively determines the cell types that survive in the adult brain (*en*-positive versus *en*-negative). The fourth lineage (MC1), however, is not affected by cell death. It is notable that cell death block in these three lineages leads to rescued lineages which appear to be phenotypically similar to MC1. The similarity is reflected in terms of *en* expression pattern, number of *en*-expressing cells as well as lineage size. This suggests the functional role of cell death in shaping lineages differentially from a similar metameric ground state and thereby, generating lineage diversity in the brain. Furthmore, in cell death block experiments, none of the lineages had additional mis-projections despite having additional cells (Kumar et al., unpublished). This also suggests a speculative role for programmed cell death in removal of redundancy in the nervous system without reconfiguring individual neuroblast lineages. Elimination of excess and redundant postmitotic cells maybe more of an energy-conserving mechanism than reprogramming individual neuroblasts for lineage-specific apoptosis.

Another central feature of the cell death events reported here is that none of the four lineages are eliminated by cell death; all four neuroblasts and a significant number of their neuronal progeny survive at the end of larval development, and these progeny are largely present in the adult. However, the lineage size and the subset of these surviving *en*-expressing progeny are different in each lineage. Thus, programmed cell death appears also to contribute to the cellular diversity of neuronal lineages in the central brain.

4.5 *engrailed*-EXPRESSING HEMILINEAGES IN THE BRAIN

Hemilineages have been hypothesized to result from the fact that the two daughter cells produced by a GMC often acquire two different cell fates (J. Truman, personal communication), thus comprising a lineage of two kinds of cells, roughly equal in number. Our findings on lineage-specific cell death support a comparable model in which each of the four brain neuroblasts can generate one *en*-positive hemilineage and one *en*-negative hemilineage. In this model, programmed cell death is then targeted in a lineage-specific manner to either the *en*-negative hemilineage (AC, PC), or the *en*-positive hemilineage (MC2) or neither hemilineage (MC1). This model makes the following predictions. First, in the AC and PC lineages, most of the surviving neurons should be *en*-positive. Second, in the MC2 lineage, most of the surviving neurons should be *en*-negative. Third, in the MC1 lineage, both *en*-positive and *en*-negative neurons should largely survive. Fourth, following apoptosis block, all four lineages should be of approximately the same size. Fifth, following apoptosis block, all four lineages should contain comparable numbers of *en*-positive and *en*-negative neurons. It is notable that these predictions are largely in accordance with our experimental results.

This model, which suggests that cell death contributes to lineage diversity in the brain by selectively affecting hemilineages, was tested further in the PC lineage. In this lineage, one half of the neural progeny express the *en* gene whereas the other half do not. That this is due to a GMC-based hemilineage effect, was confirmed by analyzing cell death blocked single and two-cell clones, with the prediction that all two-cell clones in the PC lineage will consist of one *en*-positive and one *en*-negative neuron. This was found to be largely true. On the other hand, mostly single cell *en*-positive clones were obtained in PC, very likely because programmed cell death eliminates the *en*-negative hemilineage. Similar analysis of single and

two cell clones in the other three lineages is further required to confirm the occurrence of hemilineage-specific programmed cell death and its role in generating different types of lineages in the brain.

While the hemilineage model explains most of our findings, there are several results that are not in full quantitative accord with it. First, a small number (<5) of *en*-negative neurons are found in AC and PC lineages; the model predicts that all neurons should be *en*-positive. Second, a small number (<10) of *en*-positive neurons are found in the MC2 lineage; the model predicts that all neurons should be *en*-negative. Third, a low level of cell death (involving approximately 20 *en*-negative neurons) does occur in the MC1 lineage; the model predicts that no cell death should occur.

Alternatively, *en*-positive and *en*-negative neurons in the lineages could be generated in a temporal fashion and subsequently, *en*-positive or *en*-negative neurons could be eliminated in a lineage-specific manner. However, our results point that this is unlikely. In particular, in the PC lineage, more than 80% of the two-cell clones examined were composed of one *en*-positive and one *en*-negative cell. If the above did occur, a significant number of two cell *en*-positive clones should have been obtained along with two-cell clones comprising one *en*-positive and one *en*-negative neuron. Moreover, the evidence that the cell fate determinant Notch seems to regulate *en* and determine cell fate asymmetricity during GMC division in the four lineages further corroborates the GMC based hemilineage model. Thus, the three *en*-expressing neuroblast lineages examined in the postembryonic central brain are actually composed of each surviving hemilineage whereas the fourth lineage comprises of two surviving hemilineages. This report is the first of its kind to demonstrate the existence of hemilineages in the central brain of *Drosophila*.

4.6 PUTATIVE FUNCTION OF *engrailed* DURING FLY BRAIN DEVELOPMENT

Since *en* is a transcription factor, its function very much depends on its multiple interactions with other proteins and the type of genes activated or repressed by it (Alexander and Vincent, 2003; Chanas and Maschat, 2005; Joly et al., 2007; Kobayashi et al., 2003). In general, *en* is required for both early (morphogenetic) and late (differentiative) aspects of the cells it is expressed in. The embryonic expression and function of *en* has been well studied; however, the expression, characteristics and function of postembryonic *en*-expressing cells has not been reported before. With respect to its early embryonic function, *en* appears to be involved in setting up segmental boundaries, such that adhesion molecules (Siegler and Jia, 1999) or other structural proteins that form part of the cellular mechanism for generating or maintaining segmental boundaries are under the control of *en* (Dahmann and Basler, 2000). As far as the differentiative function of *en* is concerned, loss of function studies of *en* in *Drosophila* and vertebrates show that cell types expressing *en*, such as the adrenergic and serotonergic neurons of the vertebrate brainstem, are often absent or reduced in *en* mutants (Simon et al., 2004; 2005). *en* mutants in *Drosophila* show pathfinding defects in the embryonic VNC (Joly et al., 2007) and in the cockroach cercal system (Marie et al., 2002). In most of these studies, the morphology of the neurite projections and arborizations of *en*-expressing neurons as well as the neuropile compartments they contribute to have not been analyzed in detail. It will be useful to extend the type of genetic labeling techniques utilized in the studies presented in this thesis, to investigate the precise anatomical features of mutant *en* neurons and neuronal compartments in the developing fly brain.

In the fly brain, however, the duplication of *en* gene during evolution has led to the presence of another functionally redundant gene, *invected* (*inv*) (Bhat and Schedl, 1997; Gustavson et al., 1996). Since *en* and *inv* mainly act in a concerted fashion, the loss of function of *en* alone

may sometimes not be sufficient to uncover its role due to the presence of a functional buffer, *inv*. This is in accordance with a number of similar observations (F.Maschat, personal communication; Kumar et al., unpublished). Hence, it might be useful to analyze the role of *en* and *inv* together.

In our second study, we have used *en* as a molecular marker to identify 4 lineages in the postembryonic central brain. Might *en* itself be functionally involved in regulating hemilineage specific programmed cell death in these lineages? In a set of preliminary experiments (Kumar et al., unpublished), we found that in the PC lineage, the total clone size is reduced by approximately half in loss of *en-inv* function mutants, as compared to wildtype. Although, this suggests that *en* may be involved in promoting survival of *en*-positive neurons in AC and PC, it does not explain the role of *en* in the MC2 lineage; where it would have to play an opposing role, since *en*-positive neurons die in this lineage. Thus, it seems unlikely that *en* plays a direct role in regulating hemilineage-specific cell death but it maybe interacting with other transcription factors to promote or prevent cell death in a lineage dependent context.

This does not seem unlikely; given that *en* is known to have multiple interactions with other proteins. A more complex interplay of direct and indirect targets of *en* may be responsible for the observed lineage-specific cell death. Further analysis of the involvement of such target proteins may reveal the full regulatory network in more detail.

5 Experimental Procedures

5.1 FLY STRAINS AND GENETICS

Unless otherwise indicated, all stocks have been obtained from the Bloomington Stock Centre, Indiana, USA. For generating *en*-Gal4 driven MARCM clones, a fly stock containing a recombinant chromosome was constructed: P{en2.4-Gal4}e16E, UAS *mCD8::GFP*^{LL5}, *UAS-nlslacZ*^{20b} on chromosomal arm 2R and FRT 82B, *tubP-GAL80*^{LL3} on chromosome 3. To generate MARCM clones (Lee and Luo, 1999) *+; UAS-mCD8::GFP*^{LL5}, *UAS-nlslacZ*^{20b}; *FRT82B* (Bello et al., 2003) males were crossed to females of the MARCM driver stock *hs-FLP; en-GAL4*, UAS *mCD8::GFP*^{LL5}, *UAS-nlslacZ*^{20b}; *FRT82B*, *tubP-GAL80*^{LL3} resulting in wild-type clones of *en*-expressing cells.

To generate positively marked, wild type MARCM clones driven by a ubiquitous *tub*-Gal4 driver, females of driver stock y,w,hsFLP; FRT40A, tubPGAL80^{LL10} /CyO,ActGFP^{JMR1}; tubP-GAL4^{LL7}, UAS-mCD8::GFP^{LL6}/TM6,Tb,Hu were mated to w; FRT40A, UAS-mCD8::GFP^{LL5} males. In cell death block experiments, homozygous H99 MARCM clones were generated (Bello et al., 2003) by crossing females of driver stock y,w,hsFLP; tubP-Gal4, UAS-mCD8::GFP^{LL5} / CyO, ActGFP^{JMR1}; FRT2A, tubP-Gal80^{LL9} / (TM6, Tb, Hu) with w; FRT2A, H99, kni^{ri}-2 / TM6[w^{+}] males. For loss of Notch function experiments, homozygous mutant *notch* MARCM clones were generated using a recombinant null allele- FRT19A, N^{55e11}/FM7 KrGal4 UAS-GFP (Dr. Bruno Bello; unpublished). Females of this genotype were mated with males of driver stock FRT19A, tubP-Gal80^{LL1}, hsFLP, w*; tubP-Gal4, UAS-mCD8::GFP^{LL5} / CyO, ActGFP^{JMR1}.

For all MARCM experiments, embryos of the appropriate genotype were collected on standard cornmeal/yeast/agar medium supplemented with live yeast over a 4 hour time window and raised at 25°C for 21 to 25 hours (or 44-48 hours to recover single and two cell

clones) before heat-shock treatment. Heat-shock induction of FLP was done at 37°C for 60 minutes. MARCM clones were examined in brains dissected at wandering third instar larval stage and 0 – 10 days after adult fly eclosion. Other transgenic fly lines used were rhx25lacZ (expressed in the *en* domains; Hama and Kornberg, 1990), and a151 Gal4 (expressed in subsets of sensory neurons in the adult fly; J. Simpson, unpublished).

5.2 IMMUNOHISTOCHEMISTRY

Larval and adult brains were dissected in PBS, fixed in 2% paraformaldehyde in PBL (75mM lysine HCL in sodium phosphate buffer PH 7.4) for 1 hour at room temperature (RT), washed three times for 10 minutes in PBS containing 0.5% Triton X-100 (PBT), blocked for 1 hour at RT in PBT containing 10 % normal goat serum, and incubated with primary antibodies in blocking solution overnight at 4°C. Samples were washed three times for 10 minutes in PBT at RT, and secondary antibodies were applied in blocking solution for 3 hours at room temperature. After washing three times for 15 minutes in PBS, samples were mounted in Vectashield (Vector Labs).

The following antibodies were used: mouse anti-Engrailed 4D9 (1:10; Developmental Studies Hybridoma bank {DSHB}), mouse anti-nc82 (1:20, DSHB), rabbit anti-GFP (1:450; Torrey Pines Biolabs Inc) and rat anti-Elav Mab7E8A10 (1:30; DSHB). Secondary antibodies used were Alexa- 488, Alexa-568 and Alexa-647; generated in goat (1:300; Molecular Probes).

Other antibodies used for the analysis of *en*-expressing cells in the embryonic and larval brains (see Chapter 2, V. Hartenstein) were as follows: Polyclonal rat anti DN-cadherin (DN-Cad, 1:25, DSHB), mouse anti-fasciclin II (FasII, 1:10, DSHB), mouse anti-Neurotactin (Nrt, 1:10, DSHB), rabbit anti-β galactosidase (1:1000, Cappel MP Biomedicals Inc.) and rabbit anti-Ems (1:200, gift of U. Walldorf, University of Saarland, Homburg, Germany).

5.3 MICROSCOPY AND IMAGE PROCESSING

All fluorescent images were recorded using a Leica TCS SP scanning confocal microscope. Complete series of optical sections were taken at 1 μm intervals in line average mode with picture size of 512 x 512 pixels. Digital image stacks were processed using ImageJ; all images including *en*-Gal4 and *tub*-Gal4 driven MARCM clones were recorded in above mentioned manner. In the case of ubiquitous *tub*-Gal4 driven MARCM clones, image stacks with few non-interfering clones were selected and stained processes as well as cell bodies from other clones were removed using the lasso tool in every single optical section. This was done to visualise the *en*-expressing clones more clearly. Figures were assembled using Adobe Illustrator and Adobe Photoshop.

Clone sizes were determined from confocal Z stacks using ImageJ. For cell counts, cells were marked per section and counted using the 'cell counter' plug-in of ImageJ. Means for all histograms are indicated in the text. Sample sizes for all histograms are indicated in corresponding figure legends. The error bars in all the graphs represent standard deviations.

Digital 3D-models were generated using the AMIRA software. Surface-rendered digital atlas models were created by manually labeling each lineage and neuropile compartment. For clonal analysis, the surface rendered neuropile compartments were brought into registration with the dataset of the clone by warping to the standard master template. The Amira program also allows one to adjust virtual lighting, camera angle, transparency, reflection, and other parameters in a straightforward manner.

6 References

Abrams, J. M., White, K., Fessler, L. I., Steller, H. (1993). Programmed cell death during Drosophila embryogenesis. *Development* **117**(1): 29-43.

Alexandre, C. and Vincent, J. P. (2003). Requirements for transcriptional repression and activation by Engrailed in Drosophila embryos. *Development* **130**(4): 729-39.

Awad, T. A. and Truman, J. W. (1997). Postembryonic development of the midline glia in the CNS of Drosophila: proliferation, programmed cell death, and endocrine regulation. *Dev Biol* **187**(2): 283-97.

Baba, Y. and Comer, C. M. (2008). Antennal motor system of the cockroach, Periplaneta americana. *Cell Tissue Res* **331**:751-62.

Baehrecke, E. H. (2002). How death shapes life during development. *Nat Rev Mol Cell Biol* **3**: 779-87.

Beaster-Jones, L., Kaltenbach, S. L., Koop, D., Yuan, S., Chastain, R., Holland, L. Z. (2008). Expression of somite segmentation genes in amphioxus: a clock without a wavefront? *Dev Genes Evol* **218**:599-611.

Bello, B. C., Hirth, F., Gould, A. P. (2003). A pulse of the Drosophila Hox protein Abdominal-A schedules the end of neural proliferation via neuroblast apoptosis. *Neuron* **37**(2): 209-19.

Bello, B. C., Izergina, N., Caussinus, E., Reichert, H. (2008). Amplification of neural stem cell proliferation by intermediate progenitor cells in Drosophila brain development. *Neural Dev* **3**: 5.

Bhat, K. M. (1999). Segment polarity genes in neuroblast formation and identity specification during Drosophila neurogenesis. *Bioessays* **21**(6): 472-85.

Bhat, K. M. and Schedl, P. (1997). Requirement for engrailed and invected genes reveals novel regulatory interactions between engrailed/invected, patched, gooseberry and wingless during Drosophila neurogenesis. *Development* **124**: 1675-88.

Blaschke, A. J., Staley, K., Chun, J. (1996). Widespread programmed cell death in proliferative and postmitotic regions of the fetal cerebral cortex. ***Development*** **122:** 1165-74.

Blaschke, A. J., Weiner, J. A., Chun, J. (1998). Programmed cell death is a universal feature of embryonic and postnatal neuroproliferative regions throughout the central nervous system. *J Comp Neurol* **396**: 39-50.

Bonini, N. M. and Fortini, M. E. (1999). Surviving Drosophila eye development: integrating cell death with differentiation during formation of a neural structure. *Bioessays* **21**(12): 991-1003.

Booker, R. and Truman, J. W. (1987). Postembryonic neurogenesis in the CNS of the tobacco hornworm, Manduca sexta. I. Neuroblast arrays and the fate of their progeny during metamorphosis. *J Comp Neurol* **255**(4): 548-59.

Booker, R., Babashak, J., Kim, J. B. (1996). Postembryonic neurogenesis in the central nervous system of the tobacco hornworm, Manduca sexta. III. Spatial and temporal patterns of proliferation. *J Neurobiol* **29**(2): 233-48.

Boone, J. Q. and Doe, C. Q. (2008). Identification of Drosophila type II neuroblast lineages containing transit amplifying ganglion mother cells. *Dev Neurobiol* **68**(9): 1185-95.

Bossing, T., Udolph, G., Doe, C. Q., Technau, G. M. (1996). The embryonic central nervous system lineages of Drosophila melanogaster. I. Neuroblast lineages derived from the ventral half of the neuroectoderm. *Dev Biol* **179** (1): 41-64.

Bowman, S. K., Rolland, V., Betschinger, J., Kinsey, K. A., Emery, G., Knoblich, J. A. (2008). The tumor suppressors Brat and Numb regulate transit-amplifying neuroblast lineages in Drosophila. *Dev Cell* **14**(4): 535-46.

Boyan, G. and Williams, L. (2002). A single cell analysis of engrailed expression in the early embryonic brain of the grasshopper Schistocerca gregaria: ontogeny and identity of the secondary headspot cells. *Arthropod Struct Dev* **30**:207-18.

Brand, A. H. and Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* **118**: 401-15.

Broadus, J. and Doe, C. Q. (1995). Evolution of neuroblast identity: seven-up and prospero expression reveal homologous and divergent neuroblast fates in Drosophila and Schistocerca. *Development* **121**(12): 3989-96.

Broadus, J., Skeath, J., Spana, E., Bossing, T. Techanu, G., Doe, C. Q. (1995). New neuroblasts markers and the origin of the aCC/pCC neurons in the Drosophila central nervous system. *Mech Dev* **53**: 393-402.

Brody, T. and Odenwald, W. F. (2000). Programmed transformations in neuroblast gene expression during Drosophila CNS lineage development. *Dev Biol* **226**, 34-44.

Brunet, I., **Weinl, C**., **Piper, M**., **Trembleau, A**., **Volovitch, M**., **Harris, W**., **Prochiantz, A**., **Holt, C**. (2005). The transcription factor Engrailed-2 guides retinal axons. *Nature* **438**: 94-8.

Bullock, T. H. and Horridge, G. A. (1965). Structure and function in the nervous system of invertebrates. Two volumes. Freeman, San Francisco.

Burrows, M. (1996). The Neurobiology of the Insect Brain. Oxford University Press, Oxford.

Buss, R. R., Sun, W., Oppenheim, R. W. (2006). Adaptive roles of programmed cell death during nervous system development. *Annu Rev Neurosci*. **29**: 1-35.

Callaerts, P., Leng, S., Clements, J., Benassayag, C., Cribbs, D., Kang, Y. Y., Walldorf, U., Fischbach, K. F. and Strauss, R. (2001). Drosophila Pax-6/eyeless is essential for normal adult brain structure and function. *J Neurobiol* **46**, 73-88.

Campos-Ortega, J. A. and Hartenstein, V. (1997). The embryonic development of *Drosophila melanogaster*. Berlin, Springer.

Cañestro, C., Bassham, S., Postlethwait, J. (2005). Development of the central nervous system in the larvacean Oikopleura dioica and the evolution of the chordate brain. *Dev Biol* **285**:298-315.

Cayouette, M. and Raff, M. (2002). Asymmetric segregation of Numb: a mechanism for neural specification from Drosophila to mammals. *Nat Neurosci* **5**(12): 1265-9.

Chanas, G. and Maschat, F. (2005). Tissue specificity of hedgehog repression by the Polycomb group during Drosophila melanogaster development. *Mech Dev* **122**(9): 975-87.

Chen, P., Nordstrom, W., Gish, B., Abrams, J. (1996). grim, a novel cell death gene in Drosophila. *Genes Dev* **10**(14): 1773-82.

Chen, Y. and Zhao, X. (1998). Shaping limbs by apoptosis. *J Exp Zool* **282**: 691-702.

Dahmann, C. and Basler, K. (2000). Opposing transcriptional outputs of Hedgehog signaling and engrailed control compartmental cell sorting at the Drosophila A/P boundary. *Cell* **100**:411-22.

de la Rosa, E., **de Pablo, F**. (2000). Cell death in early neural development: beyond the neurotrophic theory. *Trends Neurosci* **23**: 454-8.

DeVelasco, B., Mandal, L., Mkrtchyan, M., Hartenstein, V. (2006). Subdivision and developmental fate of the head mesoderm in Drosophila melanogaster. *Dev. Genes Evol* **216**:39-51.

Diederich, R. J., Pattatucci, A. M., Kaufman, T. C. (1991). Developmental and evolutionary implications of labial, Deformed and engrailed expression in the Drosophila head. *Development* **113**:273-81.

DiNardo, S., Kuner, J., Theis, J., O'Farrell, P. (1985). Development of embryonic pattern in D.melanogaster as revealed by accumulation of the nuclear engrailed protein. *Cell* **43**:59-69.

Doe, C. Q. (1992). Molecular markers for identified neuroblasts and ganglion mother cells in the Drosophila central nervous system. *Development* **116**(4): 855-63.

Dumstrei, K., Wang, F., Nassif, C., Hartenstein, V. (2003). Early development of the Drosophila brain: V. Pattern of postembryonic neuronal lineages expressing DE-cadherin. *J Comp Neurol* **455**(4): 451-62.

Egger, B., Boone, J. Q., Stevens, N. R., Brand, A. H., Doe, C. Q. (2007). Regulation of spindle orientation and neural stem cell fate in the Drosophila optic lobe. *Neural Dev* **2**: 1.

Ehmer, B. and Gronenberg, W. (1997). Proprioceptors and fast antennal reflexes in the ant Odontomachus (Formicidae, Ponerinae). *Cell Tissue Res* **290**:153-65.

Fjose, A., Njølstad, P. R., Nornes, S., Molven, A., Krauss, S. (1992). Structure and early embryonic expression of the zebrafish engrailed-2 gene. *Mech Dev* **39**: 51-62.

Garcia-Bellido, A. and Santamaria, P. (1972). Developmental analysis of the wing disc in the mutant engrailed of Drosophila melanogaster. Genetics **72**: 87-104.

Grenningloh, G., Rehm, E. J., Goodman, C. S. 1991. Genetic analysis of growth cone guidance in *Drosophila*: fasciclin II functions as a neuronal recognition molecule. *Cell* **67**: 45-57.

Grether, M., Abrams, J., Agapite, J., White, K., Steller, H. (1995). The head involution defective gene of Drosophila melanogaster functions in programmed cell death. *Genes Dev* **9**(14): 1694-708.

Gustavson, E., Goldsborough, A. S., Ali, Z., Kornberg, T. B. (1996). The Drosophila engrailed and invected genes: partners in regulation, expression and function. *Genetics* **142**: 893-906.

Hama, C., Ali, Z., Kornberg, T. B. (1990). Region-specific recombination and expression are directed by portions of the Drosophila engrailed promoter. *Genes Dev* **4**:1079-93.

Hanks, M., Loomis, C., Harris, E., Tong, C., Anson-Cartwright, L., Auerbach, A., Joyner, A. L. (1998). Drosophila engrailed can substitute for mouse Engrailed1 function in mid-hindbrain, but not limb development. *Development* **125**: 4521-30.

Hanström, B. (1928). Vergleichende Anatomie des Nervensystems der Wirbellosen Tiere. Berlin, Springer.

Hartenstein, V., Spindler, S., Pereanu, W., Fung, S. (2008a). The development of the Drosophila larval brain. *Adv Exp Med Biol* **628**:1-31.

Hartenstein, V., Cardona, A., Pereanu, W., Hartenstein, A. Y. (2008b). Modeling the developing Drosophila brain: Rationale, Technique and Application. **BioOne** *58*: 823-36.

Hartmann, B., Hirth, F., Walldorf, U., Reichert, H. (2000). Expression, regulation and function of the homeobox gene empty spiracles in brain and ventral nerve cord development of Drosophila. *Mech Dev* **90**: 143-53.

Hassan, B. A., Bermingham, N. A., He, Y., Sun, Y., Jan, Y. N., Zoghbi, H. Y. and Bellen, H. J. (2000). atonal regulates neurite arborization but does not act as a proneural gene in the Drosophila brain. *Neuron* **25**, 549-61.

Hidalgo A. and Constant, f.-C. (2003). The control of cell number during central nervous system development in flies and mice. *Mech Dev*.**120**(11): 1311-25.

Hirth, F., Therianos, S., Loop, T., Gehring, W. J., Reichert, H. and Furukubo-Tokunaga, K. (1995). Developmental defects in brain segmentation caused by mutations of the homeobox genes orthodenticle and empty spiracles in Drosophila. *Neuron* **15**, 769-78.

Hirth, F., Hartmann, B. and Reichert, H. (1998). Homeotic gene action in embryonic brain development of Drosophila. *Development* **125**, 1579-89.

Hirth, F., Kammermeier, L., Frei, E., Walldorf, U., Noll, M. and Reichert, H. (2003). An urbilaterian origin of the tripartite brain: developmental genetic insights from Drosophila. *Development* **130**, 2365-73.

Hitier, R., Chaminade, M. and Preat, T. (2001). The Drosophila castor gene is involved in postembryonic brain development. *Mech Dev* **103**, 3-11.

Holland, L. Z., Kene, M., Williams, N. A., Holland, N. D. (1997). Sequence and embryonic expression of the amphioxus engrailed gene (AmphiEn): the metameric pattern of transcription resembles that of its segment-polarity homolog in Drosophila. *Development* **124**:1723-32

Holmgren, B. (1928). Vergleichende Anatomie des Nervensystems der Wirbellosen Tiere. Berlin, Springer.

Homberg, U. (1994). Flight-correlated activity changes in neurons of the lateral accessory lobes in the brain of the locust Schistocerca gregaria. *J Comp Physiol A* **175**: 597-610.

Homberg, U., Christensen, T. A., Hildebrand, J. G. (1989). Structure and function of the deuterocerebrum in insects. *Annu Rev Entomol* **34**: 477-501.

Homberg, U., Hofer, S., Mappes, M., Vitzthum, H., Pfeiffer, K., Gebhardt, S., Müller, M., Paech, A. (2004). Neurobiology of polarization vision in the locust Schistocerca gregaria. *Acta Biol Hung* **55**:81-9.

Homma, S., **Yaginuma, H**., **Oppenheim, R. W**. (1994). Programmed cell death during the earliest stages of spinal cord development in the chick embryo: a possible means of early phenotypic selection. *J Comp Neurol* **345**: 377-95.

Hortsch, M., Patel, N., Bieber, A. J., Trquina, Z. R., Goodman, C. S. (1990). Drosophila neurotactin, a surface glycoprotein with homology to serine esterases, is dynamically expressed during embryogenesis. *Development* **110**: 1327-40.

Huang FD, M. H., Speese, S. D., Smith, M. A., Broadie, K. (2004). Rolling blackout, a newly identified PIP2-DAG pathway lipase required for Drosophila phototransduction. *Nat Neurosci* **7**(10): 1070-8.

Iijima, M., Takeuchi, T., Sarashina, I., Endo, K. (2008). Expression patterns of engrailed and dpp in the gastropod Lymnaea stagnalis. *Dev Genes Evol* **218**:237-51.

Imai, K. S., Satoh, N., Satou, Y. (2002). Region specific gene expressions in the central nervous system of the ascidian embryo. *Gene Expr Patterns* **2**:319-21.

Ip, Y. T., **Levine, M**., **Bier, E**. (1994). Neurogenic expression of snail is controlled by seperable CNS and PNS promotor elements. *Development* **120**: 199-207.

Iwai, Y., Usui, T., Hirano, S., Steward, R., Takeichi, M., Uemura, T. (1997). Axon patterning requires DN- Cadherin, a novel neuronal adhesion receptor, in the Drosophila embryonic CNS. *Neuron* **19**: 77-89.

Jacobs, D. K., Wray, C. G., Wedeen, C. J., Kostriken, R., DeSalle, R., Staton, J. L., Gates, R. D., Lindberg, D. R. (2000). Molluscan engrailed expression, serial organization, and shell evolution. *Evol Dev* **2**:340-7.

Jefferis, G. S., Marin, E. C., Stocker, R. F. and Luo, L. (2001). Target neuron prespecification in the olfactory map of Drosophila. *Nature* **414**, 204-8.

Jefferis, G. S., Vyas, R. M., Berdnik, D., Ramaekers, A., Stocker, R. F., Tanaka, N. K., Ito, K. and Luo, L. (2004). Developmental origin of wiring specificity in the olfactory system of Drosophila. *Development* **131**, 117-30.

Jia, X. X. and Siegler, M. V. (2002). Midline lineages in grasshopper produce neuronal siblings with asymmetric expression of Engrailed. *Development* **129**: 5181-93.

Joly, W., Mugat, B., Maschat, F. (2007). Engrailed controls the organization of the ventral nerve cord through frazzled regulation. *Dev Biol* **301**(2): 542-54.

Joyner, A. L. (1996). Engrailed, Wnt and Pax genes regulate midbrain--hindbrain development. *Trends Genet* **12**:15-20.

Kambadur, R., Koizumi, K., Stivers, C., Nagle, J., Poole, S. J. and Odenwald, W. F. (1998). Regulation of POU genes by castor and hunchback establishes layered compartments in the Drosophila CNS. *Genes Dev* **12**, 246-60.

Kammermeier, L., Leemans, R., Hirth, F., Flister, S., Wenger, U., Walldorf, U., Gehring, W. J. and Reichert, H. (2001). Differential expression and function of the Drosophila Pax6 genes eyeless and twin of eyeless in embryonic central nervous system development. *Mech Dev* **103**, 71-8.

Karcavich, R. E. (2005). Generating neuronal diversity in the Drosophila central nervous system: a view from the ganglion mother cells. *Dev Dyn* **232**(3): 609-16.

Karcavich, R. and Doe, C. Q. (2005). Drosophila neuroblast 7-3 cell lineage: a model system for studying programmed cell death, Notch/Numb signaling, and sequential specification of ganglion mother cell identity. *J Comp Neurol* **481**(3): 240-51.

Kimura, K., Ote, M., Tazawa, T. and Yamamoto, D. (2005). Fruitless specifies sexually dimorphic neural circuitry in the Drosophila brain. *Nature* **438**, 229-33.

Kloppenburg, P., Camazine, S. M., Sun, X. J., Randolph, P., Hildebrand, J. G. (1997). Organization of the antennal motor system in the sphinx moth Manduca sexta. *Cell Tissue Res* **287**:425-33.

Knoblich, J. A. (2008). Mechanisms of asymmetric stem cell division. *Cell* **132**(4): 583-97.

Kobayashi, M., Fujioka, M., Tolkunova, E. N., Deka, D., Abu-Shaar, M., Mann, R. S., Jaynes, J. B. (2003). Engrailed cooperates with extradenticle and homothorax to repress target genes in Drosophila. *Development* **130**(4): 741-51.

Komiyama, T., Johnson, W. A., Luo, L. and Jefferis, G. S. (2003). From lineage to wiring specificity. POU domain transcription factors control precise connections of Drosophila olfactory projection neurons. *Cell* **112**, 157-67.

Kornberg, T. B. (1981). Engrailed: a gene controlling compartment and segment formation in Drosophila. *Proc Natl Acad Sci USA* **78**: 1095-9.

Kosman, D., Ip, Y., Levine, M., Arora, K. (1991). Establishment of the mesoderm-neuroectoderm boundary in the Drosophila embryo. *Science* **254**: 118-22.

Kuan, C. Y., Roth, K., Flavell, R., Rakic, P. (2000). Mechanisms of programmed cell death in the developing brain. *Trends Neurosci* **23**: 291-7.

Kumar, A., Fung, S., Lichtneckert, R., Reichert, H., Hartenstein, V. H. (2009) The arborization pattern of engrailed-expressing neural lineages reveal neuromere boundaries in the Drosophila brain neuropile. *J Comp Neurol* **(in press).**

Kurusu, M., Nagao, T., Walldorf, U., Flister, S., Gehring, W. J. and Furukubo-Tokunaga, K. (2000). Genetic control of development of the mushroom bodies, the associative learning centers in the Drosophila brain, by the eyeless, twin of eyeless, and Dachshund genes. *Proc Natl Acad Sci U S A* **97**, 2140-4.

Landgraf, M., Jeffrey, V., Fujioka, M., Jaynes, J. B., Bate, M. (2003). Embryonic origins of a motor system: motor dendrites form a myotopic map in Drosophila. *PLoS Biol* **1**:E41.

Lee, T. and Luo, L. (1999). Mosaic analysis with a repressible cell marker for studies of gene function in neuronal morphogenesis. *Neuron* **22**(3): 451-61.

Lee, T. and Luo, L. (2001). Mosaic analysis with a repressible cell marker (MARCM) for Drosophila neural development. *Trends Neurosci* **24**(5): 251-4.

Lee, T., Marticke, S., Sung, C., Robinow, S. and Luo, L. (2000). Cell-autonomous requirement of the USP/EcR-B ecdysone receptor for mushroom body neuronal remodeling in Drosophila. *Neuron* **28**, 807-18.

Leptin, M. (1991). twist and snail as positive and negative regulators during Drosophila mesoderm development. *Genes Dev* **5**: 1568-76.

Lichtneckert, R., Bello, B., Reichert, H. (2007). Cell lineage-specific expression and function of the empty spiracles gene in the adult brain of Drosophila melanogaster. *Development* **134**: 1291-1300.

Lichtneckert, R., Nobs, L., Reichert, H. (2008). Empty Spiracles is required fort he development of olfactory projection neuron circuitry in Drosophila. *Development* **135**: 2415-24.

Lu, B., Jan, L., Jan, Y. N. (2000). Control of cell divisions in the nervous system: symmetry and asymmetry. *Annu Rev Neurosci*. **23**: 531-56.

Lundell, M. J., Lee, H. K., Perez, E., Chadwell, L. (2003). The regulation of apoptosis by Numb/Notch signaling in the serotonin lineage of Drosophila. *Development* **130**(17): 4109-21.

Marie, B., Cruz-Orengo, L., Blagburn, J. (2002). Persistent engrailed expression is required to determine sensory axon trajectory, branching, and target choice. *J Neurosci* **22**: 832-41.

Marin, E. C., Watts, R. J., Tanaka, N. K., Ito, K. and Luo, L. (2005). Developmentally programmed remodeling of the Drosophila olfactory circuit. *Development* **132**, 725-37.

Mathew, D., Popescu, A., Budnik, V. (2003). *Drosophila* amphiphysin functions during synaptic Fasciclin II membrane cycling. *J Neurosci* **23**: 10710-6.

Merritt, D. J., Whitington, P. M. (1995). Central projections of sensory neurons in the Drosophila embryo correlate with sensory modality, soma position, and proneural gene function. *J Neurosci* **15**:1755-67.

Miguel-Aliaga, I. and Thor, S. (2004). Segment-specific prevention of pioneer neuron apoptosis by cell-autonomous, postmitotic Hox gene activity. *Development* **131**(24): 6093-105.

Millen, K. J., Hui, C., Joyner, A. L. (1995). A role for En-2 and other murine homologues of Drosophila segment polarity genes in regulating positional information in the developing cerebellum. *Development* **121**: 3935-45.

Moshel, S. M., Levine, M., Collier, J. R. (1998). Shell differentiation and engrailed expression in the Ilyanassa embryo. *Dev Genes Evol* **208**:135-41.

Nederbragt, A. J., van Loon, A. E., Dictus, W. J. (2002). Expression of Patella vulgata orthologs of engrailed and dpp-BMP2/4 in adjacent domains during molluscan shell development suggests a conserved compartment boundary mechanism. *Dev Biol* **246**:341-55.

Noveen, A., Daniel, A. and Hartenstein, V. (2000). Early development of the Drosophila mushroom body: the roles of eyeless and dachshund. *Development* **127**, 3475-88.

Novotny, T., Eiselt, R., Urban, J. (2002). Hunchback is required for the specification of the early sublineage of neuroblast 7-3 in the Drosophila central nervous system. *Development* **129**(4): 1027-36.

Nüsslein-Volhard, C., Frohnhöfer, H. G., Lehmann, R. (1987). Determination of anteroposterior polarity in Drosophila. *Science* **238**: 1675-81.

Oppenheim, R. W. (1989). The neurotrophic theory and naturally occurring motoneuron death. *Trends Neurosci* **12**: 252-5.

Orgogozo, V., Schweisguth, F., Bellaiche, Y. (2002). Binary cell death decision regulated by unequal partitioning of Numb at mitosis. *Development* **129**(20): 4677-84.

Raff, M., Barres, B., Burne, J., Coles, H., Ishizaki, Y., Jacobson, M. (1993). Programmed cell death and the control of cell survival: lessons from the nervous system. *Science* **262**: 695-700.

Patel, N., Martin-Blanco, E., Coleman, K. G., Poole, S., Elli, M., Kornberg, T. B., Goodman, C. S. (1989). Expression of engrailed proteins in arthropods, annelids and chordates. *Cell* **58**: 955-68.

Pearson, B. J. and Doe, C. Q. (2004). Specification of temporal identity in the developing nervous system. *Annu Rev Cell Dev Biol* **20**: 619-47.

Pereanu, W. and Hartenstein, V. (2006). Neural lineages of the Drosophila brain: a three-dimensional digital atlas of the pattern of lineage location and projection at the late larval stage. *J Neurosci* **26**(20): 5534-53.

Peterson, C., Carney, G. E., Taylor, B. J., White, K. (2002). reaper is required for neuroblast apoptosis during Drosophila development. *Development* **129**(6): 1467-76.

Peterson, M.D., Popadić, A., Kaufman, T. C. (1998). The expression of two engrailed-related genes in an apterygote insect and a phylogenetic analysis of insect engrailed-related genes. *Dev Genes Evol* **208**:547-57

Pfeiffer, B., Jenett, A., Hammonds, A., Ngo, T., Misra, S., Murphy, C., Scully, A., Carlson, J., Wan, K., Laverty, T., Mungall, C., Svirskas, R., Kadonaga, J., Doe, C. Q, Eisen, M., Celniker, S., Rubin, G. M. (2008). Tools for neuroanatomy and neurogenetics in Drosophila. *Proc Natl Acad Sci USA* **105**:9715-20.

Piovant, P. and Lena, P. (1988). Membrane glycoproteins immunologically related to the human insulin receptor are associated with presumptive neuronal territories and developing neurons in Drosophila melanogaster. *Development* **103**: 145-56.

Poole, S., Kauvar, L., Drees, B., Kornberg, T. (1985). The engrailed locus of Drosophila: structural analysis of an embryonic transcript. *Cell* **40**: 37-43.

Prokop, A. and Technau, G. M. (1991). The origin of postembryonic neuroblasts in the ventral nerve cord of Drosophila melanogaster. *Development* **111**(1): 79-88.

Prud'homme, B., deRosa, R., Arendt, D., Julien, J. F., Pajaziti, R., Dorresteijn, A. W.,

Adoutte, A., Wittbrodt, J., Balavoine, G. (2003). Arthropod-like expression patterns of engrailed and wingless in the annelid Platynereis dumerilii suggest a role in segment formation. *Curr Biol* **13**:1876-81

Rajashekhar, K. P. and Singh, R. N. (1994). Neuroarchitecture of the tritocerebrum of Drosophila melanogaster. *J Comp Neurol* **349**:633-45.

Rodrigues, V. and Hummel, T. (2008). Development of the Drosophila olfactory system. *Adv Med Exp Biol* **628**: 82-101.

Rogers, B. T. and Kaufman, T. C. (1996). Structure of the insect head as revealed by the EN protein pattern in developing embryos. *Development* **122**:3419-32.

Rogulja-Ortmann, A., Luer, K., Seibert, J., Rickert, C., Technau, G. M. (2007). Programmed cell death in the embryonic central nervous system of Drosophila melanogaster. *Development* **134**(1): 105-16.

Rogulja-Ortmann, A., Renner, S., Technau, G. M. (2008). Antagonistic roles for Ultrabithorax and Antennapedia in regulating segment-specific apoptosis of differentiated motoneurons in the Drosophila embryonic central nervous system. *Development* **135**(20): 3435-45.

Rulifson, E., Kim, S. K., Nusse, R. (2002). Ablation of insulin-producing neurons in flies. *Science* **296**: 1118-1120.

Schmid A, Chiba, A., Doe, C. Q. (1999). Clonal analysis of Drosophila embryonic neuroblasts: neural cell types, axon projections and muscle targets. *Development* **126**(21): 4653-89.

Schmidt-Ott, U. and Technau, G. M. (1992). Expression of en and wg in the embryonic head and brain of Drosophila indicates a refolded band of seven segment remnants. *Development* **116**:111-25.

Schmidt, H., Rickert, C., Bossing, T., Vef, O., Urban, J., Technau, G. M. (1997). The embryonic central nervous system lineages of Drosophila melanogaster. II. Neuroblast lineages derived from the dorsal part of the neuroectoderm. *Dev Biol* **189**(2): 186-204.

Schrader, S. and Merritt, D. J. (2000). Central projections of Drosophila sensory neurons in the transition from embryo to larva. *J Comp Neurol* **425**:34-44.

Seaver, E. and Kaneshige, L. (2006). Expression of 'segmentation' genes during larval and juvenile development in the polychaetes Capitella sp. I and H. elegans. *Dev Biol* **289**:179-94.

Seaver, E., Paulson, D., Irvine, S., Martindale, M. (2001). The spatial and temporal expression of Ch-en, the engrailed gene in the polychaete Chaetopterus, does not support a role in body axis segmentation. *Dev Biol* **236**:195-209.

Shain, D., Stuart, D., Huang, F., Weisblat, D. A. (2000). Segmentation of the central nervous system in leech. *Development* **127**:735-44.

Siegler, M. and Jia, X. (1999). Engrailed negatively regulates the expression of cell adhesion molecules connectin and neuroglian in embryonic Drosophila nervous system. *Neuron* **22**: 265-76.

Simon, H., Saueressig, H., Wurst, W., Goulding, M., O'Leary, D. (2001). Fate of midbrain dopaminergic neurons controlled by the engrailed genes. *J Neurosci* **21**: 3126-34.

Simon, H., Thuret, S., Alberi, L. (2004). Midbrain dopaminergic neurons: control of their cell fate by the engrailed transcription factors. *Cell Tissue Res* **318**:53-61.

Simon, H., Scholz, C., O'Leary, D. (2005). Engrailed genes control developmental fate of serotonergic and noradrenergic neurons in mid- and hindbrain in a gene dose-dependent manner. *Mol Cell Neurosci* **28**:96-105.

Sintoni, S., Fabritius-Vilpoux, K., Harzsch, S. (2007). The engrailed-expressing secondary head spots in the embryonic crayfish brain: examples for a group of homologous neurons in Crustacea and Hexapoda? *Dev Genes Evol* **217**:791-9.

Skeath, J. B. and Doe, C. Q. (1998). Sanpodo and Notch act in opposition to Numb to distinguish sibling neuron fates in the Drosophila CNS. *Development* **125**(10): 1857-65.

Skeath, J. B. and Thor, S. (2003). Genetic control of Drosophila nerve cord development. *Curr Opin Neurobiol* **13**(1): 8-15.

Sonnenfeld, M. J. and Jacobs, J. R. (1995). Apoptosis of the midline glia during Drosophila embryogenesis: a correlation with axon contact. *Development* **121**(2): 569-78.

Sprecher, S., Reichert, H., Hartenstein, V. (2007). Gene expression patterns in primary neuronal clusters of the Drosophila embryonic brain. *Gene Expr Patterns* **7**: 584-95.

Strausfeld, N. (1976). Atlas of an Insect Brain. Berlin, Springer.

Strausfeld, N. and Gronenberg, W. (1990). Descending neurons supplying the neck and flight motor of Diptera: organization and neuroanatomical relationships with visual pathways. *J Comp Neurol* **302**:954-72.

Technau, G. M., Berger, C., Urbach, R. (2006). Generation of cell diversity and segmental pattern in the embryonic central nervous system of Drosophila. *Dev Dyn* **235**(4): 861-9.

Thomaidou, D., Mione, M., Cavanagh, J., Parnavelas, J. (1997). Apoptosis and its relation to the cell cycle in the developing cerebral cortex. *J Neurosci* **17**: 1075-85.

Truman, J. W. (1983). Programmed cell death in the nervous system of an adult insect. *J Comp Neurol* **212**(4): 445-52.

Truman, J. W. and Bate, M. (1988). Spatial and temporal patterns of neurogenesis in the central nervous system of Drosophila melanogaster. *Dev Biol* **125**(1): 145-57.

Truman, J. W., Thorn, R. S., Robinow, S. (1992). Programmed neuronal death in insect development. *J Neurobiol* **23**(9): 1295-311.

Truman, J. W., Schuppe, H., Shepherd, D., Williams, D. W. (2004). Developmental architecture of adult-specific lineages in the ventral CNS of Drosophila. *Development* **131**(20): 5167-84

Tyrer, N. and Gregory, G. (1982). A guide to the neuroanatomy of locust suboesophageal and thoracic ganglia. *Phil Trans R Soc Lond B* **297**: 91-123.

Ullmann, S. L. (1964). The origin and structure of the mesoderm and the formation of the coelomic sacs in Tenebrio molitor L (Insecta, Coleoptera). *Phil Trans R Soc Lond B* **747**:245-276.

Urbach, R. and Technau, G. M. (2003a). Segment polarity and DV patterning gene expression reveals segmental organization of the Drosophila brain. *Development* **130**(16): 3607-20.

Urbach, R. and Technau, G. M. (2003b). Molecular markers for identified neuroblasts in the developing brain of Drosophila. *Development* **130**(16): 3621-37.

Urbach, R. and Technau, G. M. (2003c). Early steps in building the insect brain: neuroblast formation and segmental patterning in the developing brain of different insect species. *Arthropod Struct Dev* **32**(1): 103-23.

Wagh, D., Rasse, T., Asan, E., Hofbauer, A., Schwenkert, I., Durrbeck, H., Buchner, S., Dabauvalle, M., Schmidt, M., Qin, G., Wichmann, C., Kittel, R., Sigrist, S., Buchner, E. (2006). Bruchpilot, a protein with homology to ELKS/CAST, is required for structural integrity and function of synaptic active zones in Drosophila. *Neuron* **49**: 833-44.

Walldorf, U. and Gehring, W. (1992). Empty spiracles, a gap gene containing a homeobox involved in *Drosophila* head development. *EMBO J*. **11**: 2247-59.

Wanninger, A. and Haszprunar, G. (2001). The expression of an engrailed protein during embryonic shell formation of the tusk-shell, Antalis entalis (Mollusca, Scaphopoda). *Evol Dev* **3**:312-21.

Wedeen, C. J. and Weisblat, D. A. (1991). Segmental expression of an engrailed-class gene during early development and neurogenesis in an annelid. *Development* **113**: 805-14.

Weeks, J. C. (2003). Thinking globally, acting locally: steroid hormone regulation of the dendritic architecture, synaptic connectivity and death of an individual neuron. *Prog Neurobiol* **70**(5): 421-42.

Weeks, J. C. and Truman, J. W. (1985). Independent steroid control of the fates of motoneurons and their muscles during insect metamorphosis. *J Neurosci* **5**(8): 2290-300.

Wenner, P., **O'Donovan, M. J**., **Matise, M. P**. (2000). Topographical and physiological characterization of interneurons that express engrailed-1 in the embryonic chick spinal cord. *J Neurophysiol* **84**: 2651-7.

White, K., Grether, M. E., Abrams, J. M., Young, L., Farrell, K., Steller, H. (1994). Genetic control of programmed cell death in Drosophila. *Science* **264**(5159): 677-83.

Wurst, W., Auerbach, A., Joyner, A. L. (1994). Multiple developmental defects in Engrailed-1 mutant mice: an early mid-hindbrain deletion and patterning defects in forelimbs and sternum. *Development* **120**: 2065-75.

Younossi-Hartenstein, A., Tepass, U., Hartenstein, V. (1993). The embryonic origin of the primordia of the adult Drosophila head. *Roux's Arch Dev Biol* **203**:60-73.

Younossi-Hartenstein, A., Nassif, C., Green, P., Hartenstein, V. (1996). Early neurogenesis of the Drosophila brain. *J Comp Neurol* **370**(3): 313-29.

Younossi-Hartenstein, A., Green, P., Liaw, G., Rudolph, K., Lengyel, J., Hartenstein, V. (1997). Control of early neurogenesis of the Drosophila brain by the head gap genes tll, otd, ems, and btd. *Dev Biol.* **182**: 270-283.

Younossi-Hartenstein, A., Salvaterra, P., Hartenstein, V. (2003). Early development of the Drosophila brain: IV. Larval neuropile compartments defined by glial septa. *J Comp Neurol.* **455**: 435-50.

Younossi-Hartenstein, A., Nguyen, B., Shy, D., Hartenstein, V. (2006). Embryonic origin of the Drosophila brain neuropile. *J Comp Neurol.* **497**: 981-98.

Zapala, M. A., Hovatta, I., Ellison, J. A., Wodicka, L., Del Rio, J. A., Tennant, R., Tynan, W., Broide, R. S., Helton, R., Stoveken, B. S. et al. (2005). Adult mouse brain gene expression patterns bear an embryologic imprint. *Proc Natl Acad Sci U S A* **102**, 10357-62.

Zaretsky, M. and Loher, W. (1983). Anatomy and Electrophysiology of individual neurosecretory cells of an insect brain. *J Comp Neurol* **216**: 253-63.

Zheng, X., Zugates, C. T., Lu, Z., Shi, L., Bai, J. M., Lee, T. (2006). Baboon/dSmad2 TGF-beta signaling is required during late larval stage for development of adult-specific neurons. *Embo J* **25**(3): 615-27.

Zhou, B., **Hutson, J.**, **Farmer, P.**, **Hasthorpe, S**., **Myers, N.**, **Liu, M**. (1999). Apoptosis in tracheoesophageal embryogenesis in rat embryos with or without adriamycin treatment. *J Pediatr Surg* **34**: 872-5.

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Zlatic, M., Landgraf, M., Bate, M. (2003). Genetic specification of axonal arbors: atonal regulates robo3 to position terminal branches in the Drosophila nervous system. *Neuron* **37**:41-51.

Appendix

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Nature INDIA **Science feature**

Give the fruit fly a chance in brain research

Abhilasha Kumar

The brain is made up of hundreds of thousands of neurons. The stem cells divide to give rise to this staggering number of neurons. How do these neurons organize themselves, connect in a way that makes sense and ultimately lead to a brain that can receive input, process information and influence behaviour of an organism?

These are questions intriguing developmental and neurobiologists alike. Concrete answers have eluded us thus far though scattered research results have helped construct a somewhat rudimentary picture. The use of a model system to best answer these questions has also been a debatable and open ended issue. What experimental feats are possible in a given model organism depends on historical, economic and biological factors.

Drosophila melanogaster, or the fruit fly, is one such widely used model system. Drosophila is arguably one of the most powerful genetic tools in the world. An easy, cost-effective animal to grow in the lab, its use in understanding genetics dates back to 1910 when Thomas Morgan isolated the first fly mutant. Since then, thousands of fly lines have been generated and made publicly available (Flybase, for instance). It was no surprise when the fly genome was sequenced in March 2000 by the Berkeley Drosophila genome project in collaboration with Celera Genomics. As a model system, sophisticated genetic calisthenics are possible in

Drosophila with reproducible and stable results, something that is unparalleled in other model systems, such as the mouse.

The fruit fly is thus, a highly amenable organism to study complex and fundamental questions about brain development and the issues surrounding it. Even though differences between insects and mammals appear overtly to be huge, research over the past decade has shown that the underlying molecular framework of the developing brain is pretty similar (1). Meaning, the molecules that build the vertebrate and invertebrate brains are conserved even though a lot of fine tuning happens later to make both brains anatomically different.

Indian scientists have done pioneering work on many aspects of brain development in the fly. The Siddiqi lab, first based at the Tata Institute of Fundamental Research (TIFR) at Mumbai and currently at the National Centre for Biological Sciences (NCBS) in Bangalore, is a much recognized and respected team the world over. Their long standing research about how flies smell and how they distinguish odors has contributed to understanding behaviours innate to living beings, such as recognizing good versus bad smells. Veronica Rodrigues and her team of researchers at TIFR and NCBS have made a serious attempt at answering questions about the cell and developmental biology of brain development in *Drosophila.* Their work on molecular processes that occur during development of sense organs such as the antennae has been highly acknowledged by peers all over the world (2). In addition, their work has shed light on how different cell types organize to function within the olfactory system or the smell system of the fly (3). The present fly brain research scenario in India is thus, a bright faced one, given the increasing number of acclaimed works coming from Indian labs.

That said, there is still a gaping void where a bridge between basic research and pharmaceutical industry should have been. Our western contemporaries, on the other hand,

are slowly but surely progressing in this direction. Neurodegenerative diseases are increasingly been studied using the fly as a model system. There are now fly models for Parkinson's and Alzheimer's disease in particular and the pharmaceutical giants are raking in the results, regarding them as a long term investment (4). Their heightened interest and investment in University based research encourages researchers and vice versa.

Indian researchers suffer from a lack of interest of the pharmaceutical industry in their research. Industries are more interested in generics and their research and development has very little to do with long term investments. Fundamental research, in their opinion, is best left to Universities. Although this might be a sound strategy to keep the money mill going for the next five years, it is certainly a myopic one. The short summary that follows highlights one such area where *Drosophila* brain research and industry could mix. An increasing importance is being attached to brain stem cells these days. Not just because stem cell is the buzzword but because stem cells have the potential to be the probable new generation of therapeutics.

One such fundamental question of high significance to the above is that of the properties of neural stem cells. Brain stem cells or neural progenitors start dividing and continue till they have cues to stop, making up the brain's definitive size. So what are these cues that tell stem cells when to stop dividing? Neural stem cells are easily recognizable in the fly nervous system at certain stages wherein complex markers are generally needed to spot them in a more complex mouse brain. Therefore, questions about their division properties and how and when neural progenitors stop to divide are comfortably addressed in *Drosophila.*

A recent work from the Gould lab in London, published this year in the journal *Cell,* has pinned down a handful of molecules that schedule the end of this dividing process, also called

neural proliferation (5). Using *Drosophila* larval and adult central nervous systems, they have identified the molecular cues that signify the end of an aging progenitor. The authors conclude that neural progenitors stop dividing by either exiting the cell cycle or by undergoing a form of programmed cell death. However, even though the strategies used to end proliferation are different, the "molecular timer" used is the same by both types of progenitors.

This molecular timer is actually a carefully orchestrated series of molecules expressed in relation to time, or in technical jargon, in a temporal series. The temporal series generated within a neural stem cell thus specifies a combinatorial code, which then acts on its respective targets, ultimately stopping the stem cell from dividing after it has reached a certain age; meaning acquired its "old age" signature of molecules. In fact, when lineages were mutated for one or several of these factors, the stem cell could no longer stop dividing. It continued to proliferate into adulthood, something that normally never happens. This probable immortalization of the stem cell without disrupting its intrinsic division properties could in the future prove to be a useful tool in stem cell therapy and regeneration.

The idea may not be as far fetched as it first sounds. Although it is a fundamental study in *Drosophila,* some of the molecules required in making stem cells stop dividing may have functional equivalents in mammals. It is also probable that even though the "molecular timer" in itself may be different, the downstream targets may be functionally conserved. Research studies such as these, besides improving the picture about fundamental processes like neurogenesis in the fly, is also in itself a stepping stone for similar research in mammals such as humans. Why? Because if scientists already have a clue about possible suspects, it narrows down the search for key control molecules, with vital therapeutic implications.

The author is a graduate student at the fag end of her PhD at the Biozentrum, University of Basel, Switzerland. Her work involves investigation of mechanisms that lead to lineage differentiation in the developing Drosophila brain.

References

1. Hirth, F. *et al.* An urbilaterian origin of the tripartite brain: developmental genetic insights from Drosophila. *Development* **130,** 2365-2373 (2003)
2. Jhaveri, D. *et al.* Mechanisms underlying olfactory neuronal connectivity in Drosophila-the atonal lineage organizes the periphery while sensory neurons and glia pattern the olfactory lobe. *Dev. Biol.* **226,** 73-87 (2000)
3. Jhaveri, D. *et al.* Positioning sensory terminals in the olfactory lobe of Drosophila by Robo signaling. *Development.* **131,** 1903-1912 (2004)
4. Alexander, J. *et al.* Drosophila models pioneer a new approach to drug discovery for Parkinson's disease. *Drug Discov. Today* **11,** 119-126 (2006)
5. Maurange, C. *et al.* Temporal transcription factors and their targets schedule the end of neural proliferation in Drosophila. *Cell* **133,** 891-902 (2008)

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- Awarded the Father Joseph Sallas prize in Zoology during undergraduate studies, St. Xavier's College, India.

- Awarded junior research fellowship by the University Grants Commission, India, 2003.

- Awarded research fellowship by the Centre for Scientific and Industrial Research, India, 2004.

LIST OF PUBLICATIONS:

1. Kumar, A., Bello, B., Reichert, H. (2009). Lineage-specific programmed cell death in the postembryonic brain development of *Drosophila*. *Development*, 136(20): 3433-42.

2. Kumar, A., Fung, S., Lichtneckert, R., Reichert, H., Hartenstein, V. (2009). The arborization pattern of *engrailed*-positive neural lineages reveal neuromere boundaries in the *Drosophila* brain neuropile. *J Comp Neurol*, 517(1): 87-104.

3. Pereanu, W., Kumar, A., Jenett, A., Reichert, H., Hartenstein, V. (2009). A development-based compartmentalization of the *Drosophila* central brain. (Submitted).

4. Kumar, A. Science feature article. Give the fruit fly a chance in brain research. Published online, August 29, 2008. Nature India.