Filling the gap between identified neuroblasts and neurons in crustaceans adds new support for Tetraconata

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The complex spatio-temporal patterns of development and anatomy of nervous systems play a key role in our understanding of arthropod evolution. However, the degree of resolution of neural processes is not always detailed enough to claim homology between arthropod groups. One example is neural precursors and their progeny in crustaceans and insects. Pioneer neurons of crustaceans and insects show some similarities that indicate homology. In contrast, the differentiation of insect and crustacean neuroblasts (NBs) shows profound differences and their homology is controversial. For *Drosophila* and grasshoppers, the complete lineage of several NBs up to formation of pioneer neurons is known. Apart from data on median NBs no comparable results exist for Crustacea. Accordingly, it is not clear where the crustacean pioneer neurons come from and whether there are NBs lateral to the midline homologous to those of insects. To fill this gap, individual NBs in the ventral neuroectoderm of the crustacean *Orchestia cavimana* were labelled *in vivo* with a fluorescent dye. A partial neuroblast map was established and for the first time lineages from individual NBs to identified pioneer neurons were established in a crustacean. Our data strongly suggest homology of NBs and their lineages, providing further evidence for a close insect–crustacean relationship.

Keywords: *Drosophila*; evolution; arthropods; neurogenesis; cell lineage

1. INTRODUCTION

Despite the overall similarities of the central nervous system (CNS) between the major arthropod groups, its early development shows some distinct differences at the cellular level. In myriapods and chelicerates, clusters of neural precursors immigrate from the ventral neuroectoderm and differentiate directly into neurons or glia cells to form the ventral CNS (figure 1; Stollewerk *et al.* 2001; Mittmann 2002; Stollewerk & Chipman 2006). In contrast, insects and crustaceans form their ventral CNS via neuroblasts (NBs), large neural precursor cells dividing in an asymmetrical stem cell mode to produce columns of ganglion mother cells (GMCs). At least in insects, each GMC in turn divides once to generate ganglion cells (GCs) that differentiate into neurons and/or glia cells (figure 1; insects: Wheeler 1891, Bate 1976, Doe & Goodman 1985, Hartenstein *et al.* 1987, Truman & Ball 1998; crustaceans: McMurrich 1895, Dohle 1976, Scholtz 1992, Gerberding 1997, Harzsch 2001).

In 1984, Thomas *et al.* (1984) detected a set of early differentiating neurons, responsible for pioneering major axon pathways in the embryonic CNS of insects and crustaceans, which led them to propose a common plan for neurogenesis in arthropods. A series of subsequent studies at the level of individually identified neurons including position, axon morphology and timing of outgrowth confirmed the existence of a set of homologous pioneer neurons in insects and crustaceans that finds no counterpart in myriapods (Whittington *et al.*

1991, 1993, 1996). Together with molecular datasets (e.g. Boore *et al.* 1998; Shultz & Regier 2000; Friedrich & Tautz 2001; Giribet *et al.* 2001; Kusche *et al.* 2002; Pisani *et al.* 2004; Petrov & Vladychenskaya 2005; Regier *et al.* 2005; Mallatt & Giribet 2006), these studies contributed considerably to the new discussion on arthropod relationships (e.g. Whittington & Bacon 1997; Dohle 2001; Richter 2002; Whittington 2004; Giribet *et al.* 2005).

Furthermore, Gerberding & Scholtz (1999, 2001) identified a midline NB that shows some similarities to corresponding cells in insects. Astonishingly, it remained controversial whether NBs in crustaceans are homologous to those in insects (convergent: Dohle & Scholtz 1988, Scholtz 1992; question unresolved: Whittington 1996, Dohle 2001, Scholtz & Gerberding 2002, Harzsch 2003; homologous: Duman-Scheel & Patel 1999, Richter 2002, Harzsch 2006). This is due to some distinct differences concerning the way they are generated and their final position in relation to the ventral neuroectoderm. However, in contrast to the insect situation, in no case has the lineage from individual NBs to identified pioneer neurons been traced in a crustacean species. To fill this gap, we used for the first time an *in vivo* labelling technique with the lipophilic fluorescent dye DiI in combination with confocal laser scanning microscopy (CLSM) and computer-aided three-dimensional reconstruction to study NBs in the ventral neuroectoderm of a higher crustacean. The amphipod crustacean *Orchestia cavimana* was chosen owing to its excellent qualities for single-cell labelling studies (e.g. Gerberding & Scholtz 1999, 2001; Wolff & Scholtz 2002, 2006).

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Figure 1. Modes of neurogenesis in euarthropods. In chelicerates and myriapods, neurons are generated via immigration of post-mitotic neural precursor cells (np), whereas in insects and crustaceans we find specialized cells, NBs, which divide in a stem cell-like manner. In insects, they bud off smaller GMCs which subsequently divide only once to generate GCs that differentiate into neurons and glia cells. There are indications for such a division pattern in higher crustaceans, but direct evidence is still missing. e, ectoderm.

2. MATERIAL AND METHODS

Specimens of the semi-terrestrial amphipod species *O. cavimana* were collected on the lakefront of the Tegeler See (Berlin). The animals were reared in a terrarium at 18–20°C and fed with carrots, cucumbers and oatmeal. Eggs in relevant stages were isolated from the ventral brood pouch (marsupium) of the females by flushing out with a glass pipette. Eggs were transferred to a saline solution that mimics the osmotic milieu in the marsupium (for details see Wolff & Scholtz 2002).

(a) *In vivo* labelling

In vivo cell labelling was done with an inverse microscope equipped with a micromanipulator (Leica DMIRB). Eggs in relevant stages were mounted on microscopic slides under small cover-slips that were equipped with plasticine feet at the corners. Positioning of the eggs was carried out by carefully shifting the cover-slip. Injection needles were made by pulling (KOPF Puller 720) glass pipettes (Hilsberg, diameter 1.0 mm, thickness 0.2 mm). After pulling, the tips of the needles were sharpened with a horizontal grinder (Bachofner) the angle of the cutting edge varying between 20° and 30°.

The fluorescent marker DiI (Molecular Probes) was used as a vital marker (2 mg ml⁻¹ dissolved in soy oil). It is lipophilic and intercalates in the cell membrane, which guarantees that the dye is exclusively restricted to the daughter cells. *In-vivo*-labelled eggs were singly kept in Petri dishes at 16°C in the saline mentioned above, which was changed every second day. Labelled eggs were checked and documented regularly with a fluorescence-microscope (Zeiss Axiophot1) equipped with digital camera (Nikon D1) using blue light or green light (strongest excitation of DiI).

(b) CLSM and three-dimensional reconstruction

For fixation and documentation with the laser scanning microscope (Leica SP2), embryos were dissected in PBS-buffered 4% formaldehyde solution, counterstained with a nucleic specific dye (Hoechst) and mounted in the anti-bleaching detergent DABCO–Glycerol (25 mg DABCO (1,4 diazabicyclo-2,2,2-octane, Merck) in 1 ml PBS to 9 ml glycerol). The image stacks produced by the laser scanning microscope were analysed with the software IMARIS v. 5.0.1 (Bitplane AG), which allows a three-dimensional reconstruction of the counter-stained cells and the clones of the

in vivo-labelled cells. The feature ‘Volume’ in the program module ‘Surpass’ creates a three-dimensional object that can be magnified and moved in all directions.

3. RESULTS AND DISCUSSION

As in all higher crustaceans studied in this respect, in *Orchestia* the NBs of the post-naupliar germ band are generated via a stereotyped cell division pattern starting with the formation of regular transverse cell rows in the ventral ectoderm before the onset of morphogenesis (figure 2a). NBs arise continuously in the two initial cell columns adjacent to the midline whereas the more lateral cells form limbs and tergites (figure 2a,b; Dohle 1976; Dohle & Scholtz 1988; Scholtz 1990, 1992). During neurogenesis, the NBs remain at the embryo’s surface intermingled with ectoderm cells. Previous studies using histological nuclear staining identified approximately 12 individual NBs within this pattern (Dohle 1976; Scholtz 1990).

The labelling of cells of the column adjacent to the midline allowed the identification of an additional 4–6 NBs per hemisegment, and we established a partial NB map (figure 3). Although we did not test it directly, there is some evidence that the second cell column next to the midline generates a similar number of NBs. Accordingly, we suggest a number of 26–30 NBs per hemisegment. This figure corresponds to that inferred for decapod crustaceans (Scholtz 1992) and in insects where 29–31 NBs are differentiated in each thoracic hemisegment (Bate 1976; Tamarelle *et al.* 1985; Hartenstein *et al.* 1987; Doe 1992; Truman & Ball 1998). In contrast to crustacean NBs, those of insects detach from the neuroectoderm early in embryogenesis, forming a layer between ectoderm and mesoderm (figure 1; Bate 1976; Tamarelle *et al.* 1985; Hartenstein *et al.* 1987; Dohle & Scholtz 1988; Doe 1992; Truman & Ball 1998).

To date, it has not been proven that crustacean GMCs show the same further fate as those of insects. Here we can definitely show that the division pattern of *Orchestia* GMCs corresponds in great detail with that in insects. The NBs in *Orchestia* produce smaller GMCs into the interior of the ganglion anlage which divide only once to give rise to neurons and/or glia cells (figure 4a–c). As in insects (Goodman & Doe 1993), the first GMC starts its

Figure 2. Invariant cell lineages in the ventral ectoderm in *O. cavimana*. (a) Scheme (modified after Wolff & Scholtz 2006) of the cell division pattern in the post-naupliar ectoderm characteristic for malacostracan crustaceans. The designation of the cells arising within the stereotyped cell division pattern in the post-naupliar germ band of *O. cavimana* follows Scholtz (1990). Each of the transverse ectodermal rows (row abcd) which form at a stage of approximately 400 cells represents a genealogical unit and undergoes the same division pattern. First, it undergoes two mitotic waves with longitudinal spindle directions resulting in a grid-like pattern of four rows (a, b, c, d). With the subsequent differential cleavages, morphogenesis starts. Within the cell columns 0 (midline) to 2, the ganglion anlagen are formed, whereas the more lateral columns give rise to the limb anlagen (orange line marks border between ganglion anlagen and limb anlagen). b1hn and d1hn (marked in red) represent the first NBs arising from the stereotyped cell divisions of the initial ectodermal rows. During each differential cleavage, new NBs are added, the NBs themselves being able to switch to the production of ectodermal cells and back to the production of GMCs. The segmental border does not match the genealogical border. The segmental border runs between the descendants of row b (black dotted line). The present study focuses on the cells of column 1 which were labelled mainly after the second mitotic wave (a1, b1, c1 and d1). After the differential cleavages, labelling is hindered by the small size of the cells. (b) Detail of two thoracic segments during the differential cleavages (SEM image). The midline (0) and cell columns 1 and 2 which give rise to the ventral ganglia are marked in orange. The first two NBs arising during the differential cleavages in cell column 1 are marked in red (b1hn and d1hn).

Figure 3. Neuroblast map of a generalized thoracic segment in *Orchestia*. From the cells of column 1 of the grid-like pattern in the post-naupliar ectoderm of *Orchestia*, 13–15 NBs arise.

division after three GMCs have been generated by the NB, and the first GCs start their differentiation when three of the GMCs have divided (figure 4c).

To test whether NBs can be identified in crustaceans which are individually homologous to those in insects, we labelled single NB precursor cells to trace their lineage up

Figure 4. Early neuroblast divisions in the thoracic segments of *Orchestia*. Anterior is to the top and median to the right. (a) DiI labelling of the cell d1v (red) with nucleic specific Hoechst counterstain (blue); stage 2 (staging according to Ungerer & Wolff 2005). Three-dimensional reconstruction (posteroventral view on the ganglion anlagen) with IMARIS (Surpass mode). To better visualize labelled cells, their nuclei are marked with orange balls. The NBs d1vin and d1veix arise from the cell d1v and produce smaller GMCs (asterisks) into the interior of the ganglion anlage. It is very probable that d1veix or one of its descendants (d1veixn) represents a neuroblast, although the assignment of GMCs was not always possible without doubt due to the close spatial relation of cells. (b) DiI labelling of a1 in stage 3 of *Orchestia* development. Digital frontal section through the ganglion anlage (IMARIS) showing the three NBs in the a1 lineage and their descendants which enter dorsomedially into the ganglion anlage. (c) Same labelling and CLSM image stack as in (b) with three-dimensional visualization of cells by setting a ball in each cell in IMARIS. White arrows point to the border of GMCs (asterisks) and GCs. After three GMCs have been produced, the first starts to divide resulting in two GCs. Again, after three divisions of GMCs, the first GCs start to differentiate into neurons (black arrow points to first axon of the neuroblast alien) or glia cells (not shown).

to the differentiation of pioneer neurons (a1: $n=27$, b1: $n=31$, c1: $n=37$, d1: $n=38$; see figure 2). Since the NB 1–1 of insects is well characterized by its position and by the pattern of pioneer neurons produced by it, we looked for a corresponding NB in *Orchestia*. Indeed, we identified the NB b1hn as sharing many characteristics with the NB 1–1 of insects (figure 5). As is the case for the latter (Goodman *et al.* 1984; Goodman & Doe 1993; Udolph *et al.* 1993; Broadus *et al.* 1995; Bossing *et al.* 1996; Schmid *et al.* 1999), the first GMC of *Orchestia* gives rise to the neurons aCC and pCC, whose early outgrowing axons act as pioneers for the establishment of the intersegmental nerve and the connective, respectively (figure 5c–f,h). In addition, the first GMCs of 1–1 in insects (Goodman & Doe 1993) and b1hn in *Orchestia* are generated in the anteriormost region of the segment and migrate anteriorly across the intersegmental border into the adjacent ganglion anlage (figure 5d). Both aCC and pCC end up in a position in the dorsalmost layer of the ganglion anlage behind the commissures and medial to the connective (figure 5e,f,h). As the insect NB 1–1 (Doe 1992; Broadus & Doe 1995), b1hn of *Orchestia* is the first NB in the segment close to the midline lying behind two rows of *en* positive NBs (figure 5a,g).

A second candidate was the NB 4–2 of *Drosophila* (figure 5g), again based on its position and its characteristic offspring, the pioneer neuron RP2 (Bossing *et al.* 1996; Landgraf *et al.* 1997; Schmid *et al.* 1999; figure 6b). We found that the NB d1hn of *Orchestia* shares the medial position in the anteroposterior axis (figure 5a) and among others the production of the well-identifiable pioneer neuron RP2 (figure 6a). However, NB 4–2 lies in the second column next to the midline whereas NB d1hn is situated directly adjacent to the midline, so a positional shift would have to be assumed.

Likewise, the *en* positive NBs aliin of *Orchestia* (figure 5a) and 7–1 of *Drosophila* (figure 5g) sharing a corresponding position at the posterior border of the segment close to the midline are putatively homologous.

Although it was not possible to identify the entire lineage of a1in, it is highly probable that it produces motoneurons sharing the characteristics of the U neurons produced by NB 7–1 of *Drosophila* (Bossing *et al.* 1996; Landgraf *et al.* 1997; Schmid *et al.* 1999; figure 6b,c).

Taken together, the position, the consistent mode of division, the correspondence of the NB cell lineages and their outcome in terms of identified neurons strongly suggest homology of some NBs in *Orchestia* and insects. Owing to the stereotyped cell division pattern characteristic for higher crustaceans (malacostracans; Dohle *et al.* 2004), it is likely that the aspects of neurogenesis investigated in *Orchestia* might be generalized for malacostracan crustaceans. Unfortunately, we do not know much about neurogenesis in non-malacostracan crustaceans. There are reports about NBs in some branchiopods showing that these remain at the surface as in malacostracan crustaceans (Gerberding 1997; Harzsch 2001; Wheeler & Skeath 2005), but no direct evidence for their division in a stem cell mode and no data about pioneer neurons yet exist. Corresponding data for the remaining crustaceans are completely lacking.

Based on molecular and morphological data, there is increasing evidence for a close relationship of insects and crustaceans forming the taxon Tetraconata (e.g. Dohle 1997, 2001; Zrzavý & Stýs 1997; Boore *et al.* 1998; Friedrich & Tautz 2001; Giribet *et al.* 2001, 2005; Regier & Shultz 2001; Kusche *et al.* 2002; Richter 2002; Pisani *et al.* 2004; Petrov & Vladychenskaya 2005; Regier *et al.* 2005; Harzsch 2006; Mallatt & Giribet 2006), with the Crustacea sometimes paraphyletic (e.g. Friedrich & Tautz 2001; Hwang *et al.* 2001; Regier & Shultz 2001; Regier *et al.* 2005; Harzsch 2006; Mallatt & Giribet 2006).

Preliminary investigations in onychophorans (Eriksson *et al.* 2003; Whittington 2006) and tardigrades (Hejnol & Schnabel 2005) did not indicate the existence of NBs but suggest that the nervous system is formed by immigration of neural precursors in these groups. This resembles to a certain extent the situation found in chelicerates and

myriapods (Stollewerk *et al.* 2001; Mittmann 2002; Stollewerk & Chipman 2006). Hence, it is reasonable to conclude that immigration of neural precursors represents the ancestral state within arthropods, and the formation of NBs in insects and crustaceans in the derived state, thus providing additional support for the Tetraconata. However, even then we are still left with the question of the position of the NBs either in a layer between ectoderm and mesoderm as in insects or in the ectodermal layer as in crustaceans.

Some of the molecular mechanisms underlying the neurogenesis in representatives of insects (Cabrera *et al.* 1987; Romani *et al.* 1987; Skeath *et al.* 1992; Wheeler *et al.* 2003), chelicerates (Stollewerk *et al.* 2001; Stollewerk 2002) and myriapods (Dove & Stollewerk 2003; Kadner & Stollewerk 2004) have been revealed. It has been shown that corresponding mechanisms are responsible for generation of neural precursor cells in all three groups. From clusters of cells expressing proneural genes (e.g. *achaete*, *scute*) in the ventral neuroectoderm either groups of neural

precursor cells (as seen in chelicerates and myriapods) or single cells (as seen in insects) are selected by lateral inhibition mediated by the transmembrane proteins Notch and Delta and translocated into the interior of the embryo (Simpson 1990; Seugnet *et al.* 1997). So far, corresponding data on crustaceans are almost completely lacking. A single study in the branchiopod, *Triops longicaudatus*, suggests a role of *achaete-scute* genes comparable to that in insects (Wheeler & Skeath 2005).

We propose that the translocation of neural precursors beneath the ectoderm in insects via lateral inhibition represents the plesiomorphic character state for the Tetraconata. In crustaceans, this mechanism must have changed in a way that allows the NBs to remain at the surface and to differentiate adjacent to each other in high density. If the preliminary data about superficial NBs in non-malacostracan crustaceans can be substantiated, then this would argue for monophyletic Crustacea, or at least a clade that minimally includes branchiopods and malacostracans.