Development 140, 1830-1842 (2013) doi:10.1242/dev.090043
© 2013. Published by The Company of Biologists Ltd

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

Institute of Genetics, University of Mainz, D-55099 Mainz, Germany.

*Author for correspondence (technau@uni-mainz.de)

Accepted 17 February 2013

DEVELOPMENT

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 *eve*^{RRK}-Gal4 (Fujioka 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); *Mz97* and *Mz360* (*eagle*-Gal4) (Ito et al., 1995); *Pox-neuro*-Gal4 (Boll and Noll, 2002) (provided by Markus Noll); UAS-*CD8::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*, *ming-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 (L3l) 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), rat anti-Elav (1:2000), mouse anti-Invected (1:2) (Patel et al., 1989), mouse anti-Sex lethal (1:10) (Bopp et al., 1991) and mouse anti-Wrapper (1:20) (Noordermeer et al., 1998) (all from Developmental Studies Hybridoma Bank); chicken anti-Beta-Gal (1:1000) (Abcam); rabbit anti-Castor (1:500) (Kambadur et al., 1998) (provided by Ward Odenwald); guinea pig anti-Dbx (1:1500) (Lacin et al., 2009) (provided by James Skeath); rabbit anti-Deadpan (1:100) (Bier et al., 1992) (provided by Harald Vaessin); rat anti-Doublesex (1:100) (Sanders and Arbeitman, 2008) (provided by Michelle Arbeitman); rabbit anti-Eagle (1:500) (Dittrich et al., 1997); rat anti-Empty spiracles (1:1000) (Walldorf and Gehring, 1992) and rabbit anti-Eyeless (1:1000) (Kammermeier et al., 2001) (provided by Uwe Walldorf); rabbit anti-Engrailed (1:100) (Santa Cruz Biotechnology); rabbit anti-Even skipped (1:1000) (Frasch et al., 1987) (provided by Manfred Frasch); mouse anti-GFP (1:250) (Roche); rabbit anti-GFP (1:500) (Torrey Pines Biolabs); rat anti-Gooseberry distal (1:2) and rat anti-Gooseberry proximal (1:2) (Zhang et al., 1994) (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-Miranda (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-Reversed 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 digoxygenin-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 Axioplan; 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 4-24 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 formed 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 (MNB), 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

DEVELOPMENT

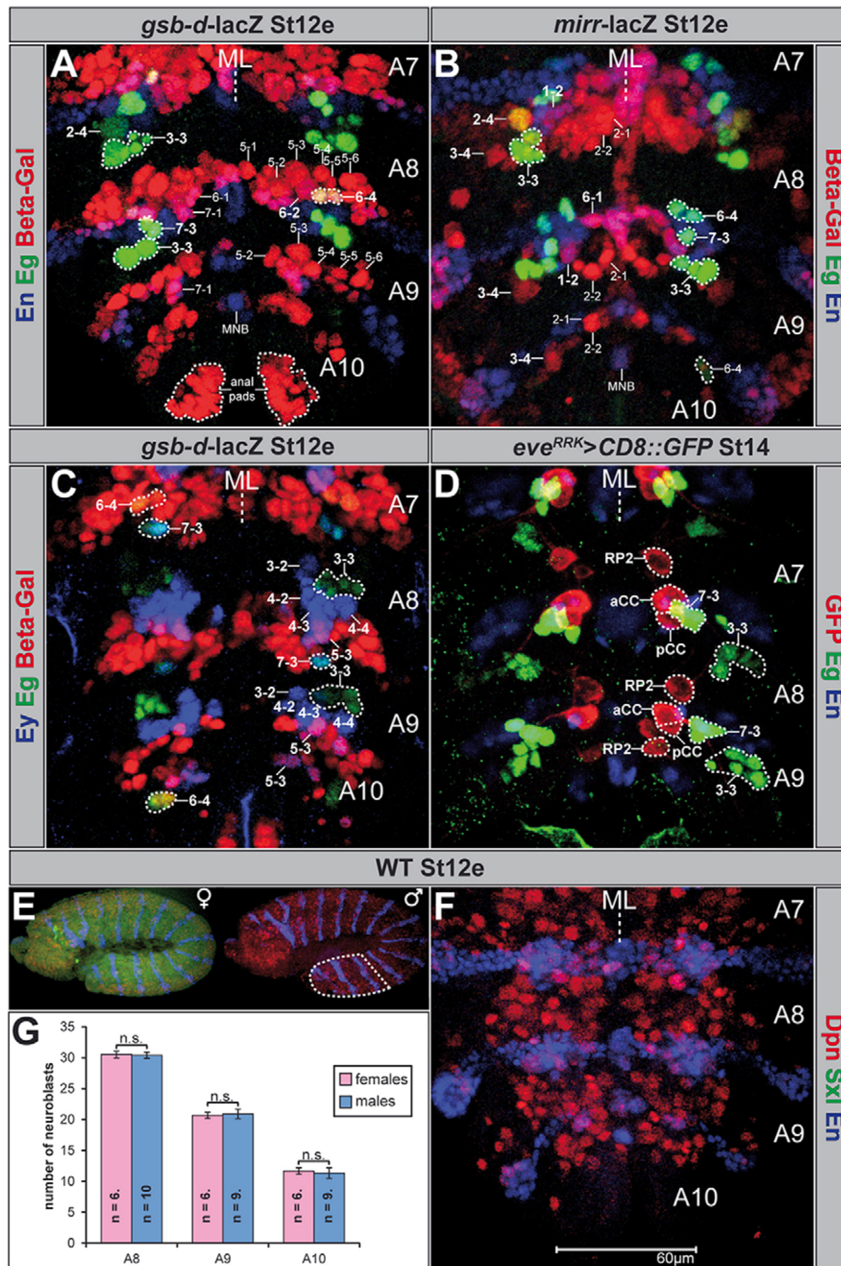

Fig. 1. Mapping and identification of neuroblasts in the terminal neuromeres A8-A10 of the *Drosophila* embryo. (**A-F**) Flat preparations (horizontal views, A-D,F) and whole mounts (lateral views, E) of early stage 12 (St12e) or St14 embryos of the indicated genotype, triple-stained against different 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) *eve* ^{RRK}-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.

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, NB3-3, 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 NB3-3 (Fig. 1A,B,D) 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 A10, and NB1-2 from A10. NB3-4 was present in all terminal neuromeres and NB3-2 appears to be present in A8 and A9, although it lacks expression of *mirr-lacZ* (Fig. 1B).

Eyeless (Ey) has been shown to be expressed in a reiterated pattern in the whole VNC (Kammermeier et al., 2001). In the thorax, Ey-expression was detected in six different NBs (NB3-2, NB4-2, NB4-3, NB4-4, NB5-3, NB7-3; R.U., unpublished). We find all of these NBs to be present in A8, NB7-3 being the only one of them missing in A9, and all of them, except NB5-3, missing in A10 (Fig. 1C).

In addition, we used markers that label characteristic parts of certain NB lineages (Fig. 1D; supplementary material Figs S2-S4). For example, the line *eve* ^{RRK}*-Gal4* (Baines et al., 1999; Fujioka et al., 2003) expresses Gal4 exclusively in the NB1-1-derived aCC and pCC and in the NB4-2-derived RP2 neurons (Landgraf et al.,

DEVELOPMENT

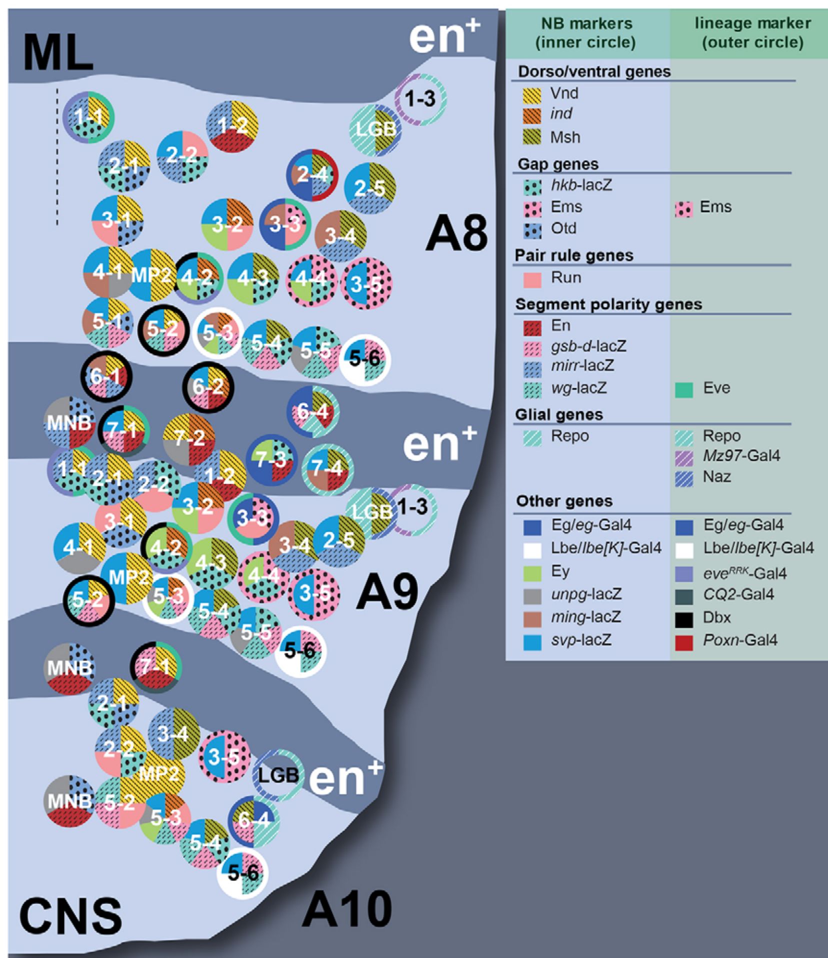

Fig. 2. A neuroblast map for the terminal abdominal neuromeres A8-A10. Cartoon illustrating all existing NBs in A8-A10 of St12e embryos; only the neuromeres of the right side are shown; segments are indicated on the right; ML, midline. The En stripes (dark grey) demarcate the posterior compartment of 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.

2003b). 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). Furthermore, we found none of these cells to express postmitotic markers, such as Reversed polarity (Repo) or Embryonic lethal

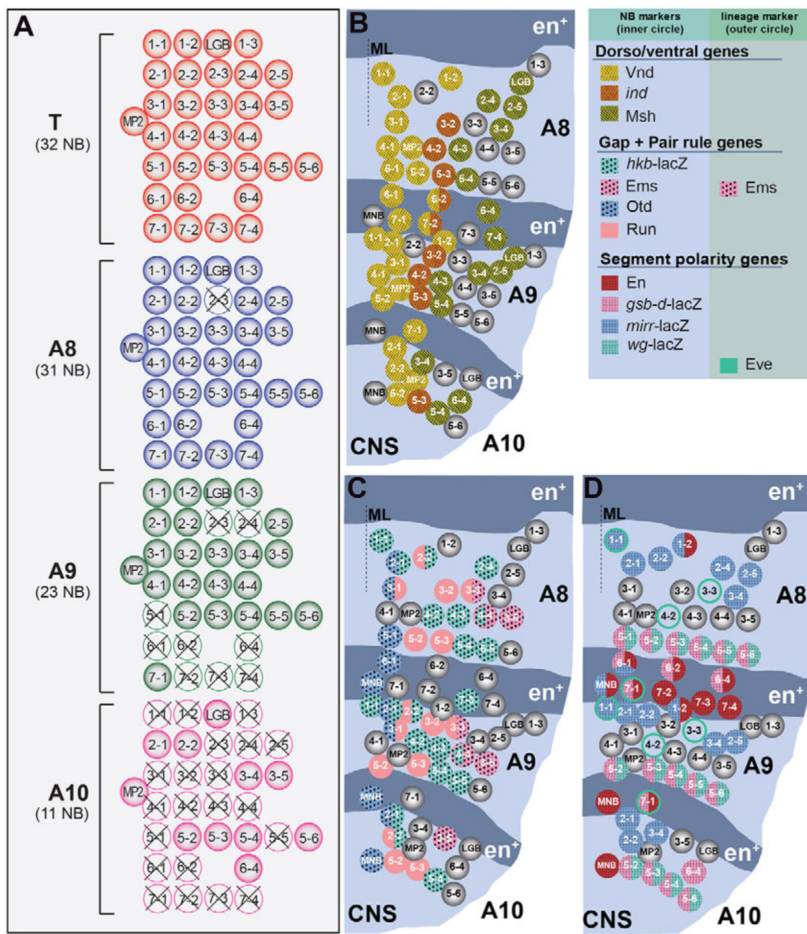

Fig. 3. Distribution of neuroblasts and their expression of A/P and D/V patterning genes in the terminal abdominal neuromeres A8-A10. (**A**) This map illustrates the NBs that are formed (coloured) and those which are missing (colourless and marked by X) in A8-A10 (right hemineuromeres) compared with the thoracic ground stage (T) [according to Doe (Doe, 1992)]. (**B-D**) Sub-patterns of marker genes shown in Fig. 2 (colour code as listed in the box). Segments are indicated on the right; ML, midline. (B) Expression of dorsoventral patterning genes. (C) Expression of gap- and pair-rule genes. (D) Expression of segment polarity genes.

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

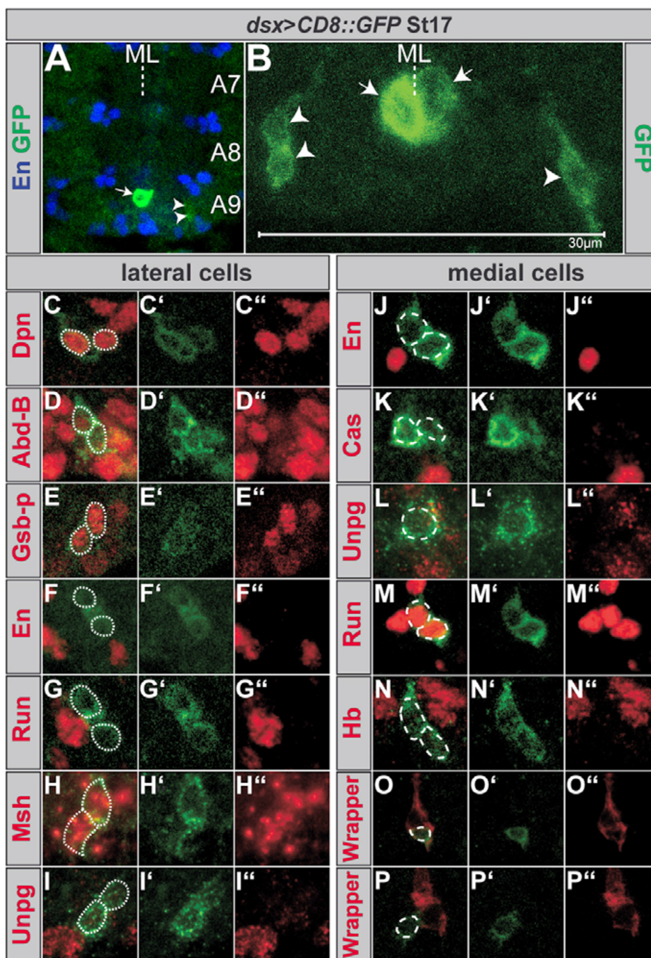

Fig. 4. The embryonic origin of sex-specific neuroblasts. (**A**,**B**) Localisation of *dsx*-Gal4-expressing cells in an overview (A) and a magnification (B) of the posterior VNC. Lateral cells are marked by arrowheads; cells in the midline are marked by arrows. ML, midline; anterior is up. (**C-P″**) 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 hemineuromere 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. S5H). 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).

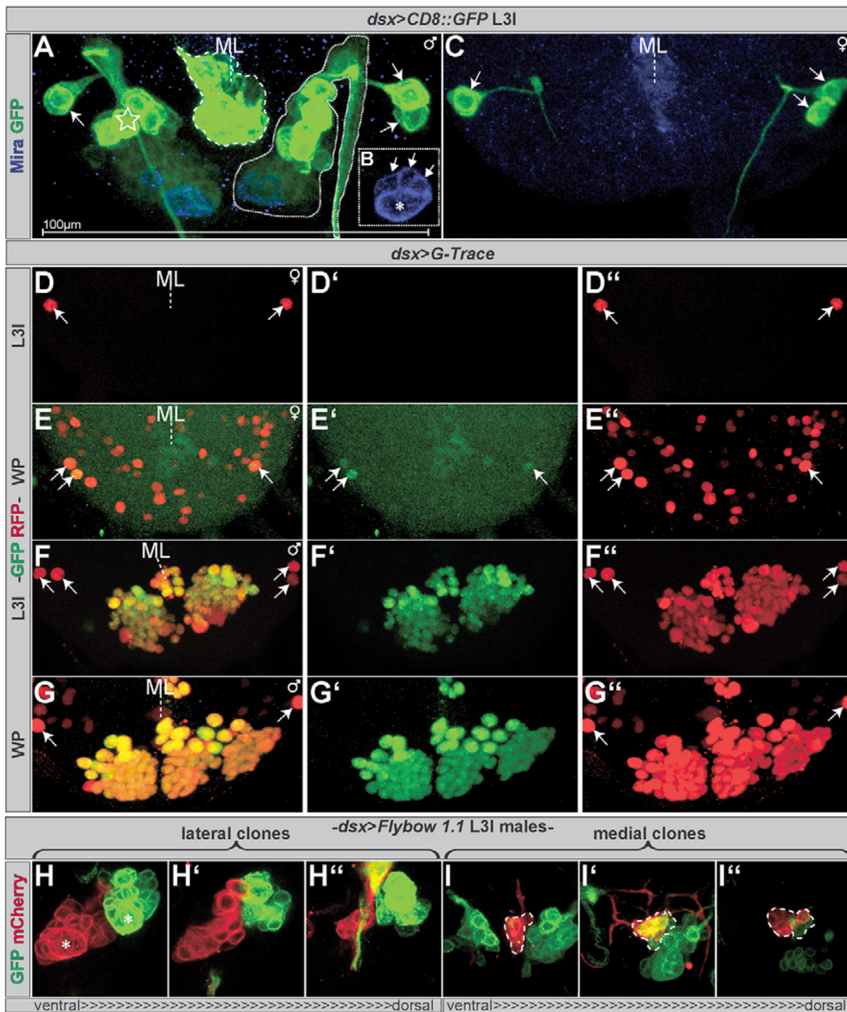

Fig. 5. Characterisation of the sex-specific neuroblasts and their lineages in wandering larvae. Horizontal views (anterior to the top) of terminal neuromeres at late L3 larval (L3l) or white pupal (WP) stage labelled with different combinations of markers as indicated. Initiator cells in all pictures are marked by arrows. ML, midline. (**A**,**C**) Maximum projections of anti-GFP and anti-Mira staining in male (A) and female (C) L3l; midline cells are surrounded by dashed lines; the right lateral cell cluster is marked by dotted lines; white star marks anterior-dorsal cells of the (left) cluster, which express *dsx* strongly. (**B**) Mira staining of a single stack, in which the NB (asterisk) and its adjacent GMCs (arrows) can be easily distinguished by their sizes. (**D-G″**) Maximum projections of *dsx >GTrace* preparations; the first column shows a merge; the second (′) and third (″) columns show separate channels for GFP and RFP, respectively. (D) Female L3l. (E) Female WP. (F) Male L3l. (G) Male WP. (**H-I″**) *dsx >Flybow1.1* L3l males stained for GFP and mCherry; three sections are shown from ventral (H,I) to dorsal (H″,I″). H shows two lateral clones; NBs are marked by asterisks. I shows two clones in the midline, which are surrounded by dashed lines.

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 L3 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 (compared with WT males), indicating a partial restoration of the male-specific *dsx*-Gal4 clusters (Fig. 8B), whereas only in one case did both lateral NBs and their lineages remain absent (not shown). This situation (3.55±0.69 NBs, n=11) is not significantly different compared with WT males (4.00±0 NBs, n=10; Fig. 8D). The midline cells were rescued in all cases. We conclude that postembryonic *dsx*-Gal4-expressing cells are not generated in females, because their precursors undergo PCD (sometime between St17 and L2l), and, when forced to survive, they form the male-specific clusters and projections (Fig. 8C).

Because PCD of sex-specific NBs occurs within the domain of strongest Abd-B expression, we investigated whether it is under the control of this Hox gene. To test this, we knocked down *Abd-B* in these cells by driving *Abd-B*-RNAi with *dsx*-Gal4. In both females and males, we found only mild effects. In two L3l females two sex-specific NBs, and in another case one of these NBs, survived; 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

DEVELOPMENT

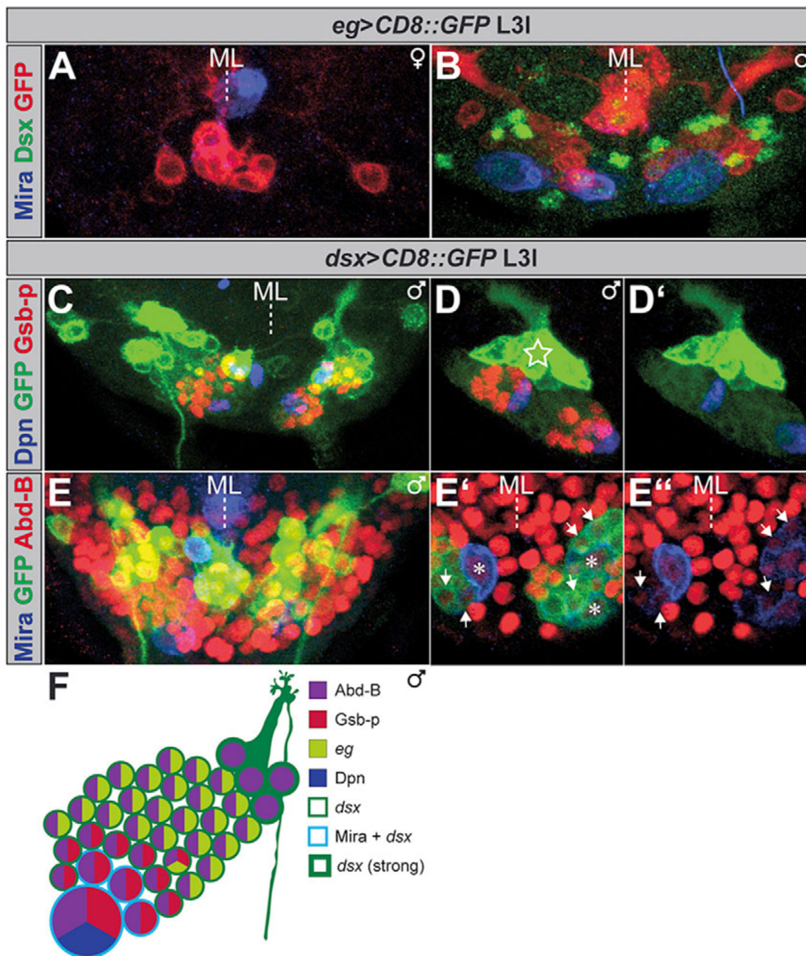

Fig. 6. Further characterisation of male-specific neuroblasts and their lineages in wandering larvae by molecular markers. Horizontal views (anterior to the top) of terminal neuromeres of L3l labelled with different combinations of markers as indicated. ML, midline. (**A**,**B**) Maximum projections of *eg >CD8::GFP* female (A) and male (B) stained for Dsx and Mira. (**C**) Maximum projection of *dsx >CD8::GFP* male stained for Gsb-p and Dpn. (**D**,**D′**) The anterior-dorsal cells, which express *dsx* strongly (white star), are negative for Gsb-p and Dpn (D′ lacks Gsb-p channel). (**E-E″**) Maximum projections of *dsx >CD8::GFP* males stained for Abd-B and Mira. (E′) Single focal plane, showing weak expression of Abd-B in NBs (asterisks) and their GMCs (arrows) (E″ lacks *dsx* channel). (**F**) Cartoon illustrating all the markers analysed in one lateral male-specific lineage of L3l.

(Hoshijima et al., 1991). It has been previously reported that *dsx* controls proliferation of sex-specific NBs. In *tra* or *tra2* mutants, which are epistatic over *dsx*, sex-specific NBs in female larvae were found to proliferate, as in WT males (Taylor and Truman, 1992). In order to test the cell-autonomous function of these factors on the level of the sex-specific NBs, we knocked down *tra* or *tra2* by driving RNAi constructs with *dsx*-Gal4. Driving *tra-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 *dsx-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

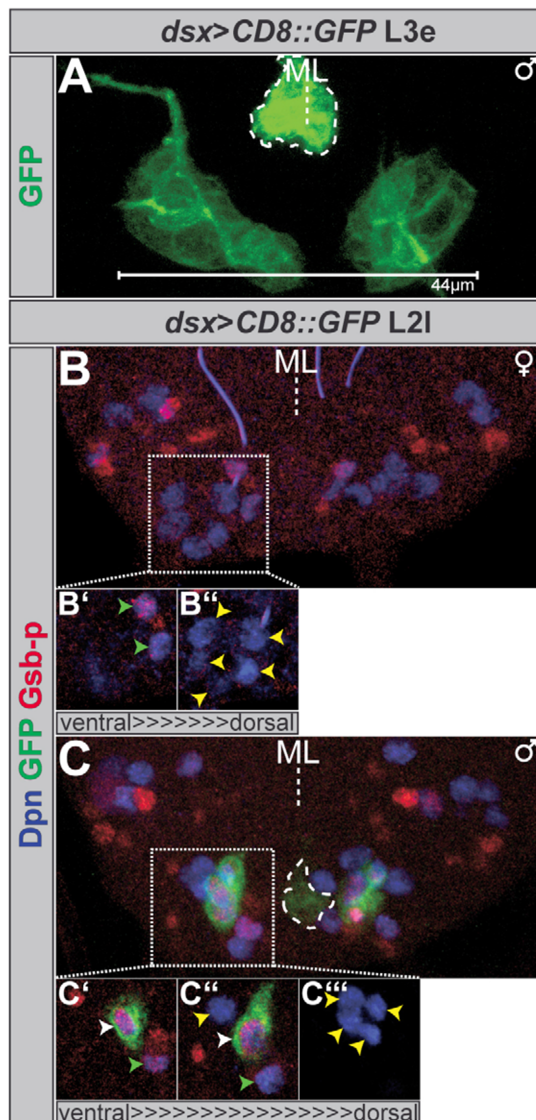

Fig. 7. Sex-specific neuroblasts at late second instar and early third instar larval stages. Horizontal views (anterior to the top) of terminal neuromeres stained against molecular markers as indicated. ML, midline. Midline cells are surrounded by dashed lines. (**A**) *dsx >CD8::GFP* in male early L3 (L3e) larva. (**B-C‴**) *dsx >CD8::GFP* in late L2 (L2l) female (B) and male (C) larvae, stained for 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.

shown for thoracic versus anterior abdominal segments, several serially homologous NBs produce segment-specific lineage variants as a result of differences in specification, PCD and/or proliferation (for reviews, see Rogulja-Ortmann and Technau, 2008; Technau et al., 2006). Correspondingly, we detected specific differences in marker gene expression among serially homologous NBs [e.g. NB3-4 expresses *ming* (*cas*)-*lacZ* in A8 and A9, but not in A10]. Such genes are candidates for being involved in the control of segmental-specific divergence of NB lineages.

The hemineuromeres in A8 show almost the same number (31) and pattern of NBs as the more anterior abdominal hemineuromeres. 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 *dsx*), are tightly attached to each other (Fig. 4) and exhibit very similar postembryonic lineages in males, it may be possible that they are generated by a symmetric division of NB5-4. Alternatively, considering the dynamics of Gsb-d (the activator of Gsb-p) expression in A9-A11 (Baumgartner et al., 1987; Gutjahr et al., 1993), and massive cell migration taking place during condensation of the VNC (Kuhn et al., 1992), it is also possible that NB5-4 of A10 may have moved anteriorly and become closely associated with NB5-4 in A9. However, we cannot exclude the possibility that the second cell represents a different precursor.

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, and Steinmann-Zwicky et al. (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

DEVELOPMENT

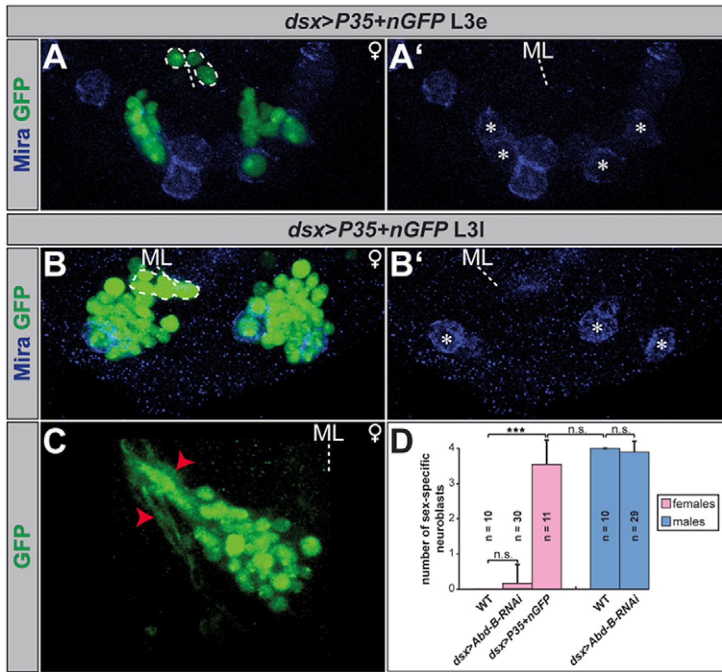

Fig. 8. In females, sex-specific neuroblasts undergo programmed cell death. (**A-C**) Horizontal views (anterior to the top) of terminal neuromeres. ML, midline. (A,B) *dsx >P35+nGFP* stained for Mira and GFP in L3l (A) and L3e (B) females; GFP-expressing midline cells are surrounded by dashed lines. (A′,B′) show only Mira expression. NBs are marked by asterisks. (C) Left terminal-lateral region of *dsx >P35+nGFP* L3l female stained for GFP; maximum projection; red arrowheads mark the male-typical projection. (**D**) Number of sex-specific NBs in L3 larvae of different genotypes (as indicated). n.s., not significant (*P*>0.05); ****P*<0.001. Error bars represent s.d.

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 *dsx* (*dsx[F]*) induces PCD of the sex-specific NBs between St17 and late L2 larval stage (L2l). Upon expression of P35 in females, sex-specific NBs survive and generate male-specific lineages. Ectopic expression of *dsx[F]* in males results in a removal of these precursors, demonstrating the pro-apoptotic effect of *dsx[F]* on sex-specific NBs. However, in a *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 *dsx* mutants sex-specific NBs are missing in both sexes (Taylor and Truman, 1992). This is compatible with the hypothesis that *dsx[M]* is required for survival of these NBs. Consequently, ectopic expression of the male-specific isoform *dsx[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 (Dsx[F]) and survival (Dsx[M]) of sex-specific NBs.

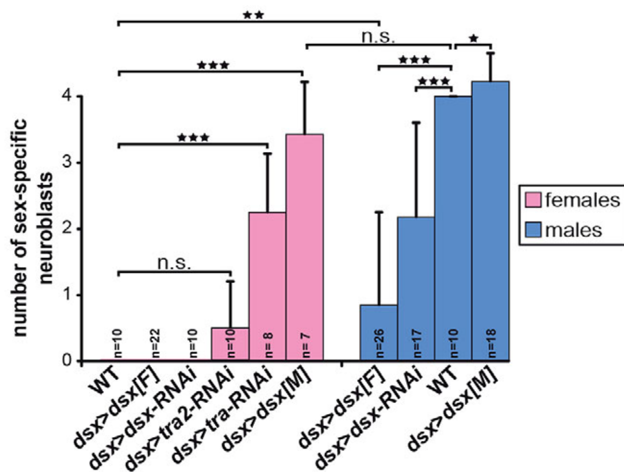

Fig. 9. A dual role for *dsx*. Statistics for sex-specific NBs in L3l larvae of different genotypes (as indicated). n.s., not significant (*P*>0.05); **P*<0.05; ***P*<0.01; ****P*<0.001. Error bars indicate s.d.

A role for *dsx[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 Dsx[F] binding site identified a locus next to the pro-apototic gene *reaper* (*rpr*), which might be a downstream target of Dsx[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 *dsx[M]* seems to be the crucial factor required for their survival. Interestingly, however, *dsx[M]* is not needed for proliferation of the sex-specific NBs or differentiation of their lineages; upon ectopic expression of *P35* in females (lacking *dsx[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 *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; Kohatsu et al., 2011; Rideout et al., 2007; Rideout et al., 2010; Sanders and Arbeitman, 2008; Technau, 1984; von Philipsborn et al., 2011). Although the Dsx transcription factors play a key role in establishing sexual dimorphism in all tissues, they appear to act at different levels (e.g. precursors versus postmitotic progeny cells) and time points of sexual differentiation. Accordingly, dynamic sexually dimorphic *dsx* expression is found throughout postembryonic CNS development (Lee et al., 2002; Rideout et al., 2010; Robinett et al., 2010; Sanders and Arbeitman, 2008; this study).

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.

Acknowledgements
We thank Ana-Rogulja-Ortmann for critically reading the manuscript; Olaf Vef and Ana-Rogulja-Ortmann for their help with fly work; Simone Renner for her help with antibody stainings; Janina Seibert for providing non-fluorescent stainings. We are very grateful to Jonathan Benito-Sipos, Chris Doe, Marta Moris-Sanz, Fernando Diaz-Benjumea, Carmen Robinett, Bruce Baker, Michelle Arbeitman, Dafni Hadjieconomou, Iris Salecker, Matthew Scott, Benjamin Altenhein, Robert Holmgren, Harald Vaessin, Uwe Walldorf, Krzysztof Jagla, Fernando Jimenez, Manfred Frasch, Juergen Knoblich, Tiffany Cook, Ward Odenwald, John Reinitz, James Skeath, Stefan Thor, Matthias Landgraf, Markus Noll, Joachim Urban, the Bloomington Stock Center, the Vienna *Drosophila* RNAi Center and the Developmental Studies Hybridoma Bank (University of Iowa) for providing flies, antibodies and *in situ* probes.

Funding
This work was supported by grants from the 'Deutsche Forschungsgemeinschaft' [TE 130/10; GRK 1044-A1] to G.M.T.

Competing interests statement
The authors declare no competing financial interests.

Supplementary material
Supplementary material available online at
http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.090043/-/DC1

References
Baines, R. A., Robinson, S. G., Fujioka, M., Jaynes, J. B. and Bate, M. (1999). Postsynaptic expression of tetanus toxin light chain blocks synaptogenesis in Drosophila. *Curr. Biol.* **9**, 1267-1270.
Baumgardt, M., Karlsson, D., Terriente, J., Díaz-Benjumea, F. J. and Thor, S. (2009). Neuronal subtype specification within a lineage by opposing temporal feed-forward loops. *Cell* **139**, 969-982.
Baumgartner, S., Bopp, D., Burri, M. and Noll, M. (1987). Structure of two genes at the gooseberry locus related to the paired gene and their spatial expression during Drosophila embryogenesis. *Genes Dev.* **1**, 1247-1267.
Bello, B., Holbro, N. and Reichert, H. (2007). Polycomb group genes are required for neural stem cell survival in postembryonic neurogenesis of Drosophila. *Development* **134**, 1091-1099.
Betschinger, J., Mechtler, K. and Knoblich, J. A. (2006). Asymmetric segregation of the tumor suppressor brat regulates self-renewal in Drosophila neural stem cells. *Cell* **124**, 1241-1253.
Bhat, K. M. (1996). The patched signaling pathway mediates repression of gooseberry allowing neuroblast specification by wingless during Drosophila neurogenesis. *Development* **122**, 2921-2932.
Bhat, K. M. (1999). Segment polarity genes in neuroblast formation and identity specification during Drosophila neurogenesis. *BioEssays* **21**, 472-485.
Bier, E., Vaessin, H., Younger-Shepherd, S., Jan, L. Y. and Jan, Y. N. (1992). deadpan, an essential pan-neural gene in Drosophila, encodes a helix-loop-helix protein similar to the hairy gene product. *Genes Dev.* **6**, 2137-2151.
Billeter, J. C., Villella, A., Allendorfer, J. B., Dornan, A. J., Richardson, M., Gailey, D. A. and Goodwin, S. F. (2006). Isoform-specific control of male neuronal differentiation and behavior in Drosophila by the fruitless gene. *Curr. Biol.* **16**, 1063-1076.
Boll, W. and Noll, M. (2002). The Drosophila Pox neuro gene: control of male courtship behavior and fertility as revealed by a complete dissection of all enhancers. *Development* **129**, 5667-5681.
Bopp, D., Bell, L. R., Cline, T. W. and Schedl, P. (1991). Developmental distribution of female-specific Sex-lethal proteins in Drosophila melanogaster. *Genes Dev.* **5**, 403-415.
Bossing, T., Udolph, G., Doe, C. Q. and Technau, G. M. (1996). The embryonic central nervous system lineages of Drosophila melanogaster. I. Neuroblast lineages derived from the ventral half of the neuroectoderm. *Dev. Biol.* **179**, 41-64.
Broadus, J., Skeath, J. B., Spana, E. P., Bossing, T., Technau, G. and Doe, C. Q. (1995). New neuroblast markers and the origin of the aCC/pCC neurons in the Drosophila central nervous system. *Mech. Dev.* **53**, 393-402.
Buenzow, D. E. and Holmgren, R. (1995). Expression of the Drosophila gooseberry locus defines a subset of neuroblast lineages in the central nervous system. *Dev. Biol.* **170**, 338-349.
Campos-Ortega, J. A. and Hartenstein, V. (1997). *The Embryonic Development of Drosophila melanogaster*. Berlin, Heidelberg, New York, NY: Springer Verlag.
Celniker, S. E., Keelan, D. J. and Lewis, E. B. (1989). The molecular genetics of the bithorax complex of Drosophila: characterization of the products of the Abdominal-B domain. *Genes Dev.* **3**, 1424-1436.
Christiansen, A. E., Keisman, E. L., Ahmad, S. M. and Baker, B. S. (2002). Sex comes in from the cold: the integration of sex and pattern. *Trends Genet.* **18**, 510-516.
Chu, H., Parras, C., White, K. and Jiménez, F. (1998). Formation and specification of ventral neuroblasts is controlled by vnd in Drosophila neurogenesis. *Genes Dev.* **12**, 3613-3624.
Colomb, S., Joly, W., Bonneaud, N. and Maschat, F. (2008). A concerted action of Engrailed and Gooseberry-Neuro in neuroblast 6-4 is triggering the formation of embryonic posterior commissure bundles. *PLoS ONE* **3**, e2197.
DeFalco, T. J., Verney, G., Jenkins, A. B., McCaffery, J. M., Russell, S. and Van Doren, M. (2003). Sex-specific apoptosis regulates sexual dimorphism in the Drosophila embryonic gonad. *Dev. Cell* **5**, 205-216.
De Graeve, F., Jagla, T., Daponte, J. P., Rickert, C., Dastugue, B., Urban, J. and Jagla, K. (2004). The ladybird homeobox genes are essential for the specification of a subpopulation of neural cells. *Dev. Biol.* **270**, 122-134.
DiNardo, S., Kuner, J. M., Theis, J. and O'Farrell, P. H. (1985). Development of embryonic pattern in D. melanogaster as revealed by accumulation of the nuclear engrailed protein. *Cell* **43**, 59-69.
Dittrich, R., Bossing, T., Gould, A. P., Technau, G. M. and Urban, J. (1997). The differentiation of the serotonergic neurons in the Drosophila ventral nerve cord depends on the combined function of the zinc finger proteins Eagle and Huckebein. *Development* **124**, 2515-2525.
Doe, C. Q. (1992). Molecular markers for identified neuroblasts and ganglion mother cells in the Drosophila central nervous system. *Development* **116**, 855-863.
Dormand, E. L. and Brand, A. H. (1998). Runt determines cell fates in the Drosophila embryonic CNS. *Development* **125**, 1659-1667.
Evans, C. J., Olson, J. M., Ngo, K. T., Kim, E., Lee, N. E., Kuoy, E., Patananan, A. N., Sitz, D., Tran, P., Do, M. T. et al. (2009). G-TRACE: rapid Gal4-based cell lineage analysis in Drosophila. *Nat. Methods* **6**, 603-605.
Frasch, M., Hoey, T., Rushlow, C., Doyle, H. and Levine, M. (1987). Characterization and localization of the even-skipped protein of Drosophila. *EMBO J.* **6**, 749-759.
Fujioka, M., Lear, B. C., Landgraf, M., Yusibova, G. L., Zhou, J., Riley, K. M., Patel, N. H. and Jaynes, J. B. (2003). Even-skipped, acting as a repressor, regulates axonal projections in Drosophila. *Development* **130**, 5385-5400.
Gorfinkiel, N., Sánchez, L. and Guerrero, I. (1999). Drosophila terminalia as an appendage-like structure. *Mech. Dev.* **86**, 113-123.
Gutjahr, T., Patel, N. H., Li, X., Goodman, C. S. and Noll, M. (1993). Analysis of the gooseberry locus in Drosophila embryos: gooseberry determines the cuticular pattern and activates gooseberry neuro. *Development* **118**, 21-31.
Hadjieconomou, D., Rotkopf, S., Alexandre, C., Bell, D. M., Dickson, B. J. and Salecker, I. (2011). Flybow: genetic multicolor cell labeling for neural circuit analysis in Drosophila melanogaster. *Nat. Methods* **8**, 260-266.
Hartenstein, V. and Campos-Ortega, J. A. (1984). Early neurogenesis in wild-type Drosophila melanogaster. *Roux's Arch. Dev. Biol.* **193**, 308-325.
Hartmann, B., Hirth, F., Walldorf, U. and Reichert, H. (2000). Expression, regulation and function of the homeobox gene empty spiracles in brain and ventral nerve cord development of Drosophila. *Mech. Dev.* **90**, 143-153.
Häsemeyer, M., Yapici, N., Heberlein, U. and Dickson, B. J. (2009). Sensory neurons in the Drosophila genital tract regulate female reproductive behavior. *Neuron* **61**, 511-518.
Hay, B. A., Wolff, T. and Rubin, G. M. (1994). Expression of baculovirus P35 prevents cell death in Drosophila. *Development* **120**, 2121-2129.

Higashijima, S., Shishido, E., Matsuzaki, M. and Saigo, K. (1996). eagle, a member of the steroid receptor gene superfamily, is expressed in a subset of neuroblasts and regulates the fate of their putative progeny in the Drosophila CNS. *Development* **122**, 527-536.

Hoshijima, K., Inoue, K., Higuchi, I., Sakamoto, H. and Shimura, Y. (1991). Control of doublesex alternative splicing by transformer and transformer-2 in Drosophila. *Science* **252**, 833-836.

Ikeshima-Kataoka, H., Skeath, J. B., Nabeshima, Y., Doe, C. Q. and Matsuzaki, F. (1997). Miranda directs Prospero to a daughter cell during Drosophila asymmetric divisions. *Nature* **390**, 625-629.

Isshiki, T., Takeichi, M. and Nose, A. (1997). The role of the msh homeobox gene during Drosophila neurogenesis: implication for the dorsoventral specification of the neuroectoderm. *Development* **124**, 3099-3109.

Ito, K., Urban, J. and Technau, G. M. (1995). Distribution, classification, and development of Drosophila glial cells in the late embryonic and early larval ventral nerve cord. *Roux's Arch. Dev. Biol.* **204**, 284-307.

Jagla, K., Jagla, T., Heitzler, P., Dretzen, G., Bellard, F. and Bellard, M. (1997). ladybird, a tandem of homeobox genes that maintain late wingless expression in terminal and dorsal epidermis of the Drosophila embryo. *Development* **124**, 91-100.

Juergens, G. (1987). Segmental organisation of the tail region in the embryo of Drosophila melanogaster. *Roux's Arch. Dev. Biol.* **196**, 141-157.

Kambadur, R., Koizumi, K., Stivers, C., Nagle, J., Poole, S. J. and Odenwald, W. F. (1998). Regulation of POU genes by castor and hunchback establishes layered compartments in the Drosophila CNS. *Genes Dev.* **12**, 246-260.

Kammermeier, L., Leemans, R., Hirth, F., Flister, S., Wenger, U., Walldorf, U., Gehring, W. J. and Reichert, H. (2001). Differential expression and function of the Drosophila Pax6 genes eyeless and twin of eyeless in embryonic central nervous system development. *Mech. Dev.* **103**, 71-78.

Kimura, K., Hachiya, T., Koganezawa, M., Tazawa, T. and Yamamoto, D. (2008). Fruitless and doublesex coordinate to generate male-specific neurons that can initiate courtship. *Neuron* **59**, 759-769.

Kohatsu, S., Koganezawa, M. and Yamamoto, D. (2011). Female contact activates male-specific interneurons that trigger stereotypic courtship behavior in Drosophila. *Neuron* **69**, 498-508.

Kosman, D., Small, S. and Reinitz, J. (1998). Rapid preparation of a panel of polyclonal antibodies to Drosophila segmentation proteins. *Dev. Genes Evol.* **208**, 290-294.

Kuhn, D. T., Sawyer, M., Packert, G., Turenchalk, G., Mack, J. A., Sprey, T. E., Gustavson, E. and Kornberg, T. B. (1992). Development of the D. melanogaster caudal segments involves suppression of the ventral regions of A8, A9 and A10. *Development* **116**, 11-20.

Lacin, H., Zhu, Y., Wilson, B. A. and Skeath, J. B. (2009). dbx mediates neuronal specification and differentiation through cross-repressive, lineage-specific interactions with eve and hb9. *Development* **136**, 3257-3266.

Landgraf, M., Jeffrey, V., Fujioka, M., Jaynes, J. B. and Bate, M. (2003a). Embryonic origins of a motor system: motor dendrites form a myotopic map in Drosophila. *PLoS Biol.* **1**, e41.

Landgraf, M., Sánchez-Soriano, N., Technau, G. M., Urban, J. and Prokop, A. (2003b). Charting the Drosophila neuropile: a strategy for the standardised characterisation of genetically amenable neurites. *Dev. Biol.* **260**, 207-225.

Lee, G., Hall, J. C. and Park, J. H. (2002). Doublesex gene expression in the central nervous system of Drosophila melanogaster. *J. Neurogenet.* **16**, 229-248.

Lewis, E. B. (1978). A gene complex controlling segmentation in Drosophila. *Nature* **276**, 565-570.

Luo, S. D., Shi, G. W. and Baker, B. S. (2011). Direct targets of the D. melanogaster DSXF protein and the evolution of sexual development. *Development* **138**, 2761-2771.

McDonald, J. A., Holbrook, S., Isshiki, T., Weiss, J., Doe, C. Q. and Mellerick, D. M. (1998). Dorsoventral patterning in the Drosophila central nervous system: the vnd homeobox gene specifies ventral column identity. *Genes Dev.* **12**, 3603-3612.

Mettler, U., Vogler, G. and Urban, J. (2006). Timing of identity: spatiotemporal regulation of hunchback in neuroblast lineages of Drosophila by Seven-up and Prospero. *Development* **133**, 429-437.

Monastirioti, M. (2003). Distinct octopamine cell population residing in the CNS abdominal ganglion controls ovulation in Drosophila melanogaster. *Dev. Biol.* **264**, 38-49.

Ni, J. Q., Liu, L. P., Binari, R., Hardy, R., Shim, H. S., Cavallaro, A., Booker, M., Pfeiffer, B. D., Markstein, M., Wang, H. et al. (2009). A Drosophila resource of transgenic RNAi lines for neurogenetics. *Genetics* **182**, 1089-1100.

Noordermeer, J. N., Kopczynski, C. C., Fetter, R. D., Bland, K. S., Chen, W. Y. and Goodman, C. S. (1998). Wrapper, a novel member of the Ig superfamily, is expressed by midline glia and is required for them to ensheath commissural axons in Drosophila. *Neuron* **21**, 991-1001.

Patel, N. H. (1994). Imaging neuronal subsets and other cell types in whole mount Drosophila embryos and larvae using antibody probes. In *Methods in Cell Biology. Drosophila melanogaster: Practical Uses in Cell Biology*, Vol. 44 (ed. L. S. Goldstein and E. Fyrberg), pp. 445-487. New York, NY: Academic Press.

Patel, N. H., Martin-Blanco, E., Coleman, K. G., Poole, S. J., Ellis, M. C., Kornberg, T. B. and Goodman, C. S. (1989). Expression of engrailed proteins in arthropods, annelids, and chordates. *Cell* **58**, 955-968.

Plickert, G., Gajewski, M., Gehrke, G., Gausepohl, H., Schlossherr, J. and Ibrahim, H. (1997). Automated in situ detection (AISD) of biomolecules. *Dev. Genes Evol.* **207**, 362-367.

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

Rezával, C., Pavlou, H. J., Dornan, A. J., Chan, Y. B., Kravitz, E. A. and Goodwin, S. F. (2012). Neural circuitry underlying Drosophila female postmating behavioral responses. *Curr. Biol.* **22**, 1155-1165.

Rideout, E. J., Billeter, J.-C. and Goodwin, S. F. (2007). The sex-determination genes fruitless and doublesex specify a neural substrate required for courtship song. *Curr. Biol.* **17**, 1473-1478.

Rideout, E. J., Dornan, A. J., Neville, M. C., Eadie, S. and Goodwin, S. F. (2010). Control of sexual differentiation and behavior by the doublesex gene in Drosophila melanogaster. *Nat. Neurosci.* **13**, 458-466.

Robinett, C. C., Vaughan, A. G., Knapp, J. M. and Baker, B. S. (2010). Sex and the single cell. II. There is a time and place for sex. *PLoS Biol.* **8**, e1000365.

Rogulja-Ortmann, A. and Technau, G. M. (2008). Multiple roles for Hox genes in segment-specific shaping of CNS lineages. *Fly (Austin)* **2**, 316-319.

Sanders, L. E. and Arbeitman, M. N. (2008). Doublesex establishes sexual dimorphism in the Drosophila central nervous system in an isoform-dependent manner by directing cell number. *Dev. Biol.* **320**, 378-390.

Schmid, A., Chiba, A. and Doe, C. Q. (1999). Clonal analysis of Drosophila embryonic neuroblasts: neural cell types, axon projections and muscle targets. *Development* **126**, 4653-4689.

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

Schütt, C. and Nöthiger, R. (2000). Structure, function and evolution of sex-determining systems in Dipteran insects. *Development* **127**, 667-677.

Shen, C. P., Jan, L. Y. and Jan, Y. N. (1997). Miranda is required for the asymmetric localization of Prospero during mitosis in Drosophila. *Cell* **90**, 449-458.

Skeath, J. B. (1999). At the nexus between pattern formation and cell-type specification: the generation of individual neuroblast fates in the Drosophila embryonic central nervous system. *BioEssays* **21**, 922-931.

Skeath, J. B., Zhang, Y., Holmgren, R., Carroll, S. B. and Doe, C. Q. (1995). Specification of neuroblast identity in the Drosophila embryonic central nervous system by gooseberry-distal. *Nature* **376**, 427-430.

Steinmann-Zwicky, M., Amrein, H. and Nöthiger, R. (1990). Genetic control of sex determination in Drosophila. *Adv. Genet.* **27**, 189-237.

Tautz, D. and Pfeifle, C. (1989). A non-radioactive in situ hybridization method for the localization of specific RNAs in Drosophila embryos reveals translational control of the segmentation gene hunchback. *Chromosoma* **98**, 81-85.

Taylor, B. J. and Truman, J. W. (1992). Commitment of abdominal neuroblasts in Drosophila to a male or female fate is dependent on genes of the sex-determining hierarchy. *Development* **114**, 625-642.

Technau, G. M. (1984). Fiber number in the mushroom bodies of adult Drosophila melanogaster depends on age, sex and experience. *J. Neurogenet.* **1**, 113-126.

Technau, G. M., Berger, C. and Urbach, R. (2006). Generation of cell diversity and segmental pattern in the embryonic central nervous system of Drosophila. *Dev. Dyn.* **235**, 861-869.

Truman, J. W. and Bate, M. (1988). Spatial and temporal patterns of neurogenesis in the central nervous system of Drosophila melanogaster. *Dev. Biol.* **125**, 145-157.

Urbach, R. and Technau, G. M. (2003a). Molecular markers for identified neuroblasts in the developing brain of Drosophila. *Development* **130**, 3621-3637.

Urbach, R. and Technau, G. M. (2003b). Segment polarity and DV patterning gene expression reveals segmental organization of the Drosophila brain. *Development* **130**, 3607-3620.

Urbach, R. and Technau, G. M. (2004). Neuroblast formation and patterning during early brain development in Drosophila. *BioEssays* **26**, 739-751.

Urbach, R., Schnabel, R. and Technau, G. M. (2003). The pattern of neuroblast formation, mitotic domains and proneural gene expression during early brain development in Drosophila. *Development* **130**, 3589-3606.

von Hilchen, C. M., Beckervordersandforth, R. M., Rickert, C., Technau, G. M. and Altenhein, B. (2008). Identity, origin, and migration of peripheral glial cells in the Drosophila embryo. *Mech. Dev.* **125**, 337-352.

von Hilchen, C. M., Hein, I., Technau, G. M. and Altenhein, B. (2010). Netrins guide migration of distinct glial cells in the Drosophila embryo. *Development* **137**, 1251-1262.

von Philipsborn, A. C., Liu, T., Yu, J. Y., Masser, C., Bidaye, S. S. and Dickson, B. J. (2011). Neuronal control of Drosophila courtship song. *Neuron* **69**, 509-522.

DEVELOPMENT

Walldorf, U. and Gehring, W. J. (1992). Empty spiracles, a gap gene containing a homeobox involved in Drosophila head development. *EMBO J.* **11**, 2247-2259.

Wang, W. and Yoder, J. H. (2012). Hox-mediated regulation of doublesex sculpts sex-specific abdomen morphology in Drosophila. *Dev. Dyn.* **241**, 1076-1090.

Weiss, J. B., Von Ohlen, T., Mellerick, D. M., Dressler, G., Doe, C. Q. and Scott, M. P. (1998). Dorsoventral patterning in the Drosophila central nervous system: the intermediate neuroblasts defective homeobox gene specifies intermediate column identity. *Genes Dev.* **12**, 3591-3602.

Wheeler, S. R., Kearney, J. B., Guardiola, A. R. and Crews, S. T. (2006). Single-cell mapping of neural and glial gene expression in the developing Drosophila CNS midline cells. *Dev. Biol.* **294**, 509-524.

White, K., Grether, M. E., Abrams, J. M., Young, L., Farrell, K. and Steller, H. (1994). Genetic control of programmed cell death in Drosophila. *Science* **264**, 677-683.

Williams, T. M., Selegue, J. E., Werner, T., Gompel, N., Kopp, A. and Carroll, S. B. (2008). The regulation and evolution of a genetic switch controlling sexually dimorphic traits in Drosophila. *Cell* **134**, 610-623.

Xie, B., Charlton-Perkins, M., McDonald, E., Gebelein, B. and Cook, T. (2007). Senseless functions as a molecular switch for color photoreceptor differentiation in Drosophila. *Development* **134**, 4243-4253.

Xiong, W. C., Okano, H., Patel, N. H., Blendy, J. A. and Montell, C. (1994). repo encodes a glial-specific homeo domain protein required in the Drosophila nervous system. *Genes Dev.* **8**, 981-994.

Zhang, Y., Ungar, A., Fresquez, C. and Holmgren, R. (1994). Ectopic expression of either the Drosophila gooseberry-distal or proximal gene causes alterations of cell fate in the epidermis and central nervous system. *Development* **120**, 1151-1161.

DEVELOPMENT