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Segment-specific generation of *Drosophila* Capability neuropeptide neurons by multi-faceted Hox cues

Anke Suska [a], Irene Miguel-Aliaga [b], Stefan Thor [a,*]

[a] Department of Clinical and Experimental Medicine, Linkoping University, Linkoping, SE-58185, Sweden
[b] University of Cambridge, Cambridge, CB2 3EJ, UK

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ABSTRACT

In the *Drosophila* ventral nerve cord, the three pairs of Capability neuropeptide-expressing Va neurons are exclusively found in the second, third and fourth abdominal segments (A2–A4). To address the underlying mechanisms behind such segment-specific cell specification, we followed the developmental specification of these neurons. We find that Va neurons are initially generated in all ventral nerve cord segments and progress along a common differentiation path. However, their terminal differentiation only manifests itself in A2–A4, due to two distinct mechanisms: segment-specific programmed cell death (PCD) in posterior segments, and differentiation to an alternative identity in segments anterior to A2. Genetic analyses reveal that the Hox homeotic genes are involved in the segment-specific appearance of Va neurons. In posterior segments, the Hox gene *Abdominal-B* exerts a pro-apoptotic role on Va neurons, which involves the function of several RHG genes. Strikingly, this role of *Abd-B* is completely opposite to its role in the segment-specific apoptosis of other classes of neuropeptide neurons, the dMP2 and MP1 neurons, where *Abd-B* acts in an anti-apoptotic manner. In segments A2–A4 we find that *abdominal A* is important for the terminal differentiation of Va cell fate. In the A1 segment, *Ultrabithorax* acts to specify an alternate Va neuron fate. In contrast, in thoracic segments, *Antennapedia* suppresses the Va cell fate. Thus, Hox genes act in a multi-faceted manner to control the segment-specific appearance of the Va neuropeptide neurons in the ventral nerve cord.

Introduction

A common feature of most nervous systems is the appearance of unique neuronal subtypes only within certain segments. Studies have shown that this may result from several developmental mechanisms, the first of which is the segment-specific generation of unique sets of progenitor cells—for example, certain segments of the *Drosophila* developing CNS (tritocerebrum (B3) and suboesophageal 1 (S1)) appear to contain fewer progenitor cells (neuroblasts) (Urbach and Technau, 2004). Second, segment-specific lineage size control has been described, such that equivalent neuroblasts may generate different sized lineages in different segments (Schmid et al., 1999; Schmidt et al., 1997). This may be due either to differences in lineage progression (by apoptosis or cell cycle exit) or to early changes in asymmetric cell division by segment-specific control of genes affecting progenitor behavior (Berger et al., 2005a). Third, once generated, segment-specific events may dictate distinct terminal cell fates in different segments (Karlsson et al., 2010). Finally, neurons may be generated throughout the neuro-axes, and be similarly specified, but may be removed in a segment-specific manner by subsequent apoptosis (Miguel-Aliaga and Thor, 2004; Rogulja-Ortmann et al., 2008). The Hox homeotic genes have been found to be involved in several of these events, but our understanding of Hox gene involvement in these processes is still rudimentary.

The *Drosophila* ventral nerve cord (VNC) contains an estimated 10,000 cells, the majority of which are neurons. Within the neuronal subset, only 200 cells specifically express neuropeptides and are often referred to as being peptidergic (Miguel-Aliaga et al., 2004; Park et al., 2008). Each peptidergic subclass typically expresses only one out of a total of some 30 neuropeptide genes (Park et al., 2008). Intriguingly, although the VNC consists of repetitive segments (neuromeres), often containing similar sets of neurons and glia, peptidergic neurons display striking segment specificity (Miguel-Aliaga et al., 2004; Park et al., 2008). Undoubtedly, this segment specificity plays important roles to control segment- and region-specific physiological output. But in addition, and most importantly for this study, the segment-specific appearance of peptidergic neurons, together with their robust expression of different neuropeptide genes, makes them excellent "model neurons" for addressing segment-specific neuronal subtype generation and differentiation. We have previously capitalized upon this notion and studied several segment-specific peptidergic neurons, such as the A6–A8-specific dMP2 and MP1 neurons (Miguel-Aliaga and Thor, 2004), as well as the thoracic-specific Nplp1 and FMRFamide neurons (Karlsson et al., 2010). These studies have identified segment-

* Corresponding author.
E-mail address: stefan.thor@liu.se (S. Thor).

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specific lineage truncation, cell fate specification, as well as apoptosis as underlying mechanisms. In all three mechanisms, homeotic genes of the Hox family were found to play critical roles.

Here, we address the segment-specific appearance of another class of peptidergic neurons, the Capability (Capa) expressing Va neurons (O'Brien and Taghert, 1998). Using a number of markers, we find that these neurons are initially generated in all VNC segments, and partially differentiate, as evident by expression of several cell-fate determinants including Capa itself. By stage 16, Va neurons in all abdominal segments posterior to A4 undergo apoptosis, while the A1–A4 Va neurons survive into larval stages. However, from these four segments, only A2–A4 contains Va neurons which express Capa. Similar to dMP2 and MP1, the RHG genes are also involved in Va neuron PCD. In addition, we find that Va PCD is under the control of *Abdominal-B*, but in contrast to its function in the related peptidergic dMP2 and MP1 neurons, in Va neurons it acts in a pro-apoptotic role. These findings reveal striking differences in Hox gene function with respect to PCD in related neurons and provide a platform for addressing context-dependent Hox function with respect to PCD. The other Bithorax-complex Hox genes, *abdominal A* and *Ultrabithorax*, in turn are important for the differentiation of Va neurons. In contrast, the *Antennapedia* Hox gene suppresses Va neuron differentiation in thoracic segments. Thus, Hox genes act in several ways to ensure the segment-specific appearance of a distinct set of neurons.

Materials and methods

Fly stocks

The following fly stocks were used: *abd-A* mutants: *abd-A*^{P10}, *abd-A*^{MX1}. *Abd-Bm* mutants: *Abd-B*^{M1}, *Abd-B*^{M2} (Sanchez-Herrero et al., 1985). *Antp* mutants: *Antp*^{12}, *Antp*^{14}. *Ubx* mutants: *Ubx*$^{9.22}$, *Ubx*$^{9.27}$; *UAS-Antp*, *UAS-Ubx*, *UAS-abd-A* (obtained from F. Hirth); *UAS-Abd-Bm* (obtained from J. Castelli-Gair); *UAS-Apoliner* (obtained from P.-L. Bardet); *dac-Gal4* (obtained from G. Mardon); *H99*, *XR38*, *X14* and *X25* deficiencies (obtained from K. White); *elav*^{GAL4} (*elavC155*). *w*^{1118} was used as a wild-type strain. Mutations were maintained over standard balancers with *GFP* markers. Mutants were identified by the absence of *GFP* expression.

Immunohistochemistry

Immunohistochemistry was carried out as previously described (Allan et al., 2003). Rabbit α-proCapa was generated by injecting rabbits (Agrisera AB, Umea, Sweden) with a synthetic peptide (CKRSVDAKSFADISKGQKELN) corresponding to the C-terminal part of proCapa (Flybase). Antibodies were affinity-purified, pre-absorbed against early embryos, and used at 1:1000. Other antibodies used were mouse α-Dac dac2-3 (1:25) (Developmental Studies Hybridoma Bank), rabbit α-caspase-3 mAb (1:50) (Cell Signaling Technology), guinea pig α-Dimm (1:500) (Baumgardt et al., 2007), mAb α-Ubx (FP3.38; 1:10) (provided by R. White), mAb α-Abd-A (1:400) (provided by I. Duncan); mAb α-Antp (1:10) and mAb α-Abd-B (1:10) (Developmental Studies Hybridoma Bank, Iowa City, IA, US). FITC-, Rhodamine-Red-X- and Cy5-conjugated secondary antibodies were obtained from Jackson Immunolabs and used at 1:200 (1:100 for the Cy5-conjugated antibody).

Confocal imaging and data acquisition

Zeiss LSM 5 or Zeiss META 510 Confocal microscopes were used to collect data for all fluorescent images; confocal stacks were merged using LSM software or Corel Paint Shop Pro Photo X2 (Ottawa, Canada). Statistical analysis was performed using Microsoft Excel.

Results

Developmental appearance of the peptidergic, Capability-expressing Va neurons

In the *Drosophila* larval VNC, a set of six peptidergic neurons expresses the Capa neuropeptides, encoded by the *capability* gene (Kean et al., 2002). In the larvae, these neurons are present only in three of the abdominal segments (A2–A4), located at the ventral surface of the nerve cord; hence they have been named ventral–abdominal (Va) neurons (O'Brien and Taghert, 1998). They can be identified already at late embryonic stages of *Drosophila* development (Fig. 1F), but their developmental appearance was hitherto not investigated in detail. To address the segment-specific generation of Va neurons, we examined the expression of Capa throughout embryonic development. Staining for proCapa revealed that peptidergic Va neurons are apparent already at stage 16. Surprisingly, at this stage, proCapa expression was not confined to segments A2, A3 and A4 (Fig. 1C). By embryonic stage 17, however, the expression of Capa was restricted to the three pairs of neurons in A2–A4 (Fig. 1E–F), similar to that observed at larval stages (not shown; Kean et al., 2002). In addition to the six Va neurons present in the VNC, a pair of Capa expressing neurons are also found in the S1 segment (Fig. 1F; Kean et al., 2002). However in this study we will address the generation of Va neurons in the VNC i.e. segments T1–T3 and A1–A9.

The bHLH transcription factor Dimmed (Dimm) is selectively expressed in the majority of peptidergic neurons (Baumgardt et al., 2007; Hewes et al., 2003; Miguel-Aliaga et al., 2004; Park et al., 2004; Park et al., 2008) and is important for the high-level neuropetide expression and high-level secretory cellular properties (Baumgardt et al., 2007; Hamanaka et al., 2011; Hewes et al., 2003; Miguel-Aliaga et al., 2004; Park et al., 2004). As anticipated, Dimm was found to be expressed also in Capa-positive neurons. Additionally, the transcriptional co-factor Dachshund (Dac) is expressed in the Va cells (Miguel-Aliaga et al., 2004). Thus, Dimm/Dac co-expression will exclusively label the Va neurons and will label these neurons prior to Capa neuropeptide expression. Using these markers, we can first observe Va neurons in mid-abdominal segments at stage 14 (Fig. 1A), and from there we observe them in progression both anteriorly and posteriorly. Interestingly, at stage 15, Dimm/Dac-positive cells were present almost in every abdominal segment, although the expression gradually weakened towards the posterior end (Fig. 1B). In thoracic segments, expression of Dimm in the same position as the abdominal Dimm/Dac cells suggested the presence of Va neurons, but in this case co-expression with Dac was not observed (Fig. 1B). At stage 16, every abdominal segment expressed Dac/Dimm, and Capa starts to appear in segments A2 to A6. In thoracic segments the expression of Dimm disappeared, and in subsequent stages no Va neurons could be identified (Fig. 1C). At stage 17 and 18 hours after egg laying (18hAEL), the expression pattern mimicked the situation in larval stages, where only three pairs of Capa-expressing neurons co-express Dimm and Dac, and the first abdominal segment, A1 shows positive staining for Dimm/Dac, but lacks the expression of the neuropeptide (Fig. 1E–F).

The temporal appearance and disappearance of Va neuron markers raised interesting questions regarding the fate of the posterior and the anterior Va neurons. Does their absence indicate a specific down-regulation of the respective transcription factors, or is it a result of selective cell death?

Va neurons are removed from posterior segments by programmed cell death

Central to programmed cell death (PCD) is the highly conserved caspase family of cysteine proteases (Bergmann et al., 1998). During development, PCD can readily be detected using an antibody against the cleaved and active form of caspase-3 (Nicholson et al., 1995). At embryonic stage 16, caspase-3 staining was observed in posterior Va