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Bithorax-complex genes sculpt the pattern of leucokinergic neurons in the *Drosophila* central nervous system

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SUMMARY

Although the Hox genes are the main factors involved in the generation of diversity along the anterior/posterior body axis of segmented organisms, it is still largely unknown how these genes act in single cells to determine specific traits at precise developmental stages. The aim of this study was to understand the mechanisms by which Hox genes of the Bithorax complex (Bx-C) of *Drosophila* act to define segmental differences in the ventral nerve cord of the central nervous system. To achieve this, we have focused on the specification of the leucokinin-expressing neurons. We find that these neurons are specified from the same progenitor neuroblast at two different developmental stages: embryonic and larval neurogenesis. We show that genes of the Bx-C acted in postmitotic cells to specify the segment-specific appearance of leucokinergic cells in the larval and adult ventral nerve cord.

KEY WORDS: *Drosophila*, CNS, Hox genes, Leucokinin

INTRODUCTION

One of the main aims of developmental neuroscience is to understand the mechanisms underlying the spatiotemporal specification of distinct classes of neurons. These are the responsibility of pattern genes and, in particular, of the Hox genes, which play a pivotal role in the regional differentiation of most organisms.

First identified in *Drosophila*, Hox genes are present in most animal phyla from hydra to chordates. In each case they are organized into gene complexes, are expressed in discrete domains along the anterior-posterior body axis and contain homeoboxes. It was originally proposed that Hox genes act to generate a unique identity for each segment of segmented organisms, and that they do so by regulating groups of genes that control general features of cell behavior, such as cell adhesion, migration, proliferation and apoptosis (Garcia-Bellido, 1975). More recently, evidence has emerged that Hox genes not only define regional differences but also act within individual cells to define very specific fates, in some cases by regulating the expression of single genes (Brodu et al., 2002; Kannan et al., 2010; Lohmann and McGinnis, 2002; Rozowski and Akam, 2002).

The expression of Hox genes defines regional differences in both the hindbrain and the spinal cord of vertebrates, and these differences are manifested in the diversity of motoneurons along the anterior-posterior axis (Carpenter, 2002; Dasen and Jessell, 2009). In *Drosophila*, Hox genes are expressed in the neuroectoderm, the presumptive region of the central nervous system (CNS), from which neural stem cells, neuroblasts (NBs), delaminate (Beachy et al., 1985; Celniker et al., 1990; Hirth et al., 1998; Karch et al., 1990; Macias et al., 1990; White and Wilcox, 1985) (Fig. 1A).

The *Drosophila* CNS is subdivided into the brain and the ventral nerve cord (VNC), which are the equivalent of the brain and spinal cord, respectively, of vertebrates. NBs undergo a series of asymmetric divisions, each division generating a set of distinct neurons and glia in a fixed temporal sequence (Hartenstein and Campos-Ortega, 1984). Each NB together with its progeny can be uniquely identified based on its position and the expression of specific cell markers (Doe and Technau, 1993). The identity of each NB is defined by two sets of genes, one expressed in stripes along the dorsal-ventral axis (columnar genes), the other along the anterior-posterior (segment polarity genes) axis (Bhat, 1999; Skeath, 1999). In addition, the lineage of a particular NB differs in different segments as a result of the expression of Hox genes (Karlsson et al., 2010; Prokop and Technau, 1994; Schmid et al., 1999; Technau et al., 2006; Tsuji et al., 2008).

NBs proliferate throughout embryogenesis and mainly generate the neurons that make up the larval nervous system. Then, following a quiescent period, NBs go through a second phase of neurogenesis that spans the larval and pupal stages, and contributes to most of the adult CNS (Hartenstein et al., 1987; Ito and Hotta, 1992; Prokop and Technau, 1991; Truman and Bate, 1988). After NBs delaminate from the neuroectoderm, Hox genes expression declines, but is later reactivated in postmitotic neurons, and their expression is crucial for neuronal fate specification, promoting apoptosis and controlling the proliferative behavior of the NBs (Bello et al., 2003; Berger et al., 2005a; Miguel-Aliaga and Thor, 2004; Prokop et al., 1998; Prokop and Technau, 1994; Rogulja-Ortmann et al., 2007; Rogulja-Ortmann et al., 2008; Schmidt et al., 1997; Tsuji et al., 2008; Udolph et al., 1993; Udolph et al., 2001). Yet the mechanisms by which Hox genes act to generate diversity among the different segments have been addressed in very few cases (Berger et al., 2005b; Karlsson et al., 2010; Suska et al., 2011).

In this work, we approached this issue by examining the pattern of expression of the neuropeptide Leucokinin (Lk), a myotropic neuropeptide found in most invertebrate species (Nässel and Winther, 2010). Lk is thought to be involved in regulating fluid secretion by Malpighian tubules (Hayes et al., 1989), as well as meal

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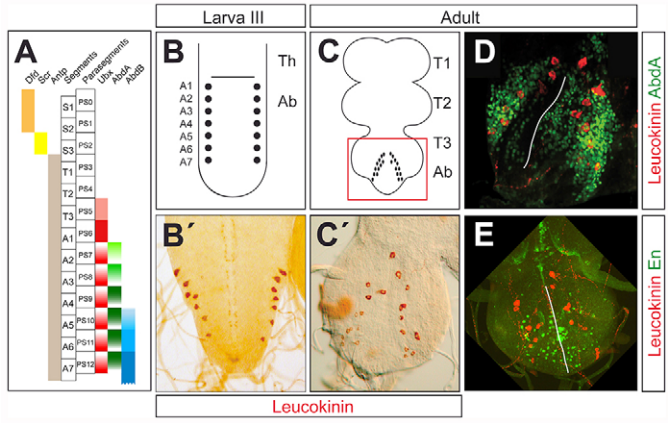

Fig. 1. Pattern of ABLKs in the larval and adult CNS. (**A**) Schematic representation of the pattern of expression of Dfd (ochre), Scr (yellow), Antp (brown), Ubx (red), AbdA (green) and AbdB (blue) in the developing CNS. The various segments and parasegments are indicated (anterior is upwards). (**B**,**C**) The pattern of ABLKs in the larval (B) and adult (C) CNS. Black circles represent ABLKs. Th, thorax; Ab, abdomen; S1-3, subesophagic segments; T1-3, thoracic segments; A1-7, abdominal segments. (**B′**,**C′**) Expression of Lk, detected using anti-Lk specific antibody, in the VNC of a third instar larva (B′) and in the abdominal ganglion of an adult CNS (C′). (C) The whole adult ventral ganglion. The red square indicates the area shown in C′. (**D**,**E**) Expression of Lk (red) and AbdA (green, D) or En (green, E) in an abdominal ganglion of an adult fly. White lines indicate the midline.

size and gustatory responses in adult flies (Al-Anzi et al., 2010; López-Arias et al., 2011).

In the VNC of *Drosophila* larvae, Lk is expressed in seven pairs of large neurosecretory cells in abdominal segments A1-7. These are named abdominal leucokinergic neurons (ABLKs; Fig. 1B-B′) (Cantera and Nässel, 1992; de Haro et al., 2010; Santos et al., 2007). Later during pupal development, the number of ABLKs increases until there are 11 per hemiganglion (right and left half of the ganglion; Fig. 1C-C′). Contrary to the situation in the larval ABLKs, the number of which is fixed, the number of ABLKs in the adult is variable. We have shown that NB5-5 gives rise to the larval ABLKs (Benito-Sipos et al., 2010), but the origin and segmental localization of the adult ABLKs is not known.

In this present work, we first identified the progenitor NB of adult ABLKs, and then analyzed how Hox genes define the segmental localization of ABLKs throughout development. We found that ABLKs are generated from the same progenitor NB at two different developmental stages, and that the Hox genes of the Bithorax Complex (Bx-C): *Ultrabithorax* (*Ubx*), *abdominal-A* (*abdA*) and *Abdominal-B* (*AbdB*) control the pattern of ABLKs temporally and spatially.

MATERIALS AND METHODS

Fly strains

The fly stocks used were as follows:

Act5C >stop >β-galactosidase, Ubx^{MX6}, $Ubx^{6.28}$, $abdA^{M1}$, $AbdB^{M1}$, $AbdB^{M5}$, *Df(3R)Ubx*^{109}, $Antp^{14}$, $Antp^{25}$, hth^{5E04}, *Df(3R)Exel6158* (referred to as hth^{6158}), Dfd^{10} and Scr^{17}.

Gal4/Gal80 lines: $elav^{C155}$-*Gal4*, *wor-Gal4*, wg^{MD758}-*Gal4*, nab^{NP1316}-*Gal4*, *ems-Gal4* and *tub-Gal80*^{ts} (Zeidler et al., 2004).

UAS lines: *UAS-GFP*, *UAS-p35*, *UAS-flp*, *UAS-Ubx*^{1A1}, *UAS-abdA*$^{20-10-1}$, *UAS-AbdB*^{m2SG19}, *UAS-Antp*, *UAS-Dfd*^{W4}, *UAS-Scr*^{EE2}, *UAS-dicer2*, *UAS-dsRNA-Ubx* (Monier et al., 2005), *UAS-abdA-RNAi*^{51900}, *UAS-AbdB-RNAi*^{12024} (VDRC) and *UAS-hth-RNAi* (long form).

lacZ lines: *hkb*^{5953}-*lacZ* and *gsb*^{01155}-*lacZ*.

Canton S was used as wild type. Embryos were staged according to Campos-Ortega and Hartenstein (Campos-Ortega and Hartenstein, 1985)

Lineage tracing and knock-down experiments

Experiment 1

Embryos of genotype *wor-Gal4*/*Act5C >stop >β-galactosidase*; *UAS-flp*/*tub-Gal80*^{ts} were collected over 2 hours at 17°C, allowed to grow to early second instar larvae and then shifted to 29°C (Fig. 2A). They were dissected and stained either as late third instar larvae or as adults.

The rationale for this procedure was as follows. *worniu* (*wor*)-*Gal4* drives the expression of *UAS-flp* in all NBs. Flipase (Flp) recombinase mediates recombination between the two FRTs (>>). After recombination, the *Act5C* promoter drives constitutive expression of β-galactosidase and its expression is maintained by lineage (Struhl and Basler, 1993). The result is that all NBs and their progeny are labeled by β-galactosidase. Conversely, Gal80 inactivates Gal4 and thus prevents FRT recombination. *tub-Gal80*^{ts} is constitutively expressed, active at 17°C and inactive at 29°C. Thus, shifting second instar larvae from 17°C to 29°C activates *β-galactosidase* expression in all NBs and their progeny from this developmental stage, while neurons generated at earlier stages remain unlabeled.

Experiment 2

Embryos of the genotype *wg-Gal4*/*Act5C >stop >β-galactosidase*; *UAS-flp*/*tub-Gal80*^{ts} were collected over 2 hours at 29°C, allowed to grow for 7 hours, shifted to 25°C, and dissected and stained as second instar larvae. In this experiment, only NBs from row 5 are labeled. Given the low efficiency of the Flipase recombinase in inducing recombination of the cassette, very few clones per ganglion are labeled.

Experiment 3

To knock down *Ubx* and *abdA* in the larval CNS, $elav^{C155}$-*Gal4*; *tub-Gal80*^{ts}/*TM3, Act>GFP* females were crossed with either *UAS-abdA-RNAi; UAS-dsUbx Df(3R)Ubx*^{109}/*TM6B* (a gift from D. Garaulet, CBM, Madrid, Spain), *UAS-abdA-RNAi* or *UAS-dsUbx Df(3R)Ubx*^{109}/*TM6B* males. Embryos from the cross were collected over 24 hours, kept for a further 20

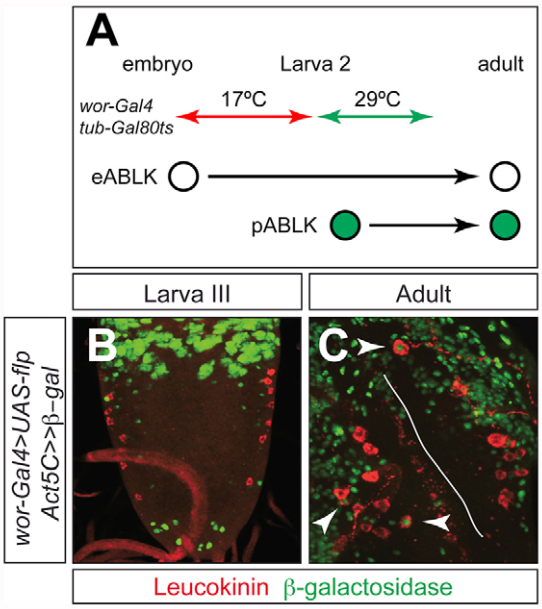

Fig. 2. Adult ABLKs originate during larval neurogenesis. (**A**) The cell lineage experiment: *wor-Gal4 >UAS-flp Act5C >stop >β-galactosidase tub-Gal80*^{ts} temperature shifted from 17°C to 29°C in the early second instar larva; only neurons generated during larval neurogenesis will express β-galactosidase. (**B**,**C**) Result of the experiment depicted in A. Expression of Lk (red) and β-galactosidase (green) in third instar larva (B) and adult (C) VNC. White arrowheads indicate ABLKs that express β-galactosidase; the white line indicates the midline.

hours at 17°C, then shifted to 29°C as first instar larvae, and dissected and stained as mature third instar larvae or adults. Heterozygosis for *Df(3R)Ubx*^{109}, which removes *Ubx* and *abdA*, increases the efficiency of the knock down. We tested RNAi efficiency by labeling AbdA and Ubx. Although AbdA was completely lost, we detected a low level of Ubx, even in *Df(3R)Ubx*^{109}/+; hence, the result of this experiment has to be interpreted with caution. In fact, we expected *Ubx* knock down to remove Lk from segment A1, but did not observe any phenotype, probably owing to the high level of *Ubx* expression in this segment.

Immunohistochemistry

Primary antibodies used were: rat anti-Lk (1:1000; this work), rabbit anti-Lk (1:50; a gift from D. Nässel, Stockholm University, Sweden), rabbit anti-Dpn (1:40) (Bier et al., 1992), mouse anti-Ubx (1:20; D.S.H.B.#FP3.38), rat anti-AbdA (1:500), rabbit anti-AbdA (1:500; a gift from M. Capovilla, Agrobiotech Institute, Sophia Antipolis, France), guinea pig anti-Hth (1:300; a gift from N. Azpiazu, CBM, Madrid, Spain), guinea pig anti-Ems (1:100; a gift from U. Walldorf, University of Saarland, Homburg/Saar, Germany), mouse anti-β-galactosidase (1:2000; Promega), rabbit anti-GFP (1:200; Invitrogen), mouse anti-AbdB (1:50; D.S.H.B.#1A2E9) and mouse anti-En (1:50; D.S.H.B.#4D9). Immunostaining was performed as previously described (Benito-Sipos et al., 2010).

Antibody production

To generate anti-Lk antibody, two rats were immunized with the peptide NSVVLGKKQRFHSWGC, corresponding to the sequence of the mature peptide. The terminal Cys residue was added to couple the peptide to keyhole limpet hemocyanin carrier protein. After five immunizations, the rats were bled and the resulting sera were tested for Lk-specific staining of the larval CNS.

Generation of *ems-Gal4*

A 7.2 kb genomic region upstream of *ems* and corresponding to position 9720183-9727447, was amplified by PCR (Primer forward: CCAGACAGAACTCCATACTCCACCC and reverse: GCGCAAAGAAGACGGCCATACTACAC), pGEM cloned, *Not*I digested and inserted into pPTGal4 P element vector. Standard methods were subsequently used for germline transformation. Transformed flies were crossed with *UAS-GFP* and double stained with anti-GFP and anti-Ems specific antibody.

RESULTS

Leucokinin expression in the ventral nerve cord is initiated at two developmental stages

Leucokinergic neurons are characterized by expressing the neuropeptide Lk. In the VNC of first instar larvae, Lk is expressed in 14 cells (ABLKs), one per hemineuromere of abdominal segments A1-7 (Fig. 1B,B′). This number persists during all larval life and starts to increase at the late pupal stage, rising to an average number of 10 per hemiganglion in 4-day-old adults (Fig. 1C,C′; supplementary material Fig. S1). There are no morphological differences between the ABLKs seen in the larva and the new ABLKs in the adult: all have the same axonal projections (de Haro et al., 2010). We tried to identify the segments from which these adult ABLKs arise by co-immunostaining with antibodies that label either segmental units (anti-Engrailed) or sets of abdominal segments (Ubx and AbdA). However, the expression of these genes in the adult CNS does not reproduce the segmental pattern observed in earlier stages (Fig. 1D,E) so it was not possible to identify from which abdominal segment these new ABLKs were generated.

ABLK neurons are generated in the embryo and larva

Before analyzing the role of Hox genes in specifying the pattern of ABLKs, it is important to know whether all of them are generated in embryonic neurogenesis and from the same progenitor NB.

Although each hemisegment in the VNC of the early embryo contains an invariant number of 30 NBs (Campos-Ortega and Hartenstein, 1985), this number is reduced in larvae. In the thorax, each larval hemisegment retains about 23 of the initial NBs, while in the central abdomen only three remain (Truman and Bate, 1988). This dramatic reduction in the number of NBs occurs late in embryogenesis and depends on cell death mediated by the proapoptotic gene *reaper* (Peterson et al., 2002). NBs that do not die at this stage enter a quiescent period and undertake a second round of neurogenesis during larval development (Prokop and Technau, 1991; Truman and Bate, 1988).

ABLKs are generated during embryonic neurogenesis, but Lk expression starts in the first instar larva. This raises the issue of whether the new ABLKs observed in the adult are also generated in the embryo but undergo delayed differentiation at adulthood, or whether they arise during larval neurogenesis. To address this, we performed a lineage-tracing experiment on postembryonic NBs (pNBs) (Experiment 1 in Materials and methods). In this experiment, we labeled the whole lineage of pNBs with β-galactosidase from the early second instar larva onwards (Fig. 2A). In the adults, we detected three or four cells labeled with the tracer and seven cells not labeled per hemiganglion (Fig. 2C). As a control, we looked at the expression of the ABLKs in third instar larvae and none of the 14 embryonic ABLKs (eABLKs) were labeled (Fig. 2B). We conclude that the three or four extra ABLKs are generated during larval neurogenesis. Hereafter, we call these neurons postembryonic ABLKs (pABLKs).

Embryonic and postembryonic ABLKs originate from the same progenitor neuroblast

As eABLKs originate from NB5-5 (Benito-Sipos et al., 2010), we tested whether this was also true for pABLKs. The three abdominal NBs can be easily recognized from their positions in the ganglion as ventromedial (vm), ventrolateral (vl) and dorsolateral (dl) pNBs, respectively (Fig. 3A,B) (Truman and Bate, 1988). The molecular markers expressed by NBs are usually maintained in their progeny. We have observed that *huckebein* (*hkb*)-*lacZ* is expressed in the vlNB and dlNB, *gooseberry* (*gsb*)-*lacZ* in the vmNB and vlNB (Almeida and Bray, 2005), and *empty spiracles* (*ems*) in the dlNB (Fig. 3E). Hence, we examined the expression of these markers in the pABLKs and found that both *hkb-lacZ* and *gsb-lacZ* were expressed, but Ems was not (Fig. 3C-G; Fig. 3I). We validated these results by tracing the lineage of *ems* expression (*ems-Gal4 >UAS-flp Act5C >stop >β-galactosidase*) and obtained the same result (Fig. 3J). These findings strongly suggest that the vlNB is the progenitor of the pABLKs.

Nevertheless, we sought to confirm this conclusion by tracing the lineage of these NBs (*nab-Gal4 >UAS-flp Act5C >stop >β-galactosidase*). However, unlike clones induced in the embryo, in which progeny usually form a cluster (Prokop and Technau, 1991), cells belonging to these NB clones become scattered in the adult ganglia, making it difficult to determine whether they belong to a single clone or are the result of several recombination events. In addition, it is not possible to identify which pNB generates each clone.

We next wondered whether vlNB corresponded to the embryonic NB5-5. To date, the identities of pNBs in relation to eNBs are not known. We therefore traced the lineage of the eNBs with a flip-in cassette and *wg-Gal4* to activate the Flipase recombinase. This driver is expressed only in the six row-5 NBs, among which is NB5-5 (*wg-Gal4 >UAS-flp Act >stop >β-galactosidase*; see Experiment 2 in Materials and methods for details). We reasoned that if the same

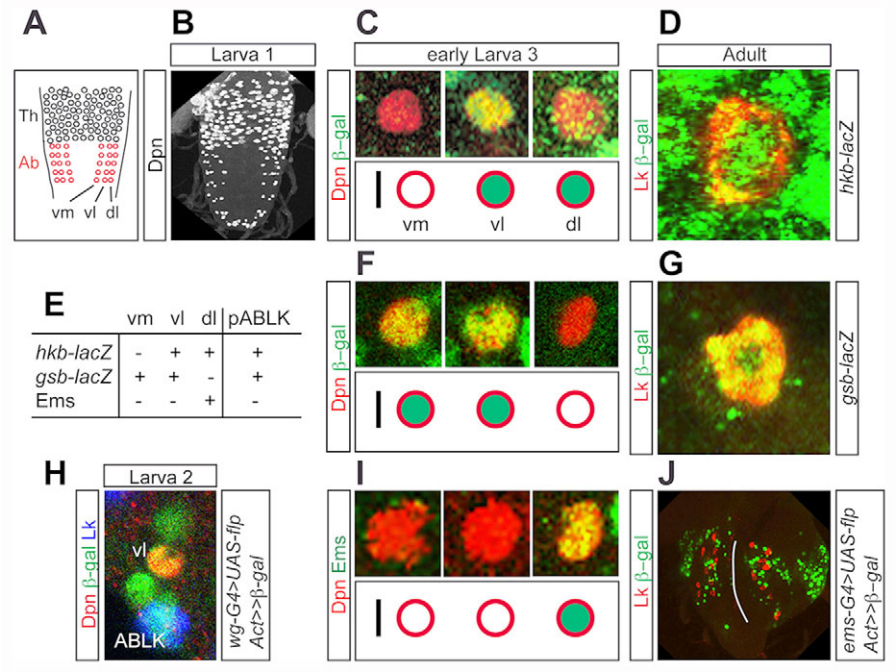

Fig. 3. Embryonic and postembryonic ABLKs share progenitor neuroblasts. (**A**) Schematic representation of neuroblasts (circles) in the larval VNC. Abdominal pNBs in the central abdomen (red): vm, ventromedial; vl, ventrolateral; dl, dorsolateral pNBs. (**B**) Dpn expression in the first instar larval VNC. (**C**,**F**,**I**) Expression of Dpn (red) and β-galactosidase (green) in the vm, vl and dl pNBs of *hkb-lacZ* (C) and *gsb-lacZ* (F), and Dpn (red) and Ems (green) in wild-type (I) early third instar larvae. A schematic representation of the results is shown at the bottom of each image. Vertical bars indicate the midline. (**D**,**G**) Expression of Lk (red) and β-galactosidase (green) in the ABLK of *hkb-lacZ* (D) and *gsb-lacZ* (G) adult ganglion. (**J**) Expression of Lk (red) and β-galactosidase (green) in clones (*ems-Gal4 >UAS-flp Act5C >stop >β-galactosidase*) that label the progeny of the dlNB. The white line indicates the midline. (**E**) Summary of the results shown in C,D,F,G,I,J. pABLKs share molecular markers with the vlNB. (**H**) Expression of Dpn (red), Lk (blue) and β-galactosidase (green) in clones (*wg-Gal4 >UAS-flp Act5C >stop >β-galactosidase*) that label the embryonic progeny of the NB precursor of the vlNB. The ABLK (blue) appears labeled, indicating that the vlNB precursor is the progenitor of eABLKs.

NB generates the eABLKs and pABLKs, clones labeling the lineage of this NB must satisfy three conditions: first, they must include the eABLK, which would indicate that the clone involves NB5-5; second, they must include a pNB, which would indicate that NB5-5 does not die and become quiescent at the end of embryogenesis; and third, the position of the neuroblast in the ganglion must correspond to the vlNB. This is indeed what we found (Fig. 3H). Only clones that included the vlNB also included the eABLK. Thus, we infer that all ABLKs are generated from the same progenitor NB, namely the embryonic NB5-5 and the postembryonic vlNB.

Ubx and AbdA are redundantly required to specify ABLK fate

Once we had established the origin of both the e- and pABLKs, we went on to explore the role of the Hox genes in defining the pattern of ABLKs in embryonic and larval neurogenesis.

Different Hox genes are expressed in the neuroectoderm of different segments (Fig. 1A), but as soon as the NBs delaminate, their expression fades away and recurs later in specific sets of postmitotic neurons in response to the combinatorial code specifying neuronal fates (Karlsson et al., 2010). In the case of NB5-6, the expression of *Antp* in thoracic segments at stage 15 and *Ubx*/*abdA* in abdominal segments at stage 12 leads to cell cycle exit and to NB death by apoptosis (Karlsson et al., 2010). In larval neurogenesis, a pulse of *abdA* precipitates the end of the abdominal pNBs by inducing apoptosis (Bello et al., 2003). Thus, Hox genes play different roles in NBs and postmitotic neurons (Rogulja-Ortmann and Technau, 2008).

As eABLKs are present only in the abdomen, we began by analyzing the roles of the Bx-C genes. We examined the expression of Lk in $Ubx^{6.28}$ and $abdA^{M1}$ mutants, and in the double mutant Ubx^{MX6} $abdA^{M1}$, and found that: in the *Ubx* mutants, ABLKs are absent from the A1 segment; in *abdA* mutants, the pattern of ABLK is unaffected; and in the Ubx^{MX6} $abdA^{M1}$ double mutant no ABLKs are present (Fig. 4A,B,J,K; supplementary material Table S1). Consistent with these results, we found that in first instar larvae, ABLKs express both Ubx and AbdA in segments A2-7 and only Ubx in segment A1 (Fig. 4H,I).

Next, we ectopically expressed *Ubx* with a pan-neural driver (*elav-Gal4 >UAS-Ubx*) and found one ABLK in each one of the thoracic hemisegments. However, ectopic expression of *abdA* (*elav-Gal4 >UAS-abdA*) generated two to four ABLKs in each hemisegment, mainly in segments T1 to A4 (Fig. 4D,J,K). These extra ABLKs were identical to the wild-type ABLKs in morphology and axonal projections (data not shown). We can envisage three different explanations for the origin of these extra ABLKs: first, they could be the sibling cells of the wild-type ABLK that normally die by apoptosis but are rescued in this experiment (Benito-Sipos et al., 2010); second, as AbdA is required for the formation of ABLKs, its ectopic expression could change the specification of some other neuron of the lineage and generate additional ABLKs; third, they could be the pABLKs that, in wild-type development, are generated in larval neurogenesis; when *abdA* is ectopically expressed, their entry into quiescence is prevented and the NB executes the full embryonic and larval program without interruption. To distinguish between these three scenarios, we performed two experiments. First,

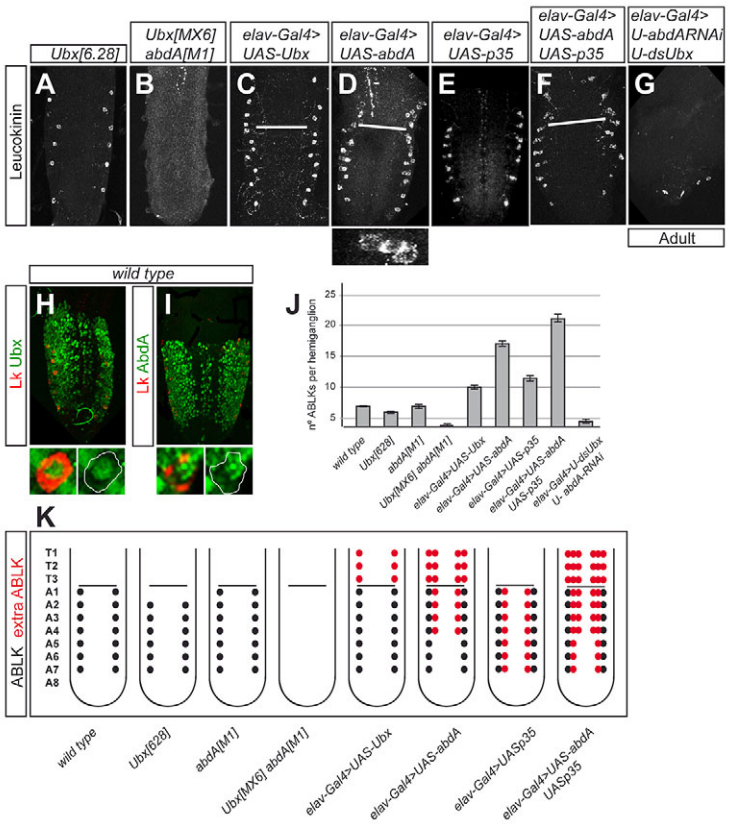

Fig. 4. Ubx and AbdA are required to specify the ABLKs. (**A-F**) Expression of Lk in *Ubx*^{628} (A), *Ubx*^{MX6} *abdA*^{M1} (B), *elav-Gal4 >UAS-Ubx* (C), *elav-Gal4 >UAS-abdA* (D), *elav-Gal4 >UAS-p35* (E) and *elav-Gal4 >UAS-abdA UAS-p35* (F) first instar larval VNCs. Horizontal bars indicate the boundary between the thorax and the abdomen. (D) A higher magnification view of hemisegment A3 is shown at the bottom of the figure. (**G**) Expression of Lk in *elav-Gal4*; *UAS-abdA-RNAi/+*; *UAS-dsUbx Df(3R)Ubx109/tub-Gal80*^{ts} adult CNS. (**H**,**I**) Expression of Lk (red) and Ubx (H) or AbdA (I) (green) in wild-type first instar larvae. A higher magnification view of an ABLK is shown at the bottom of each figure. Both Ubx and AbdA are co-expressed with Lk. (**J**) Histogram showing the number of ABLKs per hemiganglion found in the different genotypes (supplementary material Table S1). (**K**) Cartoons summarizing the most relevant phenotypes observed. Black circles, wild-type ABLKs; red circles, new ABLKs. Horizontal bars indicate the boundary between thorax and abdomen. Thoracic and abdominal segments are numbered on the left.

we ectopically expressed *abdA* and *p35* together. If the first explanation were correct, the phenotype would be the same as misexpressing *abdA* alone, as otherwise sibling cells that do not die would increase the number of ABLKs. In fact, this increased number was seen (Fig. 4F,J,K), thus eliminating the first explanation. Second, we ectopically expressed *abdA* from first instar *elav-Gal4 >UAS-abdA tub-Gal80*^{ts}. We did not note any duplication, suggesting that, although AbdA is required to activate ABLK fate, its expression in postmitotic cells of the larva is not sufficient to generate duplications. Nevertheless both scenarios are possible, i.e. AbdA could be required in postmitotic cells during embryonic development to specify the eABLK, and at the same time pABLKs, which are normally generated in larval neurogenesis and detected in the adult, could be generated in the embryo under these conditions. This would imply that if the entry into quiescence is prevented, the developmental program of NB5-5, which is normally divided into two phases, embryonic and larval, would develop without interruption.

An additional observation that favors this interpretation is that the phenotypes found in *abdA* and *p35* ectopic expression experiments were quite different. In the first, the extra ABLKs in the abdomen were restricted to segments A1-4; in the second, the extra ABLKs appeared in segments A1-7 (Fig. 4D,E,J,K); this suggests a role for *AbdB* in repressing pABLKs in segments A5-7 (see below), as *AbdB* does not participate in the specification of embryonic ABLKs.

Finally, we asked whether *Ubx* and *abdA* were required to maintain the expression of Lk. To achieve this, we knocked down the expression of both *Ubx* and *abdA* from first instar larva and tested the expression of Lk in either late third instar larvae or adults (Experiment 3 in the Materials and methods). We found that most ABLKs were lost in the adults (Fig. 4G,J). We did not observe any phenotype when we knocked down either *Ubx* or *abdA* individually. These observations indicate that Ubx and AbdA are redundantly and required in ABLK cells to sustain the expression of Lk.

To test whether Ubx and AbdA repress the expression of *Antp* in ABLKs, we examined the expression of Lk when *Antp* was mutated (*Antp*^{14}/*Antp*^{25}) or ectopically expressed (*elav-Gal4 >UAS-Antp*), but we did not observe any phenotype.

AbdB represses the expression of Lk

In the *abdA*-misexpression experiment, the extra ABLKs observed were mostly restricted to the anterior-most abdominal segments (A1-4). This points to a possible effect of *AbdB*, as *AbdB* is expressed from the posterior segments A4 to A9 (Celniker et al., 1990; Sánchez-Herrero, 1991) (Fig. 1A).

Indeed, in *AbdB*^{M1} mutants, one extra ABLK appears in each A8 hemisegment (Fig. 5A,J), and upon *AbdB* ectopic expression all ABLKs are lost (Fig. 5B,J). To test whether AbdB induces apoptosis of ABLKs, we misexpressed it together with the baculovirus caspase inhibitor *p35* (*elav-Gal4 >UAS-AbdB UAS-p35*) (Hay et al., 1994), but we obtained the same phenotype of loss of ABLKs (data not shown). These results indicate that *AbdB* represses either the ABLK fate or the expression of Lk.

In the adult an average of four ABLKs are added per hemiganglion. We reasoned that if AbdB repressed Lk in A8 during embryonic neurogenesis, it might also repress Lk in posterior segments (A5-8) in larval neurogenesis. In that case, *abdA* ectopic expression in an *AbdB* mutant background would generate, in addition to one ABLK in A8, extra ABLKs in all abdominal hemisegments, from A1 to A8. This is indeed what we observed (compare Fig. 5C,G,J and Fig. 4D,K)

To further test this hypothesis, we overexpressed *AbdB-RNAi* from first instar larva (*elav-Gal4 >UAS-AbdB-RNAi tub-Gal80*^{ts}

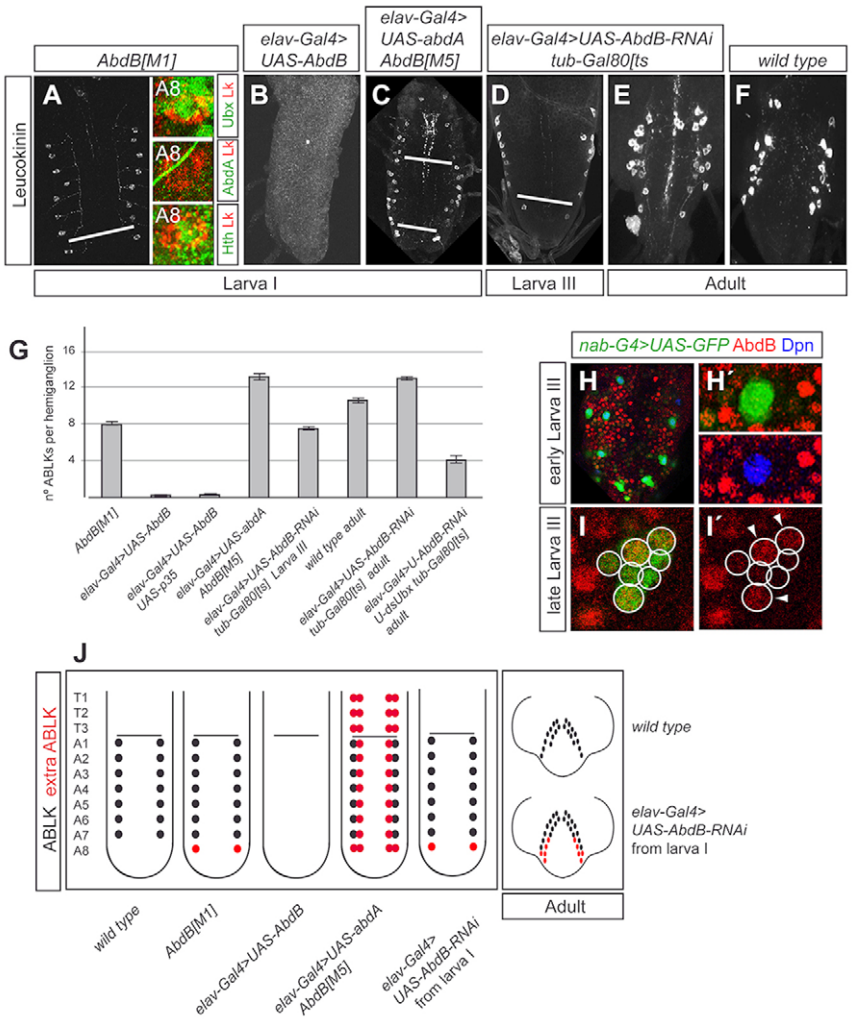

Fig. 5. *AbdB* represses the expression of Lk. (**A-F**) Expression of Lk in *AbdB*^{M1} (A), *elav-Gal4 >UAS-abdA* (B) and *AbdB*^{M5} *elav-Gal4 >UAS-abdA* (C) first instar larvae; *elav-Gal4 >UAS-AbdB-RNAi tub-Gal80*^{ts} third instar larva (D) and adult (E), and wild-type adult (F). White bars in A, C and D separate segments T3/A1 and A7/A8. To the right of A, the three insets show the expression of Ubx, AbdA and Hth in the ABLKs of segment A8. (**G**) Histogram showing the number of ABLKs per hemiganglion found in the different genotypes (see supplementary material Table S1). (**H-I´**) Expression of AbdB (red) and Dpn (blue) in *nab-Gal4 >UAS-GFP* (green) early (H) and mid-late (I) third instar larva. (H´) Higher magnification view of an area of H showing the blue and green channels separately. AbdB is excluded from the NB in the early third instar larva, but some progeny cells (white circles) express it in older larvae (arrowheads). (**J**) Cartoons summarizing the typical phenotypes in the larval and adult CNS.

grown at 17°C and shifted to 29°C at first instar larva) and observed an extra ABLK in A8 in the third instar larva (Fig. 5D,J). This suggests that *AbdB* represses the expression of Lk but not the fate of the ABLK, and, as soon as *AbdB* is removed from the neuron, Lk is de-repressed. Moreover, in adult flies, the number of ABLKs rose to 15 per hemiganglion (Fig. 5E,F), which probably corresponds to two ABLKs per hemisegment in A1-8. The number of ABLKs appears to be very sensitive to the level of expression of *AbdB*, as in *AbdB*^{M1}/+ adults we observed a significant 25% increase in the number of pABLKs (four versus 3.2 in wild-type sibling flies of the same age and grown under the same conditions).

We also observed that in the early third instar *AbdB* was expressed in many cells of the ganglia in segments A5-9, but was excluded from pNBs visualized with *nab-Gal4 >UAS-GFP* (Fig. 5H,H′). However, in late third instar larvae, several cells in the pNB clusters express AbdB (Fig. 5I,I′). We assume that one of them is equivalent to the ABLK of the most anterior segments.

homothorax/Meis1 is not required for ABLK specification

The *homothorax*/*Meis1* (*hth/Meis1*) gene encodes a homeodomain protein required for nuclear localization of Extradenticle/Pbx (Exd/Pbx), a modulator of homeotic protein activity (Abu-Shaar et al., 1999; Azpiazu and Morata, 1998; Kurant et al., 1998; Rieckhof et al., 1997; Ryoo et al., 1999). Mutations in either *hth* or *exd* strongly affect Hox gene function. It has been suggested that Hth binds to DNA together with Hox/Exd heterodimers to form Hth/Hox/Exd trimer complexes (Ryoo et al., 1999). Although *exd* is expressed both maternally and zygotically, *hth* does not appear to be maternally inherited and *hth* loss of function resembles the complete absence of *exd* (Peifer and Wieschaus, 1990; Rauskolb et al., 1993; Rieckhof et al., 1997). Therefore, we analyzed the requirement for Hth in the specification of the ABLKs.

First, we found that Hth always co-expressed with Lk in segments A1-3 and we did not detect expression in ABLKs in segments A5-7 (Fig. 6A). Segment A4 represents an intermediate situation in that we found co-expression only in some ganglia. Interestingly, the expression of Hth in ABLKs did not change in *AbdB* mutants, but the extra ABLK observed in segment A8 expressed Hth (Fig. 5A). It is known that AbdA downregulates *hth* expression (Kurant et al., 1998); thus, this result is not entirely surprising as the ABLK in segment A8 expresses Ubx but not AbdA (Fig. 5A).

To test for a requirement for Hth in ABLK specification, we looked at the expression of Lk in *hth* mutants (*hth*^{5E04}/*hth*^{6158}) and detected a very weak phenotype (Fig. 6B), although in this genetic combination the expression of other neuropeptides such as Nplp1, FMRFamide and CCAP was lost (Karlsson et al., 2010) (M.M.-S., unpublished). But when we ectopically expressed *hth* (*elav-Gal4 >UAS-hth*), we observed ectopic ABLKs in all thoracic segments but not in the more anterior segments of the CNS (Fig. 6C,F). Ubx was expressed in the ABLK in segment T3 but not T1-2, suggesting that in these segments Hth acts either alone or in combination with

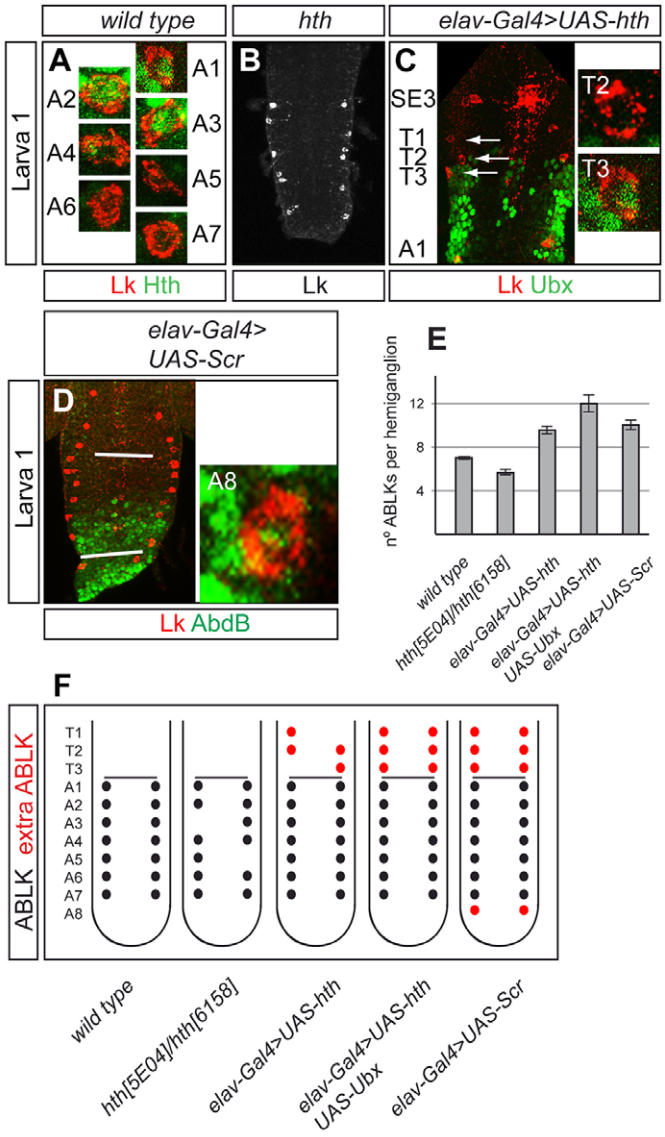

Fig. 6. Hth and Scr in ABLK specification. (**A**) Expression of Lk (red) and Hth (green) in first instar larvae. Hth expression is always found in the ABLKs of segments A1-3 and in a number of cases in segments A4. (**B**) Expression of Lk in hth^{5E04}/hth^{6158}. (**C**) Expression of Lk (red) and Ubx (green) in *elav-Gal4 >UAS-hth*. New ABLKs are found in the three thoracic segments (arrows) but only the ABLK in T3 expresses Ubx. Higher magnification views of ABLKs from thoracic segments T2 and T3 are shown to the right of the figure. (**D**) Expression of Lk (red) and AbdB (green) in *elav-Gal4>UAS-Scr*. A higher magnification view of one ABLK in the A8 segment is shown on the right. Bars indicate separation between segments T3/A1 and A7/A8. (**E**) Histogram showing the number of ABLKs per hemiganglion found in the different genotypes (see supplementary material Table S1). (**F**) The typical phenotypes observed.

Antp, the only Hox gene expressed in segments T1-2 (Hirth et al., 1998).

We next asked whether ectopic expression of *Ubx* or *abdA* together with *hth* was sufficient to activate ABLK fate in abdominal segment A8 and in more anterior segments (i.e. subesophagic segments), but the phenotype of *elav-Gal4 > UAS-hth UAS-Ubx* was similar to misexpressing *Ubx* or *hth* alone (data not shown), although the penetrance was higher and occasionally some ABLKs appear duplicated. Thus, we conclude that ectopic expression of *Ubx/hth* is not sufficient to override the function of AbdB in the posterior-most segments and to activate ABLK fate in cephalic segments.

Finally, we tested whether Hth, like Ubx and AbdA, was required to maintain Lk expression. When we knocked down *hth* during larval development (*elav-Gal4 >UAS-hth-RNAi UAS-dicer*), expression of Lk was not affected, indicating that Hth is not required to maintain Lk expression.

Sex combs reduced can activate LK expression

The Hox genes *Deformed* (*Dfd*) and *Sex combs reduced* (*Scr*) are expressed in parasegments 0/1 and 2, respectively (Fig. 1A). We assessed whether these genes played a role in subesophagic segments similar to the role that AbdB plays in segment A8, namely repressing the expression of Lk. To achieve this, we examined the expression of Lk in *Scr* and *Dfd* mutants, but there was no phenotype. When we ectopically expressed *Dfd* (*elav-Gal4 > UAS-Dfd*), the pattern of ABLKs was not affected, but when we ectopically expressed *Scr* (*elav-Gal4 > UAS-Scr*) we found extra ABLKs in segments T1-3 and A8, although *AbdB* expression in A8 segment was not affected (Fig. 6D,F).

DISCUSSION

In this report, we have analyzed how Hox genes control cell fate in the developing CNS, focusing on the pattern of expression of the neuropeptide Lk in the ABLK neurons of the *Drosophila* abdomen.

Two features characterize this pattern: first, Lk expression is initiated at two developmental stages – first instar larva and late pupae/adult; and second, in first instar larva, Lk is expressed in a single cell per hemineuromere in abdominal segments A1-7, and in the adult a variable number of ABLKs, ranging from 2 to 6, are added per hemiganglion (although it is not possible to identify the segments from which they arise).

Our findings indicate that: first, the ABLKs arising in the late pupa/adult originate in larval neurogenesis (pABLK), although the onset of Lk expression is delayed; second, the progenitor NB of these ABLKs is the vlNB; and third, this vlNB corresponds to the embryonic NB5-5, so that both embryonic and postembryonic ABLKs originate from the same NB. To our knowledge, this is the first reported case in which the same NB specifies a cell fate at different developmental stages.

Temporal factors define the competence to activate specific cell fates during the development of NBs (Maurange, 2012; Pearson and Doe, 2004), and in essence what we show here is that when NB5-5 resumes proliferation after a phase of quiescence, it maintains the same competence window. Unfortunately, cell lineage markers that allow one to follow the complete lineage of this NB are not available, but one would expect that the eABLK and pABLK are, respectively, the last and the first neurons in this lineage generated before and after quiescence, thus retaining the same temporal competence. Our analysis of pABLKs is still under way, but we have not yet found any differences between e- and pABLKs in the expression of molecular markers, genetic requirements or morphological features (P.H., unpublished).

When quiescence is prevented, as will be seen below, the NB develops the full program without interruption, which allows the generation of pABLKs in late embryogenesis. The onset of Lk expression (in the late pupa) is delayed with respect to the developmental stage at which ABLKs are generated: eABLKs are generated before stage 16 of embryogenesis but Lk is expressed in the first instar larva, whereas pABLKs are generated in the early third instar larvae but start to express the neuropeptide during pupal

stages. Such a delay in the terminal differentiation has been observed in other neuropeptidergic neurons, such as the FMRFamide- and the CCAP-expressing neurons (Schneider et al., 1993; Veverytsa and Allan, 2012). We do not know the functional relevance of this temporally tuned neuronal differentiation, but what our results suggest is that, irrespective of the factors required for the onset of Lk expression, these factors have two bursts of expression, in late embryo and late pupa, such that, when pABLKs are generated in the embryo in the *abdA*-misexpression experiment, all express Lk at the same time. Ecdysteroid hormone titers show two peaks, in late embryo and pupa (Richards, 1981), and it has been shown that ecdysone signal induces terminal differentiation of some of the CCAP-expressing neurons in pupa (Veverytsa and Allan, 2012). Our efforts to identify the factors required to drive the terminal differentiation of ABLK neurons have not so far been successful.

Ubx, AbdA and AbdB sculpt the pattern of ABLKs in embryonic neurogenesis

Once we knew the origin of the different ABLKs, we analyzed the mechanisms by which Hox genes restrict their pattern spatially and temporally. Our findings suggest that a complex interplay involving Bx-C genes controls the segment-specific appearance of ABLKs (Fig. 7).

NB5-5 delaminates from Ubx-expressing cells. Ubx is not expressed in the NB but Ubx and AbdA are expressed in segments A1-7 and A2-7, respectively. Both Ubx and AbdA are redundantly required to specify ABLKs. Ectopic expression of both *Ubx* and *abdA* generates ectopic ABLKs in the three thoracic segments. Moreover, in *AbdB* mutants an extra ABLK appears in A8, whereas *AbdB* misexpression removes all ABLKs. Together, these observations suggest that Ubx and AbdA are redundantly required to activate the ABLK fate in segments A2-7, and only Ubx is required in segment A1, whereas AbdB represses ABLK fate in A8 (Fig. 7).

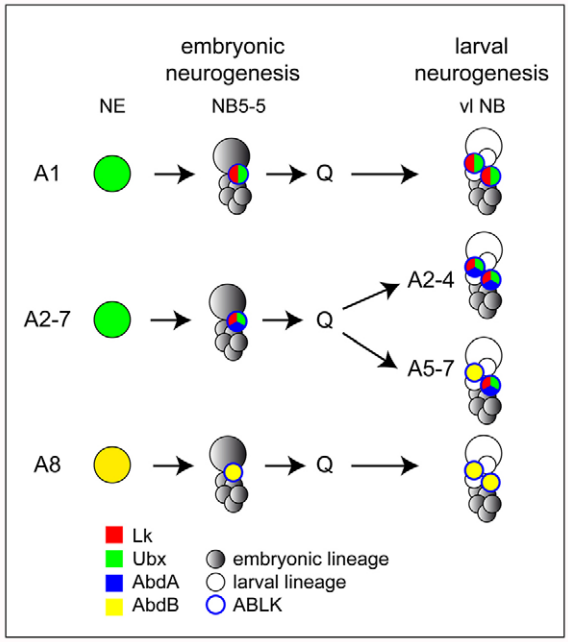

Fig. 7. Model of ABLK specification by Hox genes. The mechanisms by which Hox genes elaborate the pattern of ABLKs. NE, neuroectoderm; A1-8, abdominal segments; Q, quiescence. As there are no lineage markers for NB5-5, the number of cells represented in the cartoon and the position of the ABLK do not correspond to reality. The expression of Ubx (green), AbdA (blue) and AbdB (yellow) is shown only in the ABLK. See main text for details.

We also found that *abdA*-ectopic expression generated extra ABLKs, mostly in abdominal segments A1-4 (Fig. 4D,K), but the same experiment in an *AbdB* mutant generated extra ABLKs in most abdominal segments (A1-8) (Fig. 5C,J). These results suggest a difference between wild-type ABLKs and the new ABLKs observed in these experiments: both require AbdA, but AbdB represses wild-type ones in A8 and new ones in A5-8. We propose that, as a result of *abdA* misexpression in the late embryo, the new ABLKs observed in segments A1-4 are pABLKs. In the wild-type adult, we found an average of four pABLKs per hemiganglion that we could not assign to specific segments. When we removed *AbdB* at the early larval stage, we found as many as 15 ABLKS per hemiganglion in the adult (Fig. 5E,J); we believe that these correspond to two ABLKs per hemisegment A1 to A8. In summary, our results are compatible with a model in which AbdB represses ABLK fate in segment A8 in embryonic neurogenesis and in segments A5-8 during larval neurogenesis; when *abdA* is ectopically expressed, quiescence is prevented and pABLKs, which are generated mainly in segments A1-4 in larval neurogenesis, are generated in embryonic neurogenesis.

Hox genes are involved in the activation and the maintenance of Lk expression

In addition, the fact that removing *AbdB* in larval stages generates one extra ABLK in A8 suggests that AbdB represses Lk expression in this segment, but that the neuron is specified in embryonic neurogenesis and is competent to activate Lk once released from AbdB repression. This result also excludes a possible role for AbdB in driving apoptosis of ABLKs, unlike in other contexts, in which it can act either in a pro- or anti-apoptotic manner (Lohmann et al., 2002; Miguel-Aliaga and Thor, 2004). This reinforces the idea that the functions of Hox genes are highly dependent on cellular context, and that, in addition to specifying global properties, they are also required for a number of decisions taken at individual cellular levels.

We also found that removing Ubx and AbdA in larval development caused the absence of most ABLKs in the adult, indicating that Ubx/AbdA are required to maintain expression of Lk.

The level of expression of AbdB as a reading of the environmental conditions of the larva

eABLKs appear in a fixed number of seven per hemisegment, but pABLKs vary in number from 2 to 6. We have found that changes in the components of larval food can affect the number of pABLKs (P.H., unpublished). Taken together, these results/data suggest that changes in environmental conditions are responsible for the variability in the number of pABLKs. Our observations indicate that the expression of *AbdB* limits the occurrence of pABLKs in the posterior-most abdominal segments, and that different levels of *AbdB* (two, one or no gene copies) are associated with different numbers of pABLKs. This could indicate that the level of expression of *AbdB* could be affected by the environmental conditions of the larva. To our knowledge, evidence of plasticity in development as a result of changes in environmental conditions are few and controversial, e.g. the reported variation in the number of segments found in populations of some species of centipedes (Kettle et al., 2003); more recent reports in *Drosophila* suggest that larval development is regulated by genetic mechanisms that coordinate developmental progression with nutrient uptake (Tennessen and

Thummel, 2011). Further analysis would be necessary to determine whether the same environmental factors that affect the number of pABLKs also affect the expression level of *AbdB* in postembryonic neurogenesis.

Antp and Scr are competent to activate ABLK fate

Our findings regarding Hth indicate that is not required to specify the ABLKs. More unexpected is that *hth*-ectopic expression generated ABLKs in segments in which the only Hox gene expressed is *Antp* (T1-2), indicating that *Antp* is also competent to activate ABLK fate if enough Hth is provided. This result supports many interpretations, such as Hth competes with another co-factor to join Antp and Ubx in thoracic segments T1/2 and T3, respectively. Hth would act as co-activator and the other as co-repressor for ABLK specification. The fact that high level of Hth is also needed in thoracic segments to allow the specification of the Ap cluster neurons in NB5-6 lineage support this interpretation (Karlsson et al., 2010). Although to date, the only identified Hox co-factors are Exd and Hth, the spatial and temporal diversity of functions of the Hox genes suggests that more co-factors may exist (reviewed by Mann et al., 2009).

Interestingly, *Scr*, when ectopically expressed with a pan-neuronal driver, seems to be competent to activate ABLK fate in segments T1-3 and A8. Although there are no ABLKs in parasegment 2, where *Scr* is normally expressed. These results suggest that there are two levels at which ABLK specification is controlled: first, neuroectoderm, where different Hox genes appear to have different functions in lineage specification; and second, neuron, where *Scr*, *Antp*, *Ubx* and *abdA* seem to be able to activate the ABLK fate. Further analysis is required to establish whether the differences in the NB5-5 lineage of different segments are due to changes in lineage progression, as has been shown for NB5-6 lineage (Karlsson et al., 2010), or in the expression of factors required for cell fate specification.

Unlike *Ubx* and *abdA*, *Scr* misexpression generates ectopic ABLKs in A8 without affecting *AbdB* expression. It has been shown that a mechanism of competition for co-factor-dependent DNA-binding explains Hox phenotypic suppression (Noro et al., 2011). But contrary to the previously observed posterior prevalence (González-Reyes and Morata, 1990; Mann and Hogness, 1990), in this case, Scr shows dominance over AbdB. Further study would be required to confirm whether the Lk enhancer is a direct target of the Hox genes.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

Supplementary material available online at
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