Neuroblast pattern and identity in the *Drosophila* tail region and role of *doublesex* in the survival of sex-specific precursors

Oliver Birkholz, Christof Rickert, Christian Berger, Rolf Urbach and Gerhard M. Technau*

**SUMMARY**

The central nervous system is composed of segmental units (neuromeres), the size and complexity of which evolved in correspondence to their functional requirements. In *Drosophila*, neuromeres develop from populations of neural stem cells (neuroblasts) that delaminate from the early embryonic neuroectoderm in a stereotyped spatial and temporal pattern. Pattern units closely resemble the ground state and are rather invariant in thoracic (T1-T3) and anterior abdominal (A1-A7) segments of the embryonic ventral nerve cord. Here, we provide a comprehensive neuroblast map of the terminal abdominal neuromeres A8-A10, which exhibit a progressively derived character. Compared with thoracic and anterior abdominal segments, neuroblast numbers are reduced by 28% in A9 and 66% in A10 and are almost entirely absent in the posterior compartments of these segments. However, all neuroblasts formed exhibit serial homology to their counterparts in more anterior segments and are individually identifiable based on their combinatorial code of marker gene expression, position, delamination time point and the presence of characteristic progeny cells. Furthermore, we traced the embryonic origin and characterised the postembryonic lineages of a set of terminal neuroblasts, which have been previously reported to exhibit sex-specific proliferation behaviour during postembryonic development. We show that the respective sex-specific product of the gene *doublesex* promotes programmed cell death of these neuroblasts in females, and is needed for their survival, but not proliferation, in males. These data establish the terminal neuromeres as a model for further investigations into the mechanisms controlling segment- and sex-specific patterning in the central nervous system.

**KEY WORDS:** CNS development, Segmental patterning, Neuroblasts, Sex-specific precursors and lineages, *Doublesex*, Programmed cell death, *Drosophila*

**INTRODUCTION**

During the initial phase of CNS development in *Drosophila*, neural stem cells (called neuroblasts, NBs) delaminate from the embryonic neuroectoderm in a well-defined spatiotemporal pattern. NBs can be individually identified by their delamination time point, their characteristic subectodermal position and the expression of a unique set of molecular markers (Doe, 1992; Urbach and Technau, 2004). Furthermore, each NB generates an almost invariant and unique cell lineage (Bossing et al., 1996; Schmid et al., 1999; Schmidt et al., 1997). Along the anterior-posterior body axis, characteristic sets of NBs are generated within segmental units to form neuromeres. Detailed maps indicating the number, pattern and molecular markers of these sets of embryonic NBs have been established so far for the thoracic and anterior abdominal segments of the ventral nerve cord (VNC) (Broadus et al., 1995; Doe, 1992) and for the brain (Urbach et al., 2003; Urbach and Technau, 2003a). As opposed to the brain, the thoracic (T1-T3) and anterior abdominal (A1-A7) portion of the VNC is characterised by repetition of a largely invariant segmental set of NBs. Serially homologous NBs delaminating from corresponding neuroectodermal regions of these segments (being specified by the same positional cues) (for reviews, see Bhat, 1999; Skeath, 1999), express corresponding sets of molecular markers and generate similar lineages (for a review, see Technau et al., 2006). However, there are two regions of the embryonic VNC, which clearly exhibit a derived character (compared with the assumed developmental ground state in T2) (Lewis, 1978), and in which the NB patterns have not been analysed in detail so far. These regions comprise the three gnathal and the terminal abdominal neuromeres.

The posterior end of the abdomen (‘tail region’) is a particular developmental unit in the *Drosophila* embryo that has been shown to consist of four segments (A8-A11) and a non-segmented telson (Juergens, 1987). Here, we provide a comprehensive map of the NBs generated by the tail region. Although all NBs (except one) in more anterior neuromeres are also formed in A8, numbers are reduced by 28% in A9 and by 66% in A10. No NBs are found in A11. In both A9 and A10, NBs of the posterior compartment are almost entirely missing. All the identified NBs are serially homologous to NBs in more anterior segments as judged from the combinatorial codes of marker gene expression, delamination time points and positions. Furthermore, several characteristic progeny cells can be identified by molecular markers. The identification and description of these, so far almost disregarded, terminal NBs provides an excellent basis to study the mechanisms that control the modification of segmental CNS units (at the level of individual NBs and their lineages) in adaptation to their functional requirements. Among the region-specific circuits that need to be established in the developing terminal neuromeres are those that control the reproductive organs and process sex-specific sensory input (e.g. Häsemeyer et al., 2009; Monastirioti, 2003; Rezával et al., 2012). A set of four postembryonic NBs (two per side) in the terminal abdominal neuromeres has been shown to exhibit sex-specific proliferation behaviour during larval and early pupal stages (Truman and Bate, 1988). This behaviour depends on the sex determination gene *doublesex* (*dsx*) (Taylor and Truman, 1992), which encodes...
pivotal transcription factors controlling most aspects of male or female differentiation (reviewed by Christiansen et al., 2002). All postembryonic NBs of the VNC emerge from embryonic NBs after a period of mitotic quiescence during late embryonic/early larval stages and re-enter mitosis in the larva to produce adult-specific neurons (Prokop and Technau, 1991; Truman and Bate, 1988). We traced the embryonic origin of the sex-specific terminal postembryonic NBs and characterised their lineages during larval stages. Furthermore, we show that the female isoform of Dsx promotes programmed cell death (PCD) of these NBs, whereas the male isoform is required for their survival, but not for their proliferation.

MATERIALS AND METHODS

Drosophila strains
The following fly strains were used: wild type (Oregon R); CQ2-Gal4 (Landgraf et al., 2003a) and eveR8-Gal4 (Fujieda et al., 2003) (provided by Matthias Landgraf); doublesex-Gal4 (Robinett et al., 2010) (provided by Carmen Robinett and Bruce Baker); gooseberry-distal-lacZ (provided by Marta Moris-Sanz and Fernando Diaz-Benjumea, Universidad Autónoma de Madrid, Spain); ladybird-early [K]-Gal4 (Baumgardt et al., 2009) (provided by Stefan Thor); mFlp5 and UAS-Flybow 1.1 (Hadjieconomou et al., 2011) (provided by Dafni Hadjieconomou and Iris Salecker); Mq79 and Mq360 (eagle-Gal4) (Ito et al., 1995); Pos-neuro-Gal4 (Boll and Noll, 2002) (provided by Markus Noll); UAS-CDB8-GFP, UAS-ngFP, UAS-G-Trace (Evans et al., 2009), UAS-P35 (Hay et al., 1994), UAS-transformer-RNAi, UAS-transformer2-RNAi and UAS-Abdominal-B-RNAi (Ni et al., 2009) (all from Bloomington Stock Center); UAS-doublesex-RNAi (provided by Vienna Drosophila RNAi Center); UAS-doublesex[F] and UAS-doublesex[M] (Lee et al., 2002) (provided by Michelle Arbeitman, Florida State University, USA); unplugged-lacZ (provided by Jonathan Benito-Sipos, Universidad Autónoma de Madrid, Spain); huckebein-lacZ, minge-lacZ, mirror-lacZ, seven-up-lacZ and wingless-lacZ (Broadus et al., 1995; Doe, 1992) (all provided by Chris Doe).

RNAi-experiments were performed at 29°C; all other experiments (except for Flybow analysis) were carried out at 25°C.

Immunohistochemistry

For antibody staining, embryos [staged according to Campos-Ortega and Hartenstein (Campos-Ortega and Hartenstein, 1997)] were dechorionated, fixed and immunostained following previously published protocols (Patel, 1994). Larval CNS dissection and fixation was carried out as described previously (Bello et al., 2007). Wandering larvae (L3) were fixed for 45 minutes; early L3 (L3e) and late L2 (L2l) larvae for 30 minutes. For antibody staining, the larvae were treated in the same way as the embryos. The following primary antibodies were used: mouse anti-Abdominal B (1:20) (Celniker et al., 1989), rabbit anti-Elav (1:2000) (Plickert et al., 1999), mouse anti-Engrailed (1:100) (Santa Cruz Biotechnology); rabbit anti-Castor (1:500) (Kambadur et al., 1998) (provided by Robert Holmgren); guinea pig anti-Hunchback (1:1000) (Mettler et al., 2006) (provided by Joachim Urban); mouse anti-Ladybird early (1:2) (Jagla et al., 1997) (provided by Krzysztof Jagla); rabbit anti-mCherry (1:500) (Bio Vision); rabbit anti-Mira (1:100) (Betschinger et al., 2006) (provided by Juergen Knoblich); rabbit anti-Msh (1:500) (provided by Matthew Scott, Stanford University, USA); rabbit anti-Nazgul (1:400) (von Hilchen et al., 2010) and guinea pig anti-OrthoReversed polarity (1:10,000) (provided by Benjamin Altenhein); guinea pig anti-Orthodenticle (1:500) (Xie et al., 2007) (provided by Tiffany Cook); rabbit anti-RFP (1:500) (MBL); guinea pig anti-Runt (1:500) (Kosman et al., 1998) (provided by John Reinitz); rabbit anti-Vnd (1:2000) (McDonald et al., 1998) (provided by Fernando Jimenez).

For in situ hybridisation, we used a digoxigenin-labelled ind RNA-probe (provided by Matthew Scott). It was synthesised as described previously (Urbach and Technau, 2003b). The hybridisation on embryos was carried out as described before (Plickert et al., 1997; Tautz and Pfeifle, 1989).

As fluorescent secondary antibodies we exclusively used the DyLight (Jackson ImmunoResearch Laboratories) and Alexa (Life Technologies) series. The non-fluorescent secondary antibodies were either biotinylated or alkaline phosphatase-conjugated (Jackson ImmunoResearch Laboratories). All secondary antibodies were used according to manufacturer’s protocols.

The non-fluorescent stainings were documented on a Zeiss Axioscan; the fluorescent confocal images were acquired on a Leica TCS SP2 or SP5 and were processed by Adobe Photoshop CS4 and Adobe Illustrator CS4. 3D-models were generated using Amira 4.0.

Two-tailed t-test was performed for statistical significance (see Fig. 1G; Fig. 8D; Fig. 9).

Flybow analysis

For Flybow analysis (Hadjieconomou et al., 2011), we combined mFlp5 with doublesex-Gal4. This stock was crossed to UAS-Flybow 1.1. After egg collections (for three hours), embryos were kept for six hours at 25°C (stage 11-12). A first heat-shock was applied for two hours in a 37°C water bath. Upon recovery and further development for 13 hours at 25°C (stage 17), the embryos were subjected to a second heat-shock (two hours in a 37°C water bath). Hatching larvae were transferred into vials with Formular 2-4 Instant Medium (Carolina Biological Supply Company). The CNS of L3l was dissected and stained as described above.

RESULTS

A comprehensive neuroblast map for the terminal abdominal neuromeres

To establish a precise map for the entire population of NBs in the most posterior segments, flat preparations of fixed embryos were analysed at early stage 12 (St12e), when all NBs have delaminated from the neurogenic region of the ectoderm. In a first step, NBs were identified by their position in the subectodermal layer and by the expression of the stem cell marker Deadpan (Dpn) (Bier et al., 1992). In the trunk neuroectoderm, segment polarity genes are expressed in segmental stripes and in NBs that delaminate from these domains (e.g. Bhat, 1996; Skeath et al., 1995). Using the markers Engrailed (En), which is expressed in the posterior part of each segment (DiNardo et al., 1985; Patel et al., 1989) and gooseberry-distal (gsb-d; now known as gsb – FlyBase), which is expressed anterior to and partially overlaps posteriorly with En (Gutjahr et al., 1993), all neuromeres of the embryonic VNC can be identified, including the most posterior ones. According to these stainings, NBs are not only found in A8 and A9 as previously described (Hartenstein and Campos-Ortega, 1984), but are also found posterior to the last En stripe, i.e. in A10. The size of the neuromeres significantly decreases from A8 to A10 (Fig. 1F). Accordingly, in A8 we found ~30, in A9 ~21 and in A10 ~11 Dpn-positive cells per hemineuromere. Except for the median neuroblast (MBN), En-expressing NBs are absent in A10, whereas a row of gsb-d-expressing NBs can be identified in this segment. gsb-d is also expressed in the anal pads, which belong to A11 (Fig. 1A) (Gutjahr et al., 1993), but we did not find NBs posterior to A10.

In order to individually identify and further characterise the NBs, we combined these segmental markers with a series of additional...
molecular markers (lac-Z- or Gal4-lines, antibodies and in situ probes) that have been previously used to map NBs in more anterior neuromeres of the VNC and in the developing brain (Broadus et al., 1995; Doe, 1992; Urbach and Technau, 2003a; Urbach and Technau, 2003b) (R.U., unpublished). NBs were individually identifiable by (a combination of) these markers, their typical position and time window of delamination (Fig. 1; supplementary material Figs S1-S4). Some examples are given below.

In thoracic and anterior abdominal segments, the zinc finger transcription factor Eagle (Eg) is expressed in four NBs (NB2-4, NB3-2, NB6-4, NB7-3) and their embryonic progeny (Dittrich et al., 1997; Higashijima et al., 1996). In A8, we identified all four Eg-expressing lineages. In A9 only NB3-2 and in A10 only NB6-4 was identified (Fig. 1C).

The segment polarity gene mirror (mirr) is in thoracic and anterior abdominal segments expressed in row 1 and row 2 NBs, two NBs of row 3 (NB3-2, NB3-4) and in NB6-1 (Broadus et al., 1995). By triple-staining against mirr, Eg and En we found the following NBs to be absent: NB2-3 from A8-A10, NB6-1 from A9 and NB5-3 of A10. (D) eveRRK-Gal4 marks characteristic daughter cells of NB1-1 (aCC, pCC) and NB4-2 (RP2). (E) Male and female WT embryos are distinguishable by Sxl expression. Dpn served as a universal marker for NBs; the depicted region of a male whole-mount embryo is shown in a flat preparation in F. (G) Number of Dpn-positive NBs was counted in A8, A9 and A10 of female and male St12e embryos. Error bars represent s.d. n.s., not significant.

Fig. 1. Mapping and identification of neuroblasts in the terminal neuromeres A8-A10 of the Drosophila embryo. (A-F) Flat preparations (horizontal views, A-D,F) and whole mounts (lateral views, E) of early stage 12 (St12e) or St14 embryos of the indicated genotype, triple-stained against different combinations of molecular markers as illustrated. NBs that can be clearly identified by marker staining(s) and position are highlighted in bold letters; identified NB daughter cells are surrounded by dotted lines and highlighted in bold letters; segments are indicated on the right; ML, midline. (A) En, Eg, and gsb-d were used as segmental markers. (B) mirr-lacZ is expressed in NBs of the anterior compartment and in NB6-1. (C) Ey is expressed in six different NBs per hemineuromere in A8, five NBs in A9, and in NB5-3 of A10. (D) eveRRK-Gal4 exclusively in the NB1-1-derived aCC and pCC and in the NB4-2-derived RP2 neurons (Landgraf et al., 1999; Fujioka et al., 2003).
Driving GFP expression in these cells revealed NB1-1 and NB4-2 precursors to be formed in A8 and A9, but the progeny neurons are absent in A10 (Fig. 1D). The expression of all NB- and lineage-markers we analysed is summarised in Figs 2 and 3.

Taken together, we were able to establish a precise NB map for the terminal neuromeres A8-A10 of the *Drosophila* embryo. Each individual NB expresses a characteristic combinatorial code of molecular markers (Fig. 2). We found that all NBs previously described in thoracic and anterior abdominal segments (Broadus et al., 1995; Doe, 1992) are also present in A8, except NB2-3 (lineage only found in the thorax) (Schmid et al., 1999). In A9, the two S5 NBs 2-4 and 5-1 and all En-expressing NBs, except NB7-1, are missing. In A10, only a few NBs (belonging to rows 2, 3 and 5, and NB6-4) are generated; NBs of rows 1 (except the longitudinal glioblast, LGB), 4 and 7 are lacking (Fig. 3). Thus, although the pattern of NBs in A9 and A10 is highly derived compared with thoracic and anterior abdominal segments, NBs formed in neuromeres A8-A10 can be individually identified owing to their serial homology to NBs in more anterior segments.

**Tracing the embryonic origin of sex-specific neuroblasts**

During larval stages, four NBs (two per side) in the terminal region of the VNC have been previously reported to exhibit a sex-specific proliferation pattern as revealed by incorporation of 5-bromodeoxyuridine (BrdU). In females, these NBs stop proliferating in mid-third instar larvae (L3m), whereas they continue dividing in male larvae (Taylor and Truman, 1992; Truman and Bate, 1988). Because postembryonic NBs derive from embryonic ones (Prokop and Technau, 1991), we attempted to clarify the embryonic origin of these sex-specific lineages and to link them to our NB map.

To discriminate between sexes, we stained embryos against Sex lethal (Sxl), which is only expressed in females (Bopp et al., 1991) (Fig. 1E). First, we counted the total number of NBs per hemisegment in A8-A10 upon co-staining against Dpn and En (Fig. 1F), but could not find significant differences between males and females (Fig. 1G). Next, we tried to identify the sex-specific NBs in the embryo. The postembryonic proliferation pattern of these NBs has been shown to depend on *dsx* (Taylor and Truman, 1992). So far, no expression of *dsx* has been reported in the embryonic CNS. However, using a *dsx*-Gal4 line driving CD8::GFP and enhancing the GFP signal with an antibody, we found a weak staining in the most posterior region from St16 onwards. The GFP expression was variable: some embryos exhibited no reporter expression at all, some exclusively in the midline, some only laterally and others showed expression both in the midline and laterally (Fig. 4A,B). We could also detect weak expression using an antibody against Dsx (supplementary material Fig. S5A).

The lateral cells (zero to two per hemisegment) seem to be the sex-specific NBs as they were positive for Dpn (Fig. 4C).

**Fig. 2. A neuroblast map for the terminal abdominal neuromeres A8-A10.** Cartoon illustrating all existing NBs in A8-A10 of St12e embryos; only the neuromeres of the right side are shown; segments are indicated on the right; ML, midline. The En stripes (dark grey) demarcate the posterior compartment of a neuromere; molecular markers that are already expressed at the NB level can be found in the inner circle; molecular markers for characteristic progeny cells of particular NBs are displayed in the outer circle (for colour codes see box on the right side). The listed lineage markers (e.g. Repo) are not shown for all NBs, which give rise to progeny cells expressing these markers, but only in those for which they are useful indicators. NB identities are based on the combinations of molecular markers, delamination time points, characteristic positions and the existence of characteristic progeny cells for specific NBs.
abnormal vision (Elav) (supplementary material Fig. S5B,C). These potential sex-specific NBs are formed in both sexes (supplementary material Fig. S5D,E). Their location in the domain of strongest Abdominal B (Abd-B)-expression (posterior to A8) (Celniker et al., 1989) (Fig. 4D) and anterior to the last En stripe (Fig. 4A) suggests that they belong to segment A9. As dsx is not expressed before St16, we attempted to clarify the identities of these NBs by detecting markers that are expressed at that stage. Both cells express Gooseberry proximal (Gsb-p; now known as Gsb-n – FlyBase; Fig. 4E), which is activated by gsb-d (Buenzow and Holmgren, 1995) and thus labels NBs that once expressed gsb-d (Colomb et al., 2008). Both cells are En negative (Fig. 4F), which implies that they belong to row 5 NBs. NB 5-1 is absent in A9 and A10 (Figs 2, 3). Both cells are negative for Runt (Run) and Ey (Fig. 4G; supplementary material Fig. S5F), which rules out NB5-2 and NB5-3. Expression of Muscle segment homeobox (Msh; now known as Drop – FlyBase; Fig. 4H) indicates that they are lateral NBs of row five. We found no expression of Ladybird early (Lbe; marker for NB5-6; supplementary material Fig. S5G) or unplugged (unpg)-lacZ (marker for NB5-5; Fig. 4I) in these cells. For these reasons, NB5-4 seems to be the most likely candidate for one of the sex-specific NBs.

In the midline, we found zero to three unpaired cells to be dsx-Gal4-positive. They are located in A9 (Fig. 4A), anterior to the last En stripe, and are present in both sexes (supplementary material Fig. S6A,B). These midline cells appeared negative for En and Castor (Cas) [markers for MNB lineage and ventral unpaired median cells (VUMs); Fig. 4J,K] as well as for unpg (expressed in the MNB lineage; Fig. 4L). All of them are positive for Run, but negative for Hunchback (Hb) (Fig. 4M,N). Co-staining for Wrapper unambiguously identified at least one of them as midline glia (Fig. 4O) (Wheeler et al., 2006). As not all dsx-expressing midline cells stained positive for Wrapper (Fig. 4P), we assume a second (unknown) source for dsx expression in the midline. dsx-expressing midline cells also appear to develop sex-specific differences (see below).

**Characterisation of the sex-specific neuroblasts and their postembryonic lineages**

As the two sex-specific NBs (per side) continue proliferation beyond L3m only in males (Taylor and Truman, 1992; Truman and Bate, 1988), we set out to identify and characterise the sex-specific NBs and their lineages in the VNC of male wandering larvae (L3l). These NBs are the only terminal-lateral cells that express Dpn (supplementary material Fig. S7) and Miranda (Mira) (Ikeshima-Kataoka et al., 1997; Shen et al., 1997) (Fig. 5A,B) in L3l males, and are clearly missing in females (supplementary material Fig. S7; Fig. 5C). All sex-specific NBs, as well as the large cell clusters associated with them, express dsx-Gal4. Whereas the NBs and adjacent cells reveal moderate dsx expression, those cells located in a more distal (anterior-dorsal) position within the clusters (seven to eight cells on either side) strongly express dsx. There is also abundant expression of dsx in the midline. Furthermore, strong dsx expression is found in a group of one to four cells located anterior-laterally on either side (Fig. 5A). In L3l females, these are the only dsx-expressing cells (Fig. 5C). An antibody against Dsx reveals the
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Fig. 4. The embryonic origin of sex-specific neuroblasts. (A,B) Localisation of dsx-Gal4-expressing cells in an overview (A) and a magnification (B) of the posterior VNC. Lateral cells are marked by arrowheads; cells in the midline are marked by arrows. ML, midline; anterior is up. (C-P) The lateral cells (C-I, one hemisegment) and the midline cells (J-P) at St17 double-stained against GFP (dsx>CD8::GFP; green) and molecular markers (red, as indicated). The first column shows a merge and indicates the dsx-positive cells by dashed lines; the second (*) and the third (**) columns show the separate channels.

same patterns, suggesting that expression of the dsx-Gal4 line is specific (supplementary material Fig. S7).

Next, we performed G-Trace analysis (Evans et al., 2009) to uncover the origin of the late larval dsx-expressing cells. In L3l females, the one to four anterior-lateral cells per side expressed RFP (real-time expression), but no GFP (lineage expression), indicating that they just upregulated dsx (Fig. 5D). As they were the first cells that express dsx in female L3 larvae, we termed them ‘initiator cells’. During transition from L3l to the white pupal (WP) stage, several other cells start to express dsx (RFP expression, but no GFP expression), whereas the initiator cells now also show GFP expression (Fig. 5E). The dynamic onset of dsx expression at this time point explains the high variability in cell numbers found in L3l females (Rideout et al., 2010). In L3l males, the initiator cells also just started dsx expression (only RFP). All other cells (including the NBs) co-expressed GFP and RFP (Fig. 5F). As dsx-Gal4 is already expressed in sex-specific NBs at embryonic St16, it triggers the flipout at this time point and all subsequently formed progeny cells are labelled by GFP expression. Accordingly, the lateral cell clusters that express both reporters in L3l are male specific and represent the whole postembryonic lineages of the sex-specific NBs. The midline cells also produce a cluster of postembryonic progeny cells only in males (co-expressing GFP and RFP in L3l). In WP stage males, like in females, additional cells have started expression of RFP (Fig. 5G). As these cells do not express GFP, they are not part of the sex-specific lineages.

To analyse the male-specific lineages in more detail, we used two approaches. First, we applied the Flybow technique, which allows separation of Gal4 patterns by multicolour labelling of cells within the same individual (Hadjieconomou et al., 2011). Using the dsx-Gal4 driver we differentially labelled the sex-specific lineages. In the lateral hemisegments of L3l males (Fig. 5H), we found one cell cluster (~50 cells) generally located more ventrally than the other (~45 cells). Both lineages show axonal projections into the neuropile, from where they turn to project out of the VNC (supplementary material Fig. S8A). In the midline, we found a smaller ventral clone (approximately six cells) and a bigger dorsal clone (approximately ten cells) sending projections in different directions (Fig. 5I; supplementary material Fig. S8B). This confirms our assumption (see above) that two different sources of dsx expression exist in the midline.

Second, we analysed the expression of molecular markers. In L3l, we detected eg-Gal4 expression in several abdominal midline clusters and in up to three lateral cells per hemiseguomere of both sexes (Fig. 6A,B). Male larvae additionally revealed eg-Gal4 expression in most cells of the male-specific lateral NB lineages. G-Trace analysis in L3l shows that eg is downregulated in the male-specific NBs, ganglion mother cells (GMCs) and adjacent cells (representing the youngest progeny cells; supplementary material Fig. S9A) and becomes restricted to cells in an intermediate position of the clusters (Fig. 6B,F). eg expression in these lineages comes up in postembryonic stages as we found no eg-expression in the sex-specific NBs in the embryo (supplementary material Fig. S9H). In contrast to eg, Gsb-p is already found in the embryonic progenitors of the male-specific lineages. In male L3l, Gsb-p is expressed in the NBs, their GMCs and in ventral cells in close vicinity to these (10-14 cells in total per lineage; Fig. 6C,F). The dorsal cells, showing strong dsx-expression, never express Gsb-p. All Gsb-p-positive cells in the terminal ganglia express dsx (Fig. 6D; there is no expression of Gsb-p in the terminal ganglia of L3l females, not shown). Although a few cells co-express Gsb-p and eg (supplementary material Fig. S9B), expression of the two markers in these lineages is mutually exclusive. Thus, these markers label three distinct subsets of cells within the male-specific lateral NB-lineages (as summarised in Fig. 6F): (1) NB, GMCs and proximal (late-born) progeny cells (Gsb-p expression), (2) intermediate progeny cells (eg expression), and (3) most distal (earliest-born) progeny cells (no eg and no Gsb-p, but strong dsx expression). All of the dsx-expressing cells in male L3l co-express Abd-B (only weak expression in NBs and GMCs; Fig. 6E).

Sex-specific neuroblasts in females are undergoing programmed cell death

In early third instar larval (L3e) males, we found clusters of dsx-expressing cells in the lateral regions and one cluster in the midline (Fig. 7A) that consist of smaller numbers of cells, but show the same spatial arrangement as observed in L3l (Fig. 5A). In L3e females, we found no dsx expression (not shown), suggesting that sex-specific NBs in females do not produce postembryonic lineages. This is in contrast to a previous report showing that the sex-specific NBs in females are proliferating until L3m (Taylor and Truman, 1992).
As mentioned above, at the L3l stage Gsb-p is expressed in male-specific lineages, but is not found in the terminal ganglia of females. Surprisingly, at the late second instar larval stage (L2l), Gsb-p is expressed in both sexes (Fig. 7B,C). Posterior to a bulk of Gsb-p-expressing cells, there is a group of terminal Dpn-positive NBs (on either side). In L2l females, we found six to seven NBs per hemisegment in this region (Fig. 7B). Two of them express Gsb-p, four to five do not. By contrast, L2l males possess eight to nine NBs in this region (Fig. 7C). Two of them express Gsb-p, another two (the male-specific NBs) co-express Gsb-p and \( dsx \), and four to five do not express these markers. These data suggest that in females the sex-specific NBs disappear before L2l and do not give rise to any postembryonic daughter cells.

As we were not able to detect the sex-specific NBs in L2l females, we wondered if these NBs undergo PCD. To test this, we ectopically expressed P35 using \( dsx \)-Gal4, which should suppress apoptosis (Hay et al., 1994) in the sex-specific NBs. In L3l females, we found that in seven cases the complete male-specific \( dsx \)-Gal4 clusters were restored in the lateral VNC. In these cases, the lateral clusters were associated with two \( dsx \)-expressing, Mira-positive NBs on either side (Fig. 8A), which are never present in wild-type (WT) L3 females (0 NBs, \( n=10 \); Fig. 8D). In three cases, one lateral NB and its lineage was missing; in all other female larvae, sex-specific NBs were not detectable (0.16±0.53 NBs, \( n=30 \); Fig. 8D). In L3l males, we found one of the sex-specific NBs to be missing in three individuals; all others showed the WT pattern (3.89±0.30 NBs, \( n=29 \); Fig. 8D). These weak effects might be due to poor knockdown efficiency. Alternatively, or in addition, further factors might act in parallel with \( Abd-B \) to control survival of these NBs.

A dual role for \( doublesex \)

\( transformer (tra) \) and \( transformer2 (tra2) \) are necessary for the female-specific splicing of \( dsx \) pre-mRNA, which results in the generation of \( Dsx[F] \) protein. Loss of \( tra \) or \( tra2 \) leads to default splicing which generates the male-specific \( Dsx[M] \) protein.
controls proliferation of sex-specific NBs. In tra/Hoshijima et al., 1991). It has been previously reported that tail region Drosophila Neuroblasts in the significant reduction of sex-specific NBs in males (2.7±1.42 NBs, four sex-specific NBs; Fig. 9). Finally, we knocked down cases (except for four individuals, which revealed five instead of function by driving tra/dsx-dsRNAi constructs with -Gal4. Driving RNAi constructs with -Gal4 to drive dsx RNAi resulted in a rescue of one to four sex-specific NBs in all female L3l individuals (2.25±0.88 NBs, n=8); driving tra2-RNAi rescued one or two sex-specific NBs in four cases (0.5±0.70 NBs, n=10; Fig. 9). The rescue of NBs in these knockdowns might have different reasons: It could be due to the loss of dsx[F] (which might be necessary to trigger PCD in sex-specific NBs), or to the generation of dsx[M] in females (which might be sufficient to rescue sex-specific NBs), or both.

To distinguish between these possibilities, we ectopically expressed dsx[F] or dsx[M]. Using dsx-Gal4 to drive dsx[F] in males resulted in the sex-specific NBs being entirely missing in most larvae (0.85±1.41 NBs, n=26; Fig. 9). In a control experiment, dsx >dsx[F] females (n=22) showed the WT phenotype (no sex-specific NBs; Fig. 9). Conversely, upon driving dsx[M] with dsx-Gal4 in females, the number of surviving sex-specific NBs did not differ significantly from WT males (3.43±0.79 NBs, n=7; Fig. 9). dsx >dsx[M] in males (n=18) did not affect NB numbers in most cases (except for four individuals, which revealed five instead of four sex-specific NBs; Fig. 9). Finally, we knocked down dsx function by driving tra-RNAi with dsx-Gal4, and observed a significant reduction of sex-specific NBs in males (2.7±1.42 NBs, n=17), whereas females displayed no differences compared with WT (n=10; Fig. 9) (see Discussion).

Taken together, these results demonstrate that dsx plays a dual role in sex-specific NBs: whereas dsx[F] promotes PCD, dsx[M] is required for the survival of sex-specific NBs.

DISCUSSION

Pattern and identities of embryonic neuroblasts in the terminal neuromeres

Previous work based on UV-laser ablation and the examination of epidermal mutant phenotypes identified four segmental anlagen, A8-A11, and a non-segmented telson in the so-called tail region of the Drosophila embryo (Juergens, 1987). The complete metameric caudal units are only displayed in the extended germ band stage. After this stage, owing to morphogenetic movements, condensation and fusion of segmental primordia, the tail region assumes an aperiodic and highly derived appearance compared with the trunk region (Kuhn et al., 1992).

We present here the first comprehensive map of NBs derived from the tail region. The map refers to early stage 12 (St12e), when all terminal NBs have been formed (slightly later than their more anterior homologues) (Doe, 1992) and the metameric units are still distinguishable. Importantly, all NBs that are formed can be individually identified owing to serial homology to NBs in other segments of the VNC as reflected by the combinatorial codes of marker gene expression, similar delamination time points and positions (Broadus et al., 1995; Doe, 1992) (R.U., unpublished), or the presence of characteristic progeny cells. However, as previously
In A9, we found 23 NBs per hemineuromere occupying lateral, intermediate and ventral positions. Strikingly, all NBs of rows 1 to 5 (except NB2-4 and NB5-1) are present, whereas all En-positive NBs of the posterior compartment (rows 6 and 7, except of NB7-1) are missing in A9. Thus, the last En stripe, which belongs to parasegment 15, appears to demarcate a border for the absence of many NBs. In segment A10, we found 11 NBs (per side) to be generated. To our knowledge, the existence of NBs and formation of a neuromere in A10 have not been described before. However, some neurons have been identified that express Gsb-p and derive from a gsb-d stripe located posterior to the gsb-d stripe of A9 (Gutjahr et al., 1993). Similar to A9, almost all NBs of the posterior compartment are missing (except the MNB, which is the only En-positive NB in A10). As we were not able to identify NBs posterior to row 5 and NB6-4 (lateral CNS) and the MNB (midline) in A10, these NBs represent the most caudal progenitor cells of the CNS. The reduction in the number of embryonic NBs in A9 (by 28%) and A10 (by 66%) is not due to PCD, as we found no significant differences in their number and pattern in apoptosis-deficient H99 (White et al., 1994) mutant embryos (O.B., O. Vef, A. Rogulja-Ortmann, C.B. and G.M.T., unpublished). Instead, in agreement with the observation that the segments of the tail anlage (A9, A10 and A11, but not A8) are progressively reduced (Juergens, 1987), formation of lower numbers of NBs appears to be due to smaller sizes (A9, A10) or absence (A11) of neuroectoderm. According to our NB map, size reduction mainly affects the anterior-posterior (A/P) axis: specific A/P rows of NBs are almost completely missing (row 6 and 7 in A9; rows 1, 4, 6 and 7 in A10), whereas representatives of all three dorsal-ventral (D/V) columns are found in terminal neuromeres. In addition, we noticed that diameters of the NBs in A9 and A10 are often reduced, which suggests a lower number of mitoses and, thus, the production of smaller lineages compared with those of their more anterior homologues.

**Embryonic origin of the sex-specific neuroblasts**

We show that *dsx* is already expressed in the embryonic CNS. The pattern of expression is the same in both sexes. We provide evidence that the sex-specific NBs are located anterior to the last En stripe (which belongs to A9) and that at least one of them corresponds to NB5-4. Considering that at St17 both cells (per side) express the same combination of markers (Gsb-p and Msh, in addition to Dpn and Gsb-p expression). The left region of terminal NBs is marked by a white square and different focal planes of this region are shown (B’-B”, C’-C”). NBs that are negative for *dsx* and Gsb-p are marked by yellow arrowheads; NBs negative for *dsx* but positive for Gsb-p are marked by green arrowheads; NBs positive for *dsx* and Gsb-p by white arrowheads.

**Dual role for doublesex in controlling the fate of the sex-specific precursors**

Although the embryo is sexually determined by the expression of the master control gene *Sxl* prior to cellularisation (Bopp et al., 1991) [for reviews on sex determining genes, see Schütt and Nöthiger, 2000; Steinmann-Zwicky et al., 1990], sexual differentiation of the CNS via the *dsx* pathway occurs much later. In both sexes, we detected *dsx* expression in the sex-specific NBs from St16 onwards. Temperature shift and BrdU incorporation experiments previously...
revealed that commitment of these NBs to sex-specific postembryonic proliferation behaviour occurs at the end of the first larval stage (L1l), but they do not express their different behaviours before the mid-third larval stage (L3m). At this stage, female NBs stop dividing, whereas in males sex-specific NBs continue dividing until 12 hours after puparium formation (Taylor and Truman, 1992). As expected, G-Trace analysis disclosed prominent postembryonic lineages of sex-specific NBs in males. However, in females we were not able to detect postembryonic lineages of the corresponding cells. Instead, our experiments suggest that the expression of the female-specific isoform of $\text{dsx}[\text{F}]$ induces PCD of the sex-specific NBs between St17 and late L2 larval stage (L2l). Upon expression of $\text{P35}$ in females, sex-specific NBs survive and generate male-specific lineages. Ectopic expression of $\text{dsx}[\text{F}]$ in males results in a removal of these precursors, demonstrating the pro-apoptotic effect of $\text{dsx}[\text{F}]$ on sex-specific NBs. However, in a $\text{dsx}$ knockdown experiment (affecting both isoforms) we found no surviving sex-specific NBs in female, and a reduced number of sex-specific NBs in male L3l larvae, in agreement with a previous report showing that in loss-of-function $\text{dsx}$ mutants sex-specific NBs are missing in both sexes (Taylor and Truman, 1992). This is compatible with the hypothesis that $\text{dsx}[\text{M}]$ is required for survival of these NBs. Consequently, ectopic expression of the male-specific isoform $\text{dsx}[\text{M}]$ in females rescues the sex-specific NBs from PCD. Thus, these gain- and loss-of-function experiments suggest a dual role for Dsx in PCD ($\text{dsx}[\text{F}]$) and survival ($\text{dsx}[\text{M}]$) of sex-specific NBs.

A role for $\text{dsx}[\text{F}]$ in mediating PCD has been also reported in other contexts, e.g. in embryonic somatic gonadal precursors (DeFalco et al., 2003), in P1 interneurons of the adult brain (Kimura et al., 2008) and in the pupal and adult TN1 cluster of the thoracic VNC (Sanders and Arbeitman, 2008). Additionally, whole-genome screening for the perfect consensus $\text{Dsx}[\text{F}]$ binding site identified a locus next to the pro-apototic gene $\text{reaper}$ ($\text{rpr}$), which might be a downstream target of $\text{Dsx}[\text{F}]$ (Luo et al., 2011).

The sex-specific postembryonic lineages seem to be functionally required in adult male flies as the precursors continue proliferation in the early pupa, and progeny cells appear to persist into the adult (Taylor and Truman, 1992). To generate these lineages, it is a prerequisite that their precursors survive and $\text{dsx}[\text{M}]$ seems to be the crucial factor required for their survival. Interestingly, however, $\text{dsx}[\text{M}]$ is not needed for proliferation of the sex-specific NBs or differentiation of their lineages; upon ectopic expression of $\text{P35}$ in females (lacking $\text{dsx}[\text{M}]$), surviving sex-specific NBs generate a complete male-specific larval lineage that forms typical projections. Thus, in both sexes the stem cells are able to carry out the entire intrinsic programme for the generation of the same type of postembryonic lineage. Sex-specific existence versus absence of this lineage is controlled by $\text{dsx}$, which acts at the stem cell level to decide whether the cell survives or not.

In addition to sex-specific lineage development in the terminal ganglia, several other sexual differences become established in the VNC and brain of female and male flies regarding cell numbers, neural circuits and behaviour (e.g. Billeter et al., 2006; Kimura et al., 2008).
It has been shown that dsx acts in concert with the Hox gene Abd-B to regulate the expression of their common downstream target bric à brac (bab) (Williams et al., 2008). Furthermore, it has been postulated that Abd-B sculpts sex-specific abdomen morphology by positively regulating dsx during pupal development (Wang and Yoder, 2012). Our data suggest that Abd-B participates in controlling survival of sex-specific abdominal NBs, but the mechanisms of its putative interaction with dsx and other factors still need to be clarified.

The data and tools available now establish the terminal neuromeres of Drosophila as an attractive model system for further investigations into the mechanisms controlling segment-specific and sex-specific differences in the CNS.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

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References


Fig. S1. Expression of seven-up-lacZ, wingless-lacZ, ming-lacZ, huckebein-lacZ and unplugged-lacZ in neuroblasts of the tail region. Flat preparations (horizontal views; anterior to the top) of mid stage 11 (St11m), late stage 11 (St11l) or early stage 12 (St12e) embryos of the indicated genotype triple-stained against different combinations of molecular markers as illustrated. Location of the NBs is indicated; NBs which can be clearly identified by marker staining(s), position and/or delamination time point are highlighted in bold letters; identified NB daughter cells are surrounded by broken lines and highlighted in bold letters; segments are indicated on the right; ML, midline. (A) Most NBs transiently express seven-up (svp)-lacZ (Doe, 1992). Therefore, svp can serve as a marker to identify NBs during different delamination time points. The big lateral S1 NBs are all present in A8 and A9. Runt (Run) is expressed in a subset of only seven NBs (Dormand and Brand, 1998). Double labelling reveals that NB5-2 and NB5-3 are present in A8 and A9. There is also one svp/Run-positive NB in A10 (NB5-3). Note the absence of svp-positive NBs in the En stripe of A9. The NB7-1 in A9 does not express svp. (B) wingless (wg)-lacZ is expressed in all row 5 NBs (Doe, 1992). Double labelling with Run shows that both NB5-2 and NB5-3 are also present in A10. NB5-1 is missing in A9 and A10. In A9, all other row 5 NBs are present. (C) ming-lacZ is expressed in many NBs in the thorax and in anterior abdomen (Doe, 1992). However, in A9 and A10 ming becomes variably expressed in only few cells. (D) Staining for Run in huckebein (hkb)-lacZ (Doe, 1992) shows NB2-1 (Run negative) and NB2-2 (Run positive) to be present in A8 and A9. (E) We could also detect these two NBs in A10 and found that all hkb-positive NBs of row 4 (NB4-2, NB4-3, NB4-4, which are Gsb-d negative) and five (NB5-4, NB5-5, which are Gsb-d positive) are present in A8 and A9. NB2-4 is clearly missing in A9 and A10, but is present in A8. Note the hkb and Gsb-d-positive NB in A10 (NB5-4). (F) unplugged (unpg)-lacZ-expression identifies NB4-1, NB5-3 and NB5-5 (Doe, 1992) in A8 and A9, and the MNB down to A10 (double labelling with En).
Fig. S2. Expression of Empty spiracles, Orthodenticle, Ladybird early, Eyeless and Reversed polarity in neuroblasts and their progeny of the tail region. Flat preparations (horizontal views; anterior to the top) of St11e or St12e embryos of the indicated genotype stained against different combinations of molecular markers as illustrated. Location of the NBs is indicated; NBs which can be clearly identified by marker staining(s), position and/or delamination time point are highlighted in bold letters; identified NB daughter cells are surrounded by broken lines and highlighted in bold letters; segments are indicated on the right; ML, midline. (A) The gap-gene Empty spiracles (Ems) is expressed in NB3-5, NB4-4 (and some of their progeny) and in NB3-3 (Hartmann et al., 2000). We identified these NBs down to A9. In A10, it is only one Ems-positive NB, which is also positive forsvp (NB3-5). (B) In the thorax, Orthodenticle (Otd) is expressed in four ventral NBs (NB2-1, NB3-1, NB5-1 and NB6-1; R.U., unpublished). All of them are found in A8. In A9, NB2-1 and NB3-1 can be identified, whereas NB5-1 and NB6-1 are missing. In A10, there is one Otd-positive NB (NB2-1). (C) Ladybird early (Lbe) is expressed in the newly formed NB5-6 and some of its daughter cells (De Graeve et al., 2004). We found these cells to be present down to A10, although their number is reduced in the most terminal segments. Note the expression in the anal plate (Jagla et al., 1997). (D) lbe[K]-Gal4 driven RFP-expression corresponds to the pattern in C, except that expression in the anal plate is missing. (E) In the thorax, Eyeless (Ey) is expressed in a set of six different NBs per hemisegment (NBs 3-2, 4-2, 4-3, 4-4, 5-3, 7-3; R.U., unpublished). All of them are also present in A8, and all except NB7-3 are present in A9. Only one Ey-positive NB (NB5-3) is found in A10. (F) At St12e, the NB6-4-derived glial cells [co-expressing Eg, En and the glial-specific marker Reversed polarity (Repo)] are present in A8, but missing in A9. Please note that there is also one early-born glia cell (on either side) in A10 (derived from the LGB, NB5-6 or NB6-4).
**Fig. S3. Expression of columnar genes and glial-specific genes in the terminal abdominal neuromeres.** Flat preparations (horizontal views; anterior to the top) of St10, St12e or St13 embryos of the indicated genotype stained against different combinations of molecular markers as illustrated. Location of the NBs is indicated; NBs which can be clearly identified by marker staining(s), position and/or delamination time point are highlighted in bold letters; identified NB daughter cells are surrounded by broken lines and highlighted in bold letters; segments are indicated on the right; ML, midline. (A-D) Expression of dorso/ventral patterning (columnar) genes. (A) Ventral nervous system defective (Vnd) is expressed in the most ventral neuroectodermal column and in the NBs that delaminate from this region (Chu et al., 1998). In A8, Vnd expression is found in ventral NBs of which NB2-1, NB3-1, NB4-1, MP2, NB5-1 and NB5-2 are clearly identifiable. (B) In A9 and A10, some NBs are missing in the ventral region. (C) In more anterior segments of the trunk, intermediate neuroblasts defective (ind) is expressed for a short period in five different NBs (Weiss et al., 1998). All of them are present in A8 (NB3-2, NB4-2, NB5-3, NB6-2, NB7-2). In A9, NB6-2 and NB7-2 are clearly missing. Only one ind-expressing NB is found in A10 (NB5-3). (D) Muscle segment homeobox (Msh) is expressed in the lateral NBs of the VNC (Isshiki et al., 1997). In A8, we could clearly identify NB2-4, NB4-3 and NB5-4 by their co-expression of hkb and NB6-4 as well as NB7-4 by co-expression of En. NB4-3 and NB5-4 are also present in A9, whereas NB2-4, NB6-4 and NB7-4 are missing in this segment. In A10, three Msh-expressing NBs are found (NB3-4, NB5-4, NB6-4). (E) At St10, the early-born glial progeny of NB7-4 can be identified down to A8, but is missing in A9 and A10. The LGB can also be identified owing to its Repo expression (Xiong et al., 1994) down to A9. (F) Nevertheless, at St13 we also identified a longitudinal glia cell in A10. This cell is located in a typical dorsal position, and appears a bit distant from the longitudinal glia in A8 and A9. Beside Repo it expresses the marker Nazgul (Naz), which is specifically expressed in longitudinal glia (von Hilchen et al., 2010).
**Fig. S4. Expression of markers for characteristic progeny cells of specific neuroblasts.** Flat preparations (horizontal views; anterior to the top) of St14-St17 embryos of the indicated genotype stained against different combinations of molecular markers as illustrated. Identified daughter cells of specific NBs are surrounded by broken lines and highlighted in bold letters; segments are indicated on the right; ML, midline. (A,B) Even skipped (Eve) is expressed in NB4-2-derived RP2 (A) (Bossing et al., 1996), in NB1-1-derived aCC and pCC (Broadus et al., 1995), NB7-1-derived U-neurons (Bossing et al., 1996) and NB3-3-derived EL-neurons (B) (Schmidt et al., 1997). All of these characteristic daughter cells are present down to A9, but missing in A10. There is also Eve expression in the hindgut (Gorfinkiel et al., 1999). (C) CQ2-Gal4 drives expression in the NB7-1-derived U-neurons (Landgraf et al., 2003) and confirms their existence in A8 and A9 and absence in A10. (D) Pox neuro (Poxn)-Gal4 (Boll and Noll, 2002) drives expression in the NB2-4 lineage (Rogulja-Ortmann et al., 2008). We found this lineage to be present in A8, but missing in A9 and A10. (E) In more anterior segments, five different NBs (NB4-2, NB 5-2, NB6-1, NB6-2, NB7-1) have been shown to give rise to Dbx-expressing neurons (Lacin et al., 2009). We found all of these typical cells in A8, whereas in A9 the cells derived from NB6-1 and NB6-2 are missing. (F) Mz97-Gal4 drives expression in some peripheral glia cells, which derive from NB1-3 (von Hilchen et al., 2008). These cells are identifiable down to A9, but appear to be missing in A10.
Fig. S5. Characterisation of doublesex (dsx)-Gal4-expressing lateral cells. (A-K″) The lateral cells (one hemisegment) at St17 double-stained against GFP (dsx >CD8::GFP; green) and molecular markers (red; as indicated); the first column shows a merge and indicates the dsx-positive cells by dashed lines; the second (′) and the third (“) columns show the separate channels.
Fig. S6. Characterisation of doublesex-Gal4-expressing medial cells. (A-C″) Medial cells at St17 double-stained against GFP (dsx >CD8::GFP; green) and molecular markers (red; as indicated); the first column shows a merge and indicates the dsx-positive cells by dashed lines; the second (′) and the third (″) columns show the separate channels.

Fig. S7. Comparison of doublesex-Gal4 and Doublesex antibody expression in wandering larvae and white pupae. (A-D) Horizontal views of terminal neuromeres (anterior to the top) of dsx >CD8::GFP females (A,C) and males (B,D) at late L3 (L3l) or white pupal (WP) stages. All images are maximum projections. ML, midline. Initiator cells are marked by arrows. Some (predominantly dorsal) cells (marked by double-headed arrows) are detected by the antibody, but reveal no GFP expression (which appears with some delay).
Fig. S8. 3D models of Flybow clones. 3D reconstructions generated by the Amira software. ML, midline. (A) Dorsal view of the two lateral clones shown in Fig. 5H. (B) Ventral view of the dorsally located midline clone shown in Fig. 5I.

Fig. S9. Characterisation of male-specific lineages in wandering larvae. Horizontal views of terminal neuromeres (anterior to the top). ML, midline. (A) Maximum projection of \textit{eg}>G-Trace L3I male (merge); sex-specific NBs are marked by asterisks. (B) Maximum projection of \textit{eg}>CD8::GFP L3I male (merge). (A′,B′) Without red channel; (A″,B″) without green channel.