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Evolutionary variations in the expression of dorso-ventral patterning genes and the conservation of pioneer neurons in *Tribolium castaneum*

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ABSTRACT

Insects are ideally suited for gaining insight into the evolutionary developmental mechanisms that have led to adaptive changes of the nervous system since the specific structure of the nervous system can be directly linked to the neural stem cell (neuroblast) lineages, which in turn can be traced back to the last common ancestor of insects. The recent comparative analysis of the *Drosophila melanogaster* and *Tribolium castaneum* neuroblast maps revealed substantial differences in the expression profiles of neuroblasts. Here we show that despite the overall conservation of the dorso-ventral expression domains of *muscle segment homeobox*, *intermediate neuroblasts defective* and *ventral nervous system defective*, the expression of these genes relative to the neuroblasts in the respective domains has changed considerably during insect evolution. Furthermore, functional studies show evolutionary changes in the requirement of *ventral nervous system defective* in the formation of neuroblast 1-1 and the correct differentiation of its presumptive progeny, the pioneer neurons aCC and pCC. The inclusion of the expression data of the dorso-ventral genes into the recently established *T. castaneum* neuroblast map further increases the differences in the neuroblast expression profiles between *D. melanogaster* and *T. castaneum*. Despite these molecular variations, the Even-skipped positive pioneer neurons show an invariant arrangement, except for an additional Even-skipped positive cluster that we discovered in *T. castaneum*. Given the importance of these pioneer neurons in establishing the intersegmental nerves and the longitudinal tracts, which are part of the conserved axonal scaffold of arthropods, we discuss internal buffering mechanisms that might ensure that neuroblast lineages invariantly generate pioneer neurons over a wide range of molecular variations.

Introduction

Q2 Insects belong to the most diverse and species rich animal phylum with over 1 million described species. Despite the high variation in shape and behaviour, the nervous system shows a conserved structure consisting of a tripartite brain, which is connected to the rope-ladder-like axonal scaffold of the gnathal and trunk ganglia (Anderson, 1973; Cong et al., 2014; Scholtz and Edgecombe, 2006). Although in many cases ganglia are fused in the adults, the conserved axonal pattern is initially established in all insect embryos. This raises the question of which mechanisms determine the developmental robustness of axonal scaffold formation on one hand and how evolutionary modifications have been implemented to accommodate structural and functional morphological changes in the individual species on the other hand.

In insects the nervous system is generated by neural stem cells (neuroblasts), which arise from the neuroepithelium. In the part of the neuroepithelium which gives rise to the ventral nerve chord (thoracic and abdominal ganglia), the arrangement of individual neuroblasts has been compared in several different insect species (Bate, 1976; Breidbach and Urbach, 1996; Doe, 1992; Doe and Goodman, 1985; Hartenstein and Campos-Ortega, 1984; Shepherd and Bate, 1990; Tamarelle et al., 1985; Truman and Ball, 1998). Neuroblast maps have been established mainly for thoracic hemineuromeres and show a striking conservation in their arrangement. Since neuroblasts have evolved in the last common ancestor of hexapods and crustaceans, they can be considered as homologous structures (Eriksson and Stollewerk, 2010). The homology has even been extended to individual neuroblasts in several cases where neuroblasts located in similar positions were shown to produce the same lineages in different insect and crustacean species (Thomas et al., 1984; Ungerer and Scholtz, 2008). Even-skipped (Eve), for example, is one of the early motor and interneuronal markers that revealed conserved subsets of pioneer neurons in hexapods and crustaceans (Duman-Scheel and Patel, 1999; Thomas et al., 1984).

In *Drosophila melanogaster* detailed molecular and morphological data are available for every single ventral neuroblast and its lineage (Doe, 1992; Landgraf et al., 1997; Schmid et al., 1999;

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Schmidt et al., 1997). We have recently established a neuroblast map for the flour beetle *Tribolium castaneum* based on parameters that have been used for the published neuroblast map of *D. melanogaster* (Biffar and Stollewerk, 2014; Doe, 1992). The data show that for almost all neuroblasts the relative positions in the ventral hemi-neuromeres are conserved; however, in over half of the neuroblasts the time of formation as well as the gene expression profile has changed (Biffar and Stollewerk, 2014). Thus, there seems to be a strong developmental constraint to maintain the precise arrangement despite molecular changes. The *T. castaneum* neuroblast map now provides a framework for comparing individual neuroblasts and their lineages in *D. melanogaster* and *T. castaneum* as well as for understanding the correlation between molecular variation and evolution of morphology.

In *D. melanogaster* pre-patterning mechanisms in the ventral neuroectoderm (VNE) result in the precise positioning of neuroblasts in each neuromere (reviewed by Skeath (1999)). In addition, the patterning mechanisms result in the generation of unique neuroblast expression profiles, which in turn determine the number and identity of progeny produced in each lineage (e.g., Skeath and Thor, 2003; Thor and Thomas, 1997). For example, the combinatorial expression of *huckebein*, *fushi tarazu*, *prospero* and *pdm1* is required in the neuroblast lineage 4-2 for the activation of *eve* expression and thus the correct differentiation of the RP2 motoneurons which emerge from this lineage (McDonald et al., 2003). Furthermore, the importance of the pre-patterning mechanism for the identity and composition of neuroblast lineages was demonstrated by heterochronic transplantation experiments (Berger et al., 2001).

Here we focus on the dorso-ventral patterning genes *ventral nerve cord defective* (*vnd*), *intermediate neuroblast defective* (*ind*) and *muscle segment homeobox* (*msh*). The genes are expressed in three longitudinal columns in the ventral neuroectoderm both in *D. melanogaster* and *T. castaneum* (Skeath, 1999; Wheeler et al., 2005). In *D. melanogaster*, the boundaries between the expression domains of the medial dorso-ventral patterning gene *vnd* and the intermediate gene *ind* are maintained by mutual repression. This is not the case in *T. castaneum*. Although *Tc-vnd* seems to repress the medial expansion of *Tc-ind* expression domain in the neuroectoderm of *T. castaneum*, *Tc-vnd* expression is largely unchanged in *Tc-ind* loss of function embryos (Wheeler et al., 2005). Furthermore, the separate expression domains of *ind* and the lateral dorso-ventral patterning gene *msh* are maintained by *ind* repressing *msh* in *D. melanogaster*, while this seems not to be the case in *T. castenum* (McDonald et al., 1998; Weiss et al., 1998; Wheeler et al., 2005). However, in both insects 80–90% of neuroblasts are missing in the respective domains in *vnd* and *ind* loss of function embryos, while *msh* seems not to be required for the formation of neuroblasts (Chu et al., 1998; Weiss et al., 1998; Isshiki et al., 1997; Wheeler et al., 1995).

Although the expression of the dorso-ventral patterning genes has been described in *T. castaneum*, it has not been related to individual neuroblasts. Using the previously established *T. castaneum* neuroblast map (Biffar and Stollewerk, 2014), we show here significant variations in the expression of the dorso-ventral patterning genes in individual neuroblasts between *T. castaneum* and *D. melanogaster*. In order to elucidate the consequences of these variations, we compare the pattern of pioneer neurons that establish the axonal tracts in these insect species and analyse the role of *Tc-vnd* in the formation of the Eve pattern by RNA interference (RNAi).

Materials and methods

In situ detection of expression patterns and documentation

Antibody and nuclei staining as well as in situ hybridisation and documentation were performed as described in Biffar and Stollewerk (2014). In addition to the standard antibodies described in Biffar and Stollewerk (2014), we used the anti-Even-skipped antibody 3C10 (1:20) produced against grasshopper Eve (Patel et al., 1992), which cross-reacts with the Eve protein in *T. castaneum* (Brown and Denell, 1996). Furthermore, we used the anti-Engrailed antibody (4D9, 1:20), produced against *D. melanogaster* Engrailed/Invected, which has been shown to detect Engrailed antigens in various arthropods and other invertebrates (Patel et al., 1989).

PCR cloning

Standard procedures were used for PCR cloning. Primer sequences were designed using the freely available Primer3 software (primer3.sourceforge.net). The sequenced *T. castaneum* genome (Tribolium Genome Sequencing Consortium et al., 2008) allowed for designing specific primers for amplification of the desired gene fragments from cDNA. Primers were ordered from Sigma Aldrich Ltd. UK.

Embryonic RNA interference

RNA interference was performed as described by Posnien et al. (2009). The following primers were used for amplifying DNA fragments of *Tc-vnd* which were used to produce double-stranded RNA:

Number	Forward	Reverse	Length (bp)
1	TTACACTCC-CATCACCAGCA	GAACATCGGC-CACTGAATTT	800
2	CCATCCTGCC-CAACTCAG	TGACCTGACC-GAAACAACAA	726
3	AAAACAA-CAACCCCTTGC-CG	TTGTACAATT-GAGAACGAAGC	504
4	GGCAAGCCCT-GTCTCAGTG	ATGTAGACTA-GAACATCGGC-CAC	401
5	ACTGGACC-GATGG-GAAATCC	CTAGCTTGCTG-GTCGGTGAG	398

Primer pairs 1 and 2 amplify fragments that include the homeodomain, while the remaining primers exclude the conserved region. Primer pair 5 is located in the ORF upstream of the homeodomain, 3 and 4 are located in the ORF downstream of the homeodomain.

Results and discussion

In the following we compare our results in *T. castaneum* mainly to the published data in *D. melanogaster* because our knowledge on the dorso-ventral patterning genes in other arthropods is fragmentary. This is followed by overall conclusions on the contribution of our findings to understanding the evolution of the arthropod nervous systems.

Comparative analysis of the expression of the dorso-ventral patterning

Ventral nervous system defective (vnd)

We analysed the expression patterns of the columnar genes in order to correlate their expression with specific neuroblasts.

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