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Evolution of Developmental Control Mechanisms

How can conserved gene expression allow for variation? Lessons from the dorso-ventral patterning gene muscle segment homeobox

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ABSTRACT

Arthropods are common in marine, freshwater, terrestrial, and even aerial environments. The arthropod nervous systems must be adjusted to the highly diverse behaviour and requirements of the individual arthropod species. This raises the question of how the underlying patterning mechanisms have changed during arthropod evolution to produce the characteristic axonal scaffold on the one hand and allow for variations in neuronal networks on the other hand. Here we show that the overall number of the neural precursor groups/neuroblasts as well as their spatial arrangement in rows and columns is similar in all four arthropod groups indicating a common origin of this pattern. Furthermore, we demonstrate differences in the expression pattern of the columnar gene *muscle segment homeobox* and both differences in the expression and regulation of the neural subtype specific genes *even-skipped* and *islet*. This variation may underlie the evolutionary variations in neural identity in the individual arthropod groups. Furthermore, we discuss to what extent the stereotyped pattern of neural precursors is required for the conserved axonal scaffold and thus might have been constrained along with the underlying patterning mechanisms.

Introduction

The central nervous system (CNS) of arthropods consists of the brain and the ventral trunk ganglia which are connected by two longitudinal fascicles that extend along the entire length of the ventral nerve cord (Anderson, 1973). In addition, two fascicles cross each ganglion which results in the typical ladder-like appearance of the arthropod axonal scaffold. Despite this conserved ground plan, the arthropod nervous system must be adjusted to the highly diverse behaviour and requirements of the individual arthropod species. This raises the question of how the underlying patterning mechanisms have changed during evolution to produce the conserved spatial pattern of the axonal scaffold on the one hand and allow for variations that have facilitated modifications of the neuronal networks on the other hand.

Arthropods consist of four groups: insects, crustaceans, chelicerates and myriapods. We have shown recently that neurogenesis in chelicerates and myriapods is significantly different from insects and crustaceans (Chipman and Stollewerk, 2006; Dove and Stollewerk, 2003; Kadner and Stollewerk, 2004; Pioro and Stollewerk, 2006; Stollewerk, 2002; Stollewerk and Chipman, 2006; Stollewerk et al., 2001, 2003). While in insects and higher crustaceans (malacostracans) the nervous system is generated by single stem-cell like cells (neuroblasts), in chelicerates and myriapods groups of neural precursors (NPGs) are specified for the neural fate, which directly differentiate into neural cells (Dove and Stollewerk, 2003; Goodman and Doe, 1993; Stollewerk et al., 2001; Ungerer and Scholtz, 2008).

Interestingly, regardless of the different modes of neurogenesis, the pattern of NPGs and neuroblasts is similar in all arthropod species that have been analysed. In all species about 30 neuroblasts/NPGs per hemi-segment are arranged in 7 transverse rows (e.g., Doe and Goodman, 1992; Dove and Stollewerk, 2003; Stollewerk et al., 2001; Ungerer and Scholtz, 2008). One possible reason for the stereotyped arrangement of neural precursors is the connection of neural precursor identity with spatial cues that confer anterior–posterior and dorso-ventral identities within a segment. These spatial cues are mediated by the expression of segment polarity and dorso-ventral patterning genes which subdivide the segments into anterior–posterior and dorso-ventral domains. The genes continue to be expressed during neurogenesis so that each proneural cluster shows a unique expression profile, which is maintained in the neuroblast that derives from the cluster (reviewed by Skeath (1999)). Thus, in *Drosophila* each neuroblast has a distinct spatial and temporal identity which is reflected in a unique expression profile and the production of a stereotyped number and identity of progeny. As a result of the unique expression profile of each neuroblast, different combinations of neural differentiation genes are expressed in each lineage in *Drosophila* (Skeath and Thor, 2003). *Tailup* (*islet 1*) and *even-skipped*

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(*eve*) are among the early neuronal differentiation markers which are expressed in distinct subsets of motor- and interneurons in the *Drosophila* embryo (Landgraf et al., 1999; Thor and Thomas, 1997). These genes are not expressed in neuroblasts but the origin of the motor- and interneurons that express *tailup* and *eve* has been traced back to specific neuroblasts (Landgraf et al., 1997, 1999; Thor et al., 1991; Thor and Thomas, 1997).

Comparative studies of the mechanisms that determine neural precursor identity in the different arthropod groups are few and incomplete (reviewed by Stollewerk and Simpson (2005)). However, from the limited data available, the expression of the segment polarity genes appears to have been conserved in arthropods (e.g. Chipman et al., 2004; Damen, 2002). These genes are expressed during neurogenesis suggesting an additional (or even primary) function in neural precursor identity (Chipman and Stollewerk, 2006; Duman-Scheel and Patel, 1999; Eriksson et al., 2009; Patel, 1994; Patel et al., 1989, 1992, 2001). Studies on the segment polarity gene *engrailed* have indeed revealed that this gene is specifically expressed in neuroblasts/NPGs of rows 1, 6 and 7 in all arthropod groups (Chipman and Stollewerk, 2006; Duman-Scheel and Patel, 1999; Fabritius-Vilpoux et al., 2008; Patel, 1994; Patel et al., 1989). Within the arthropods, the expression pattern and function of the dorso-ventral patterning genes *ventral nerve cord defective (vnd)*, *intermediate nerve cord defective (ind)* and *muscle segment homeobox (msh)* have only been studied in *Drosophila melanogaster* and *Tribolium castaneum* (Skeath, 1999; Wheeler et al., 2005). The overall expression of these genes in three longitudinal columns seems to be conserved, although differences in the temporal expression were observed between the species.

If we assume that the number and arrangement of neural precursors as seen in the extant arthropod groups has been present in their last common ancestor, we would have to conclude that either neuroblast lineages have evolved from NPGs or vice versa. But does the pattern of neuroblasts and NPGs correspond in detail? Or is the arrangement of neuroblasts/NPGs only superficially similar indicating that the pattern could have evolved independently in the individual arthropod lineages? Do neuroblasts and NPGs that are located in the same area express the same spatial identity genes? Do they give rise to progeny with similar identities?

Here we address these questions by analysing the pattern of NPGs in two representatives of chelicerates and one representative of myriapods in detail. We demonstrate that evolutionary modifications in the expression of the patterning gene *msh* in individual NPGs/neuroblasts and their progeny as well as functional changes in the regulation of neural subtype specific genes might have allowed for variations in the composition of the arthropod neuromeres.

Materials and methods

Cupiennius salei stock

Fertilised female spiders of the Central American wandering spider *Cupiennius salei* Keyserling (Chelicerata, Arachnida, Araneae, Ctenidae) were obtained from Ernst-August Seyfarth's laboratory at the Institute for Cell Biology and Neuroscience, J. W. Goethe-Universität, Frankfurt am Main, Germany. Embryos were collected as described before (Stollewerk et al., 2001).

Immunohistochemistry and Phalloidin staining

We used the polyclonal primary antibody rat anti-*C. salei* Prospero to label neural cells (1:200; (Weller and Tautz, 2003). Double-staining by in situ hybridisation with a Digoxigenin-labelled *C. salei Prospero* mRNA-probe and the polyclonal rat anti-*C. salei* Prospero antibody confirmed co-localisation of the staining in the same cells (Weller and Tautz, 2003).The following primary antibodies were used to stain the cytoskeleton and the neural precursor groups after in situ hybridisation: anti-*D. melanogaster* α-Tubulin, anti-*D. melanogaster* β-Tubulin, anti-*D. melanogaster* α-Spectrin (1:5; DSHB). The fluorescent secondary antibodies (anti-rat Cy5, Jackson Immunoresearch, 712-175-153 and anti-mouse Alexa 488, PromoKine, PK-PF488P-AK-M1) were used in a 1:500 dilution. The dye Phalloidin-FITC (1:20) was purchased from Molecular Probes. Phalloidin-FITC staining and antibody staining were performed on whole-mounts as described before (Stollewerk et al., 2001). Fluorescent images were analysed under the Leica TCS SPII confocal microscope and processed using Leica Confocal software and Adobe Photoshop. The brightness and contrast of basal horizontal confocal sections has been adjusted to the same level as the apical optical sections.

PCR cloning

C. salei msh and *islet* and *Glomeris marginata msh* (Dove, 2005) were amplified by RT-PCR on RNA prepared from embryos at neurogenesis stages. We used the following degenerate primers which were complementary to conserved positions of the homeodomains: (*Cs msh*, *Gm msh*) forward, AAY MGN AAR CCN MGN ACN CC, ACN CCN TTY ACN ACN CAR CA; reverse GCN GCC ATY TTN ADY TTY TC, TTY TCD ATY TCN GCY TCY; (*Cs isl*) forward, YTN GAR TGG CAY GCN GCN TG, MGN GAY GGN AAR ACN TAY TG; reverse, ACN ARY TGY TGR AAN GCN GG, ARN GCY TTC CAN GGN GGY TG; TG. Larger fragments of the genes were obtained by rapid amplification of cDNA ends (cDNA amplification kit, Roche). The DNA fragments were cloned into the pZero vector system (Invitrogen). The sequences were deposited in the NCBI database. The sequences have been submitted to GenBank. Accession numbers: *Gm msh*, GU974343; *Cs msh*, GU974344; *Cs isl*, GU974345. *Cupiennius even-skipped* was amplified by using primers specific to the published sequence (GenBank accession number: AJ252155).

In situ hybridisation

The *Cs msh*, *isl*, *eve* and *Gm msh* fragments were used to generate antisense and sense digoxigenin-11-UTP-labelled RNA-probes with the in vitro transcription system from Roche (Roche Diagnosticcs GmbH, Roche Applied Science, Mannheim, Germany). None of the three sense digoxigenin-11-UTP-labelled RNA-probes gave a label after in situ hybridization and staining against sheep anti-digoxigenin alkaline phosphatase conjugated Fab fragments, while specific staining was obtained with the antisense probes. In situ hybridizations were performed on whole-mounts as described before (Stollewerk et al., 2001). The RNA-probes were diluted in the following way: *Cs msh*, *Cs isl*, *Cs eve*: 1:250; *Gm msh*: 1:500.

RNA interference

Double-stranded (ds) RNA was prepared from a *Cs msh* cDNA clone by in vitro transcription using both SP6 and T7 RNA polymerases in one reaction to generate sense and antisense RNA.

The RNA strands were annealed by heating the sample to 80 °C and afterwards cooling it down slowly. The production of ds RNA was verified by gel electrophoresis. The 5′ region of the open reading frame was used as a template for the generation of a 100 bp *Cs msh* ds RNA fragment, since it has been shown that siRNA sequences from within 100 to 200 nucleotides of the 5′ termini of coding sequences had low chances for off-target reactivity (Qiu et al., 2005). 2 μg of ds *Cs msh* RNA were use for injection of approximately 300 embryos per RNA interference experiment. Ds *GFP* RNA was used for control injections. Embryos were injected before germ band formation and fixed and analysed after formation of all neural precursor groups at 190 to 200 hours of development (stages after Seitz (1966)). We stained ds *Cs msh* RNA injected embryos with a DIG labelled *Cs msh* RNA-probe to verify that the mRNA transcripts were significantly