

The *labial* gene is required to terminate proliferation of identified neuroblasts in postembryonic development of the *Drosophila* brain

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Summary

The developing brain of *Drosophila* has become a useful model for studying the molecular genetic mechanisms that give rise to the complex neuronal arrays that characterize higher brains in other animals including mammals. Brain development in *Drosophila* begins during embryogenesis and continues during a subsequent postembryonic phase. During embryogenesis, the Hox gene *labial* is expressed in the developing tritocerebrum, and *labial* loss-of-function has been shown to be associated with a loss of regional neuronal identity and severe patterning defects in this part of the brain. However, nothing is known about the expression and function of *labial*, or any other Hox gene, during the postembryonic phase of brain development, when the majority of the neurons in the adult brain are generated. Here we report the first analysis of Hox gene action during postembryonic brain development in *Drosophila*. We show that *labial* is expressed initially in six larval brain neuroblasts, of which only four give rise to the *labial* expressing neuroblast lineages present in the late larval brain. Although MARCM-based clonal mutation of *labial* in these four neuroblast lineages does not result in an obvious phenotype, a striking and

unexpected effect of clonal *labial* loss-of-function does occur during postembryonic brain development, namely the formation of two ectopic neuroblast lineages that are not present in wildtype brains. The same two ectopic neuroblast lineages are also observed following cell death blockage and, significantly, in this case the resulting ectopic lineages are *Labial*-positive. These findings imply that *labial* is required in two specific neuroblast lineages of the wildtype brain for the appropriate termination of proliferation through programmed cell death. Our analysis of *labial* function reveals a novel cell autonomous role of this Hox gene in shaping the lineage architecture of the brain during postembryonic development.

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Key words: *labial*, Hox, Homeotic, Neuroblast, Neural stem cell, Programmed cell death, Lineage, Brain, Postembryonic, *Drosophila*

Introduction

The neural cells of the *Drosophila* central brain develop from a set of approximately 100 neural-stem-cell-like neuroblasts which derive from the cephalic neuroectoderm in the early embryo (reviewed by Urbach and Technau, 2004; Technau et al., 2006; Hartenstein et al., 2008). During embryogenesis, these neuroblasts divide in an asymmetric manner to self-renew and produce ganglion mother cells which generally give rise two postmitotic neural progeny (reviewed by Skeath and Thor, 2003; Doe, 2008; Knoblich, 2008). This initial phase of embryonic neurogenesis gives rise to the functional brain of the *Drosophila* larva. Towards the end of embryogenesis, most neuroblasts enter a reversible cell cycle arrest called quiescence, which separates the initial phase from the subsequent secondary phase of neurogenesis (Isshiki et al., 2001; Tsuji et al., 2008; Egger et al., 2008). In response to intrinsic and extrinsic factors involving nutritionally activated mitogens and glial cell-dependent interactions, neuroblasts resume proliferation during early larval stages (Chell and Brand, 2010; Sousa-Nunes et al.,

2011). During this postembryonic phase of neurogenesis the majority of the adult-specific neurons of the brain are generated (Truman and Bate, 1988; Prokop and Technau, 1991). The adult-specific neural cells produced postembryonically by each individual neuroblast form a lineage-related cluster of immature neurons which differentiate in the pupal phase and contribute to the functional adult brain circuits (Truman et al., 2004; Pereanu and Hartenstein, 2006; Hartenstein et al., 2008).

Timely, precise and irreversible termination of postembryonic neuroblast proliferation is crucial to ensure that the correct number of neural progeny is generated and to avoid the danger of uncontrolled overgrowth (reviewed by Neumüller and Knoblich, 2009; Weng et al., 2010; Knoblich, 2010; Reichert, 2011). This process varies in temporal and spatial respects in the developing brain, but is largely finished by the end of metamorphosis as no identifiable neuroblasts are present at adult stages (Truman and Bate, 1988; Ito and Hotta, 1992). For most of the neuroblasts of the central brain and thoracic ganglia, termination of proliferation is achieved by series of cellular adjustments, involving shrinkage,

lengthening of the cell cycle, expression of nuclear *prospero* and then cell cycle exit via a symmetric final division (Maurange et al., 2008). In contrast, for neuroblasts in the abdominal ganglia, which cease dividing in larval stages, termination of proliferation involves another mechanism, namely induction of programmed cell death in neuroblasts through expression of Hox gene-encoded transcription factors (reviewed by Pearson et al., 2005; Rogulja-Ortmann and Technau, 2008; Miguel-Aliaga and Thor, 2009; Sousa-Nunes et al., 2010). More specifically, in all neuroblasts of the central abdomen, the Hox gene *abdominal-A* (*abd-A*) is expressed in a short pulse during larval development in order to trigger programmed cell death (Bello et al., 2003). This ability of Hox genes to trigger programmed cell death in the abdominal ganglia is tightly regulated by epigenetic mechanisms involving the Polycomb group of genes (Bello et al., 2007).

Hox genes have also been shown to act in the development of the central brain in *Drosophila*, and notably for the Hox gene *labial*, loss-of-function has been associated with severe patterning defects in embryonic brain development (Diederich et al., 1989; reviewed by Lichtneckert and Reichert, 2008; Reichert and Bello, 2010). During embryogenesis, *labial* is expressed throughout the tritocerebrum anlage; all thirteen neuroblasts of the tritocerebrum as well as two neuroblasts of the deutocerebrum are Labial-positive (Younossi-Hartenstein et al., 1996; Urbach and Technau, 2003). If *labial* is inactivated, postmitotic cells are generated; however, they do not extend neurites and lack the expression of neuronal markers, indicating that *labial* is required to establish neuronal identity in the embryonic tritocerebrum (Hirth et al., 1998). Interestingly, these defects can be rescued by targeted misexpression by all Hox genes except *Abd-B* (Hirth et al., 2001). Moreover, expression of *labial* in the tritocerebrum can be subject to cross-regulatory interactions among Hox proteins during embryonic brain development (Sprecher et al., 2004).

In contrast to the extensive information on the role of the *labial* gene in embryonic brain (tritocerebrum) development, virtually nothing is known about the expression and function of *labial*, or any other Hox gene, in postembryonic brain development of *Drosophila*. Hence, it is unclear if Hox genes have any influence on the development of the adult-specific, secondary neurons that make up the bulk of the neuronal circuitry in the adult brain. Here we show that the Hox gene *labial* is expressed in late L3 larval stage brain in four neuroblasts that give rise to the identified *labial* expressing neuroblast lineages BA1p4, BA1v, TRdm and TRld. Moreover, we demonstrate that two additional *labial* expressing neuroblasts are present in the late L2 stage – but not in the early L3 stage. Remarkably, while MARCM-based clonal mutation of *labial* in the BA1p4, BA1v, TRdm and TRld neuroblast lineages does not result in any obvious mutant phenotype, a striking effect of clonal *labial* loss-of-function does occur, namely the formation of two ectopic neuroblast lineages that are not present in wildtype brains. These two ectopic neuroblast lineages are also observed following MARCM-based block of cell death and, significantly, these ectopic lineages are Labial-positive. Since both clonal cell death block and clonal *labial* inactivation result in the formation of the same two ectopic neuroblast lineages, these findings imply that *labial* is required in these two postembryonic brain neuroblast lineages for termination of proliferation through programmed cell death. This analysis of *labial* function reveals a novel cell autonomous role of a Hox gene in shaping the lineage architecture of the brain during postembryonic development.

Materials and Methods

Fly strains and genetics

Unless otherwise stated fly stocks were obtained from the Bloomington Stock center. Wildtype was *w*¹¹¹⁸. Gal4-Lines that were used: GAL4^{MZ1407}, UAS-mCD8::GFP^{LL5} (Luo et al., 1994; Betschinger et al., 2006) (B.C.B., unpublished); *w*; *wor*-GAL4, UAS-mCD8::GFP^{LL5}/CyO, ActGFP^{JMR1} (Albertson et al., 2004). One recombinant chromosome was constructed: FRT82B, *lab*¹⁴. For *labial* mutant and wildtype MARCM analysis (Lee and Luo, 1999; Lee and Luo, 2001), FRT82B, *lab*¹⁴/TM3, *actGFP*^{JMR2}; or FRT82B males were crossed to *y,w,hsFLP*¹²², *tubP*-GAL4, UAS-mCD8::GFP^{LL5}/CyO, *actGFP*^{JMR1}; FRT82B, *tubP*-GAL80^{LL5}/(TM6,Tb,Hu) females (B.C.B., unpublished). For *labial* mutant “rescue” analysis UAS-*labial*; FRT82B, *lab*¹⁴/TM3, *actGFP*^{JMR2} males were crossed to *y,w,hsFLP*¹²², *tubP*-GAL4, UAS-mCD8::GFP^{LL5}/CyO, *actGFP*^{JMR1}; FRT82B, *tubP*-GAL80^{LL5}/(TM6,Tb,Hu) females. For H99 and wildtype control MARCM analysis, *w*; FRT2A, Df(3L)H99, *kni*¹-1/TM6 [*w*+] or *w*; FRT2A were crossed to *y,w,hsFLP*¹²²; *tubP*-GAL4, UASmCD8::GFP^{LL5}/CyO; FRT2A,*tubP*GAL80^{LL5}/(TM6,Tb,Hu) females (B.C.B., unpublished). For *labial* misexpression analysis UAS-*labial*; FRT82B males were crossed to *y,w,hsFLP*¹²²; *tubP*-GAL4, UAS-mCD8::GFP^{LL5}/CyO, ActGFP^{JMR1}; FRT82B, *tubP*-GAL80^{LL5}/(TM6,Tb,Hu) females. For 24 hour ALH heatshock MARCM experiments, embryos were collected on standard medium over a 4 hour time window, raised at 25°C for 48 hours and then heat-shocked for 1 hour at 37°C. For embryonic heatshock MARCM experiments, embryos were collected for an 8 hour time window on standard medium at 18°C, raised at 18°C for 15 hours and afterwards heatshocked for 1 hour at 37°C. For RNAi experiments, UAS-*labRNAi*²⁹⁹⁰ (obtained from VDRC) was crossed to GAL4^{MZ1407}, UAS-mCD8::GFP^{LL5} and *wor*-GAL4, UAS-mCD8::GFP^{LL5}/CyO, *actGFP*^{JMR1}. For p35 cell death block experiments, UAS-p35^{BH2} was crossed to GAL4^{MZ1407}, UAS-mCD8::GFP^{LL5}.

Immunolabeling

Embryos were dechorionated, fixed and labeled according to standard protocols (Patel, 1994). Larval brains were fixed and immunostained as previously described (Bello et al., 2007). The following antibodies were used: guineapig-anti-Dpn (1:1000) (J. Skeath), mouse-anti-BP016 Neurotactin (1:20) (DSHB), mouse-anti-nc82 Bruchpilot (1:20) (DSHB), rabbit-anti-Labial (1:200) (F. Hirth and H.R., unpublished), rat-anti-Labial (1:200) (F. Hirth and H.R., unpublished). Alexa fluorescence-conjugated secondary antibodies (Molecular Probes) were used at 1:200.

Microscopy and image processing

All fluorescent images were recorded using a Leica SP confocal microscope. Optical sections range from 1–2 μm with a picture size of 1024×1024 pixels. Collected images were arranged and processed using ImageJ, Adobe Photoshop and Adobe Illustrator. Cell counts were performed with the ImageJ plugin “cell counter”. For highlighting specific MARCM clones, cell bodies and neurites from other clones in the vicinity were removed in every single optical section. 3D models (superposition) were generated with the Fiji 3D viewer tool by selecting structures of interest such as MARCM clones or the neurite scaffold. Schemes were drawn in Adobe Illustrator.

Results

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

To investigate the expression of the Hox gene *labial* in postembryonic brain development, we performed an immunocytochemical analysis of whole-mount brains of wandering third-instar (late L3 stage) larvae using a Labial-specific antibody (LAB) in combination with an anti-Bruchpilot (NC82) antibody to visualize neuropile structures. Expression of the *labial* gene was detected in two bilaterally symmetrical groups of cells located posterior to the antennal lobe and adjacent to the SOG (subesophageal ganglion) in the general region of the posterior central brain that corresponds to the developing tritocerebrum (Fig. 1). In confocal single optical sections, these *labial* expressing neural cells were observed in spatial association with a small number of secondary axon tracts labeled by MZ¹⁴⁰⁷-Gal4 suggesting that these neurons might correspond to a small set of neuroblast lineages (Luo et al., 1994; Betschinger et al., 2006). In addition to the labeled neuron groups, *labial* expression was also observed in four larger cells which co-expressed the marker *deadpan* (DPN) indicating that they were neuroblasts (San-Juán and Baonza, 2011). These four neuroblasts were also located in the same posterior

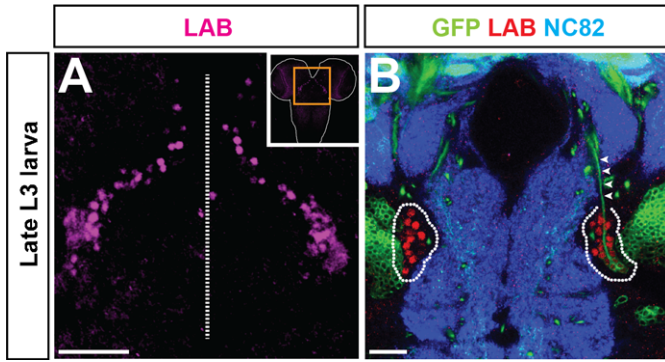


Fig. 1. Regionalized expression of *labial* in the posterior central brain at the late larval stage. (A) Overview of the late L3 larval brain. Two bilaterally symmetric cell clusters express *labial*. Labeled cells are shown in a Z-projection of multiple optical sections of a whole-mount brain. Dotted line indicates midline. Inset shows total larval CNS with box indicating region of *labial* expression. (B) Single optical section showing *labial* expressing cells (red), nc82 immunolabeled neuropile (blue) and MZ¹⁴⁰⁷-Gal4 driven and membrane-targeted GFP expression (green). Dotted lines indicate position of the *labial* expressing cells. Arrowheads indicate secondary axon tract of *labial* expressing cells. Scale bars: 50 μ m in A; 20 μ m in B. In this and all subsequent figures, ventral views of the brain are presented and, with the exception of Fig. 3E–H', anterior is always to the top.

central brain region and were invariably associated with the *labial*-expressing cell clusters (Fig. 2). Several other Deadpan-positive neuroblasts were located in the vicinity of these four *labial*-expressing neuroblasts, but none of these were Labial-positive.

To identify the postembryonic lineages generated by the four *labial* expressing neuroblasts, we performed a MARCM-based clonal analysis with an ubiquitous *tub*-Gal4 driving UAS-mCD8::GFP (Lee and Luo, 1999; Lee and Luo, 2001). Clones were induced at random at 24 hours after larval hatching (ALH) and recovered at the late L3 larval stage and, therefore, only secondary (adult-specific) lineages of individual neuroblasts were labeled. MARCM-labeled clones were co-labeled with the anti-Labial antibody and with an anti-Neurotactin (NRT; BP106) antibody that is specific for secondary lineages. We recovered four neuroanatomically distinct neuroblast lineages that had Labial-immunopositive neuroblasts.

For further identification of these neuroblast lineages, we determined the projection patterns of each of their secondary axon tracts relative to the ensemble of secondary axon tracts in the late larval brain based on anti-NRT immunolabeling and compared these patterns to those documented in previous lineage mapping studies (Pereanu and Hartenstein, 2006; Spindler and Hartenstein, 2010). Since all of these neuroblast lineages had an invariant and unique projection pattern of their secondary axon tracts (SAT), we were able to unambiguously assign the four *labial* expressing neuroblast lineages to four previously identified postembryonic lineages, namely BALp4, BALv, TRdm and TRld (Fig. 3).

For a more detailed characterization of these four identified neuroblast lineages, we performed cell counts on the corresponding Labial-immunolabeled MARCM clones. The BALp4 lineage contained an average of 64 cells (s.d.=8, n=12) of which an average of 62 cells (s.d.=7, n=12) were Labial-immunopositive, the BALv lineage contained an average of 81 cells (s.d.=7, n=6) of which an average of 19 cells (s.d.=4, n=6) were Labial-immunopositive, the TRdm lineage contained an average of 59 cells (s.d.=6, n=5) of which an average of 12 cells (s.d.=8, n=5) were Labial-immunopositive, and the TRld lineage contained an average of 86 cells (s.d.=4, n=2) of which an average of 31 cells (s.d.=3, n=2) were Labial-immunopositive. This analysis indicates that the BALp4 (basoanterior lineages, posterolateral subgroup) lineage expresses *labial* in most if not all cells while the BALv (basoanterior lineages, ventrolateral subgroup), TRdm (dorsomedial tritocerebral lineage) and TRld (dorsolateral tritocerebral lineage) lineages express *labial* only in subset of their cells including the neuroblast.

Mutational inactivation of *labial* does not affect cell number and secondary axon tract projections in the BALp4, BALv, TRdm and TRld lineages

To investigate the role of *labial* in the development of the BALp4, BALv, TRdm and TRld lineages, we compared the wildtype and *labial* mutant MARCM clones, induced at 24 hours ALH and recovered at late L3 larval stages, for each of these neuroblast lineages. Mutant GFP-labeled clones were homozygous for *lab*¹⁴, an embryonic lethal loss-of-function allele of *labial* (Merrill et al., 1989). All of the recovered *labial* mutant clones of the BALp4,

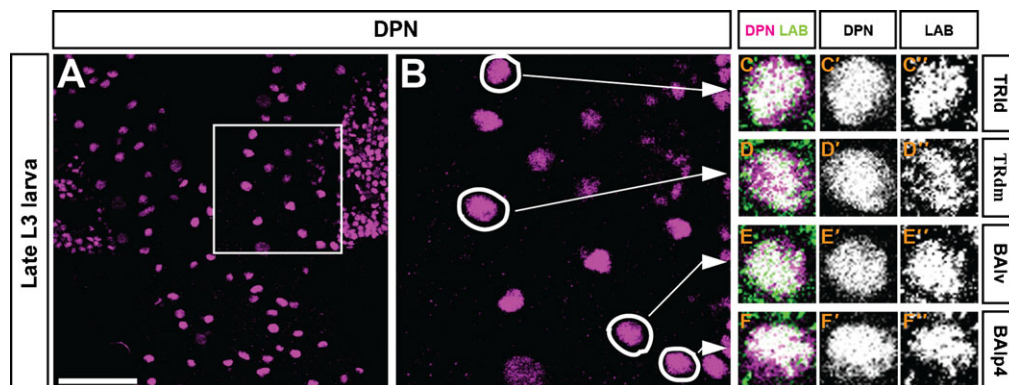


Fig. 2. *labial* is expressed in four neuroblasts of the late L3 larval brain. Labeled cells are shown in a Z-projection of multiple optical sections. (A) Overview of anti-Deadpan immunolabeled cells in the L3 larval brain. Box delimits cells a region in one hemisphere of the posterior central brain. (B) Magnified view of the boxed region shown in A. Neuroblasts co-immunolabeled with anti-Deadpan and anti-Labial are indicated by circles. (C–F'') Single optical sections of each of the four Deadpan-immunolabeled neuroblasts that express *labial* at the late L3 stage. Magnified view of the circled cells shown in B. Anti-Deadpan immunolabeling is in magenta. Labial immunolabeling is in green. Based on their relative position, each of these neuroblasts can be assigned to four lineages: TRld, TRdm, BALv, BALp4. Scale bar: 50 μ m in A.

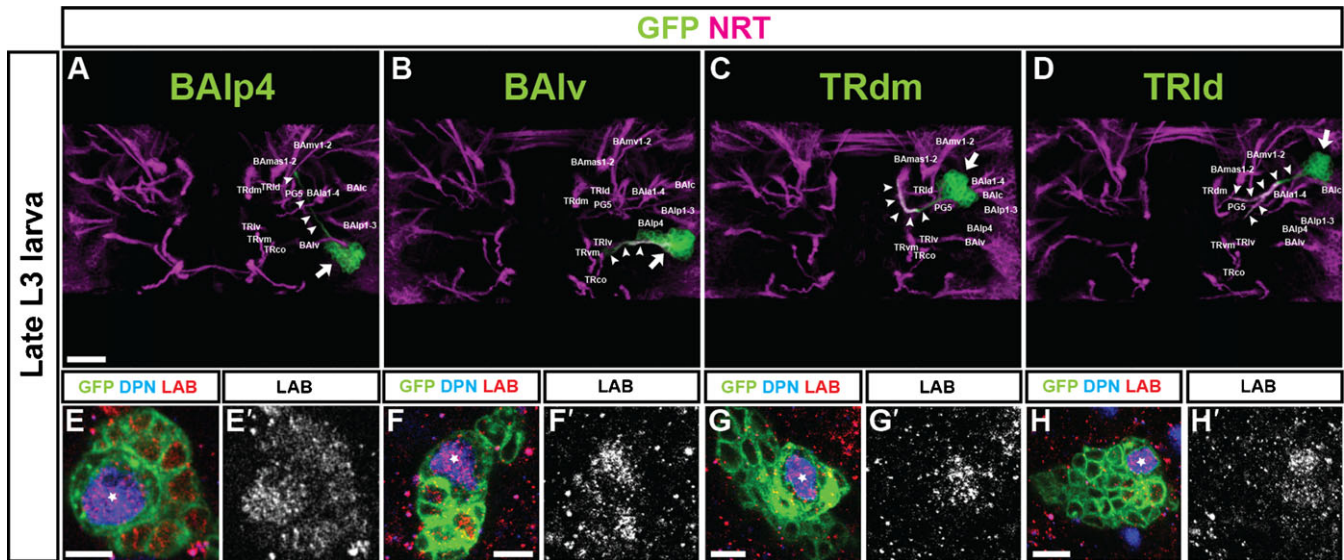


Fig. 3. The four *labial* expressing neuroblasts give rise to the BAIp4, BALv, TRdm and TRld lineage. (A–D) Individual identified neuroblast clones are shown for each lineage together with the array of identified secondary axon tracts in the posterior central brain region of interest. GFP-labeled MARCM clones of the four neuroblast lineages are in green. Anti-Neurotactin labeling of secondary axon tracts secondary lineages is in magenta. Arrows indicate position of the cell bodies of the BAIp4, BALv, TRdm and TRld lineages. Arrowheads indicate secondary axon tracts of the BAIp4, BALv, TRdm and TRld lineages. Figures are superposition of multiple optical sections in late L3 brains. (E–H') Neuroblasts in each of these four lineages express *labial*. Deadpan immunolabeling (neuroblasts) is in blue. Labial immunolabeling is in red. Single optical sections of BAIp4 (E,E'), BALv (F,F'), TRdm (G,G') and TRld (H,H'). Stars indicate the neuroblast. Scale bars: 20 μ m in A–D; 5 μ m in E–H'.

BALv, TRdm and TRld lineages were similar in their general neuroanatomical features to the respective wildtype clones. They all comprised a single large cell corresponding to the neuroblast as well as an associated cluster of labeled cells corresponding to the secondary neurons, and the secondary axon tracts formed by the secondary neurons had an appropriate, wildtype-like projection pattern in all cases (supplementary material Fig. S1).

To determine if the number of cells in the *labial* mutant clones was comparable to that of the corresponding wildtype clones, we performed cell counts for each of the four lineages. For all four lineages, the total cell number was not significantly different in wildtype versus *labial* mutant clones (supplementary material Fig. S1). Thus, average cell counts for wildtype versus mutant were 66 versus 66 (BAIp4), 70 versus 71 (BALv), 65 versus 62 (TRdm) and 80 versus 88 (TRld). We conclude that clonal mutation of *labial* does not alter cell number and secondary axon tract projection in the BAIp4, BALv, TRdm and TRld lineages.

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

In contrast to the lack of overt mutant phenotype in *labial* mutant BAIp4, BALv, TRdm and TRld lineages, a striking and unexpected effect of clonal *labial* loss-of-function was observed in the developing L3 larval brain, namely the formation of ectopic neuroblast lineages that were not present in wildtype brains. Ectopic lineages were recovered in about 50% of all brains containing randomly induced *lab*¹⁴ mutant clones. These ectopic neuroblast lineages could be unambiguously identified based on the projection patterns of their ectopic secondary axon tracts within the ensemble of secondary axon tracts of late larval brains (Fig. 4A,B). Morphologically they could be assigned to two different types, which we refer to as

Ectopic1^{lab} (Ect1^{lab}) and Ectopic2^{lab} (Ect2^{lab}) lineages. Ect1^{lab} was located between the BAIp4 and the BALv lineages, had an average cell number of 107 cells (s.d.=24, n=3) and formed several secondary axon tract projections, of which one always followed an axon tract of the BALc lineage (Fig. 4C–D'). Ect2^{lab} was located close to the TRdm and TRld lineage, had an average cell number of 25 cells (s.d.=1, n=3) and projected its secondary axon tract towards the midline (Fig. 4E–F'). These ectopic lineages were only seen in the late larval (L3) brain. Moreover, they were never observed in MARCM-based genetic rescue experiments (clone induction: 24 hours ALH) in which a UAS-*labial* transgene under the control of the *tub*-GAL4 driver was used to express the *labial* gene in *labial* loss-of-function mutant clones (n=16).

To further confirm that the formation of ectopic neuroblast lineages was indeed due to *labial* loss-of-function, we performed genetic knockdown experiments in which *worniu*-Gal4 and MZ¹⁴⁰⁷-Gal4 were used to drive UAS-*labRNAi*²⁹⁹⁰ in all developing neuroblasts (Albertson et al., 2004). Ectopic neuroblast lineages comparable to those induced by *lab*¹⁴ mutant clones resulted (Fig. 5A,B). These ectopic lineages were recovered in 50% of the late larval brains for the *worniu*-Gal4 driver (n=23) and in 20% of the late L3 larval brains for the MZ¹⁴⁰⁷-Gal4 driver (n=14). In accordance with the *lab*¹⁴ mutant clonal analysis (loss-of-function and genetic rescue), these findings indicate that the appearance of ectopic lineages is a *labial*-specific loss-of-function effect. Moreover, since the targeted knockdown of *labial* driven by *worniu*-Gal4 and MZ¹⁴⁰⁷Gal4 is largely neuroblast-specific, these findings also suggest that the ectopic lineage phenotype was due to the absence of Labial protein in the neuroblasts themselves rather than in their neural cell progeny. This assumption is supported by the observation that both types of ectopic lineages recovered in

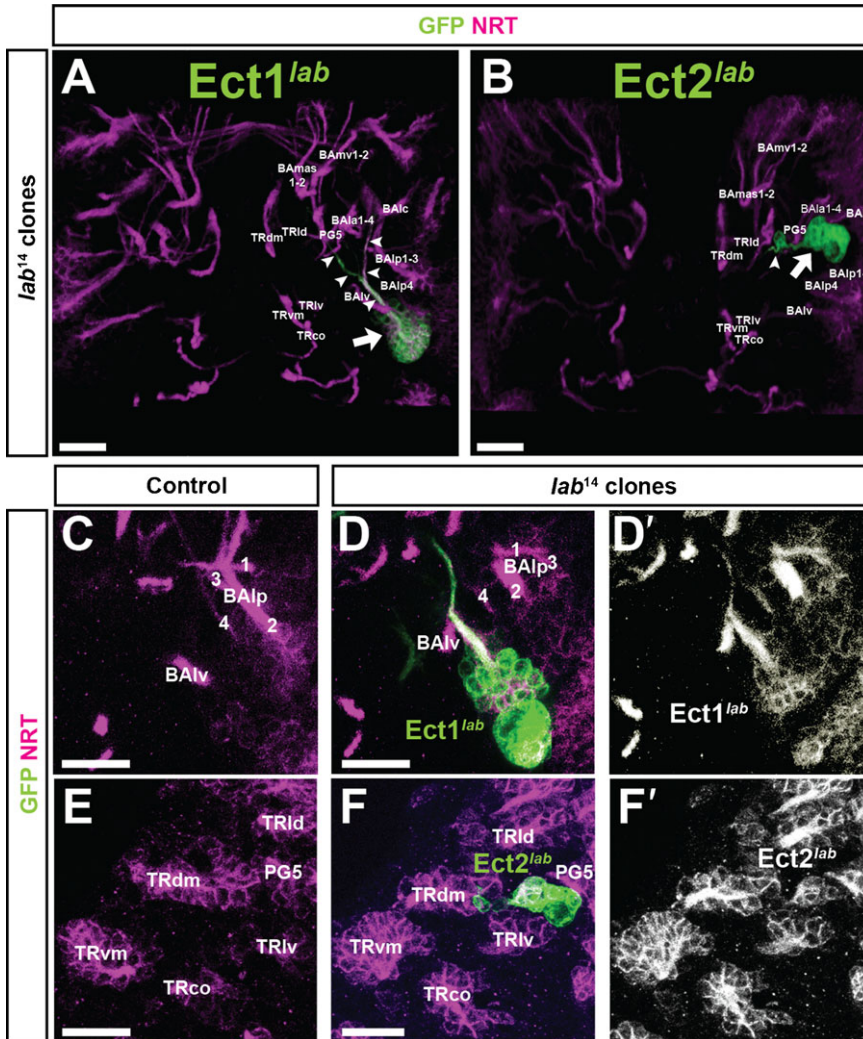


Fig. 4. Clonal loss-of-function of *labial* leads to the formation of ectopic neuroblast lineages. Late L3 brains; GFP-labeled mutant *lab*¹⁴ MARCM clones are in green; secondary axon tracts labeled by anti-Neurotactin are in magenta. (A,B) Two identified ectopic neuroblast clones, *Ect1*^{lab} and *Ect2*^{lab} (arrows), are recovered in *labial* clonal loss-of-function experiments. Arrowheads indicate secondary axon tracts of *Ect1*^{lab} and *Ect2*^{lab}. Figures are superposition of multiple optical sections. (C–F') Both of these ectopic lineages can be identified by the projection patterns of their ectopic secondary axon tracts relative to the surrounding wildtype secondary axon tract scaffold. (C,E) Control showing corresponding wildtype axon tracts in two different optical sections. (D,D') *Ect1*^{lab} is present between the *labial* expressing lineages BAlp4 and BAlv and projects several axon bundles in anterior-medial direction. Single optical section. (F,F') *Ect2*^{lab} is close to the *labial* expressing lineages TRdm and TRld. Single optical section. Scale bars: 20 μ m in A,B; 10 μ m in C–F'.

*lab*¹⁴ clonal MARCM experiments invariably contained a single large Deadpan-positive neuroblast (Fig. 5C–D').

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

The cell-autonomous induction of MARCM-based mutant neuroblast clones is only possible in mitotically active progenitor cells (Lee and Luo, 1999; Lee and Luo, 2001). This implies that additional *labial*-expressing neuroblasts must be present and mitotically active during early larval brain development when the *lab*¹⁴ mutant ectopic clones were induced. To investigate this, we determined the number of *labial*-expressing neuroblasts in the wildtype second larval instar stage (L2) by double immunolabeling with anti-Labial and anti-Deadpan. These experiments revealed the presence of six double-labeled cells indicating that six *labial*-expressing neuroblasts are present at the L2 stage (Fig. 6A–H'). These six neuroblasts were arranged in the L2 brain in a spatial pattern which is comparable to that of the BAlp4, BAlv, TRdm, TRld, *Ect1*^{lab} and *Ect2*^{lab} neuroblasts in the *labial*-mutant late larval brain. Interestingly, an average of six *labial*-expressing neuroblasts were also present in the late embryonic brain implying that the number of

labial-expressing neuroblasts does not change from the late embryonic stage to the second larval instar stage (supplementary material Fig. S2). Given that only four *labial*-expressing neuroblasts (the BAlp4, BAlv, TRdm, and TRld neuroblasts) are present in the wildtype late L3 larval brain, these findings suggests that two of the six neuroblasts present in the L2 larval stage are missing in the L3 stage. In accordance with this assumption, double immunolabeling experiments with anti-Labial and anti-Deadpan at the early L3 stage (immediately after the L2/L3 molt) revealed only four *labial*-expressing neuroblasts, and these were arranged in a spatial pattern corresponding to the BAlp4, BAlv, TRdm, TRld neuroblasts characterized above in late (wandering) L3 larval stages (Fig. 6I–O').

What is the fate of the two *labial*-expressing neuroblasts that are present in L2 but are no longer observed in L3 wildtype larval brains? While it is conceivable that these two neuroblasts are still present in L3 but have terminated their proliferative activity and at the same time ceased to express *labial*, a simpler explanation is that they are eliminated by programmed cell death at late larval stages. To investigate this possibility, we performed a MARCM clonal analysis of neuroblast lineages in the general region of the developing tritocerebral region using H99, a deficiency removing

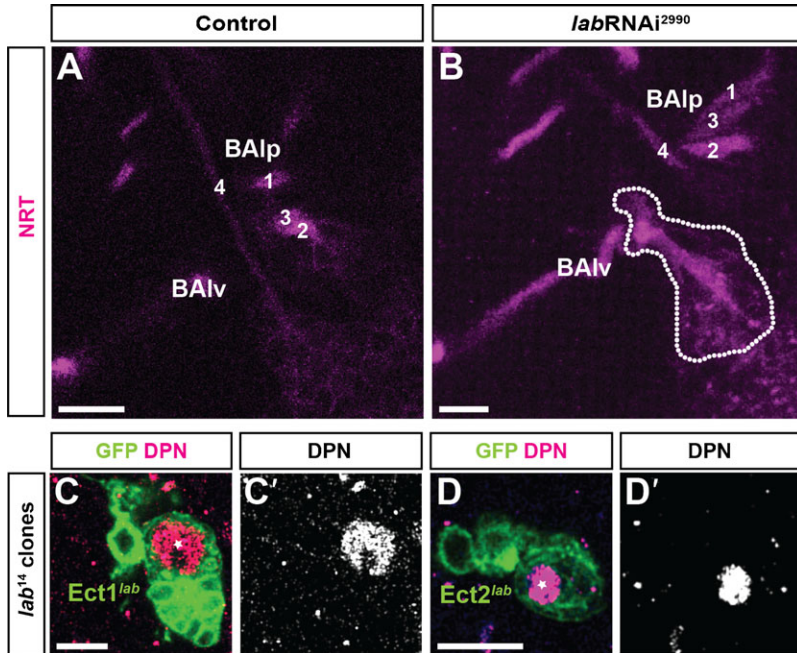


Fig. 5. Targeted RNAi knockdown of *labial* leads to ectopic neuroblast lineages comparable to those induced by *labial* loss-of-function mutation. Late L3 brains. (A) Wildtype control showing the secondary axon tracts of the BA1p1–4 and BA1v lineages. Anti-NRT immunolabeling, Z-projection of optical sections. (B) UAS-*labRNAi²⁹⁹⁰* driven by MZ¹⁴⁰⁷-Gal4 to knockdown *labial* results in ectopic lineages. Dotted lines indicate position of Ect1^{lab} ectopic lineage relative to the secondary axon tracts of the BA1p1–4 and the BA1v lineages. Anti-NRT immunolabeling, Z-projections of optical sections. (C,D) Ectopic lineages contain a single Deadpan-positive neuroblast. GFP labeled *lab¹⁴* MARCM mutant clones of Ect1^{lab} and Ect2^{lab} (green) immunostained with anti-Deadpan (magenta). Single optical sections. Stars indicate ectopic neuroblasts. Scale bars: 10 μ m in A–D'.

the proapoptotic genes *reaper*, *grim* and *head involution defective*, in an otherwise wildtype background (White et al., 1994). Homozygous H99 mutant clones were induced at 24 hours ALH and recovered in late L3 larval brains. In these experiments, a number of supernumerary ectopic lineages were observed in the corresponding region linking the central brain and the SOG. Among these, we consistently recovered two ectopic lineages that were comparable in terms of location and secondary axon tract projection pattern to the Ect1^{lab} and Ect2^{lab} lineages recovered in the clonal *lab¹⁴* mutant assays. We refer to these lineages as Ectopic1^{H99} (Ect1^{H99}) and Ectopic2^{H99} (Ect2^{H99}). Ect1^{H99} was located between the BA1p4 and the BA1v lineage, had several secondary axon tracts of which one always projected in a straight medial direction and manifested an average cell number of 80 cells (s.d.=11, n=9) of which an average of 70 (s.d.=12, n=9) were Labial-positive (Fig. 7A,C–D'). Ect2^{H99} was located posterior-laterally to the TRdm and TRld lineage, extended several secondary axon tracts that projected medially and had an average cell number of 62 cells (s.d.=8, n=12) of which an average of 31 were Labial-positive (Fig. 7B,E–F'). Importantly, both ectopic lineages, Ect1^{H99} and Ect2^{H99}, also consistently expressed *labial* in their neuroblast of origin (Fig. 7G–H'). Comparable results were obtained by targeted apoptosis block in experiments in which MZ¹⁴⁰⁷-Gal4 was used to drive UAS-p35^{BH2} in larval brain neuroblasts; ectopic *labial*-expressing neuroblast lineages that strongly resemble Ect1^{H99} and Ect2^{H99} in terms of location and secondary axon tract projection pattern were observed (data not shown).

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

terminate their proliferation through programmed cell death during late larval development.

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

Previous studies of Hox gene action in ventral nerve cord development have shown that the Hox genes *Antp*, *Ubx* and *abd-A* are able to trigger programmed cell death in neuroblasts in which they are not normally expressed (Bello et al., 2003). To determine if the Hox gene *labial* is also able to induce programmed cell death in central brain neuroblast lineages other than Ect1 and Ect2, we performed a clonal MARCM misexpression assay of *labial*. GFP-labeled *labial* mutant clones were induced at embryonic stage 12–15, recovered in late L3 larval brains, and were co-labeled with the neuroblast marker anti-Deadpan. No effect of *labial* misexpression on neuroblast survival was observed in the following lineages of the central brain: TRlv (ventrolateral tritocerebral lineage) (n=11), BA1p2 (n=7), BA1p3 (n=4), BAMv1 (n=9), BAMv2 (n=3), BAmas1 (n=3), BAmas2 (n=2), PG5 (n=7). Similarly, no effect of *labial* misexpression on neuroblast survival was seen in the *labial* expressing lineages BA1p4 (n=9), BA1v (n=8), TRld (n=8). Thus, the ability of *labial* to terminate neuroblast survival is likely to be restricted to the two neuroblast lineages Ect1 and Ect2.

In the TRvm and PG5 lineages, *labial* misexpression did result in aberrant secondary axon tract projection patterns. The TRvm lineage normally projects posteriorly and its secondary axon tract terminates close to where the TRco lineage SAT forms a commissure. In the *labial* misexpression assay, the secondary axon tract of the TRvm lineage projects posteriorly but then turns laterally to terminate close to the secondary axon tract of the BA1v lineage (supplementary material Fig. S3A,B). The PG5 lineage is located medial to the BA1a1–4 lineages and its secondary axon tract normally projects medially to terminate in between the bifurcating secondary axon tract of the TRld lineage. (The PG5 lineage has not been included in previous mapping

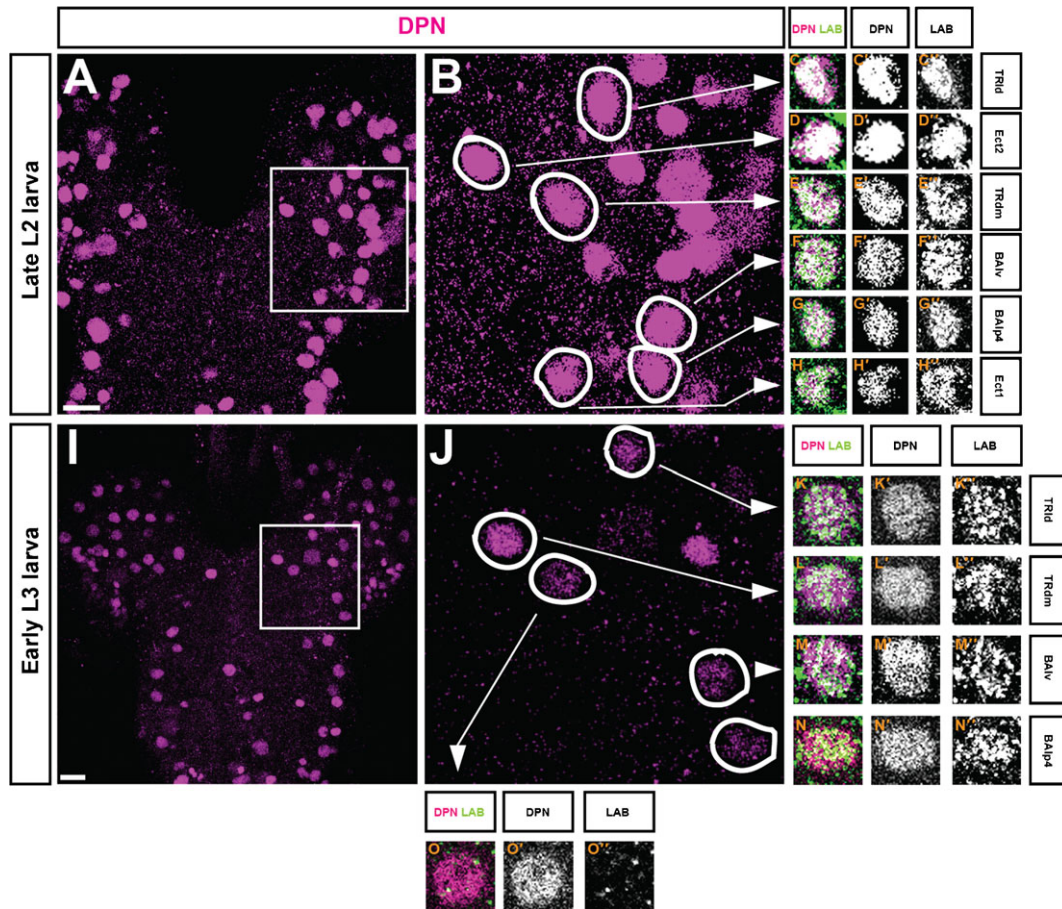


Fig. 6. Six *labial* expressing neuroblasts are present at the L2 larval stage. (A) Overview of anti-Deadpan immunolabeled cells in the late L2 larval brain. Labeled cells are shown in a Z-projection of multiple optical sections. (B) Magnified view of the region in the box of A. Neuroblasts co-immunolabeled with anti-Deadpan and anti-Labial are indicated by circles. (C–H) Single optical sections of each of the six anti-Deadpan immunolabeled neuroblasts that express *labial* at the late L2 stage. Magnification of the circled cells shown in B. Based on their relative position and appearance, each of these neuroblasts can be assigned to the BALp4, BALv, TRdm, TRld, Ect1^{lab}, and Ect2^{lab} lineages. Deadpan immunolabeling is in magenta. Labial immunolabeling is in green. (I) Overview of anti-Deadpan immunolabeled cells in the early L3 larval brain. (J) Magnified view of the region in the box of I. Neuroblasts co-immunolabeled with anti-Deadpan and anti-Labial are indicated by circles. (K–O) Single optical sections of each of the four anti-Deadpan-immunolabeled neuroblasts that express *labial* at the early L3 stage. Magnification of the circled cells shown in J. Based on their relative position, each of these neuroblasts can be assigned to the BALp4, BALv, TRdm, and TRld lineages. Deadpan immunolabeling is in magenta. Labial immunolabeling is in green. Scale bars: 20 μ m in A,I.

studies (Pereanu and Hartenstein, 2006; Spindler and Hartenstein, 2010).) In the *labial* misexpression assay, the PG5 lineage secondary axon tract projects medially, but then turns posteriorly to terminate close to the secondary axon tract of the BALv lineage (supplementary material Fig. S3C,D). Thus, while *labial* misexpression does not appear to affect neuroblast survival, it can result in aberrant secondary axon tract projection patterns in central brain lineages during postembryonic development.

Discussion

Our findings on the role of *labial* in postembryonic brain development are in accordance with a model in which *labial* is cell autonomously required for the stage-specific programmed cell death of two of the six postembryonic neuroblasts that express *labial* during larval stages (Fig. 8). This model is supported by expression studies which indicate that six *labial*-expressing neuroblasts are present in the developing brain at the late embryonic stage (stage 17) and at the end of the second larval stage (L2), while only four *labial*-expressing neuroblasts continue to be present during the third larval stage (L3).

Moreover, the model is supported by functional studies which indicate that this reduction in *labial*-expressing neuroblast number is due to Labial-dependent programmed cell death of two neuroblasts during postembryonic brain development, since clonal *labial* loss-of-function leads to the recovery of two ectopic neuroblast lineages and these two ectopic neuroblast lineages are also recovered (and express *labial*) following clonal cell death block.

Given that six Labial-positive neuroblasts are present at late L2 stages, and only four Labial-positive neuroblasts are present at early L3 stages, we posit that the *labial*-dependent apoptosis of the two affected neuroblasts (Ect1, Ect2) is associated with the L2/L3 transition. Since that the L2/L3 transition involves molting that is associated with elevated levels of steroid hormones such as ecdysone, it is possible that ecdysis-triggering endocrine signals participate in the *labial*-dependent apoptotic event in Ect1 and Ect2 (reviewed by Truman, 2005). Alternatively, transiently expressed temporal transcription factors might regulate the competence of the affected neuroblasts to undergo apoptosis in a *labial*-dependent manner (Maurange et al., 2008; Chell and

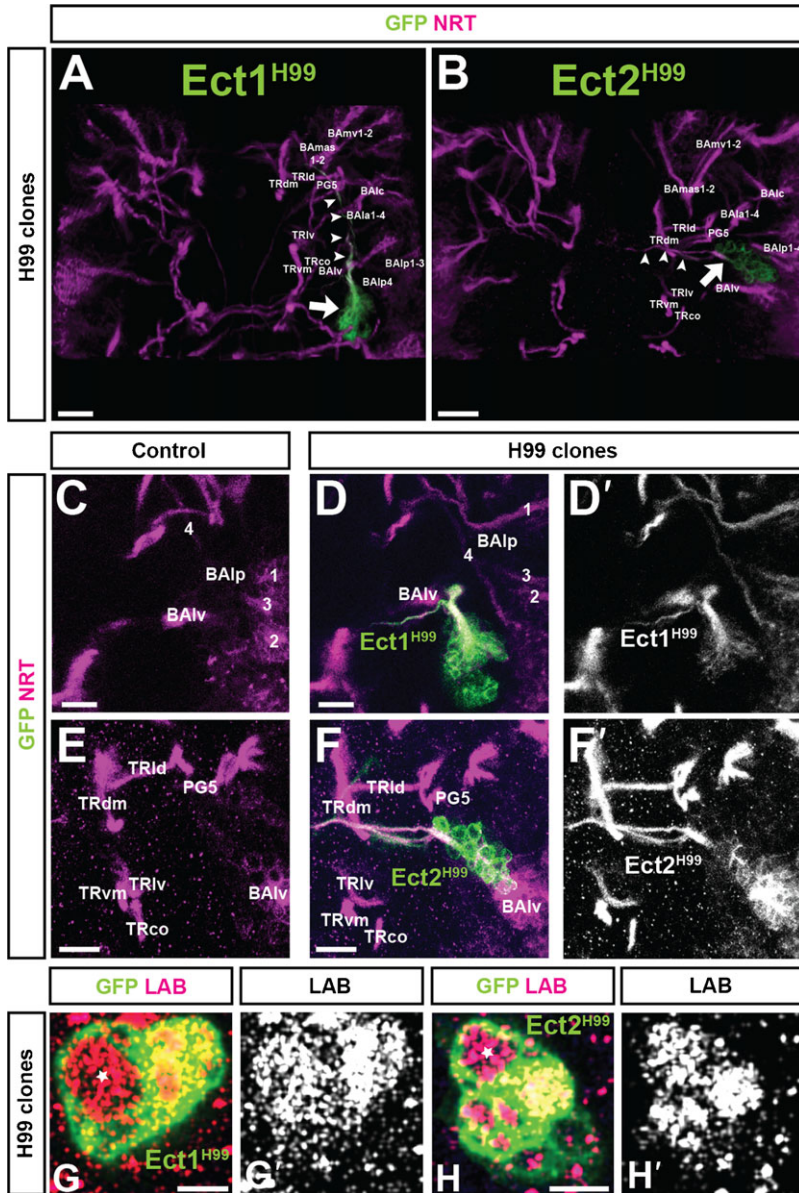


Fig. 7. Blocking of cell death leads to *labial* expressing ectopic neuroblast lineages. GFP-labeled H99 MARCM mutant clones are green; secondary axon tracts labeled by anti-Neurotactin are in magenta. (A,B) Two identified ectopic neuroblast clones, *Ect1^{H99}* and *Ect2^{H99}* (arrows) are recovered after cell death block; both are similar in terms of position and secondary axon projection pattern (arrowheads) to *Ect1^{lab}* and *Ect2^{lab}* found in the *labial* mutant assay. Superposition of multiple optical sections. (C,E) Control showing corresponding wildtype axon tracts in two different optical sections. (D,D',F,F') Following clonal cell death block, both ectopic lineages can be identified by the projection patterns of their ectopic secondary axon tracts relative to the surrounding wildtype secondary axon tract scaffold. Single optical sections. (G,H) The ectopic *Ect1^{H99}* and *Ect2^{H99}* lineages express *labial* in the neuroblast (star). Labial immunolabeling is in magenta. Scale bars: 20 μm in A,B; 10 μm in C–F'; 5 μm in G–H'.

Brand, 2008). While the molecular nature of these signals is currently not known, they are apparently not sufficient to elicit programmed cell death in all *labial*-expressing neuroblasts, since the *labial*-expressing BAAlp4, BAAlv, TRdm, and TRld neuroblasts are not affected.

The neuroblast-specific requirement of the Hox gene *labial* in programmed cell death during postembryonic brain development reported here is novel and differs in several respects from the type of Hox-gene dependent programmed cell death that occurs in the abdominal ganglia during postembryonic development of the ventral nerve cord (Bello et al., 2003). In the larval abdominal ganglia, the Hox gene *abd-A* is expressed in a short pulse during the mid-L3 stage and results in the cell autonomous programmed cell death of all neuroblasts that express the *abd-A* pulse. In contrast, in the larval brain, the Hox gene *labial* is expressed during early larval development in six larval neuroblasts and this only results in the cell autonomous programmed cell death in two

of these neuroblasts around the L2/L3 transition. Moreover, in contrast to the general apoptotic effect of clonal misexpression of *abd-A* (as well as *Antp* or *Ubx*) in larval neuroblasts of the ventral nerve cord as reported by Bello and coworkers (Bello et al., 2003), our studies indicate that the clonal misexpression of *labial* in larval neuroblasts of the brain does not result in apoptosis. Misexpression of *labial* does, however, result in axonal projection defects in central brain lineages. Interestingly, genetic misexpression of vertebrate Hox genes, including misexpression of the *labial* ortholog *Hoxb1*, has been shown to result in axonal projection defects of developing motoneurons (reviewed by Butler and Tear, 2007; Guthrie, 2007).

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

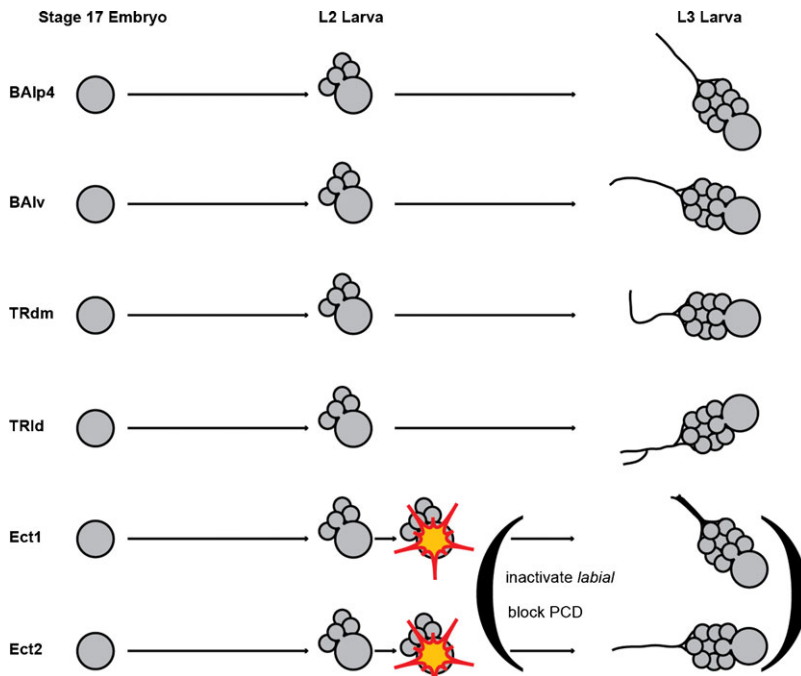


Fig. 8. Model for *labial*-dependent termination of specific postembryonic neuroblasts. In this model, *labial* is cell autonomously required for the stage-specific programmed cell death of two of the six postembryonic neuroblasts that express *labial* during larval stages. The neuroblasts of the BAlp4, BAlv, TRdm and TRld lineages express *labial* and persist to late L3 larval stages. The neuroblasts of the Ect1 and Ect2 lineage express *labial* during early larval stages, and *labial* is required for their termination through apoptosis at the L2 to L3 transition. If *labial* is inactivated or cell death is blocked, these two neuroblasts give rise to ectopic lineages which persist to late L3 larval stages.

tritocerebral neuroblasts and their neural progeny, and functional inactivation of *labial* does not terminate neuroblast proliferation since postmitotic cells are generated in the mutant domain. However, the generated cells do not express neuronal markers and do not manifest neuronal morphology implying that *labial* is required to establish regional neuronal identity in the embryonic tritocerebrum. Interestingly, there is a marked decrease in the number of neuroblasts that express *labial* during embryonic development. At embryonic stage 11, *labial* is expressed in fifteen neuroblasts of which thirteen are of tritocerebral and two of deutocerebral origin (Urbach and Technau, 2003). In contrast, at the end of embryogenesis (stage 17) only six *labial*-expressing neuroblasts were present in the developing brain. The fate of the remaining nine embryonic neuroblasts is not known. They may simply cease to express *labial* and remain present or they may terminate proliferation via cell cycle exit or apoptosis as it has been reported for neuroblasts in the embryonic ventral nerve cord (Abrams et al., 1993; White et al., 1994; Peterson et al., 2002).

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

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

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

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Competing Interests

The authors have no competing interests to declare.

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