Early steps in ventral nerve cord development in chelicerates and myriapods and formation of brain compartments in spiders

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1 Introduction

The nervous system of higher organisms serves many functions like reception of different stimuli, transmission of these signals and triggering and execution of adequate reactions. The functional complexity is reflected in the complex structure of the nervous system. It can be subdivided into two major parts, the central nervous system (CNS) and the peripheral nervous system (PNS). In invertebrates, the CNS consists of the ventral nerve cord (VNC) and the brain and is responsible for the processing of information. The PNS conducts incoming signals and outgoing reactions.

In all four arthropod subphyla (chelicerates, crustaceans, insects and myriapods) the nervous system consists of segmentally organised ganglia which are assembled in a rope ladder-like manner. The different subphyla and also the different species within one group show a huge variation in morphology and behaviour. Hence, the organisation and function of these nervous systems is adapted to the specific demands of the different arthropod groups. The adaptive changes in form must have led to evolutionary changes in the developmental processes that generate the corresponding structures. The investigation of evolutionary modifications and adaptations in developmental processes, as for example organogenesis, body axis determination and segmentation (for example heart development (Janssen and Damen, 2008) or segmentation (Damen *et al.*, 2000; Damen 2001; Stollewerk *et al.*, 2003) in the spider *Cupiennius salei*) is the field of research of Evolutionary Developmental Biology (EvoDevo). The comparative analysis of neurogenesis of different representatives of arthropods might reveal which mechanisms are conserved and which have diverged during evolution of the distinct nervous systems.

The main focus of this work was to uncover and analyse evolutionary modifications during neurogenesis in chelicerates and myriapods. Therefore, specific aspects of neural development were analysed in the millipede *Glomeris marginata* (*Glomeris*) and the spiders *Cupiennius salei* (*Cupiennius*) and *Achaearanea tepidariorum (Achaearanea)*. The results were then compared with data from neurogenesis of other arthropods but mainly with the insect *Drosophila melanogaster (Drosophila)*, because this model organism provides the most detailed data.

Two aspects of how neural development can be modified to generate diverse adult nervous systems were analysed in this thesis. The first aspect was to examine modifications during early neurogenesis, thereby investigating and comparing neural precursor formation and identity in the ventral neuroectoderm (VNE). Since there are similarities as well as modifications in early neurogenesis among different arthropod groups, further analyses of the involved mechanisms came into focus. The second aspect of this work was the analysis and comparison of brain development in spiders. These results gave insights in the mechanisms that lead to the formation of specialised brain centres which arise from neural precursors.

1.1 Early neurogenesis in arthropods

In all arthropods the CNS is located ventrally and comprises of the VNC and brain. The neural cells of the VNC are generated by progenitor cells which are recruited from within the VNE (reviewed in Campos-Ortega and Hartenstein, 1997). After the formation of neural precursors they give rise to different types of neural cells which then differentiate to execute their specific function. Differentiation is for example reflected by the generation and directed outgrowth of axons and dendrites, which are necessary to build the complex neuronal circuits (Goodman and Doe, 1993; Landgraf *et al.*, 1997; Landgraf *et al.*, 2003). To allow these complex networks to operate properly, every developing cell has to have its unique identity and has to fulfil its assigned fate. The mechanisms that are involved in these steps of neurogenesis will be presented in the next paragraphs.

1.1.1 Formation of neural precursors

For the *Drosophila* VNC, about 30 neuroblasts (NBs) are generated in the NE of each hemisegment. Initially, clusters of equivalent proneural cells (proneural clusters) are specified by the expression of proneural genes of the *acheate scute* complex (Fig.1-2 A, B) (Campuzano and Modolell, 1992; Romani *et al.*, 1989; Skeath, 1999). The proneural genes have also been identified in other representatives of insects, such as *Tribolium castaneum*, *Anopheles gambiae*, *Schistocerca gregaria*, *Tribolium castaneum*, *Precis coenia*, *Ceratitis capitata*, *Calliphora vicina* and *Phormia terranovae*, reflecting their possible conserved role (Broadus and Doe, 1995; Galant

et al., 1998; Pistillo *et al.*, 2002; Skaer *et al.*, 2002; Wheeler *et al.*, 2003; Wulbeck and Simpson, 2000; Wulbeck and Simpson, 2002). In *Drosophila* only one cell of the proneural cluster differentiates into a NB by a mechanism called lateral inhibition which is conveyed by the interaction of the neurogenic genes *Delta* and *Notch*. The remaining cells of the proneural cluster adopt an epidermal fate (Bourouis *et al.*, 1989; Campos-Ortega and Hartenstein, 1997; Lehmann *et al.*, 1981). This mechanism eventually leads to the recruitment of about 30 NBs per hemisegment, which delaminate from the apical towards the basal layer in five distinct waves shortly after gastrulation (Fig.1-1 A (NBs of the first wave), Fig.1-2). After delamination the NBs start to divide asymmetrically and generate ganglion mother cells (GMCs), which then divide to produce neurons and/or glial cells (Fig.1-1 A, Fig.1-2 B). The NBs are arranged in a stereotypic pattern (Cartesian coordinate system) of seven rows with three to six NBs per row. Since all the NBs are individually identifiable by position and expression of molecular markers, they were named according to their positions within the coordinate system. The rows are named with numbers from one to seven in anterior to posterior direction and the second number marks the position of NBs in each row from the midline towards lateral. In other insects like *Tribolium castaneum (Tribolium)* and *Schistocerca gregaria (Schistocerca)* NBs were observed in a very similar arrangement (Doe and Goodman, 1985a; Jimenez and Campos-Ortega, 1990; Skeath *et al.*, 1994; Wheeler *et al.*, 2003; Wheeler *et al.*, 2005).

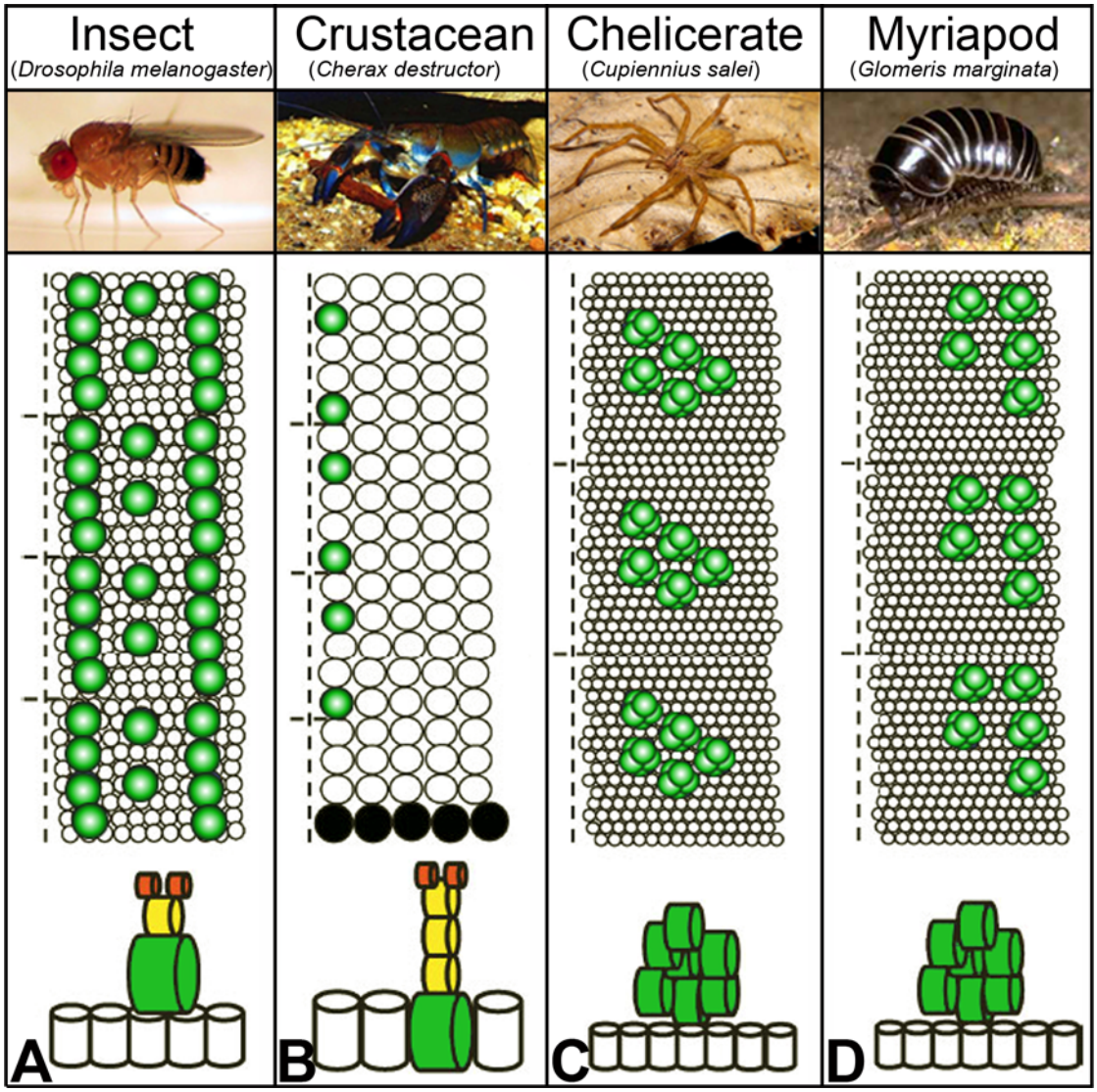

Figure 1-1: Schematic drawing of neural precursor formation in arthropods.

Comparison of different modes of neural precursor formation in four hemineuromeres in insects (**A**) and crustaceans (**B**) and in three hemineuromeres in chelicerates (**C**) and myriapods (**D**). (**A**) In insects about 30 single neural stem cells (NBs) adopt a neural cell fate in the apical layer of the NE and delaminate towards basal in five distinct waves (in green are presented the NBs of the first wave). After delamination the NBs (green) undergo asymmetric divisions in which ganglion mother cells (GMC, yellow) are generated and the NB is self-renewed. The GMCs divide once to generate neurons and/or glial cells (red). (**B**) In some representatives of crustaceans the single neural stem cells (NBs, green) follow a similar division pattern as the NBs in insects; they divide asymmetrically and generate GMCs (yellow), which then divide to generate neurons and/or glial cells (red). Unlike in insects, the NBs in the analysed crustaceans do not delaminate but remain in the apical layer. (**C, D**) In chelicerates and in myriapods whole groups of cells adopt a neural cell fate and invaginate from the apical layer as groups. Dotted lines represent the midline and segmental boundaries. Modified after Stollewerk and Simpson, 2005.

In crustaceans the mechanisms underlying early neurogenesis are only partially described and detailed molecular data is still missing. In Malacostraca, the higher crustaceans, the nervous sytem is generated by single neural stem cells (NBs), similar to insects (Gerberding, 1997; Harzsch, 2001). In contrast to insects, the NBs

do not delaminate but stay in the apical layer while they divide to produce GMCs towards the interior of the embryo. When the GMCs divide asymmetrically they generate neurons and/or glial cells, like it has been shown in *Drosophila* (Fig.1-1 B) (Dohle, 1976; Gerberding, 1997; Harzsch, 2001; Scholtz, 1992; Ungerer and Scholtz, 2008). Despite this similarity in the generation of neural lineages there is a difference concerning the produced cells: crustacean NBs can give rise to neural and epidermal progeny, whereas NBs in studied insects only generate neural cells.

Similar to *Drosophila*, about 30 neural precursors are sequentially generated at stereotyped positions in the VNE of the spider *Cupiennius salei* (Stollewerk *et al.*, 2001). Whereas in insects single neural stem cells delaminate from the NE, in *Cupiennius* whole groups of cells adopt the neural fate and invaginate from the apical layer (Fig.1-1 C) (Stollewerk *et al.*, 2001). Previous data suggested a connection between cell proliferation in the apical layer and the groups of cells which adopt neural cell fate (Barth, 1985). But Dr. A. Stollewerk and co-workers could show that there is no obvious correlation between the mitotic divisions in the NE and the invaginating NPGs (Stollewerk *et al.*, 2001), indicating that the cells within one NPG are not clonally related. Furthermore, no asymmetric cell divisions could be detected within the NPGs, confirming that there are no single neural stem cells in chelicerates (Stollewerk *et al.*, 2001). The neuroectodermal regions from which NPGs delaminate are prefigured by the expression of the *achaete-scute homologue ASH1* (Stollewerk *et al.*, 2001). This proneural gene is responsible for the formation of neural precursors in the CNS and PNS. The second identified *acheate-scute* homologue, *Cupiennius ASH2*, is expressed exclusively in the neural precursor cells and is involved in their differentiation (Stollewerk *et al.*, 2001). Expression data and functional analysis of the neurogenic genes *Cupiennius Delta1*, *Cupiennius Delta2* and *Cupiennius Notch* suggest that these genes mediate lateral inhibition similar to the mechanism found in *Drosophila*. However, in the spider this process generates groups of neural precursors rather than single NBs like in *Drosophila* (Stollewerk, 2002). In contrast to *Drosophila* where the neuroectodermal cells produce neural and epidermal cells, all the cells of the NE in the spider contribute to the nervous system (Campos-Ortega, 1990; Musakvich, 1994; Stollewerk, 2002). In chelicerates the epidermis overgrows the neural tissue from lateral and medial after the differentiation of the neural cells. The epidermal cells which cover the developing nervous system from medial are the same cells which have covered the widened midline before the

two halves of the embryo meet again at the ventral midline. Other epidermal cells were generated lateral to the neuromeres before they spread medially to overgrow the CNS from the lateral side (Stollewerk, 2002). However, about 30 NPGs are recruited in *Cupiennius* and invaginate in four waves which is similar to what has been observed for the NBs in insects (Fig.1-1 C (NPGs of the first wave)). Although the NPGs are recruited at different time points, they all stay attached to the apical surface (Stollewerk *et al.*, 2001).

The formation of neural precursors in myriapods was mainly analysed in the diplopod *Glomeris marginata* and the chilopods *Lithobius forficatus (Lithobius)*, *Strigamia maritima (Strigamia)* and *Ethmostigmus rubripes (Ethmostigmus*) (Chipman and Stollewerk, 2006; Dove and Stollewerk, 2003; Kadner and Stollewerk, 2004; Whitington P.M., 1991). Early neurogenesis in myriapods seems to share several characteristics with chelicerates, since there also whole groups of cells adopt the neural cell fate in the VNE and invaginate as groups (Dove and Stollewerk, 2003; Kadner and Stollewerk, 2004; Mittmann, 2002; Stollewerk et al., 2001; Stollewerk A. , 2006). Furthermore the number and the arrangement of the invaginating NPGs in seven rows are similar between myriapods and chelicerates (Dove and Stollewerk, 2003; Kadner and Stollewerk, 2004; Stollewerk A. , 2006). Like in insects and spiders, the formation of neural precursors takes place in four distinct waves in *Lithobius* and *Glomeris* (Dove and Stollewerk, 2003; Kadner and Stollewerk, 2004). This common feature was not observed in *Strigamia* where the NPGs are specified sequentially (Chipman and Stollewerk, 2006). In addition to this difference, the NPGs in *Strigamia* are initially arranged in three rows and do not rearrange into the common seven rows pattern until later stages (Chipman and Stollewerk, 2006). A third striking difference is given by the morphology of the NPGs, as they are not attached to the apical surface like in the other analysed myriapods and spiders, but to a single cell of the precursor group (Chipman and Stollewerk, 2006). Like in *Cupiennius*, the regions of the NPGs are predetermined by the expression of *acheate-scute* homologues in *Glomeris* and *Lithobius* (Dove and Stollewerk, 2003; Kadner and Stollewerk, 2004). But in contrast to the spider, these regions are furthermore associated with an increased mitotic activity in *Glomeris* (Dove and Stollewerk, 2003). The expression patterns of *Delta* and *Notch* in *Glomeris* and *Lithobius* hint at a role similar to that in *Cupiennius*, where the two genes are not involved in the decision between a neural or epidermal fate. Instead, the epidermis

overgrows the VNC after the determination of the neural cells as has been shown in spiders (Dove and Stollewerk, 2003; Kadner and Stollewerk, 2004; Stollewerk *et al.*, 2001).

Even if there are different modes of neural precursor formation among the arthropod groups, there also exist several similarities such as the number and the arrangement of the neural precursors or the expression of homologous genes in the NE. A striking difference in neural precursor formation between the different arthropod groups is the specification of single neural precursor cells (NBs) in insects and crustaceans, which generate stereotyped lineages, whereas in chelicerates and myriapods whole groups of cells (NPGs) adopt the neural cell fate in the NE. Furthermore, most of these neural precursors directly differentiate into neural cells in spiders and myriapods.

1.1.2 Neuroblast lineages versus neural precursor groups

Since the nervous system is generated by single neural precursors in insects and crustaceans and by neural precursor groups in chelicerates and myriapods, the question arises how these different modes of neural precursor formation correspond to each other.

The NBs in insects and crustaceans generate cells of the CNS by dividing in a stem cell like mode. In insects the NBs start to divide asymmetrically after delamination, whereas the NBs in crustaceans remain in the apical layer (Gerberding and Scholtz, 1999; Gerberding and Scholtz, 2001; Ungerer and Scholtz, 2008). Through a number of sequential asymmetric divisions NBs then generate ganglion mother cells which will divide once to produce glial cells and/or neurons. As a result, each NB gives rise to a cell lineage with clonally related cells. The number of divisions and the identity of their progeny depend on the identity of the NB and therefore every NB gives rise to an individual cell lineage (Bossing *et al.*, 1996; Doe and Goodman, 1985b; Schmidt *et al.*, 1997).

Recent clonal studies about NB cell lineages in crustaceans revealed that some NBs and their corresponding lineages give rise to similar lineages in crustaceans and insects and thus might be homologous (Ungerer and Scholtz, 2008). According to the assumption that the NPGs represent the ancestral pattern, which would include that the NBs (NB lineages) evolved from the NPGs in the branch leading to insects and

crustaceans, it is very likely that at least some of the NB/NPG identity mechanisms are conserved. Analysing if and to what extend the NPGs in spiders and myriapods are homologous to the NB lineages in insects was part of this doctoral thesis project. In these experiments mechanisms which are involved in NB identity in insects were compared with the mechanisms used in chelicerates and myriapods. In insects spatial and temporal patterning genes determine the identity of the NBs which consequently leads to the generation of specific lineages (see paragraph 1.3.3). The analysis of the expression pattern and function of a selection of known patterning genes should reveal if the NBs and the NPGs are specified in a similar way. The results then allowed drawing conclusions about a possible homology between the cells of the NPGs in chelicerates and myriapods and the NB lineages in insects and crustaceans.

1.1.3 Identity of neural precursors

The nervous system consists of a variety of different cells, which all have their own identity. *Drosophila* NBs acquire their specific identity by spatial and temporal cues and every NB then gives rise to an individual cell lineage (Broadus and Doe, 1995; Doe, 1992; Skeath, 1999). The temporal mechanism is activated after delamination of the NBs and is conveyed by the sequential expression of genes in individual NBs and their progeny (Doe, 1992) (Fig.1-2 B). As this thesis concentrates on the mechanisms of spatial but not temporal identity, the mechanisms of temporal identity will not be described further.

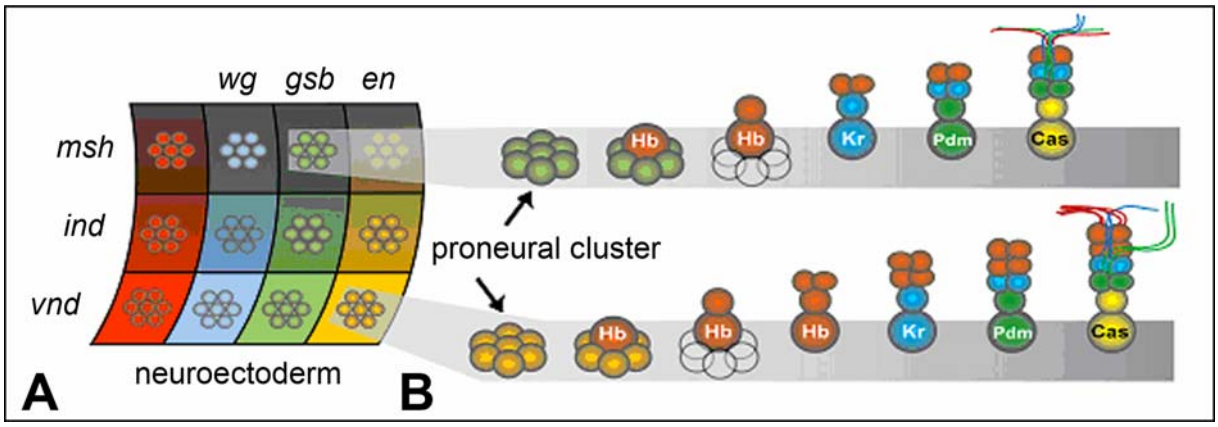

Figure 1-2: Neuroblast identity in *Drosophila melanogaster*.

In *Drosophila* the NBs get their specific identities by spatial (**A**) and temporal (**B**) mechanisms. (**A**) Schematic drawing of one hemineuromere; anterior is towards the left, dorsal is towards the top. The VNE is subdivided in a grid like pattern by the expression of dorsoventral (DV) patterning genes in columns (*msh*, *ind*, *vnd*) and anteroposterior (AP) patterning genes in transverse stripes (for example: *wg*, *gsb*, *en*). The identity of the NB depends on the position of the proneural cluster from which it is selected and thus on the combination of patterning genes which are expressed at this position. (**B**) A temporal mechanism determines the identity of the NBs and their progeny after delamination (presented for two NBs and their lineages). After the NBs are specified within the proneural clusters they delaminate and start to sequentially express temporal patterning genes (Hb, Kr, Pdm, Cas). The expression of these temporal factors determines the NB identity at a certain point in time and as their expression is maintained in the generated progeny also the identity of the daughter cells. ***Cas***, Castor; ***en***, *engrailed*; ***gsb***, *gooseberry*; ***ind***, *intermediate neuroblast defective*; ***Hb***, Hunchback; ***Kr***, Krüppel; ***msh***, *muscle segment homeobox*; ***Pdm***, POU domain protein; ***vnd***, *ventral neuroblast defective*; ***wg***, *wingless*. Schematic drawing by courtesy of Dr. J. Urban.

The spatial mechanism is controlled by the expression of anteroposterior (AP) and dorsoventral (DV) patterning genes in the NE (Fig.1-2) (Bhat, 1996; Skeath, 1999; Skeath and Carroll, 1994; Skeath and Doe, 1996; Skeath *et al.*, 1992; Truman and Bate, 1988). Their expressions subdivide the NE into a grid-like pattern in which every field of the grid expresses an individual combination of patterning genes. The segment polarity genes (for example *wingless*, *gooseberry* and *engrailed*) are expressed in transverse stripes whereas the columnar genes *muscle segment homeobox* (*msh*), *intermediate neuroblast defective* (*ind*) and *ventral neuroblast defective* (*vnd*) are expressed in three longitudinal columns from dorsal to ventral (Fig.1-2 A). The identity of each NB depends on its position within the NE, and thus on the combination of patterning genes expressed in this region.

Expression data of some of these segment polarity genes in other arthropods hint at a similar mechanism of neural precursor identity, but their actual function concerning neurogenesis has not yet been analysed. The data on AP patterning genes in segmentation shows a rather conserved expression pattern in several analysed

arthropods (insects: *Tribolium castaneum*, *Schistocerca gregaria*, crustaceans: *Artemia franciscana*, chelicerates: *Achaearanea tepidariorum*, *Cupiennius salei*, *Archegozetes longisetosus*, *Tertranychus urticae*, myriapods: *Lithobius forficatus*, *Strigamia maritima*, *Glomeris marginata*) (Brown *et al.*, 1997; Copf *et al.*, 2003; Dawes *et al.*, 1994; Eckert *et al.*, 2004; McGregor *et al.*, 2008; Mouchel-Vielh *et al.*, 2002; Patel, 1994; Patel *et al.*, 1992). The DV patterning genes, which are known to be involved in neural precursor formation and identity in *Drosophila* were also analysed in *Tribolium* and shown to play a similar role in the formation of neural precursors (Wheeler *et al.*, 2005). Furthermore *ind* and *msh* expression was already examined in *Glomeris*, but functional data about these genes are still missing in all arthropods with the exception of *Drosophila* and *Tribolium* (Dove, 2003).

Considering the already existing data on DV patterning genes in different arthropod groups and their function in NB formation and identity in *Drosophila*, the analysis of these genes in spiders and myriapods could serve as a starting point to compare the formation of the nervous system and neural identity mechanisms and to uncover evolutionary changes. Results of these investigations should then help to answer the question if the cells of the NPGs are homologous to the NB lineages. The expression patterns and gene function of the DV patterning genes will be described in more detail in the next paragraph.

1.1.3.1 DV patterning of the developing nervous system in vertebrates and invertebrates

As mentioned before, the NE of *Drosophila* can be divided into three longitudinal columns by the expression of the DV patterning genes *msh*, *ind* and *vnd* (Isshiki *et al.*, 1997; McDonald *et al.*, 1998; Skeath, 1999; Weiss *et al.*, 1998). *msh* expression covers the lateral, *ind* the intermediate and *vnd* the medial column. Transcriptional repression between these genes restricts their expression pattern to the respective columns: *vnd* represses *ind* and *msh* in the medial column and *ind* represses *msh* in the intermediate column (McDonald *et al.*, 1998; Skeath, 1999; Urbach and Technau, 2003c; Weiss *et al.*, 1998). *ind* and *vnd* are necessary for NB formation and identity, whereas the NBs in *msh* mutants are formed normally but show changes in cell fate

(Chu *et al.*, 1998; Isshiki *et al.*, 1997; Jimenez and Campos-Ortega, 1990; Jimenez *et al.*, 1995; McDonald *et al.*, 1998; Skeath and Carroll, 1994; Weiss *et al.*, 1998).

In vertebrates the neural tube is also separated in three longitudinal columns by the expression of homologues of the *Drosophila* columnar genes. In both vertebrate model organisms and some invertebrate representatives like for example *Drosophila melanogaster*, *Tribolium castaneum*, *Platynereis dumerilii* the medial column is marked by the expression of *vnd*/*nkx* homeobox gene family members, the intermediate column by *ind*/*gsh* homeobox genes and the lateral column by *msh*/*msx* homeobox genes (Cornell and Ohlen, 2000; Deschet *et al.*, 1998; Ekker *et al.*, 1997; Hsieh-Li *et al.*, 1995; Pabst *et al.*, 1998; Qiu *et al.*, 1998; Valerius *et al.*, 1995). In vertebrates multiple orthologues of each DV patterning gene exist, which are often expressed in overlapping domains. This impedes the analysis of these genes for neural regionalisation in vertebrates, due to functional redundancy (Cornell and Ohlen, 2000; Satokata and Maas, 1994; Wang *et al.*, 1996b). One example is given by the overlapping expression of *msx1*, *msx2* and *msx3* in the dorsal neural tube in *mus musculus*. There is no convincing phenotype in *msx1* knock out mice because *msx2* and *msx3* can most likely substitute for *msx1* gene function (Satokata and Maas, 1994; Wang *et al.*, 1996b). Nevertheless, there are some studies showing that these genes play similar roles in vertebrates and invertebrates in the DV regionalisation of the developing nervous system (Briscoe *et al.*, 1999; Cornell and Ohlen, 2000; Foerst-Potts and Sadler, 1997; Sussel *et al.*, 1999). Briscoe *et al.* could show that in *nkx2.2* mutants the cell fates are shifted from ventral to intermediate in the ventral spinal cord (Briscoe *et al.*, 1999).

Although the mechanisms of DV patterning in the developing nervous system seem to be highly conserved, there must be evolutionary modifications that result in differences in the neural composition of the nervous systems in individual lineages. To analyse evolutionary changes the expression patterns and functions of the DV patterning genes are compared among different species. In this thesis the issue is examined with regard to arthropods. Specifically, representatives of chelicerates and myriapods were analysed and findings then compared to insects. Apart from *Drosophila*, some data about DV patterning of the NE in other insects already exist. Morphological studies revealed that the neural precursors are arranged in three columns in other insects, like *Schistocerca* and *Tribolium* (Doe and Goodman, 1985c; Wheeler *et al.*, 2005). In *Tribolium* the patterning mechanisms involving the

expression and function of the DV patterning genes *Epidermal growth factor* (*Egfr*), *vnd*, *ind* and *msh* showed that these are conserved (Wheeler *et al.*, 2005). However, the authors also found alterations in the spatiotemporal expression, which could cause species-specific morphological and functional differences within the nervous system. Even though the differences between *vnd* and *ind* expression in *Drosophila* and *Tribolium* are rather subtle, the expression of *msh* starts clearly later in *Tribolium* as compared to *Drosophila*.

1.1.4 Marker gene expression in postmitotic differentiating neural cells

Since the individual NBs in *Drosophila* generate specific lineages, they produce stereotypic progeny, which differentiate into specific glial cells, moto- or interneurons. Individual single or subsets of cells are distinguishable by the expression of differentiation markers. In *Drosophila* all glial cells and most of the motoneurons are identifiable according to differentiation marker gene expression and their specific axonal projections. Furthermore, it is known from which NBs they originate. The major group of cells in the CNS are interneurons. For these only a few markers allow the identification of individual cells, but by marker gene expression, clonal analyses and morphological studies more and more cells are identified. This feature of characterising lineages by certain differentiation markers offers another opportunity to examine if the NPGs in chelicerates and myriapods are homologous to the NB lineages in *Drosophila*. Therefore the positions of differentiation marker gene expressions in the lineages of *Drosophila* are compared to the expression of these genes in the NPGs in *Cupiennius*. For this comparative approach, differentiation markers which are known to be expressed in early generated neurons (for example motoneurons) in *Drosophila* are useful, because the origin of these cells can then be traced back to individual NPGs which are still present at early stages of neurogenesis in *Cupiennius*. Furthermore, these markers should be expressed in a known subset of cells in *Drosophila* and the NB lineage which gives rise to these cells should be known. Both preconditions are achieved by the differentiation markers *even-skipped* (*eve*) and *tailup* (*islet* (*isl*)), as they are both expressed in known subsets of moto- and interneurons and the NBs from which these cells originate these cells are known as well. Both *even-skipped* and *islet* are not expressed in NBs. Whereas *islet* is expressed in identified motoneurons, which innervate ventral body wall muscles,

even-skipped is expressed in identified motoneurons, which innervate dorsal muscles. Furthermore, *islet* is expressed in dopaminergic and serotonergic interneurons and *even-skipped* in other identified interneurons, which are the so-called U-neurons and the neurons of the *eve*-lateral cluster (Certel and Thor, 2004; Landgraf *et al.*, 1997; Landgraf *et al.*, 1999; Thor *et al.*, 1999; Thor *et al.*, 1991; Thor and Thomas, 1997). Therefore, it might be possible to compare the positions of the NBs giving rise to *islet* or *even-skipped* expressing daughter cells to the positions of NPGs expressing these differentiation markers in the spider *Cupiennius*. Hence, these expression studies could help to uncover whether the NB lineages in insects and the NPGs in chelicerates are homologous. Furthermore, the function of these patterning genes in neural precursor identity can be studied by investigating cell fate changes in embryos with impaired patterning gene expression.

1.2 Brain development in arthropods

The formation of the VNE in spiders has been described in several publications and in this thesis (Stollewerk, 2002; Stollewerk, 2004; Stollewerk *et al.*, 2001). However, until now there is almost no data about brain development in these animals. The organisation of the adult brain has already been investigated, but how these brain structures emerge was not analysed. Like in other arthropods the adult spider brain is composed of distinct brain centres with specific neurological functions and a three-dimensional organisation (Barth, 2002; Strausfeld *et al.*, 1998; Strausfeld *et al.*, 1993; Weygoldt, 1985). Since there are similarities as well as differences in brain centres and their functions in *Drosophila* and *Cupiennius*, it is worthwhile to analyse how these differences are generated during development. Until now most data about brain development in invertebrates have come from insects. The only known fact about brain development in spiders is that the brain in *Cupiennius* is generated by invaginating NPGs (Stollewerk, 2004). But how the three-dimensional structures are built from the flat germ band remained unknown. This raises the question of how the NPGs from the procephalic NE can form these structures and how the neural precursors contribute to the distinct brain centres, which are further subdivided in compartments. To get an insight into brain development, the formation of the brain centres by NPGs was analysed in detail by tracing the morphogenetic movements in the procephalic region and the behaviour of the NPGs during this process.

Furthermore the data were compared with *Drosophil*a brain development to investigate if this aspect of neurogenesis is similar between these two arthropods. Detailed comparative studies of the developmental process of brain centre formation were done to reveal which features in this process are conserved or have diverged in the arthropod groups. In *Drosophila* the brain is generated from NBs of the procephalic NE. It is subdivided in distinct centres with specialised functions, like the central body, mushroom body or optic neuropiles (Fig.1-3) (Ito and Hotta, 1992; Sprecher *et al.*, 2006; Urbach and Technau, 2003a; Younossi-Hartenstein *et al.*, 2006). Development of the mushroom body (MB) has been analysed in detail and the origin of the neural cells which contribute to the larval mushroom body was traced back to four identified brain NBs (Ito and Hotta, 1992; Noveen *et al.*, 2000; Prokop and Technau, 1991; Truman and Bate, 1988). Thereby marker gene expression helped to identify the mushroom body NBs (Ito and Hotta, 1992; Kurusu *et al.*, 2000; Noveen *et al.*, 2000; Urbach and Technau, 2003b; Younossi-Hartenstein *et al.*, 2006). For example, the marker gene *dachshund* (*dac*) is expressed in the four mushroom body NBs and their progeny which facilitated the analysis of mushroom body development from single NBs to the late larval brain centre. Furthermore *dachshund* is expressed in the developing optic lobes (Chen *et al.*, 1997; Chen *et al.*, 1999; Mardon *et al.*, 1994; Martini *et al.*, 2000; Tavsanli *et al.*, 2004). The analysis of mushroom body development offers a way to investigate which individual lineages contribute to specialised brain centres. As the aim of this work was not only to analyse the contribution of the NPGs to the brain centres, but also to identify the brain centres during development, marker gene expression could reveal additional information about brain centres in the spider. Due to the expression pattern of *dachshund* in *Drosophila* the expression of this gene was analysed in *Cupiennius* to investigate how the mushroom bodies and the optic neuropiles are generated here. *Cupiennius dachshund* and other marker genes, for example *Cupiennius ASH1*, *Cupiennius netrin*, *Cupiennius islet*, should help to identify the developing brain centres and to trace back from which regions and NPGs in the early two-dimensional procephalic NE they are generated.

1.2.1 Organisation of the brain

The organisation of the brain into different centres with different functions is conserved in vertebrates and invertebrates. Arthropods have specialised centres with specific neurological functions contributing to cognition, behaviour and memory (Heisenberg, 2003; Strauss, 2002). In most of the arthropods, the major centres of the brain include the central body, the mushroom bodies and the optic neuropiles. But there are also differences in the function and morphology of the brain centres, which possibly reflect different requirements of the nervous system in different arthropods. One example for strong morphological differences which need modified processing is the different types and amounts of eyes in arthropods. Whereas insects have one pair of compound eyes and three ocelli (simple eyes), spiders have eight simple eyes that are grouped in six secondary and two principle eyes.

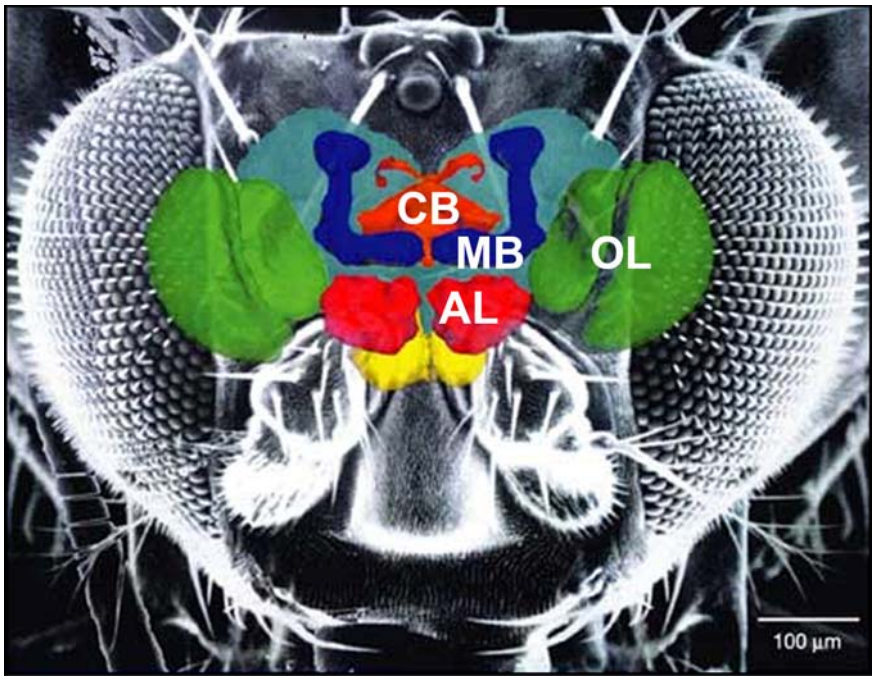

Figure 1-3: Brain centres in the adult *Drosophila melanogaster*.

In insects the brain is organised in distinct brain centres with different functions (coloured fields indicate a frontal view of the adult brain). The positions and morphology of the brain centres, like for example the antennal lobes (red), the optic lobes (green), the central body (orange) and the mushroom bodies (blue) are very well described in *Drosophila*. ***AL***, antennal lobes; ***CB***, central body; ***MB***, mushroom bodies; ***OL***, optic lobes. Modified after Heisenberg, 2003.

The central body is a distinct unpaired midline neuropile, which is considered homologous for insects, crustaceans and myriapods with exception of one myriapod group, the diplopods (Fig.1-3, orange) (Heisenberg, 2003; Homberg, 2008; Loesel *et*

al., 2002). Although this structure has also been found in chelicerates, where it is called arcuate body, there are several differences compared to the central body. The arcuate body in chelicerates is not embedded in the protocerebrum like in insects and crustaceans, but is located in a more superficial position in the dorsal most posterior part of the brain (Strausfeld *et al.*, 2006).

The bilateral mushroom bodies, which are positioned in the anterior-dorsal part of the brain, are very well studied and are associated mainly with olfactory learning and memory (Fig.1-3, blue) (Davis, 1993; de Belle and Heisenberg, 1994; Heisenberg, 1998; Strausfeld *et al.*, 1998; Strausfeld and Hildebrand, 1999; Strausfeld *et al.*, 2009). Therefore, the mushroom bodies can be functionally compared to the mammalian hippocampus, which is also involved in learning and memory (Howland and Wang, 2008). In spiders, the mushroom bodies have been identified, but unlike in insects they do not represent the olfactoric neuropile, but rather play a role in visual information processing (Barth, 2002).

In insects, crustaceans and chelicerates the visual information is transmitted and processed in three optic neuropiles. The first and second optic neuropiles are comparable in the three mentioned arthropod groups, whereas there are differences in the third neuropile. In all three arthropod groups each eye projects to a first and second optic neuropile (ON1 and ON2). From there the information is transmitted to the third optic neuropile (lobula complex) and then to higher order centres of the brain (for example mushroom bodies) in insects and crustaceans. In spiders the visual information from the secondary eyes is transmitted to a common third neuropile (mushroom bodies), after it has been processed by the first and second neuropile. The secondary optic neuropiles of the principal eyes project to the arcuate body, which demonstrates another modification as compared to the insect brain, where the central body does not receive visual information (Barth, 2002).

1.2.2 Brain development in insects

Similar to the VNC, the neural tissue of the insect brain is also generated by specialised stem cells, the NBs. Although the recruitment and delamination of NBs appear similar in several representatives of insects, in *Drosophila* additional modes of NB formation in the procephalic region have been observed (Doe and Goodman, 1985a; Hartenstein V., 1984; Poulson, 1950; Urbach et al., 2003a; Urbach and Technau, 2003a; Urbach and Technau, 2003b; Younossi-Hartenstein et al., 1996; Zacharias D., 1993). This is reflected by cell proliferation in some regions of the procephalic NE prior to NB formation (Foe, 1989; Urbach *et al.*, 2003a; Urbach and Technau, 2003a). As described by Urbach and Technau in 2003 two of these special mitotic domains (domain 1 and 5) are prefigured by horizontal divisions of most of the neuroectodermal cells in this region (Urbach *et al.*, 2003a; Urbach and Technau, 2003a). Apart from these differences in formation of some NBs, the NB identity mechanisms are similar between the VNC and the brain. Different marker genes are expressed in brain NBs which regulate NB fate (Urbach *et al.*, 2003a; Urbach and Technau, 2003a; Urbach and Technau, 2003b; Urbach and Technau, 2004; Urbach and Technau, 2008; Urbach *et al.*, 2003b; Urbach *et al.*, 2006). But some regulatory networks show modifications, for example the regulation of DV patterning (Sprecher *et al.*, 2006; Urbach *et al.*, 2006).

After delamination the approximately 100 brain NBs divide and produce progeny as was described for the VNE (Bauer, 1904; Malzacher, 1968; Panov, 1963; Poulson, 1950; Schrader, 1938; Urbach *et al.*, 2003a; Urbach and Technau, 2003a; Younossi-Hartenstein *et al.*, 1996). However, the progenitors of the optic lobe are an exception, as they do not share the same mode of division (Meinertzhagen, 1973; Meinertzhagen, 1993; Nordlander, 1969; Panov, 1957; Panov, 1960; Schrader, 1938; Urbach and Technau, 2003a).

The proliferation mode of the brain NBs in *Drosophila*, which reflects the division mode in the VNE, takes place mainly in two waves. After the first wave each lineage contains about 10-20 primary neurons in the late embryo. The second wave leads to a ten-fold increase of cells during the larval stages. The cells of these large individual lineages are grouped together and ensheated by glial cells, respectively, which allows to distinguish between the single lineages. Furthermore, the axons that originate from single groups fasciculate together as still distinguishable bundles,

which extend towards the developing neuropile (Pereanu and Hartenstein, 2004; Younossi-Hartenstein *et al.*, 2006). Thus, each larval lineage represents a larval compartment of the developing brain. The analysis of morphogenetic movements and the local activation of molecular markers which are expressed continuously from embryonic to late larval stages helped to link the embryonic brain NBs with the larval brain compartments (Noveen *et al.*, 2000; Sprecher *et al.*, 2007; Urbach and Technau, 2003b; Younossi-Hartenstein *et al.*, 2006). However, until now it has not been described how the larval compartments correspond to the main centres of the adult brain.

1.3 Aims

A significant difference in neural precursor formation between insects, crustaceans, myriapods and chelicerates is reflected by the recruitment of single neural stem cells (NBs) in the first two arthropod groups and the determination of whole groups of invaginating neural precursors (NPGs) in the latter. Analysing and comparing the mechanisms which confer neural precursor identity should reveal if they share a similar mechanism and furthermore if the NB lineages can be homologised to the cells of the NPGs. Comparing the expression pattern and gene function of the columnar genes in different arthropods should give new insights in species-specific differences of neural development. As their function in determining the expression borders between each other and in regulating the formation and fate of the neural precursors in the respective columns is very well studied in *Drosophila*, further studies about these genes in other arthropods offer a good opportunity to investigate evolutionary modifications of neurogenesis. Based on these assumptions, one major issue of this doctoral thesis was to analyse the expression and function of the columnar genes in the myriapod *Glomeris marginata* and the chelicerate *Cupiennius salei*.

The brain consists of a large number of different neural cells, which build morphologically and functionally distinguishable centres. These centres are further subdivided into compartments, which are generated by single or grouped neural precursors. In insects these structures are specified by a combination of developmental processes, which are both similar to the development of the VNC, and

at the same time different in utilising modified mechanisms. As the brains of the different arthropod groups have similar and modified functions and morphologies, the analysis of compartmentalisation and generation of brain centres by the cells of the NPGs in the spider was the second approach of this thesis. To get detailed information about this issue, morphogenetic movements in the precheliceral NE and the differentiation of neural cells were analysed during several stages of development, to subsequently show how neural precursors are arranged into compartments and which compartments then differentiate into brain centres. Furthermore *in situ* hybridisations against marker genes should help to identify the developing compartments and brain centres.

2 Materials and Methods

2.1 Equipment

The equipment used is listed in the appendix.

2.2 Chemicals

All chemicals used and the recipes for the solutions are listed in the appendix.

2.3 Biomolecular methods

2.3.1 Phenol-chloroform precipitation

This technique is used to remove contamination, like for example enzymes, from DNA solutions. The use of Phase Lock Caps (Eppendorf) enables a more convenient handling.

For the precipitation 1 volume phenol-chloroform is mixed thoroughly with the sample and pipetted in a Phase-Lock Cap. To separate the two phases it is centrifuged for 5min at 14000rpm. Then one volume chloroform is added to the upper aqueous DNA containing phase and centrifuged like before. The DNA in the upper phase can then be transferred to a new tube.

The phenol-chloroform precipitation is often followed by an ethanolic precipitation to concentrate the DNA in a smaller volume.

2.3.2 Ethanolic precipitation

To concentrate DNA or RNA a precipitation with ethanol and salt can be done. The ethanol dehumidifies the DNA or RNA and a following centrifugation separates the DNA or RNA from the surrounding liquid.

For the precipitation 1/10 volume sodium acetate (3M, ph 5.2) and 2,5 volumes 100% ethanol are added to the DNA solution and then incubated at least for 1h at -20°C. When RNA needs to be precipitated, LiCl (4M) instead of sodium acetate is used as the salt component. To segregate the precipitated DNA or RNA from the liquid, the mixture is centrifuged for 30min at 14000rpm at 4°C. The supernatant is discarded and the pellet is washed with 500µl 70% ethanol and centrifuged like before for 15min. After drying the DNA pellet it is dissolved in sterile water, DEPC water or elution buffer. In order to dissolve the DNA thoroughly the solution is briefly incubated at 37°C. The RNA pellets are dissolved in DEPC water and briefly heated to 55°C facilitating dissolving of the pellets.

To determine the amount of DNA or RNA after precipitation the concentration can be measured photometrically.

2.3.3 Gel electrophoresis

This technique is used to separate DNA or RNA according to their size.

Both RNA and DNA gels contain agarose, but their further composition is different. The agarose molecules are cross-linked and build a net, the mesh size of which depends on the concentration of agarose. Agarose concentrations between 0.7% and 1.75% were inserted depending on the sizes of the fragments which should be separated.

To separate the fragments, the gel is energised (between 70V and 120V) and the negative charged DNA and RNA fragments migrate towards the positive pole.

2.3.3.1 DNA-Gel

For the assembly of the gel the required amount of agarose is added in 50ml 1x TAE buffer and the mixture is boiled up until the agarose is completely dissolved. When the mixture has cooled down 20µl ethidium bromide (1mg/ml) are added and the liquid gel is transferred to the gel chamber. The gel chamber is equipped with a comb, which moulds pockets in the gel. After hardening of the gel the comb is removed and the samples can be added to the pockets. Before loading, the samples are mixed with loading dye. The loading dye makes the samples drop into the

pockets and the colouring of the dye allows tracking their migration. Then ultraviolet radiation shows the DNA fragments, because it visualises the ethidium bromide, which intercalates in the DNA.

To determine the sizes of the separated DNA fragments a DNA ladder with fragments of known sizes is loaded onto the gel. 1µl ladder (100bp+ (Fermentas) or 2 log ladder) was mixed with 1µl 6x loading dye and 4µl sterile water before loading to the gel pocket.

2.3.3.2 RNA-Gel

A gel with a volume of 50ml is composed of agarose, 1x MOPS buffer, DEPC-H_2O and formaldehyde.

30ml DEPC-H_2O are boiled with 10x MOPS buffer and the required amount of agarose until the agarose has dissolved completely. After cooling down to about 50°C 6.8ml 37% formaldehyde is added to the mixture and it is filled onto the gel chamber (see 2.3.3.1). Before filling the RNA samples to the gel pockets, they are incubated with loading buffer for 5min at 70°C to denaturate the RNA sample. This buffer contains ethidium bromide and therefore enables a later detection of the RNA bands with ultraviolet radiation. After the denaturation loading dye is added to the mixture to weight the sample and to allow tracking it in the gel.

2.3.4 Working with RNA

To avoid degradation of RNA by in the environment encountered RNase, it is necessary to note some special procedures. RNA may only be in contact with sterile equipment, RNase-free solutions and should always be kept on ice.

2.3.5 RNA-Isolation

For the isolation of total RNA from *Cupiennius salei* and *Glomeris marginata*, embryos of selected stages were chosen to ensure that the total RNA contains the mRNA of interest. The embryos were dechorionised (see 2.5.1) and frozen or used for isolation immediately after dechorionisation. The isolation was done with Trizol

solution and without isolation kits. This promises a higher rate of yield, because in the kits columns are used from which long RNA fragments cannot be diluted and get lost.

About 250µl embryos are homogenised with 750µl Trizol solution on ice under the hood. After the suspension was centrifuged for 10min at 14000rpm at 4°C, the supernatant is transferred to a new tube and kept at RT for 5min. Then 20 µl chloroform is added and the solutions is mixed and kept for further 15min at RT. The mixture is centrifuged for 10min at 14000rpm at 4°C and the supernatant is transferred to a new tube, where 500µl isopropanol are mixed with the solution. After 10min at RT, the solution is centrifuged for 15min at 14000rpm at 4°C and the supernatant is removed on ice. The pellet is covered with 500µl -20°C cold ethanol and centrifuged for 10min at 14000rpm at 4°C. After removing the supernatant on ice the RNA containing pellet is dried and then solved in 50µl DEPC-H_2O.

The quality of the isolated RNA is checked with an RNA or a DNA gel and the rate of yield is determined photometrically.

2.3.6 cDNA synthesis

The cDNA is produced using a reverse transcriptase (Superscript III, Invitrogen) which synthesises DNA from an RNA template (2.3.3). To generate cDNA from an RNA template, Oligo dT or Random primers can be used, but the reaction was mostly realised with Oligo dT primers.

For this reaction 5µg RNA template are mixed with 1µl Oligo dT or Random primer (3µg/µl) in a volume of 10µl and incubated for 10min at 72°C. After the mixture is cooled down to 4°C, 4µl 5x first strand buffer, 4µl 5mM dNTPs, 1µl 0,1M DTT and 1µl RNase out are added and kept at 42°C for 2min to prepare the RNA template for the reverse transcriptase. 1µl of the enzyme (200u) is added to the reaction and incubated for 50min at 42°C before it is inactivated by putting the mixture on 72°C for 10min. Finally the RNA template is removed by adding 1µl DNase-free RNaseH to the reaction mix and the cDNA can be stored at -20°C. Enzymes, buffers and primers which were used are from Invitrogen.

2.3.7 PCR

The PCR (Polymerase Chain Reaction) is used to amplify defined DNA sequences from a cDNA template using a DNA polymerase. If the sequence of the DNA which should be amplified is already known, specific primers are designed which frame the regarding sequence. If the gene sequence has to be identified, degenerated primers, which show sequence similarities to the homologue gene in other species, are designed.

2.3.7.1 PCR reactions

The RT-PCR is carried out in a volume of 30µl, which is composed of 1-2u Taq-polymerase, 1x Taq polymerase buffer, 12.5mM 3‘primer, 12.5mM 5‘primer, 0,6µl 10mM dNTPs, 2µl cDNA and sterile water.

For the Nested PCR the same composition is used, but instead of the cDNA, 1µl PCR-product from the RT-PCR is used as a template.

The different steps of the PCR reaction are conducted at different temperatures:

1. 94°C for 60sec
2. 94°C for 30sec
3. 56-63°C for 60sec
4. 72°C for 90sec
5. 72°C for 5min

Steps 2.-4. are repeated 30 times.

The high temperatures of the first two steps are necessary to denaturate the DNA. In the third step the annealing of the primers to the single-stranded DNA is taking place. The ideal temperature for the annealing depends on the GC-level of the primers and hence is chosen individually for each primer combination. The temperature of the last two steps is the optimal temperature for the Taq polymerase, which synthesis the new DNA strands in these steps.

2.3.8 5‘ PCR

This technique is used to elongate known fragments of a gene towards the 5‘ end. Therefore the 5‘PCR Kit from Invitrogen was used like it is described in the instructions. It starts with the generation of defined cDNA from total RNA using a specific primer, which is designed with the known gene fragment. Then sequential PCRs with specific 3’primers of the gene fragment and 5’primers of the kit lead to the elongation of the gene towards the 5‘ region.

2.3.9 Cloning

Cloning is a strategy to integrate a defined DNA sequence in a vector plasmid, which is inserted in bacteria cells. The features of the vector than allow to amplify the DNA fragment or to generate RNA probes or double-stranded RNA of the fragment. The ligation of the fragments in the vector was realised with the pZero Cloning Kit from Invitrogen. For the transformation of the vector in bacteria chemocompetent and electrocompetent cells were used.

2.3.9.1 Preparation of vector

Before the DNA fragments can be integrated in the pZero vector with blunt ends some steps to prepare the vector have to be done. To ensure that the plasmid has blunt ends it is digested with a restriction enzyme. This is done in a 10µl reaction mix with 1µl pZero stock, 1µl EcoRV and 1x enzyme buffer, which is incubated for 30min at 37°C. To remove all the enzymes and other unwanted components of the vector the reaction mix is precipitated with phenol-chloroform and ethanol and salt (see 2.3.1 and 2.3.2). After these two precipitations the pellet is dissolved in 90µl sterile water and stored at -20°C. To check the result of the preparation 1µl of the dissolved vector is loaded to a DNA gel (see 2.3.3.1).

2.3.9.2 Preparation of insert

Like the vector, the DNA fragment which should be inserted into the vector must have blunt ends. Therefore the klenow fragment, which fills in recessed 3‘ ends and

digests away protruding 3‘ overhangs and so produces blunt ends, is used. The 60µl reaction mix contains 50µl PCR-product, 1x klenow buffer, 4mM dNTPs, 2u klenow fragment and sterile water and is incubated for 40 min at 37°C.

If the fragment showed some background in the gel after the PCRs, it had to be loaded on an elution gel, which has a low agarose concentration (about 0.7%) and runs for at least 1.5h at maximally 70V. These parameters ensure a good separation of the bands. The band of the DNA fragment is cut out and extracted from the gel using a Gel Extraction Kit (QIAGEN or Peqlab), like it is described in the respective instruction manuals. To increase the yield of DNA the fragment is dissolved in two steps with 30µl prewarmed EB buffer (40°C - 45°C) each. Finally the fragment is purified and the volume is reduced to 10 -15µl with an ethanolic extraction (see 2.3.1).

If the fragment showed no background in the gel after the PCRs, it could be directly purified either with the PCR purification kit from QIAGEN or with a phenol chloroform precipitation (see 2.3.1). The Purification Kit is used as described in the manual and the last step the yield of DNA is increased by diluting it in two steps with 30µl prewarmed EB buffer (40°C – 45°C) each. After both possibilities of extraction the fragment is purified further and concentrated with an ethanolic extraction.

2.3.9.3 Ligation

During the ligation reaction, which is catalysed by the T4 ligase, the DNA fragment is inserted into the vector.

For this approach 7,5µl of the precipitated PCR-product (see 2.3.9.2), 1µl of the precipitated pZero vector (see 2.3.9.1), 1x ligation buffer, 2.5u T4 ligase and sterile water are mixed in a volume of 10µl and incubated for 1h at 16°C. The vector with the inserted DNA fragment can be stored at -20°C or can directly be transformed into bacteria (see 2.3.9.4).

2.3.9.4 Transformation with electrocompetent cells

Electroporation is a technique which is used to transfer plasmids into bacteria. The electrocompetent TOP10 cells (Invitrogen), which are stored at -80°C, should be

defrosted slowly on ice. 2µl of the ligation are mixed with about 50µl cells and put in a sterile cuvette. Then the cuvette is charged with 2kV/ms, what enables a penetration of the plasmids in the cells. Directly after that 250µl SOC medium are added to the bacteria and the mixture is incubated for 1h at 37°C on a shaker. Afterwards the bacteria are plated on LB plates, which contain 50 µg/ml kanamycin and incubated at 37°C over night. Due to the kanamycin only bacteria carrying the plasmid with the kanamycin resistance gene form colonies on the plate.

2.3.9.5 Transformation with chemocompetent cells

With this technique plasmids are transferred to bacteria cells with the help of a heat shock. Chemocompetent TOP10 (Invitrogen) cells are defrosted slowly on ice and then mixed with 4-10µl plasmid. Before they get the heat shock for 2min at 42 °C, they are incubated for 30min on ice. Right after the heat shock they are directly put back on ice and 250µl SOC medium are added. The growing and plating of the cells is accomplished like it is described for electrocompetent cells.

2.3.9.6 Colony PCR

To analyse which colonies on the plates carry the plasmid with the designated insert colony PCRs are done. Therefore between 24 and 96 single colonies are picked, dipped into the PCR reaction mix and streaked onto a kanamycin containing (50µg/ml) replication plate in labelled fields. For one 10µl PCR reaction mix 1x Taq-buffer, 1µl dNTPs (2mM), 1µl M13 forward primer (10mM), 1µl M13 reverse primer (10mM), 0,2u Taq polymerase and the missing volume sterile water are mixed. The template is then added in form of the picked colony.

PCR program:

1. 96°C for 10min
2. 94°C for 60sec
3. 55°C for 30sec
4. 72°C for 60sec
5. 72°C for 7min

The steps 2.-4. are repeated 35 times.

The success of the transformation can be checked with a DNA gel (see 2.3.3.1) and it can be estimated which colonies carry the plasmid with the insert of interest.

2.3.9.7 Bacterial cultures

The colonies with the designated insert, which were identified like described in 2.3.9.6, are transferred from the replication plates into 3ml LB medium (50µg kanamycin/ml) by picking them with a tip and adding the whole tip to the 15ml falcon. The colonies are incubated with the medium at 37°C over night on a shaker to give best conditions for cell proliferation.

2.3.9.8 Isolation of plasmid DNA from bacteria

To isolate plasmid DNA from the bacteria cells, one half of each over night culture (1.5ml) is pipetted in a 1.5ml Eppendorf tube and centrifuged for 3min at 8000rpm. The supernatant is discarded, the residual 1,5ml bacteria are pipetted to the pellet and the centrifugation is repeated. For the isolation of the plasmids the Qiaprep Miniprep Kit (QIAGEN) is used like it is described in the instruction manual. This Kit makes use of the alkaline lyses to solubilise the cells and to separate the plasmid DNA from the bacterial chromosome which stays connected to the cell wall of the bacteria. After isolation the plasmid DNA is dissolved in 50µl prewarmed EB-buffer (40°C – 45°C). Before the insert of the plasmid is sequenced, the success of the isolation can be checked with a DNA gel (see 2.3.3.1) and by measuring the concentration photometrically.

2.3.10 Generation of RNA probes

For the detection of mRNA of specific genes with help of *in situ* hybridisations antisense RNA probes with digoxigenin (DIG) or fluorescin (FITC) label were generated. Therefore in vitro transcription with a special RNA-polymerase is utilised. The RNA polymerases start the transcription of complementary RNA from a DNA template of their corresponding promoter sequences. The promoter sequences flank

the insert in the plasmid and thus the orientation of the insert determines which RNA-polymerase is used to generate antisense RNA. To label the emerging RNA, DIG- or FITC labelled UTPs are inserted.

For the in vitro transcription a 20µl reaction mix containing 1µg template, 2µl NTPs (DIG- or FITC labelled, Roche), 20u RNAse inhibitor, 1time transcription buffer, 40u T7- or SP6-polymerase (Roche) and DEPC water is prepared. The mixture is then incubated for 2h at 37°C. After the reaction, 20u RNase-free DNase I are added and incubated for 15min at 37°C to get rid of the DNA template. To finally stop all enzymatic reactions 2µl 0.2M EDTA are mixed with the sample. For the purification of the RNA probe it is ethanolic precipitated (see 2.3.2) and the pellet is dissolved in 50 µl DEPC water. To avoid degradation of the RNA probe 20u RNase inhibitor can be added. The success of the in vitro transcription is checked with an RNA or a DNA gel (see 2.33) and the probes are stored at -20°C.

2.3.11 Generation of double-stranded RNA interference

RNAi interference is a 21-23 nucleotide long double-stranded RNA, which can downregulate the expression of specific genes (Baulcombe, 1999; Sharp, 1999). The short RNAi fragments are produced by the cleavage of longer ds RNAs by an RNase III nuclease, named Dicer. This reaction is comparable with the answer to viral infections to protect the organism (Bernstein *et al.*, 2001). The RNAi can downregulate gene expression specifically by guiding a multiproteincomplex (RISC) to the complementary mRNA. Together with RISC the RNAi leads to a degradation of mRNA and therefore to a reduced gene expression.

Although the downregulation is sequence specific, off-target effects can occur, if some restrictions concerning the applied dsRNA are not obeyed. Mainly, the dsRNA which is injected should not be much longer than 100-200bp, the sequence should not be in the 3‘ portion of the gene and it should not cover the first 100bp of the 5‘ end (Qiu *et al.*, 2005). Therefore, fragments which confirm these conditions were cloned to produce the dsRNAi for the required genes.

Like the RNA antisense probes, the dsRNA interference for specific sequences is also generated with in vitro transcription (see 2.3.10). The protocol is modified as both, the T7 and the SP6 polymerase are used in one reaction. Furthermore

unlabelled nucleotides are used to assemble the two strands. The concentration of dsRNAi is increased as the pellet is dissolved in only 10µl after precipitation. Before the dsRNA is tested in a gel it is incubated for 5min at 80°C and then very slowly cooled to RT allowing the two complementary strands to attach to one another.

2.3.12 Sequences and Primers

For the generation of probes and dsRNA for *Cupiennius muscle segment homeobox* (*msh*), *Cupiennius islet* (*isl*), *Glomeris intermediate neuroblast defective* (*ind*) and *Glomeris muscle segment homeobox* (*msh*), clones and primers which were described in Hilary Doves thesis were used (Dove, 2003). The other sequences and primers which were used to generate probes have been published earlier: *Cupiennius acheate-scute homologue 1* (*ASH1*) (AJ309490; (Stollewerk *et al.*, 2001)), *Cupiennius dachshund* (*dac*) (AJ518942; (Prpic *et al.*, 2003)), *Cupiennius engrailed* (*en*) (AJ007437; (Damen, 2002)), *Cupiennius even-skipped* (*eve*) (AJ252155; (Damen *et al.*, 2000)).

2.4 Animal stocks

2.4.1 *Achaearanea tepidariorum* animals

The spiders establishing the breed were kindly provided by H. Oda and Y. Akiyama-Oda. The adult animals are kept solitary in small glasses, which bottoms are covered with humid soil and whose tops are closed with air permeable foam plastic lids. Depending on their size and age, the animals were fed with *Drosophila melanogaster*, *Drosophila pseudoobscura* or small *Acheta domesticus*. After mating the females produce up to 15 cocoons in intervals of about one week. The cocoons contain at most 300 eggs and the quality and quantity of the embryos decreases the later a cocoon is build. The cocoons can be taken away from the mothers with featherweight forceps and as many embryos as needed can be removed for further experiments. The cocoon can be closed again and kept in a humid chamber enabling the remaining embryos to continue their development. Cocoons which were used for further breeding were kept in humid Petri dishes and the hatched spiders were kept in these dishes until the spiders were separated and kept solitary in glasses.

2.4.2 *Cupiennius salei* animals

In the beginning fertilised females were kindly provided by Ernst August Seyfarth. Later on an own breed was established with animals from the same source. Each spider was kept in a big cucumber jar with a layer of moist soil on the bottom and an air permeable lid on top at 25°C and 70% humidity. *Acheta domesticus* was fed to the spiders two times per week and nearly every day to fertilised females. About three weeks after fertilisation, the spiders produce up to five cocoons sequentially in intervals of two to four weeks. The cocoons are carried by the mothers and can be taken away not earlier than on the third day by dazing the female with CO_2 and picking the cocoon carefully with long tweezers. The cocoons, which contain up to 1000 embryos, are opened and the needed amount of embryos are removed. If different stages are needed the cocoon can be closed again and kept in a humid chamber that the remaining embryos continue their development. If the embryos of the cocoon were used for the breed, the cocoon was left by the mother for about eight days, then taken away and kept in a jar until the spiders hatched. After hatching they were kept together for at least three weeks before they were separated to keep them solitary. During development they were fed with *Drosophila melanogaster* and *pseudoobscura* and different sizes of *Acheta domesticus*.

2.4.3 *Glomeris marginata* animals

Adult *Glomeris* were collected in the Ober-Olmer forest close to Mainz, Germany, during their reproduction season, which starts in March and can extend until end of June. They were kept for about three weeks in the lab to collect their eggs and then were brought back to the natural habitat, because they did not produce eggs for a longer period. About 10 males and 20 females were kept in plastic boxes (10cm x 20cm) that were covered with humid paper towels which themselves were covered with soil and leaves from the same forest where the animals were collected. The animals were kept at RT and at least once a day some water was sprayed in the boxes to keep their living space moist enough. The eggs, which are covered with dirt to protect them from injuries and drying-out, were collected every to every second day with pincers and kept in moist chambers until the required stage.

2.5 Preparation of embryos for *in situ* hybridisation and immunohistochemistry

To use the embryos for Phalloidin staining, antibody staining, *in situ* hybridisation or combinations of them, they have to be prepared appropriate. The following paragraphs describe the respective preparation.

2.5.1 Dechorionisation

First, the chorion which sheets the embryos has to be removed. Therefore the embryos are transferred into 15ml falcons and covered with bleach. For spiders 6% chlorine bleach and for *Glomeris* 4% bleach is used. After incubating the embryos with bleach for around 2min the bleach is removed and the embryos are rinsed thoroughly with tap water.

2.5.2 Fixation

The fixation is used to crosslink proteins, RNA and DNA in perpetuity. The protocol is adapted to the experiments which were planned with the embryos.

2.5.2.1 *Cupiennius* and *Achaearanea* for Phalloidin staining

The fixation mixture is composed of 1 volume heptane and 1 volume 5.5% methanol-free formaldehyde (Polyscience, Inc) in PBS or PEMS. For the fixation the embryos are incubated with the fixative for 30min on an overhead-shaker.

2.5.2.2 *Cupiennius* for *in situ* hybridisation

The fixation mixture is composed of 1 volume heptane and 1 volume 5.5% formaldehyde (VWR) in PEMS. For the fixation the embryos are incubated with the fixative over night on an overhead-shaker.

2.5.2.3 *Glomeris* for Phalloidin staining

The fixation mixture is composed of 1ml heptane and 100µl methanol–free formaldehyde (16%). For the fixation the embryos are incubated with the fixative for 20min on a shaker.

2.5.2.4 *Glomeris* for *in situ* hybridisation

The fixation mixture is composed of 1ml heptane and 50µl formaldehyde (37%). For the fixation the embryos are incubated with the fixative for 4h on a shaker.

After fixation the embryos are transferred to either MeOH, if they were fixed for *in situ* hybridisation or EtOH, if they were fixed for Phalloidin staining, by washing them several times. The embryos can be stored in MeOH or EtOH at -20°C until they are used for staining.

2.5.3 Devitellenisation

Finally, the vitellinmembrane has to be removed manually using thin and sharp pincers to enable probes, Phalloidin or antibodies to enter the embryonic tissue.

2.6 Injection of dsRNA interference

To study the functions of specific genes the technique of downregulation by dsRNA interference was utilised. In *Cupiennius* the dsRNA is directly injected into the embryos, whereas in *Glomeris* the technique of parental RNAi is used.

2.6.1 RNAi injection in *Cupiennius salei* embryos

The embryos are injected on the third day after the mother produced the cocoon. After dechorionisation (see 2.5.1), the embryos are pipetted with tap water on a coloured agar plate. Using tweezers they are arranged in rows in small squares (ca.15mm x 20mm) which also help to suck up the spare water. Then the embryos dry for about 10min before they are glued on a cover slip (18mm x 24mm). A cover

slip that was coated with heptane glue on one side is put onto the arranged embryos with the sticky side pointing downwards. The cover slip is then turned around and put in a small Petri dish. Beforehand a drop of oil was added in the dish to avoid the cover slip from moving. When the embryos have again dried for about 10min they are covered with 3A voltalef oil so that only a small part of them breaks through the surface.

Then a capillary is filled with 2µg dsRNA (for the specific gene or for GFP as control) and food dye. This filling is enough to inject about 50 embryos. For the injections the capillary should penetrate the embryo with an ankle as steep as possible from above through the region which is not covered with oil. After the injections the embryos are completely covered with oil and kept in a humid chamber at 25°C until they reach the developmental stage in which they should be fixed for further experiments. Before they can be fixed, they have to be removed from the cover slip with heptane.

2.6.2 Parental RNAi in *Glomeris marginata*

Previous studies on *Glomeris* revealed that direct injections of RNAi in the embryos does not work (Dove, 2003). Therefore the technique of parental RNAi was used in this PhD project. This technique was successfully established in *Tribolium* and *Achaearanea* and thus promising to work in *Glomeris* as well (Akiyama-Oda and Oda, 2006; Bucher *et al.*, 2002).

Therefore the dsRNA was injected in adult females and their progeny was collected and kept until the proper developmental stage before it was fixed. To inject the dsRNA a small hole was made in the dorsal part beneath the heart in the cuticle with help of scalpel and needle. As the animals roll up when they are disturbed they were fixed with modelling clay in this coiled up state and could not move during this procedure. The injections were done with a capillary through the hole in the cuticle and repeated every second day for four times. With every injection about 0.4µg dsRNA were brought in the females and for each gene more than 30 females were injected. As control water was injected in 10 females.

2.7 Immunohistochemistry

2.7.1 Phalloidin staining

Phalloidin binds to F-actin and as it is labelled with FITC enables to visualise the cell shapes with a fluorescent microscope.

Before the staining procedure starts the embryos are transferred from EtOH in PBS by rinsing and washing them with PBS. Then the embryos are blocked for 1h in 1% BSA in PBS to reduce background staining. 10µl Phalloidin-FITC solution are vaporised and then resolved in 200µl PBS with 1% BSA. This staining solution is then incubated with the embryos over night at 4°C. To remove the spare Phalloidin-FITC the embryos are rinsed and washed with PBS for several times. To store the embryos at -20°C they are transferred to a 1:1 solution of 70% glycerol in PBS and Vecta Shield mounting medium what protects the fluorescence. Furthermore the whole procedure is done in as dark as possible conditions to protect the fluorescence.

2.7.2 Antibody staining

Antibody staining is used to detect specific proteins. This is accomplished by primary antibodies, which bind to a specific epitope and labelled secondary antibodies which bind specifically to the primary antibodies. The labelling of the secondary antibody allows the detection of the specific protein by a direct fluorescent label.

For an antibody staining a detergent has to be added to the washing and incubation buffer to enable the 1st and the 2nd antibody to enter the tissue and to reach their epitopes. However, if an antibody staining is combined with a Phalloidin staining the proportion of the detergent should be as low as possible, since it impairs the Phalloidin staining. To make both stainings as good as possible, 0.02% PBTween are used as washing and incubation buffer during the antibody staining and then the Phalloidin staining follows as described above. The following paragraph describes the general protocol of the antibody staining, which can also be done after an *in situ* hybridisation.

The fixed embryos are transferred from EtOH in 0.3% PBTx by rinsing and washing them several times. To reduce background the embryos are incubated with 1% sera

from the corresponding animal for 2h at RT. Then the primary antibody in the adequate dilution (Tab.2-1) in 1% serum in 0.3% PBTx is pipette to the embryos and incubated over night at 4°C. Several rinsing steps and eight 10min washing steps with 0.3% PBTX should remove the spare antibody before the embryos are incubated with the secondary antibody in the adequate dilution in 0.3%PBTx for 2h at RT. If the secondary antibody is labelled with a fluorescent dye the last and all following steps are done in as dark as possible conditions. To remove the spare secondary antibody the washing procedure is repeated before the embryos are transferred over PBS in 70% glycerol in PBS or a 1:1 mixture of 70% glycerol and Vectashield Mounting medium.

Antibody	Species	Dilution	Source
anti-*Dm* α-Tubulin	mouse	1:5	DSHB
anti-*Dm* β-Tubulin	mouse	1:5	DSHB
anti-*Dm* α-Spectrin	mouse	1:5	DSHB
anti-*Cs* Prospero	rat	1:200	Weller
anti-DIG-alalic phosphatase	sheep	1:1000	Roche
anti-FITC- alkalic phosphatase	sheep	1:1000	Roche
anti-FITC- alkalic phosphatase	rabbit	1:200	DAKO

Table 2-1: Used primary antibodies.

Antibody	Label	Species	Dilution	Source
anti-mouse	Alexa	donkey	1:500	Dianova
anti-mouse	Cy3	donkey	1:500	Dianova
anti-mouse	Cy5	donkey	1:500	Dianova
anti-rat	Cy5	donkey	1:500	Dianova
anti-rat	FITC	donkey	1:500	Dianova

Table 2-2: Used secondary antibodies.

2.7.3 *In situ* hybridisation

With this method it is possible to detect transcripts of a specific gene using labelled RNA antisense probes (see 2.3.10). The probe binds to its specific complementary

sequence and as it is labelled (DIG/FITC) can be detected with an additional antibody staining against the label of the probe. The antibody itself is tagged with an enzyme (for example alkalic phosphatase) which catalyses the generation of a detectable product and thus is visualised after adding the substrates for that reaction. As the *in situ* hybridisation needs the assignment of RNA probes the rules of handling RNA have to be followed and only autoclaved solutions with DEPC water can be used.

The devitellinised embryos are transferred from MeOH in 0.1% PBTw (PBTw) by washing those 5min in 50%, 5min in 30% MeOH and 5min in PBTw. Then the embryos are post fixed in 1ml PBTw and 140µl formaldehyde (37%) for 20min at RT, washed again 5min in PBTw and incubated for 5min with proteinase K (2.5µl 1:10 diluent in 1ml PBTw). The proteinase K solution is substituted by PBTw for further 5min before a second postfixation follows as described above. After the fixation the embryos are washed 2x 5min in PBTw and 5min in DEPC water before they are incubated with 1ml TEA buffer and 2.5µl acete anhydride for 1h at RT. Then they are washed and transferred to hybridisation solution B (hybB) by washing them 2x 5min in PBTw, 5min in PBTw:hybB (1:1) and 5min in hybB. hybB is then substituted by hybridisation solution A (hybA) and prehybridised for 4h at 65°C. The probes are prepared by mixing them with hybA in the appropriate dilution (Tab.2-3) and are heated to 100°C for 5min before they are cooled down on ice. By heating the probes, they denaturate and obtain their single stranded form. For the hybridisation, the probes are incubated with the embryos for at least 18h at 65°C.

To remove the proportion of probe with weak or no binding, the embryos are washed at 65°C in different preheated solutions. They are successively washed each time 15min in hybB with 25% 2xSSC, hybB with 50% 2xSSC, hybB with 75% 2xSSC, 2XSSC and 2x 30min in 0,2xSSC. This is followed by several 10min washing steps at RT in 0.2xSSC with 25% PBTw, 0.2xSSC with 50% PBTw, 0.2xSSC with 75% PBTw and just in PBTw. After this washing procedure the antibody staining against the label of the probe is prepared. Therefore the embryos are blocked for 2h at RT with 2% sheep serum in PBTw. Then the antibody (anti-DIG-alkalic phosphatase or anti-FITC-alkalic phosphatase) is incubated with the embryos in 1:1000 dilutions in 2% sheep serum in PBTw over night at 4°C.

The spare antibody is removed by washing with PBTw as described in 2.7.2. Since the antibody is linked to the enzyme alkalic phosphatase it is possible to detect it by adding substrate for this enzyme which is then turned into a visible product. Before the staining reaction takes place, the embryos are transferred into alkalic phosphatase staining buffer and washed twice for 5min. Then they are covered with staining solution which consists of 1ml alkalic phosphatase staining buffer, 3.5µl BCIP and 4.5µl NBT. The staining reaction should be done under dark conditions and its progress has to be observed. If the staining takes very long, the staining solution has to be exchanged at least every 90min. When the staining is strong enough the staining reaction is stopped by rinsing the embryos 3x in PBTw and postfixed them for 20min in 1ml PBTw and 140µl formaldehyde (37%). To get rid of the reddish proportion of the staining and to make it clearer the embryos are transferred in MeOH and incubated for 10min. If no more staining follows, the embryos can be stored in MeOH or 70% glycerol at -20 °C.

If an antibody staining follows the *in situ* hybridisation the MeOH step can impair the binding of the antibody to its epitope, especially when the epitope is a cell membrane component. In this case the incubation with MeOH is skipped and the embryos are transferred in 0.3% PBTx by rinsing and washing them with that buffer after the postfixation. Then the antibody staining follows like it is described in 2.7.6.

RNA probe	Label	Dilution
Cupiennius achaete-scute homologue 1 (*ASH1*)	DIG	1:250
Cupiennius dachshund (*dac*)	DIG	1:250
Cupiennius engrailed (*en*)	DIG	1:250
Cupiennius even-skipped (*eve*)	DIG	1:250
Cupiennius muscle segment homeobox (*msh*)	DIG	1:250
Cupiennius islet (*isl*)	DIG	1:250
Glomeris intermediate neuroblast defective (*ind*)	DIG / FITC	1:500
Glomeris muscle segment homeobox (*msh*)	DIG / FITC	1:500

Table 2-3: Used RNA antisense probes.

2.8 Documentation and evaluation of Phalloidin staining, *in situ* hybridisation and antibody staining

2.8.1 Flat preparations of embryos

For detailed studies of the stainings it was necessary to make flat preparations of the embryos. Therefore they were transferred to an object slide in a drop of 70% glycerol or an 1:1 mixture of 70% glycerol and Vectashield Mounting medium. On the slide the yolk is removed from the embryos with very thin needles. In the spiders *Achaearanea* and *Cupiennius* the legs cover the neuroectoderm (NE) and thus avert evaluations in the NE. Hence, the appendages are cut off with the needles. When the germ band is free of yolk and other impedimental tissues the NE can be covered with a cover slip and the edges are sealed with nail polish.

2.8.2 Documentation

Whole mount pictures of the embryos with visible staining are done with a stereo microscope which is equipped with a camera. Flat preparations are documented using transmission microscope pictures and scans with the confocal laserscanning microscope (Leica, LSM, TCS SP2). The laserscanning microscope enables the detection of visible and fluorescent staining at once. Furthermore it is able to capture all layers from basal to apical of the NE.

2.8.3 Evaluation

For the evaluation of the stacks of scans of the confocal microscope the Leica Lite software and the Imaris software were used. For the documentation and editing of the pictures of the fluorescent and visible stainings Adobe Photoshop and Adobe Illustrator were used.

3 Results

3.1 Neural precursor identity in chelicerates and myriapods

3.1.1 Number and arrangement of neural precursor groups in the chelicerates *Achaearanea tepidariorum*, *Cupiennius salei* and the myriapod *Glomeris marginata*

Until now, the number and arrangement of NPGs in *Cupiennius* and *Glomeris*, have not been analysed in detail (Dove and Stollewerk, 2003; Stollewerk *et al.*, 2001).

A prerequisite for the comparative study of neural precursor specification is a precise description of the numbers and the arrangement of NPGs in wild types of the analysed species. For this detailed analysis Phalloidin staining was used, because Phalloidin binds to the cytoskeleton component F-actin and therefore visualises the cell shapes. As the cells of the NPGs are bottle-shaped with their thinner parts in the apical layer, these bundles of thinner cellular extensions appear as dots in horizontal optical sections stained with Phalloidin-FITC (Fig.3-1). In all studied species (*Achaearanea*, *Cupiennius* and *Glomeris*) the number and arrangement of the NPGs was examined in a developmental stage, where all the NPGs should be present (in the leg hemineuromeres 1-4 in spiders and 1-3 in myriapods, see Fig.3-1 B, C, F, G, J, K). For the detection of NPGs flat preparations of embryos were analysed using a confocal laser-scanning microscope and evaluated in detail (Fig.3-1).

For further investigations it was reasonable to address the NPGs individually. Therefore, the NPGs in *Achaearanea*, *Cupiennius* and *Glomeris* were named in a similar fashion to the nomenclature for NBs in *Drosophila*. However, to avoid the impression of homology between NBs and NPGs in chelicerates and myriapods letters instead of numbers were used to label the rows (Fig.3-2). Although the numbers of the NPGs in *Achaearanea*, *Cupiennius* and *Glomeris* and the NBs in *Drosophila* differ, the arrangement of the neural precursors is similar in all the species with seven transverse rows and three to seven precursor groups per row (Fig. 3-2).

In the two spider species, the number and arrangement of the NPGs is very uniform in the studied hemineuromeres (60 hemineuromeres of 15 *Cupiennius* embryos, 30 hemineuromeres of eight *Achaearanea* embryos) within each species. Furthermore there are no segment-specific differences in the analysed leg neuromeres (Fig.3-1 B, C, F, G). Each leg hemineuromere in *Achaearanea* contains 37 NPGs and in *Cupiennius* the corresponding hemineuromeres contain 38 NPGs (Fig.3-1 C, D, G, H). Both species show a very similar arrangement of NPGs in seven transverse rows with three to seven invagination groups per row. Although the NPGs within a hemineuromere of *Achaearanea* are positioned in a more rectangular pattern compared to the hemineuromeres of *Cupiennius* the almost identical arrangement of the NPGs in these two spider species is obvious (Fig.3-1 C, G). Due to this striking similarity the NPGs in the two spiders seem to be comparable at single NPG level. Thus, the NPG which is missing in *Achaearanea* compared to *Cupiennius* is most likely NPG **g-4** (Fig.3-1 D, H; Fig. 3-2).

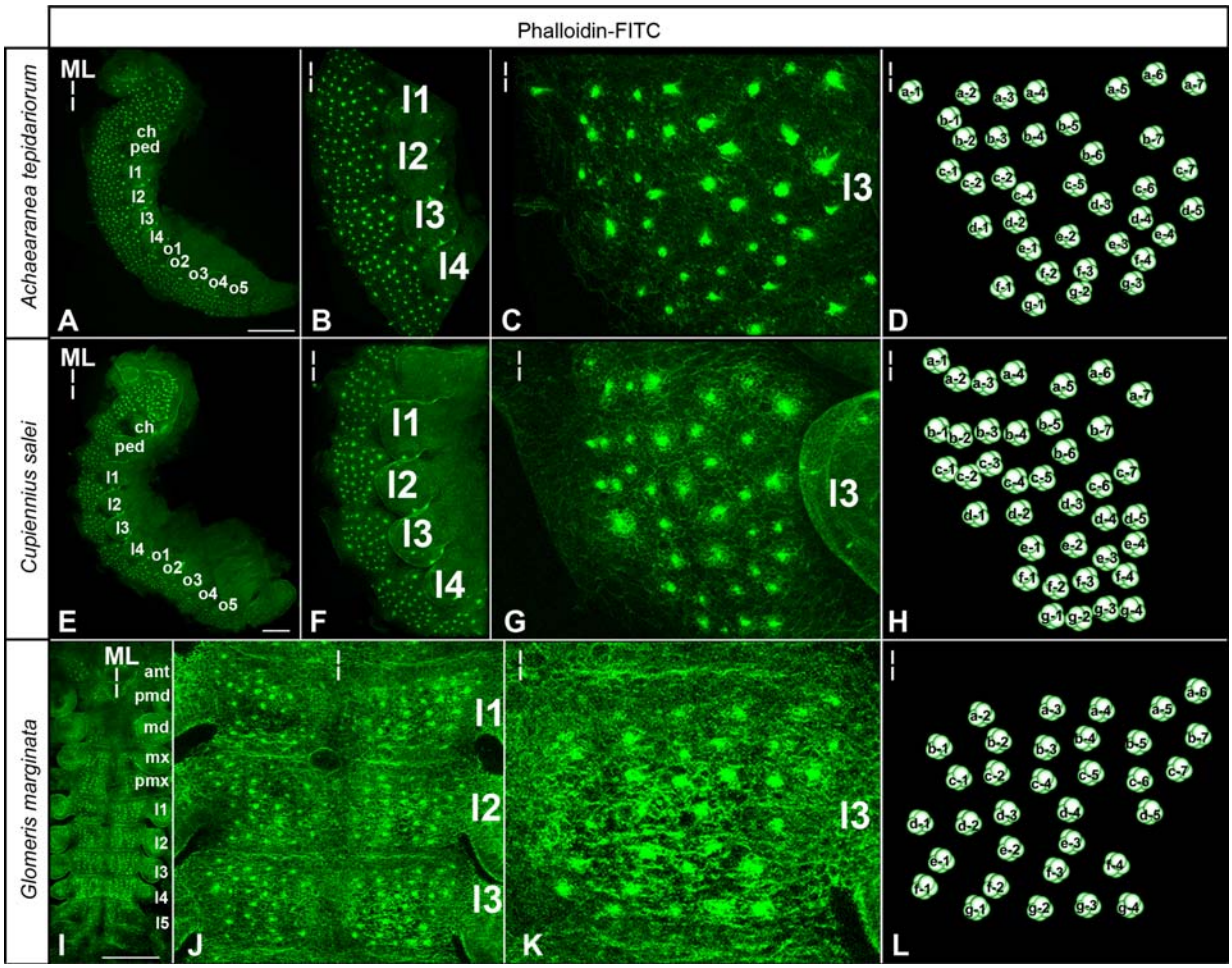

Figure 3-1: Neural precursor groups in *Achaearanea tepidariorum*, *Cupiennius salei* and *Glomeris marginata*.

Maximum projections of Phalloidin stainings in flat preparations of *Achaearanea* (**A-C**), *Cupiennius* (**E-G**) and *Glomeris* (**I-K**) visualise the NPGs; ventral views. Schematic drawings of the number and the arrangement of the NPGs in one leg hemineuromere in *Achaearanea* (**D**), *Cupiennius* (**H**) and *Glomeris* (**L**). The lateral halves of an *Achaearanea* embryo in a stage which is comparable a *Cupiennius* embryo after 200 hours of development (**A**) and a 200 hours old *Cupiennius* embryo (**E**) show NPGs in all developing hemisegments. The NPGs are arranged quite similar in all leg hemineuromeres (**F**) and each leg hemineuromere contains 37 NPGs (**C**, **D**) in *Achaearanea*. In *Cupiennius* the 38 NPGs (**G, H**) also show a similar pattern in all leg hemisegments (**H**). Due to the very similar number and arrangement of NPGs in the two spider species the NPGs can be compared at single NPG level and the one which is missing in *Achaearanea* compared to *Cupiennius* is identified as **g-4** (**D**, **H**). Although there is an obvious developmental gradient between the segments in *Glomeris*, all the segments contain invaginating NPGs at stage 3-4 (stages after Dohle, 1964) (**I**). These NPGs show a uniform arrangement in the leg hemineuromeres 1-3 (**J**), containing 33 NPGs each (**K**, **L**). Despite the slightly different shapes of the hemineuromeres in *Glomeris* and the two spiders, the arrangement of the NPGs is very similar. Thus, the invagination groups can be compared at single NPG level and the NPGs which are missing in *Glomeris* compared to the spiders are most likely **a-1**, **a-7**, **b-6**, **c-3** and **e-4** (**D**, **H**, **L**). ***an***, antennal segment; ***ch***, cheliceral segment; ***l1-l5***, leg segments 1-5; ***md***, mandibular segment; ***ML***, midline; ***mx***, maxillar segment; ***ped***, pedipalpal segment; ***pmd***, premandibular segment; ***pmx***; postmaxillar segment; ***o1-o5***, opisthosomal segments 1-5. Anterior is to the top. Scale bars in (A) 160μm in panel A, E, and I.

In *Glomeris* the detection of the NPGs is more difficult, because the cells of the neuroectoderm (NE) are smaller than compared to these cells in the spider. This makes it more difficult to distinguish between the staining of the NPGs and that of the surrounding cells. Furthermore, the NPGs are staggered and on top of each other which makes it harder to detect them all (Dove and Stollewerk, 2003). But detailed examination and comparison of a large number of hemisegments (n=100) show that in the leg hemineuromeres 1-3 in *Glomeris* 33 NPGs delaminate from the apical layer in seven transverse rows with three to six NPGs per row (Fig.3-1 J, K, L). Most of the NPGs are detectable in more than 90% of the hemineuromeres, but some NPGs in the posterior medial region are not easy to identify. These NPGs are **d-1** and **g-3**, which are detectable in 40% of the hemineuromeres, **c-1** and **f-3** which can be found in 60% and **e-1**, **f-1**, **g-1**, **g-2**, **f-2** and **d-2** which are detectable in 80-85% of the hemineuromeres. However, a detailed comparison of the arrangement of the NPGs in the two analysed spiders shows a striking similarity in the positioning of the NPGs. This impression is enhanced if the slightly different shapes of the hemineuromeres are included into the comparison of the arrangements. Thus, the five NPGs which are fewer in *Glomeris* than compared to *Cupiennius* are most likely **a-1**, **a-7**, **b-6**, **c-3** and **e-4** (Fig 3-1 D, H, L; Fig. 3-2).

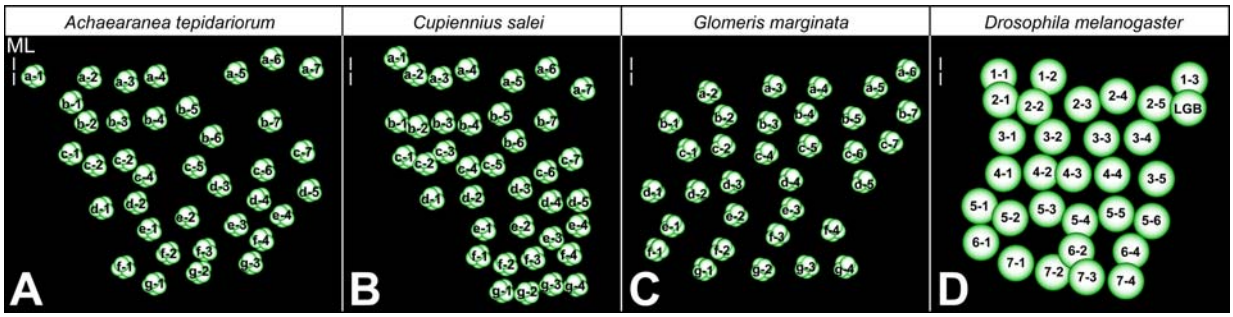

Figure 3-2: Nomenclature of neural precursor groups in *Achaearanea tepidariorum*, *Cupiennius salei*, *Glomeris marginata* and *Drosophila melanogaster.*

Schematics of NPGs in leg hemineuromeres in the spider species *Achaearanea tepidariorum* (**A**) and *Cupiennius salei* (**B**), the myriapod *Glomeris marginata* (**C**) and the insect *Drosophila melanogaster* (**D**, modified after Doe, 1992). (**D**) In *Drosophila* each hemineuromere contains about 30 NBs that are arranged in seven rows with three to six NBs per row. Two numbers name each NB; the first number marks the row and the second number the position within the row from ventral to dorsal. (**A**, **B**, **C**) The NPGs in *Achaearanea*, *Cupiennius* and *Glomeris* were named according to the nomenclature system in *Drosophila*, but with a letter instead of the first digit. (**A**) In *Achaearanea* 37 NPGs are arranged in seven rows with three to seven NPGs per row. (**B**) Even though the hemineuromeres are more elongated along the AP axis in *Cupiennius*, the 38 NPGs are arranged in an almost identical pattern with seven rows and three to seven NPGs per row. (**C**) In the more square-shaped hemineuromeres of *Glomeris* the arrangement of the 33 NPGs is more similar to the arrangement of NBs in *Drosophila*. (**A-D**) With regard to the different shapes of the hemineuromeres in the presented arthropods, the overall arrangement of neural precursors is very similar among all analysed representatives. ***LGB***, lateral glioblast; ***ML***, midline. Anterior is to the top.

Having obtained the exact information about number and arrangement of the NPGs, it was now possible to investigate if chelicerates and myriapods share a similar mechanism of neural precursor identity with *Drosophila*. Therefore the expression patterns and gene function of spatial patterning genes were analysed with regard to their expression within the NPGs and their possible role in transferring cell identity to the NPGs in the chelicerate *Cupiennius salei* and the myriapod *Glomeris marginata*.

3.1.2 In the expression domain of the DV patterning gene *msh* in *Glomeris marginata* seven to eleven identified NPGs are recruited

As mentioned above the dorsoventral (DV) patterning gene *muscle segment homeobox* (*msh*) is expressed in the lateral column of the VNE in *Drosophila* and its expression has also been studied in *Tribolium*. In *Drosophila* it has been shown that this gene is not required for NB formation but plays an essential role in giving the lateral NBs a specific identity. In *Glomeris msh* is expressed in the most lateral part of the NE (Dove, 2003). The classification of the NPGs now made it possible to analyse which of the 33 NPGs express *msh*. Double-stainings with *msh* and α-Spectrin

facilitated the simultaneous analysis of the *msh* expression domain and the position of the NPGs (Fig.3-3). α-Spectrin is located at the inner side of the plasma membrane to form a dimer with β-Spectrin. Since this epitope is located at the cell membrane, the staining with α-Spectrin antibody visualises the cell shapes including the apical cell processes of the NPGs.

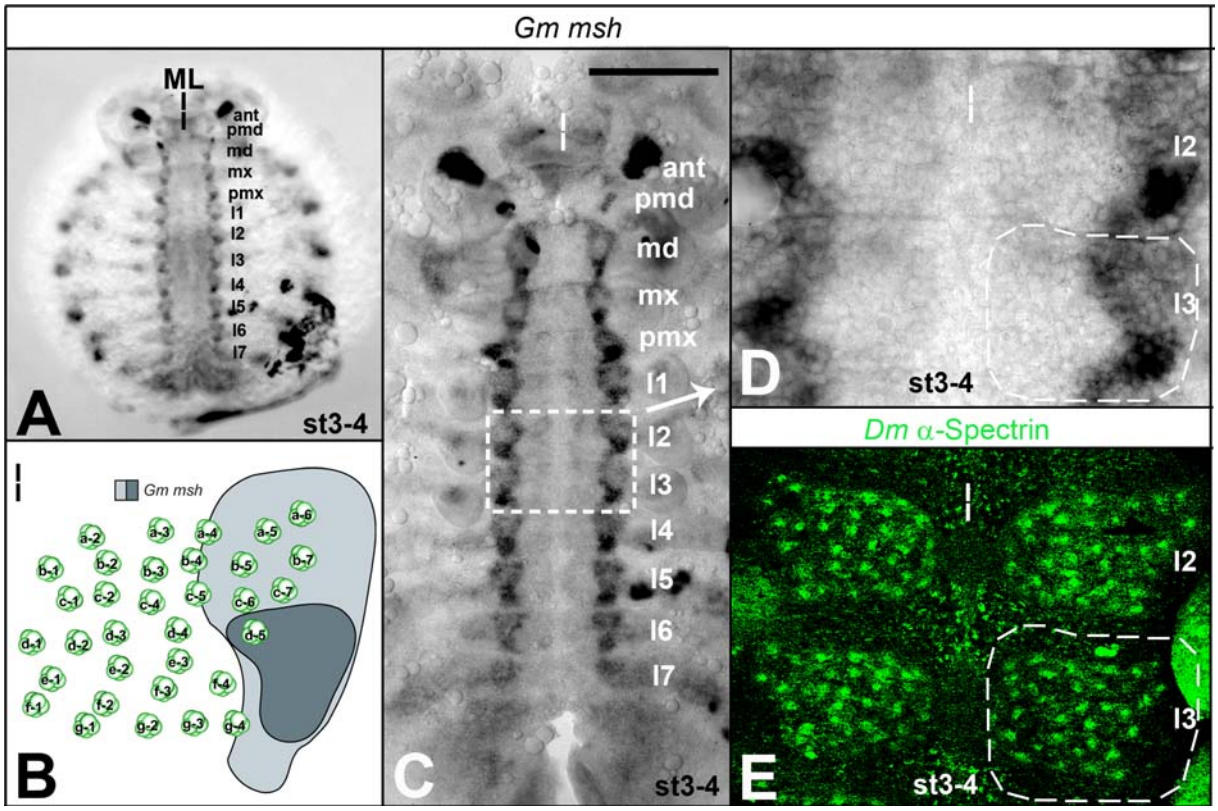

Figure 3-3: *Glomeris marginata msh* expression.

(**A**) Whole mount *Glomeris* embryo stage 3-4 stained with a DIG-labelled *Glomeris msh* RNA antisense probe, ventral view. (**C**, **D**) Flat preparations of stage 3-4 *Glomeris* embryos stained with a DIG-labelled *Glomeris msh* RNA antisense probe; ventral views. (**E**) Fluorescent anti-α-Spectrin staining visualises the NPGs in the same hemineuromeres which are presented in D. (**B**) Schematic drawing of the NPGs and *msh* expression domain in one leg hemineuromere. (**A**, **C**) *msh* is expressed in developing segments in the lateral column of the NE in a rather invariant pattern with the exception of the antennal segment, where *msh* is quite strongly expressed and in the premandibular segment, where *msh* expression is weaker. (**A**, **C**, **D**) In all other segments *msh* is expressed in the lateral part of the NE in a more extended domain with weaker expression in the anterior proportion and a narrower domain with stronger expression in the posterior proportion. (**D**, **E**) Double-staining of *msh* and the NPGs reveals which NPGs are recruited within the *msh* expression domain. (**B**) Seven NPGs (**a-5**, **a-6**, **b-5**, **b-7**, **c-6**, **c-7**, **d-5**) are clearly recruited from the region with *msh* expression and further four NPGs (**a-4**, **b-4**, **c-5**, **g-4**) are located at the border of *msh* expression. ***an***, antennal segment; ***Dm***, *Drosophila melanogaster*; ***Gm***, *Glomeris marginata*; ***l1-l7***, leg segments 1-7; ***md***, mandibular segment; ***ML***, midline; ***msh***, *muscle segment homeobox*; ***mx***, maxillar segment; ***pmd***, premandibular segment; ***pmx***, postmaxillar segment. Anterior is to the top. Scale bar: (C) 160μm.

As described above, in *Glomeris msh* is expressed in the lateral part of the NE in all segments from the antennal segment onwards (Fig.3-3 A, C). Apart from the head segments, where it is very strongly expressed in the antennal segment and very weakly in the premandibular segment, expression level is equal in the remaining segments (Fig.3-3 A, C). However there are different expression levels within the single neuromeres. In the anterior parts of the hemineuromeres *msh* is expressed in a broader domain compared to the more restricted domain in the posterior parts of the hemineuromeres. Within this narrow domain *msh* is clearly stronger expressed than in the broader anterior domain (Fig.3-3 C, D). The *msh* and Spectrin stainings in one and the same embryo revealed that seven to eleven NPGs invaginate from the *msh*-positive region (Fig.3-3 D, E). Seven of these groups are located clearly in the *msh* expressing region and four of these groups invaginate at the border of the *msh*-positive area. Since the NPGs are individually addressable due to their position within the NE and relative to each other, the seven NPGs in the *msh* expressing region were determined as **a-5**, **a-6**, **b-5**, **b-7**, **c-6**, **c-7** and **d-5** and the NPGs at the expression border as **a-4**, **b-4**, **c-5** and **g-4** (Fig.3-3 B). In contrast, ten NBs delaminate from the *msh* expression domain and maintain *msh* expression in *Drosophila*.

3.1.3 Two to six NPGs are recruited in the expression domain of the DV patterning gene *ind* in *Glomeris marginata*

The second analysed DV patterning gene is *intermediate neuroblast defective* (*ind*). In *Drosophila ind* is expressed in the intermediate column and is important for NB formation and identity. The expression pattern of *ind* was also studied in *Tribolium*, where it is expressed in a cell cluster in the posterior part of the neuromeres and not in a continuous column like in *Drosophila*.

It has already been demonstrated that *Glomeris ind* is expressed in the intermediate domain of the hemineuromeres (Dove, 2003), but it was not shown which NPGs invaginate from the *ind*-positive region. The aim to reveal possible homologies between the NPGs in *Glomeris* and the NB lineages in *Drosophila* demanded a more detailed study of *ind* expression with respect to the individual NPGs in *Glomeris* (Fig.3-4).

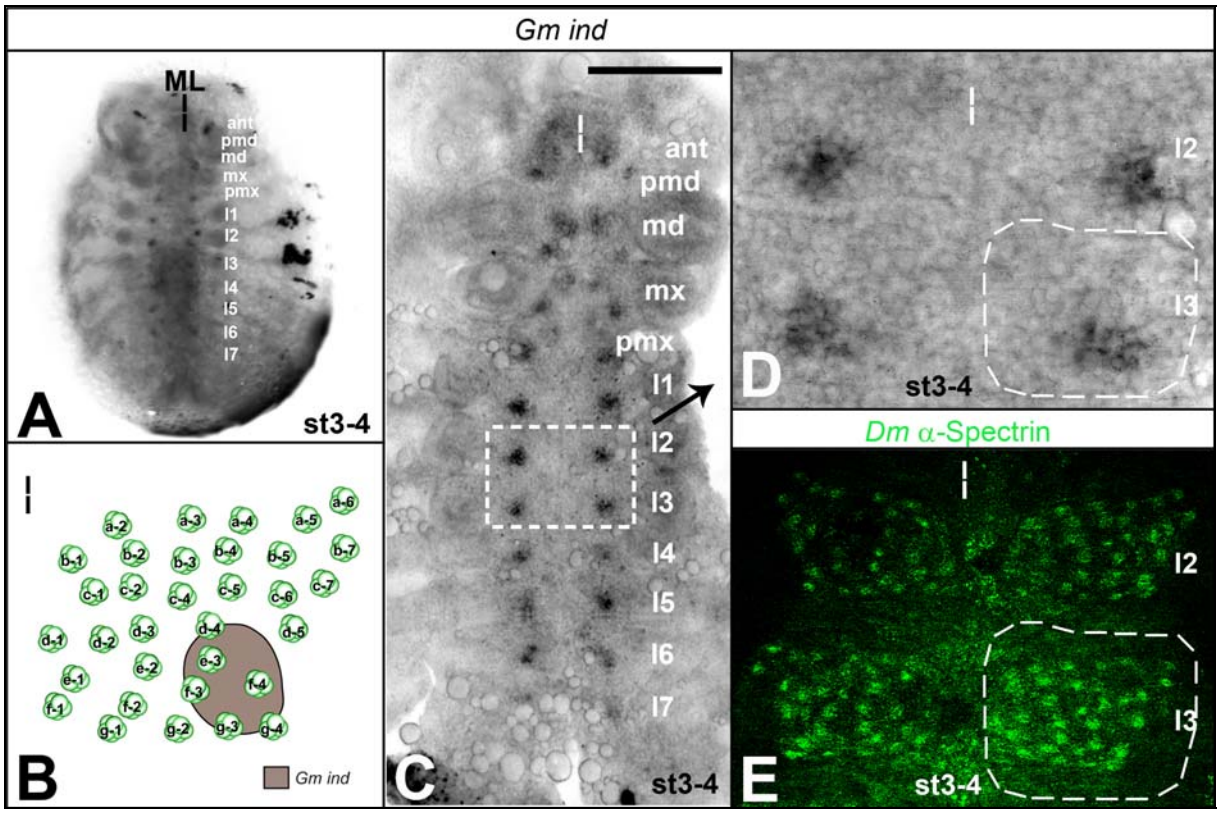

Figure 3-4: Expression pattern of *Glomeris marginata ind*.

(**A**) Whole mount *Glomeris* embryo stage 3-4 stained with a DIG-labelled RNA antisense probe against *Glomeris ind*, ventral view. (**C**, **D**) Flat preparations of stage 3-4 *Glomeris* embryos stained with a DIG-labelled *Glomeris ind* RNA antisense probe; ventral views. (**E**) Spectrin staining visualising the NPGs in the same hemineuromeres which are presented in D. (**B**) Schematic drawing of the NPGs and *ind* expression domain in one leg hemineuromere. (**A**, **B**) *ind* is expressed in a circular expression domain in the posterior-median part of the NE in all developing hemisegments. (**C**) In the gnathal segments an additional *ind* domain is found, which is positioned anterior-medial to the described *ind*-positive cells. (**C**, **D**) *ind* expression in the leg hemineuromeres is located in the intermediate column and restricted to the posterior part of the neuromeres. (**D**, **E**) Labelling of NPGs and *ind* together allows analysing which NPGs are located in the *ind*-positive region. (**B**) 4 NPGs (**d-4**, **f-3**, **g-3**, **g-4**) are located in the *ind*-positive region and two more (**e-3**, **f-4**) invaginate at the anterior or posterior border of *ind* expression, respectively. ***an***, antennal segment; ***Dm***, *Drosophila melanogaster*; ***Gm***, *Glomeris marginata*; ***ind***, *intermediate neuroblast defective;* ***l1-l7***, leg segments 1-7; *md*, mandibular segment; ***ML***, midline; ***mx***, maxillar segment; ***pmd***, premandibular segment; ***pmx***, postmaxillar segment. Anterior is to the top. Scale bar: (C) 160μm.

The *in situ* hybridisation of *ind* is consistent with earlier data and shows that *ind* is expressed in a cell cluster in the posterior medial region of the hemineuromeres (Fig.3-4 A, C). This expression domain is found in all the segments from the most anterior (antennal segment) to the most posterior (newly built leg segment in posterior growth zone) segment. In addition, further *ind*-positive cells are found in a more anterior and further medial position in the gnathal neuromeres (Fig.3-4 C). This has not been described before and will be discussed further in paragraph 3.1.5. In summary, these experiments could show which NPGs are recruited from within the

ind expressing region in one and the same embryo (Fig.3-4 D, E). Between two and six NPGs delaminate from the *ind*-positive region; the NPGs **e-3** and **f-4** are located in the centre of *ind* expression, whereas the NPGs **d-4**, **f-3**, **g-3** and **g-4** are positioned at the border of this region (Fig.3-4 B). In comparison to this, in *Drosophila* five NBs are recruited from the intermediate column expressing *ind.*

3.1.4 The expression of *msh* and *ind* seem to be mutually exclusive in the leg segments of *Glomeris marginata*

In the VNC of *Drosophila* there are three columnar genes: *msh* is expressed lateral, *ind* intermediate, and *vnd* expression spans the ventral column. There, DV patterning is proposed to follow a strict hierarchy of ventral dominance, where ventral genes repress more dorsal genes (Cowden and Levine, 2003). Therefore *vnd* represses *ind* and *vnd* and *ind* repress *msh*. Due to this regulation of expression the columnar genes are never coexpressed in the VNE in *Drosophila*.

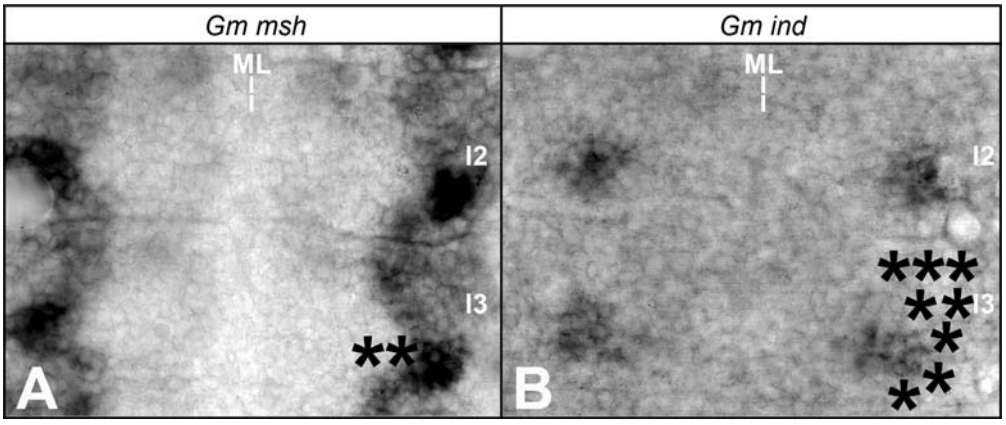

Figure 3-5: *msh* and *ind* expression seem to exclude one another in stage 3-4 *Glomeris marginata* leg segments.

Flat preparations of leg neuromeres 2 and 3 in stage 3-4 *Glomeris* embryos stained with a DIG-labelled *Glomeris msh* (**A**) and *Glomeris ind* (**B**) RNA antisense probe; ventral views. (**A**) *msh* is expressed in the most lateral part of the NE with a broader expression domain in the anterior portion and a thinner expression domain in the posterior part of the hemisegments. (**B**) *ind* is expressed more medial compared to *msh* and its expression is restricted to the posterior halves of the hemineuromeres. (**A**, **B**) The asterisks mark the expression domains of *ind* (A) or *msh* (B), respectively, and thus show that the expressions of both genes seem to exclude one another. ***Gm***, *Glomeris marginata*; ***ind***, *intermediate neuroblast defective*; ***I2*** *and* ***I3***, leg segments 2 and 3; ***ML***, midline; ***msh***, *muscle segment homeobox*. Anterior is to the top.

Double *in situ* hybridisation of both genes was done in *Glomeris* to find out if expression patterns of *ind* and *msh* are mutually exclusive, but the staining was difficult to evaluate since the two dyes used were not easily distinguishable due to unspecific staining (data not shown). But the comparison of both single stainings in the leg segments suggests very strongly, that the genes are not coexpressed (Fig.3-5). The asterisks mark the areas where the respective other gene is expressed (Fig.3-5 A, B). This is further supported by the comparison of the NPGs that invaginate from the *msh*- or *ind*-positive region, because the NPGs invaginate from either the *ind* or *msh* expressing domain (Fig.3-4 B, Fig.3-3 B). The only exception is NPG **g-4**, which invaginates at the border between *ind* and *msh* expression and can not be assigned clearly to either of the two expression domains.

3.1.5 The expression of *msh* and *ind* might overlap in the anterior segments of *Glomeris marginata*

To further investigate the assumption that *ind* and *msh* are not coexpressed in *Glomeris,* as is known for *Drosophila*, the expression of the two genes was analysed in all segments at various developmental stages.

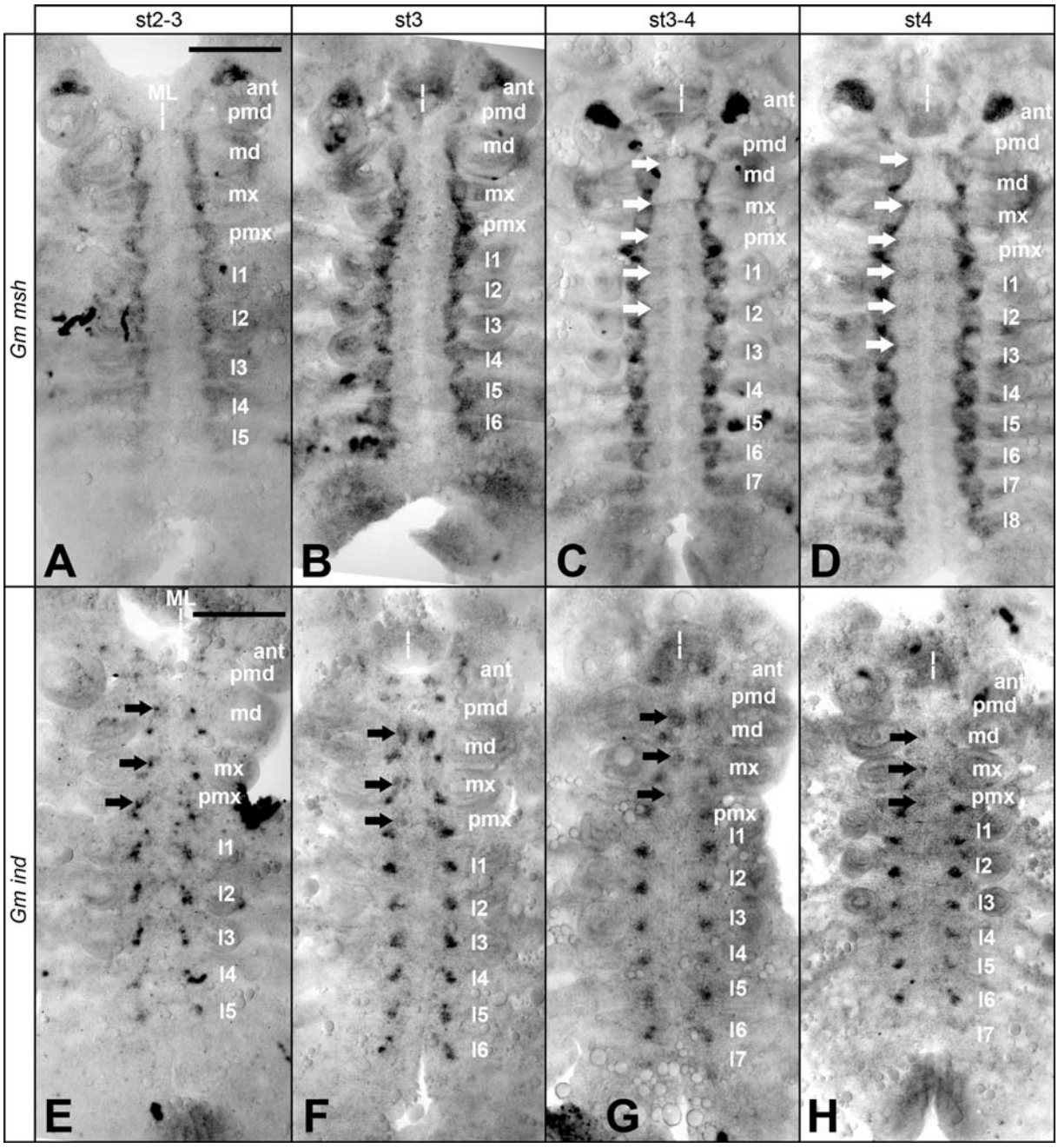

Figure 3-6: Expression of *msh* and *ind* in different stages of *Glomeris marginata*.

Legend see next page (51).

Legend to Fig.3-6: Flat preparations of *Glomeris* embryos stained with a DIG-labelled *Glomeris msh* RNA antisense probe at stage 2-3 (**A**), stage 3 (**B**), stage 3-4 (**C**) and stage 4 (**D**)*;* ventral views. Flat preparations of *Glomeris* embryos stained with a DIG-labelled *Glomeris ind* RNA antisense probe at stage 2-3 (**E**), stage 3 (**F**), stage 3-4 (**G**) and stage 4 (**H**); ventral views. (**A**) *msh* expression is already present in stage 2-3 in the lateral part of the NE in all developing segments. At this stage expression is uniformly within the segments, but staining in the antennal segment is already stronger compared to the other segments, especially compared to the premandibular segment. (**B**) The continuous lateral *msh* expression increases and within this expression domain transcripts strongly accumulate in the posterior parts in stage 3. In the newly generated posterior segments *msh* expression starts immediately after their formation. (**C**) In stage 3-4 *msh* is expressed in an additional cell cluster in a further anterior medial position in the gnathal hemineuromeres and in the leg hemineuromeres 1 to 3 (arrows). (**D**) Until stage 4 the cells in the medial anterior region show an increase in *msh* expression in the gnathal hemineuromeres and in the leg hemineuromeres 1 to 3 (arrows) and furthermore *msh* is expressed in the more posterior segments. (**E**) At stage 2-3 *ind* is already expressed in several domains in the VNE. In the gnathal hemineuromeres *ind* is expressed in two separate clusters, in the intermediate posterior cluster and in a more anterior medial cluster (arrows). In the leg hemineuromeres *ind* expression is found in a small diagonal domain which extends from posterior intermediate to anterior medial. (**F**) While *ind* expression in the leg hemisegments becomes more circular, the expression in the gnathal hemisegments in the anterior medial region extends and becomes stronger (arrows) in stage 3. Furthermore *ind* starts to be expressed in the newly built posterior segments. (**G**) In stage 3-4 *ind* is continuously expressed in the medial anterior domain in the gnathal hemineuromeres (arrows). (**C**, **G**) The expression domains of *msh* and *ind* in the anterior medial region in the gnathal hemisegments might overlap which is most obvious in the mandibular and maxillar hemineuromeres. (**D**, **H**) Apart from its circular expression in the posterior intermediate part of the hemisegments, *ind* remains expressed in the additional anterior medial cluster in the gnathal hemisegments in stage 4 (arrows). The mandibular and maxillar hemisegments show a possible coexpression of *ind* and *msh* in the anterior medial region of the hemisegments. ***an***, antennal segment; ***Gm***, *Glomeris marginata*; ***ind****, intermediate neuroblast defective;* ***I1-I7***, leg segments 1-7; ***md***, mandibular segment; ***ML***, midline; ***msh***, *muscle segment homeobox*; ***mx***, maxillar segment; ***pmd***, premandibular segment; ***pmx***, postmaxillar segment. Anterior is to the top. Scale bars: (A, E) 160μm in panel A-H.

msh expression is detectable in stage 2 - 3 in the lateral part of the NE in the segments already generated (Fig.3-6 A). This lateral expression becomes stronger by stage 3 and a more intense staining in the posterior part of the neuromeres becomes visible (Fig.3-6 B). During all other analysed stages the expression pattern of *msh* in the lateral part of the NE is constant in all the segments which are sequentially generated one after another (Fig.3-6 C, D). However, apart from this constant expression in the lateral column during all the stages, other cells in a more medial region in the anterior part of the neuromeres start to express *msh* as well. This additional expression is clearly detectable at stage 3 - 4 in all anterior segments up to the second leg segment and also (but weaker) in the third leg segment (Fig.3-6 C, arrows). With ongoing development the additional expression in the third leg hemineuromeres increases to an intensity which is comparable to the further anterior segments and furthermore the additional expression starts in the fourth leg segment (Fig.3-6 D, arrows).

Similar to *msh*, *ind* can already be detected in stage 2 - 3 in all segments generated. In this early stage, *ind* shows a more extended expression domain from the medial posterior region to the further medial anterior part of the hemineuromeres. In the leg hemisegments 1 - 3 this seems to be one domain, but in the further anterior hemisegments, like the mandibular and the maxillar hemisegments this domain is clearly separated into two spots of *ind*-positive cells (Fig.3-6 E, arrows). In stage 3, this additional expression in the anterior part of the hemineuromere disappears in the leg segments and they show the already described circular *ind*-expression domain in the medial posterior part of the hemineuromeres. However, the anterior *ind*-expression extends within the gnathal segments at the same time (Fig.3-6 F, arrows). This expression pattern is then maintained throughout development. The newly formed segments initially express *ind* in a more extended domain before it is restricted to the rather circular domain in the posterior medial part of the hemineuromeres (Fig.3-6 F, G, H). The gnathal segments are an exception as they maintain, or even increase, *ind*-expression in the additional region in the anterior medial part of the hemisegments (Fig.3-6 E, F, G, H, arrows).

Comparing *msh* and *ind* expression in the leg neuromeres strongly suggests that the two genes exclude one another, because as the additional *msh* expression in the anterior medial part of the hemisegments starts, *ind* expression is already restricted to the posterior medial region (Fig.3-5, Fig.3-6). This is different in the gnathal neuromeres where *ind* is expressed in the anterior medial region. When *msh* expression starts in the additional anterior medial domain the expression of *ind* rather extends than disappears in this domain. This suggests that the two columnar genes might be coexpressed in the anterior medial part of the gnathal segments in stage 3 - 4 and stage 4. This possibility is most obvious in the mandibular, the maxillar and the postmaxillar segments (Fig.3-6 C, D, G, H, arrows).

These results suggest a different mode of regulation of *ind* and *msh* in the gnathal segments of *Glomeris* as compared to *Drosophila.* However, double-stainings are required to prove this hypothesis.

3.1.6 RNA interference experiments did not reveal reliable data about the gene functions of *msh* and *ind* in *Glomeris marginata*

To analyse the function and regulation of *msh* and *ind*, the consequences of a knock-down of these genes were of interest. Therefore, parental RNA interference (RNAi) was utilised since earlier studies showed that direct injection of dsRNAi into the embryos does not work (Dove, 2003). Targeted gene knock-down using parental RNAi was successfully established in *Tribolium* and *Achaearanea*, and thus promising to work in *Glomeris* as well. dsRNA for *msh* or *ind* was injected in adult females and their progeny were tested for phenotypes. Those embryos were used to analyse the effect of a possible downregulation. Since cross-repressive interaction between *ind* and *msh* seems possible, *ind* RNAi embryos were stained for *msh* and conversely *msh* RNAi embryos were stained for *ind* expression. However, for the analysis of *msh* RNAi embryos it was not possible to collect a sufficient amount of embryos to perform this analysis. Also, the *msh* staining of the *ind* RNAi embryos did not show a phenotype. In the neuromeres of all analysed *ind* RNAi embryos (n=11) *msh* was expressed similar to untreated embryos (see above). Therefore it can not be excluded that *msh* and *ind* regulate one another, especially because in *Drosophila* *msh* is repressed by *ind* and *vnd*, and therefore *vnd* could be the candidate which keeps *msh* expression in its normal boundaries in *ind* RNAi embryos. But for reliable conclusions about the regulation and functions of the DV patterning genes, these experiments have to be repeated and further experiments including the third DV patterning gene *vnd* have to be done.

3.1.7 All NPGs of rows f and g and some NPGs of row a are recruited in the expression domain of the segment polarity gene *engrailed* in *Cupiennius salei*

In addition to analysing the expression and function of the DV patterning genes, one AP patterning gene (segment polarity gene) was analysed for its expression in the NPGs of *Cupiennius*.

It is already known that expression of the segment polarity gene *engrailed* is conserved in the neural precursor rows 1 (=**a**), 6 (=**f**) and 7 (=**g**) (Stollewerk A. , 2006). But *in situ* hybridisation of this AP patterning gene together with labelling of the NPGs with antibodies against α- and β-Tubulin allows a more detailed analysis. Furthermore the newly acquired data about the detailed number and arrangement of NPGs would allow addressing exactly which NPGs of row **a**, **f** and **g** are recruited in the *engrailed* expressing region (Fig.3-7 C, D). In *Cupiennius engrailed* is expressed in segmental stripes in the NE and the appendages (Fig.3-7). These stripes cover the most posterior part of the segments and extend into the anterior part of the following segment during neurogenesis (Fig.3-7 C, E, F). Detailed studies and comparisons of the *engrailed* expression and the staining of the NPGs reveal that all the NPGs of row **f** (**f-1**, **f-2**, **f-3**, **f-4**) and **g** (**g-1**, **g-2**, **g-3**, **g-4**) and four of the seven NPGs of row **a** (**a-1**, **a-2**, **a-3**, **a-4**) are recruited in the *engrailed* stripe (Fig.3-7 C, D, F). This is very similar to *Drosophila* where all the NBs of rows 6 and 7 and one NB of row 1 delaminate from the *engrailed* expressing transversal stripe.

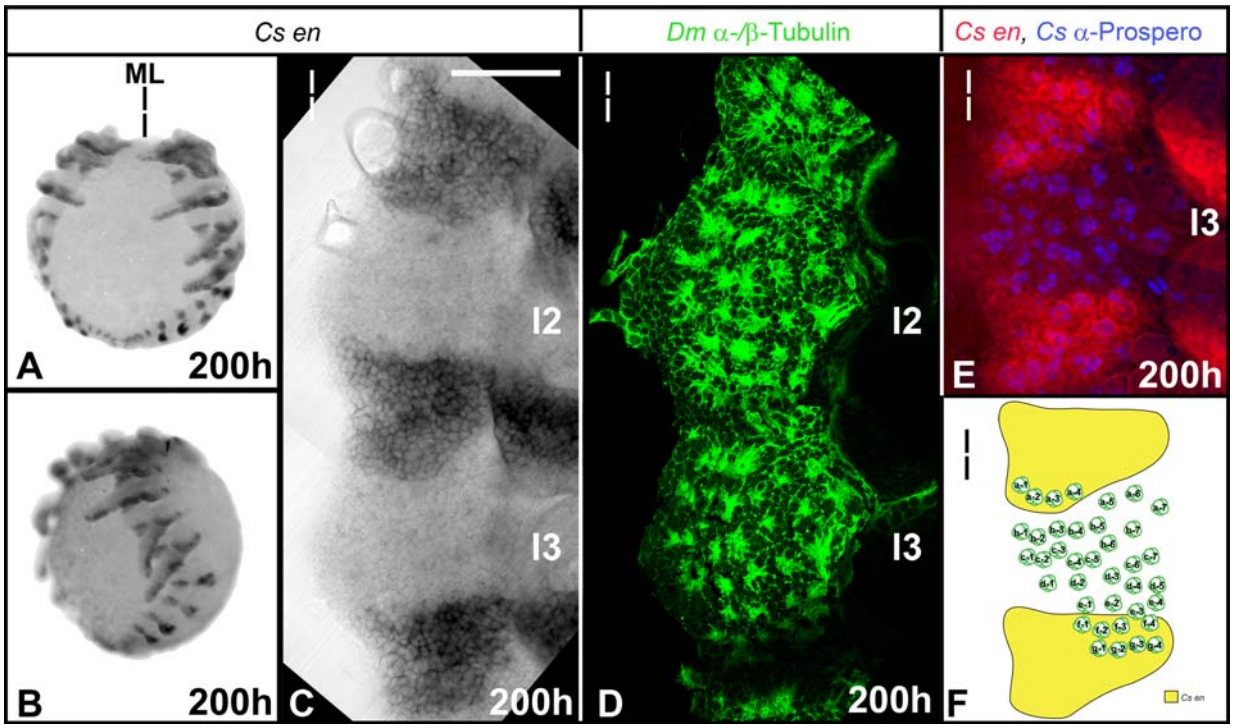

Figure 3-7: *Cupiennius salei engrailed* expression in the NE and neural precursors.

(**A**, **B**, **C**, **E**) *In situ* hybridisation with a DIG-labelled *Cupiennius engrailed (en)* RNA antisense probe in *Cupiennius* embryos after 200 hours of development. (**A**) Whole mount, ventral view. (**B**) Whole mount, lateral view. (**C**) Flat preparation, leg hemineuromeres 2 and 3. (**E**) Leg hemineuromere 3 with an *en*/Prospero double-staining. (**D**) The NPGs are visualised with a combined α- and β- Tubulin antibody staining in leg hemineuromeres 2 and 3. (**F**) Schematic drawing of NPGs and *en* expression in one leg hemineuromere. (**A**, **B**) *en* is expressed in transverse stripes in the posterior region in all segments and in the posterior half of the appendages. (**C**) At 200 hours of development the *en* stripe covers the posterior part of the hemisegments and extends into the anterior part of the following hemisegments. (**D**) The staining of the NPGs and *en* in the same hemisegments reveals which NPGs are recruited in the *en*-positive domain. (**E**) The differentiating neural precursors maintain *en* expression after invaginating from the *en*-positive transverse stripe. (**F**) The *en*-positive domain contains the first four NPGs of row **a** (**a-1**, **a-2**, **a-3**, **a-4**) and all NPGs of rows **f** (**f-1**, **f-2**, **f-3**, **f-4**) and **g** (**g-1**, **g-2**, **g-3**, **g-4**). ***ch***, cheliceral segment; ***Cs***, *Cupiennius salei*; ***Dm***, *Drosophila melanogaster*; ***en***, *engrailed*; ***l1-l4***, leg segments 1 to 4; ***ML***, midline; ***o1-o5***, opisthosomal segments 1 to 5; ***ped***, pedipalpal segment. Anterior is to the top. Scale bar: (C) 80μm in panel C, D and E.

The NPGs are not only generated in the *engrailed* expressing region, but furthermore the expression is maintained in the differentiating neural cells since they coexpress *engrailed* and the differentiation marker Prospero (Fig.3-7 E).

3.1.8 Eleven NPGs are recruited in the expression domain of the DV patterning gene *msh* in *Cupiennius salei*

Like in the insects *Drosophila* and *Tribolium* and in the myriapod *Glomeris*, the DV pattering gene *msh* is expressed in the most lateral part of the VNE in *Cupiennius*

(Fig.3-8). In *Drosophila* the expression is maintained in most of the NBs recruited in this region (Isshiki *et al.*, 1997). *msh* expression in *Cupiennius* is also restricted to the lateral part of the NE in all segments from the cheliceral segment towards posterior in a stage where all the NPGs are detectable in the VNE (Fig.3-8 A, B). However, this expression is not continuous in a complete lateral column, but rather shows intersegmental gaps (Fig.3-8 C). Furthermore it is not expressed in a regular stripe with an even width, but shows a wider expression in the anterior part of the hemisegment and a more narrow expression in the posterior part (Fig.3-8 C). Within the segmental expression domain, *msh* is stronger expressed in the posterior area, similar to *Glomeris* (Fig.3-8 C).

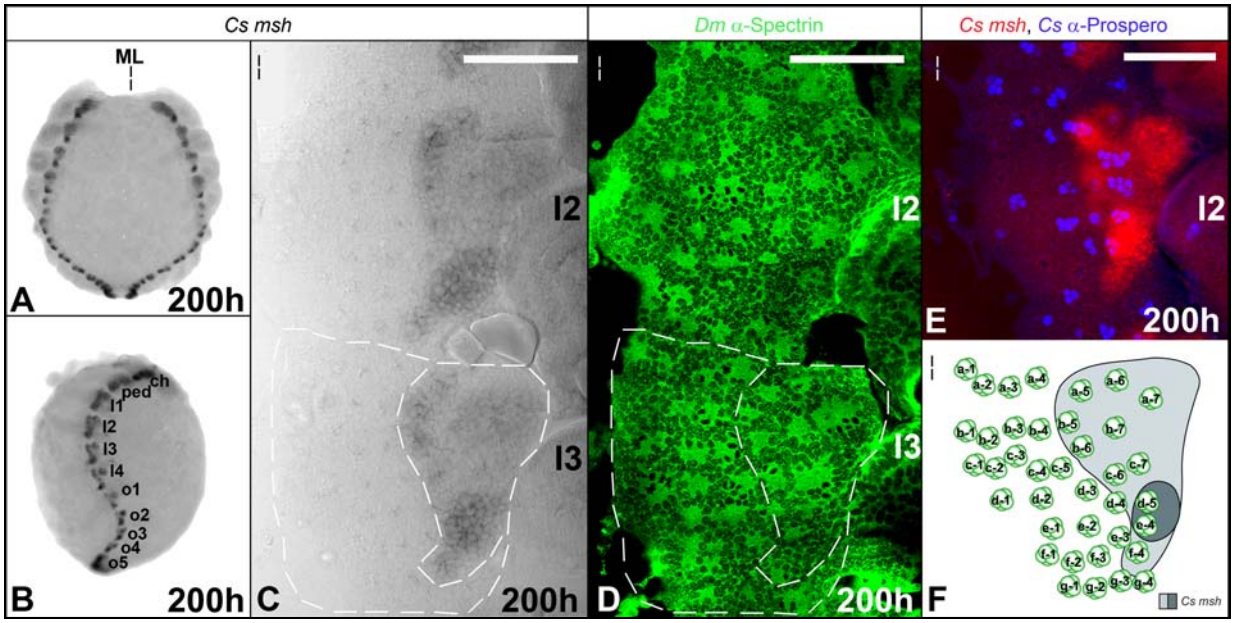

Figure 3-8: *Cupiennius salei msh* expression in the NE and the neural precursors.

(**A**, **B**, **C**, **E**) *In situ* hybridisation with a DIG-labelled *Cupiennius msh* RNA antisense probe in a *Cupiennius* embryo after 200 hours of development. (**A**) Whole mount, ventral view. (**B**) Whole mount, lateral view. (**C**) Flat preparation, leg hemineuromeres 2 and 3, ventral views. (**D**) The NPGs in the leg hemineuromeres 2 and 3 are marked by Spectrin antibody, ventral view. (**E**) Double-staining of *msh* and Prospero in the second leg hemineuromere, ventral view. (**F**) Schematic drawing of *msh* expression and NPGs in one leg hemineuromere. (**A**, **B**) *msh* is expressed in the lateral part of the NE in all segments. (**C**) The expression levels differ within the hemisegments, as there is broader and weaker expression in the anterior part of the neuromeres and narrower and stronger expression in the posterior part of the hemineuromeres. The dotted staining over the whole hemisegments is background which occurs when dye accumulates in the small groves formed by the invaginating NPGs. (**C**, **D**) The staining of the NPGs and *msh* in the same hemisegments shows which NPGs are recruited in the *msh*-positive VNE. (**E**) Co-expression of *msh* (red) and Prospero (blue) shows that *msh* is expressed in the differentiating cells of the NPGs (**F**) Eleven lateral NPGs (**a-5**, **a-6**, **a-7**, **b-5**, **b-6**, **b-7**, **c-6**, **c-7**, **e-4**, **f-4**) are formed in the *msh*-positive region. ***ch***, cheliceral segment; ***Cs***, *Cupiennius salei*; ***Dm***, *Drosophila melanogaster;* ***I1-I4***, leg segments 1 to 4; ***ML***, midline; ***msh***, *muscle segment homeobox*; ***o1-o5***, opisthosomal segments 1 to 5; ***ped***, pedipalpal segment. Anterior is to the top. Scale bars in C, D and E: 80μm.

In *Drosophila* about ten NBs maintain *msh* expression after delamination from the *msh* expressing region in the NE (Isshiki *et al.*, 1997). Eleven of the 38 NPGs of *Cupiennius* are recruited in the region with *msh*-expression. These are NPGs **a-5**, **a-6**, **a-7**, **b-5**, **b-6**, **b-7**, **c-6**, **c-7**, **e-4** and **f-4**. None of the NPGs of row **g** arise in the *msh*-domain, because of the gap in *msh* expression between segments (Fig.3-8 C, D, F). Furthermore, *msh* is not only expressed in neural precursors but expression is maintained in the differentiating neural cells which is shown by the coexpression of the neural differentiation marker Prospero (Fig.3-8 E).

Although in *Drosophila*, *Tribolium* and *Cupiennius*, *msh* expression is restricted to the most lateral part of the NE, there are differences in the shape of the expression domains and the temporal expression of the gene. The expression in the lateral column in *Drosophila* is relatively continuous and begins early. In contrast the onset of expression in *Tribolium* is much later (Isshiki *et al.*, 1997; Wheeler *et al.*, 2005). In *Cupiennius msh* expression in the lateral parts of the VNE lasts from the beginning of neurogenesis until the end of embryogenesis (Fig.3-9).

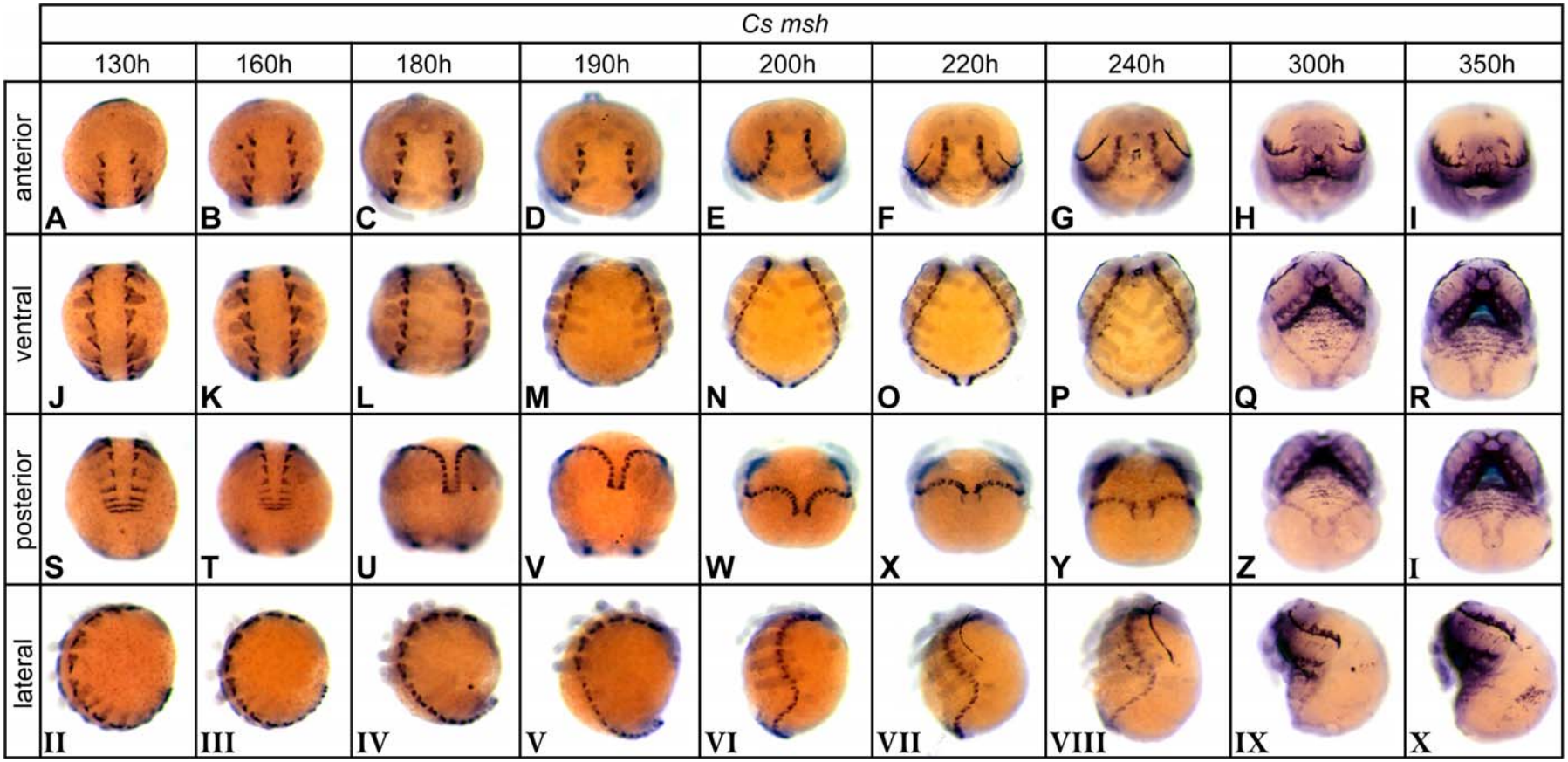

Figure 3-9: *msh* is continuously expressed in the lateral column of the NE during embryogenesis in *Cupiennius salei*.

In situ hybridisation with a DIG-labelled *Cupiennius msh* RNA antisense probe in whole mount *Cupiennius* embryos at different stages of development from 130 hours to 350 hours from anterior (**A**-**I**), ventral (**J**-**R**), posterior (**S**-**I**) and lateral (**II**-**X**). (**A**, **J**, **S**, **II**) *msh* expression is detectable from early stages onward in the lateral part of the NE. (**S**, **T**) The newly built segments in the posterior region show *msh* expression in transverse stripes which becomes restricted to the lateral part of the neuromeres as the segments develop. (**A**-**X**) Later in development *msh* is slightly stronger expressed in the lateral column of the NE, but the spatial expression remains the same in all observed stages. (**H**, **Q**, **Z**, **IX**, **I**, **R**, **I**, **X**) Even in late embryonic stages (300h-350h), *msh* is still expressed in the lateral part of the VNE. This expression is obscured by newly formed cuticle in the anterior segments but is still visible in the more posterior opisthosomal segments. ***Cs***, *Cupiennius salei*; ***msh***, *muscle segment homeobox*.

Although there are slight changes in the shape of the expression domain during development, the expression in the lateral part is maintained during all observed developmental stages (Fig.3-9).

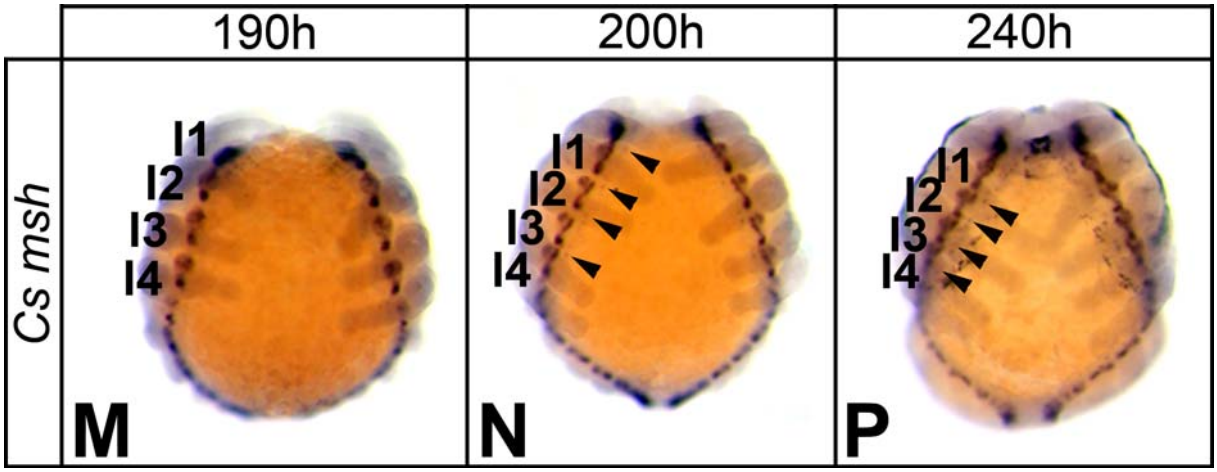

Figure 3-10: *msh* is also expressed in anterior medial cells in *Cupiennius salei*.

(**M**, **N**, **P**) Magnifications from Fig.3-9. Ventral views of whole mount *Cupiennius* embryos stained with a DIG-labelled *Cupiennius msh* RNA antisense probe after 190 hours (**M**), 200 hours (**N**) and 240 hours (**P**) of development. (**M**) At 190 hours *msh* is expressed exclusively in the lateral column of the NE. (**N**) Ten hours later there are additional *msh*-positive cells in a more medial position in the anterior part of each segments (arrowheads). (**P**) During further development *msh* transcripts accumulate in this anterior medial expression domain (arrowheads). ***Cs***, *Cupiennius salei*; ***I1-I4***, leg segments 1 to 4; ***msh***, *muscle segment homeobox*. Anterior is to the top.

An additional small cluster of *msh* expressing cells is found in the anterior medial part of the hemisegments between 190 and 200 hours of development (Fig.3-10 M, N, arrowheads) and persists throughout development (Fig.3-10 P, arrowheads). The cells of the medial cluster seem to originate form the lateral *msh* expression domain. At about 200 hours of development they seem to separate from the lateral domain and migrate medially along a deeper layer of differentiated cells (Fig.3-11 E, F, G, H arrowheads). Most of the *msh* expressing cells in the lateral domain are positioned further apical than the cells of the additional medial cell cluster (Fig.3-11). The apical lateral expression domain contains some differentiating neural cells, but the majority of the differentiating Prospero-positive cells are located further basal (Fig.3-11 A, B, C, D, F, H, Fig.3-8 E). In later stages (about 240 hours of development) *msh* is expressed in an additional medial cell cluster, which is positioned in the posterior part of the hemisegments (Fig.3-11 G, arrows). Like the medial cluster in the anterior part

of the segment, the posterior medial cluster is also located in a more basal layer, which contains differentiating neural cells (Fig.3-11 G, H).

Thus, the expression of the DV patterning gene *msh* in the lateral part of the NE is very stable during all studied stages of *Cupiennius salei* neurogenesis. The only alteration in the *msh* expression pattern occurs from 200 hours onwards, where the expression is extended towards other cell clusters located at a more medial basal position.

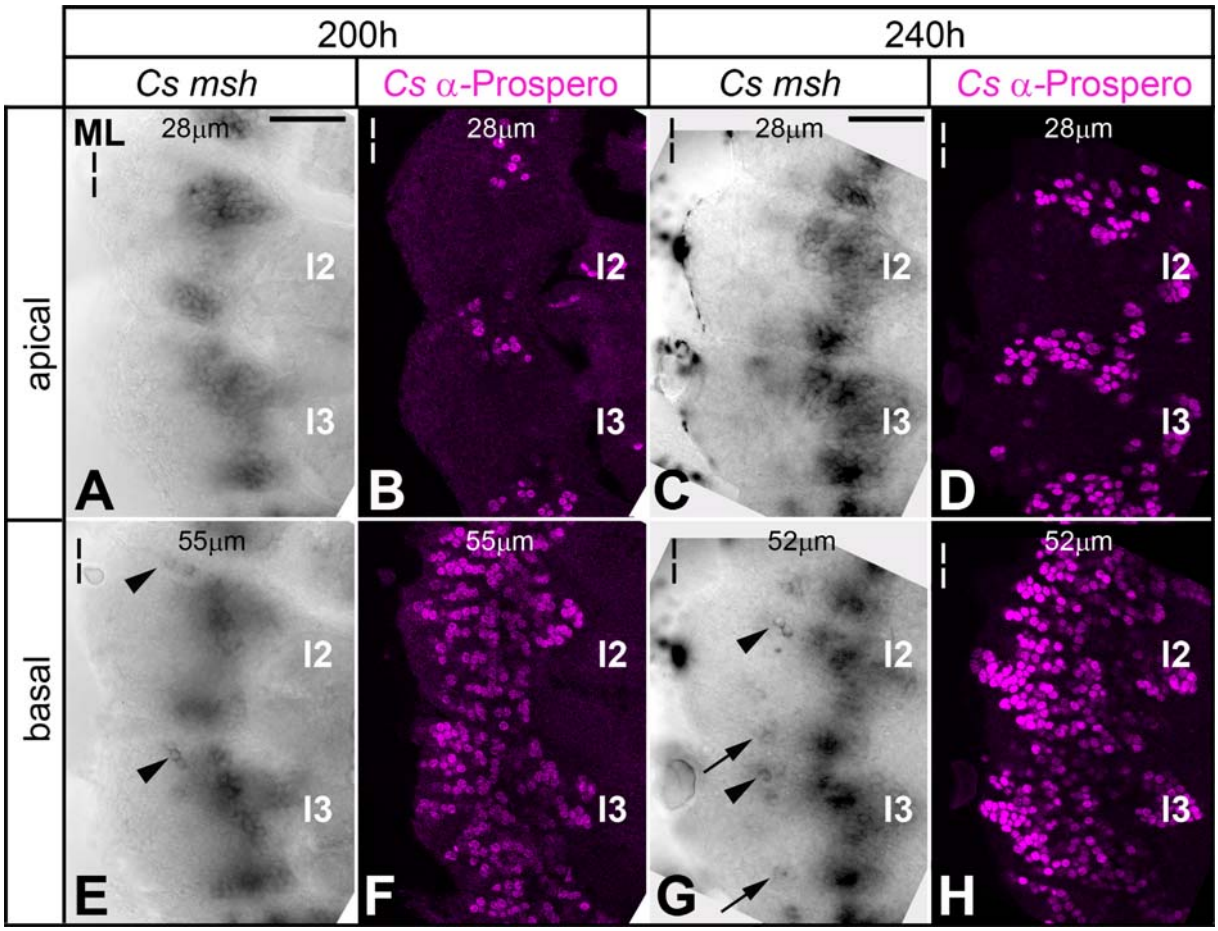

Figure 3-11: The anterior medial *msh*-positive cells are located in a basal position in *Cupiennius salei*.

Legend see next page (61).

Legend to Fig.3-11: (**A**, **E**) *In situ* hybridisation with a DIG-labelled *Cupiennius msh* RNA antisense probe in a flat preparation of leg hemineuromeres 2 and 3 in different apico-basal positions after 200 hours of development, ventral views. (**B**, **F**) *Cupiennius* Prospero antibody staining in the same hemineuromeres and horizontal-layers as in A, E. (**C**, **G**) Flat preparations of leg hemineuromeres 2 and 3 stained with a DIG-labelled *Cupiennius msh* RNA antisense probe after 240 hours of development. (**D**, **H**) Differentiating neural precursors stained with the *Cupiennius* Prospero antibody in the same hemineuromeres and apico-basal positions like in C, G. (**A**, **B**) *msh* expression in the lateral column of the NE is mainly positioned in an apical layer with only a few differentiating neural precursors. (**E**, **F**) The *msh*-positive cells in the anterior medial cluster are positioned more basally (arrowheads) compared to the major part of the *msh*-positive cells of the lateral column. The basal layer which contains the *msh*-positive cells also contains more differentiating neurons after 200 hours of development. (**D**) After 240 hours of development more neural precursors started to differentiate in the apical part of the NE. Most of the differentiating cells in this layer are positioned at the segmental borders. (**C**) The main portion of *msh*-positive cells in the lateral column is located in the apical part which does not contain the described further medial *msh* expressing cells. (**E**, **G**) The more basal part of the hemisegments shows weaker *msh* expression in the lateral column. This basal part contains the anterior medial *msh*-positive cell cluster, which seems to be more medial as the corresponding cell cluster after 200 hours of development (arrowheads). (**G**) In addition to the *msh*-positive cell cluster in the anterior medial region (arrowheads) another cluster shows *msh* expression in the posterior medial region (arrows) after 240 hours of development. (**F**, **H**) Like in younger stages, the basal part of the NE contains more differentiating neural precursors compared to the apical part after 240 hours of development. ***Cs***, *Cupiennius salei*; ***I1+I2***, leg hemisegments 1 and 2; ***msh***, *muscle segment homeobox*. Anterior is to the top. Scale bars: (A, C) 80μm in panel A-H.

3.1.9 The homologue for the *Drosophila* DV patterning gene *ind* could not be identified in *Cupiennius salei*

In order to analyse mechanisms underlying neurogenesis in the spider it was of great interest to investigate the expression pattern and function of the DV patterning gene *ind* in *Cupiennius.* It is known that this gene is expressed in the intermediate column of the NE in *Drosophila*, *Tribolium* and *Glomeris*, but until now there are no data about this gene in spiders. A lot of effort was made to identify the sequence of the gene to generate probes and to check its expression.

The PCRs with degenerated primers (see paragraph 2.3.7) amplified short fragments in the conserved domain of the homeodomain. The attempts to amplify longer fragments did not lead to the identification of *ind*, but to the identification of other homeodomain proteins, for example the homologue of *Drosophila bagpipe*, which is expressed in the developing heart (data not shown). Using other degenerated primers, additional longer fragments were obtained. One of these fragments showed strong homology to *Drosophila plexin A*, which is important for axonal pathfinding (data not shown).

3.1.10 The differentiation marker *even-skipped* is expressed in a small cell cluster in the NE of the posterior segments in *Cupiennius salei*

The pair-rule gene *even-skipped* (*eve*) is expressed in a known subset of differentiated moto- and interneurons in *Drosophila*. Although this gene is not expressed in neural stem cells in *Drosophila* it is known that the four NBs 1-1, 3-3, 4-2 and 7-1 give rise to *even-skipped*-positive lineages (Fig.4-2 B) (Doe *et al.*, 1988; Landgraf *et al.*, 1997). In order to compare NB lineages and NPGs the expression pattern of this differentiation marker was analysed in *Cupiennius* and compared to the pattern in *Drosophila*. Motoneurons which express *even-skipped* project via the intersegmental nerve (ISN) to dorsal muscles and furthermore *even-skipped* plays an essential role in the pathfinding process of these neurons (Doe *et al.*, 1988; Landgraf *et al.*, 1999). Apart from expression in these identified motoneurons *even-skipped* is also observed in eight to ten interneurons which represent the *eve*-lateral cluster (Frasch *et al.*, 1987).

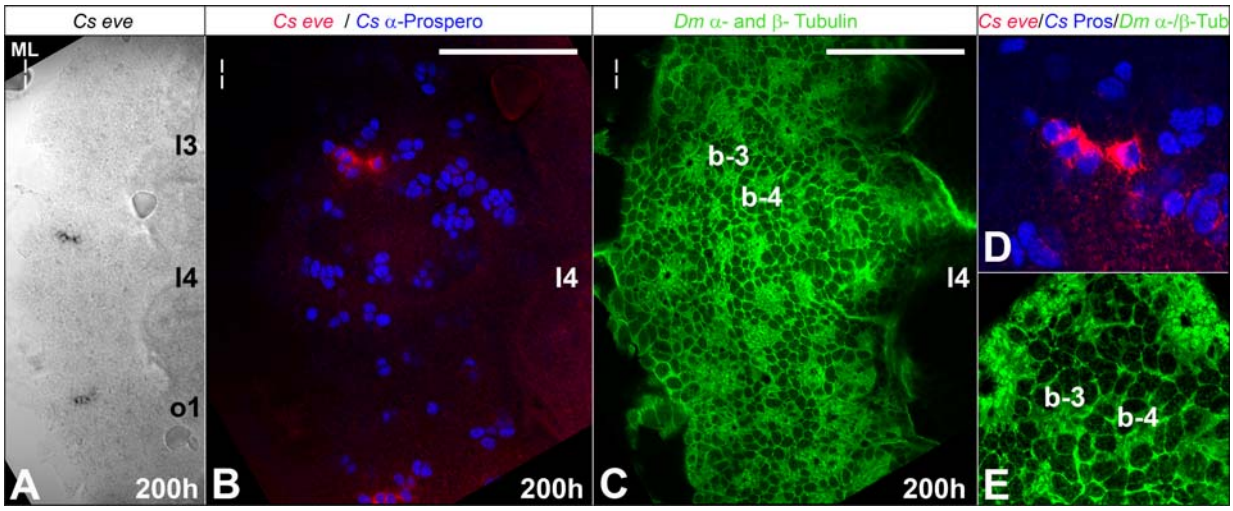

Figure 3-12: Expression of the differentiation marker *even-skipped* in *Cupiennius salei*.

Legend see next page (63).

Legend to Fig.3-12: (**A**) *In situ* hybridisation with a DIG-labelled *Cupiennius even-skipped* (*eve*) RNA antisense probe in leg hemineuromeres 2 and 3 and the first opisthosomal segment in a flat preparation of a *Cupiennius* embryo after 200 hours of development; ventral view. (**B**) Double-staining with a DIG-labelled *Cupiennius eve* RNA antisense probe (red) and the *Cupiennius* Prospero antibody (blue) in the fourth leg hemineuromere after 200 hours of development; ventral view. (**C**) Maximum projection of a *Drosophila* α- and β-Tubulin antibody staining in the same hemineuromere as presented in B; ventral view. (**D**, **E**) Magnifications of the pictures B and C present the region which contains the *eve*-positive cells. (**A**) After 200 hours of development *eve* is expressed in a small cell cluster in the anterior medial region in all the opisthosomal hemisegments and the fourth leg hemisegment. The more anterior neuromeres do not express *eve* during this developmental stage. (**B**-**E**) Triple-stainings of *eve*, the differentiating neural precursors (Prospero) and the invaginating NPGs (Tubulin) reveal, that *eve* is expressed in differentiating neural cells which correspond to either both the NPGs **b-3** and **b-4** or just one of them. (**D**) At this developmental stage the *eve*-positive cluster contains 4 cells. ***b-3***/***b-4***, NPG **3** or **4** of row **b**; ***Cs***, *Cupiennius salei*; ***Dm***, *Drosophila melanogaster*; ***eve***, *even-skipped*; ***l3***/***l4***, leg hemisegments 3 and 4; ***o1***, opisthosomal segment 1. Anterior is to the top. Scale bars in B and C: 80μm.

In the spider *Cupiennius salei* the expression of the differentiation marker *even-skipped* is very dynamic during development. At about 200 hours of development an *even-skipped*-positive cell cluster is found in an anterior position in the NE in the opisthosomal hemineuromeres and in the fourth leg segments (Fig.3-12). During further development, *even-skipped* is not only expressed in the fourth leg hemineuromere and in the opisthosomal neuromeres but also in the more anterior (prosomal) segments. In addition to that extension of expression over all hemineuromeres, the expression also spreads within the hemineuromeres (data not shown). Apart from its presence in the VNE, *even-skipped* is also expressed in the developing heart of the spider (data not shown) (Janssen and Damen, 2008).

At about 200 hours of development, when all NPGs are detectable in the leg neuromeres, *even-skipped* is expressed in a small cell cluster in the anterior medial region of the NE in the opisthosomal hemisegments and in the fourth leg hemisegment (Fig.3-12 A). These cells are most likely neural cells of the NPGs **b-3** and **b-4**. However, it is possible that they derive from only one of these precursor groups (Fig.3-12 B, C, D, E, Fig.3-14)

As mentioned above, the number of *even-skipped* expressing cells increases with ongoing development. The expansion of *even-skipped* expression in the VNE starts around the time in point when the primary invagination sites detach from the apical surface and the secondary invagination sites are formed. In addition to the four waves during which the primary NPGs are generated in the spider, secondary NPGs form later. Many of the secondary NPGs do not differentiate during embryogenesis but persist as epithelial vesicles and consist of more cells than the primary NPGs

(Stollewerk, 2004). There is no clear separation between the progeny of the individual secondary NPGs because the progeny of all NPGs get concentrated in the centre of the hemineuromeres during formation of the segmental ganglia. Therefore it can not be shown in detail which of the secondary NPGs contain *even-skipped*-positive cells in later stages of development.

3.1.11 The differentiation marker *islet* is expressed in several cell clusters in the lateral half of the NE in *Cupiennius salei*

Like *even-skipped*, the differentiation marker *tailup (islet)* is expressed in a well-known subset of moto- and interneurons in *Drosophila*, but the two genes are expressed in distinct subsets of cells. Thus, in contrast to *even-skipped*, *islet (isl)* is expressed in the motoneurons which innervate the ventral muscles (Thor and Thomas, 1997). The *islet*-positive interneurons are identified as dopaminergic and serotonergic neurons. It has also been shown that the NBs 3-1, 4-2, 5-2, 7-1 and 7-3 generate the *islet*-positive cells (Landgraf *et al.*, 1997; Lundell and Hirsh, 1994; Thor and Thomas, 1997). Again, these NBs do not express the differentiation marker themselves. Therefore the expression of *islet* was analysed in *Cupiennius* to get more information concerning how NB lineages in insects can be compared to the NPGs in chelicerates.

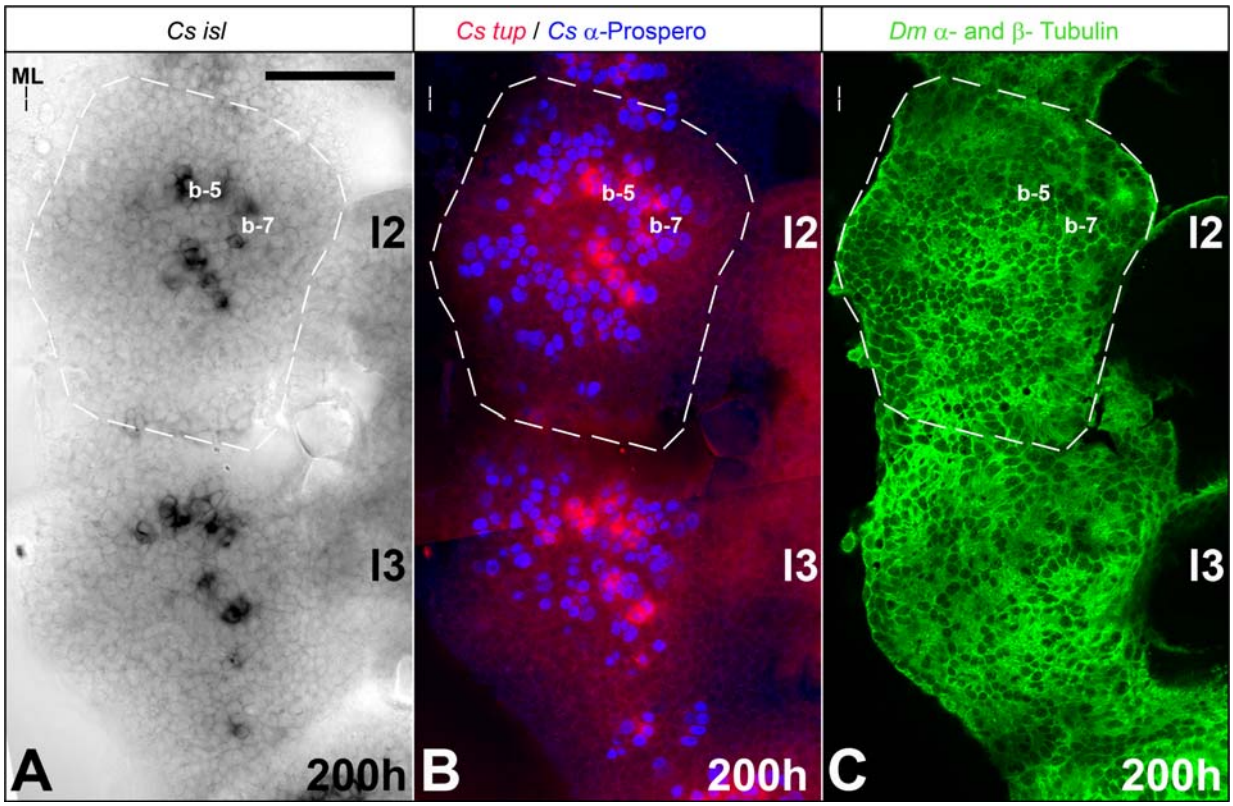

Figure 3-13: Expression of the differentiation marker *islet* in *Cupiennius salei*.

(**A**) Flat preparation of leg hemineuromeres 2 and 3 of a *Cupiennius* embryo after 200 hours of development stained with a DIG-labelled *Cupiennius islet* (*isl*) RNA antisense probe; ventral view. (**B**) Double-staining with a DIG-labelled *Cupiennius isl* RNA antisense probe (red) and the *Cupiennius* Prospero antibody (blue) in the same hemineuromeres as in A; ventral view. (**C**) Combined antibody staining of *Drosophila* α– and β-Tubulin shows the NPGs in the same hemineuromeres as in A and B. (**A**) The differentiation marker *isl* is expressed in several cell clusters in the lateral anterior part of the hemisegments after 200 hours of development. (**B**) Some of the differentiating neural precursors in this area express *isl* at this stage. (**A**-**C**) Triple-stainings for *isl*, the differentiating neural precursor cells (Prospero) and the invaginating NPGs (Tubulin) reveal that the *isl*-positive differentiating neural precursors arise from seven NPGs (**a-5**, **b-5**, **b-6**, **b-7**, **c-6**, **d-3**, **d-4**); note that only two of the NPGs (**b-5**, **b-7**) are marked as an example. ***b-5***/***b-7***, NPG **3** or **5** of row **b**; ***Cs***, *Cupiennius salei*; ***Dm***, *Drosophila melanogaster*; ***I2***/***I3***, leg hemisegments 2 and 3; ***isl***, *islet*. Anterior is to the top. Scale bar: (A) 80μm in panel A-C.

In the spider, *islet* shows a rather dynamic expression pattern. Expression starts in a few small clusters in the anterior lateral part of the NE (data not shown) and then extends into additional cell clusters where *islet* expression is stronger. Within the NE this expression is restricted to the lateral half. In addition, *islet* also shows a very intense expression in the precheliceral lobes and in the developing heart (Fig.3-19 A, Fig.3-20 A)

When all NPGs are detectable, *islet* is expressed in several cell clusters, which are located in the anterior lateral part of the hemineuromeres (Fig.3-13 A). In this stage *islet* expression is restricted to the anterior third of the segments. The seven NPGs

containing *islet*-positive neural cells are identified as **a-5**, **b-5**, **b-6**, **b-7**, **c-6**, **d-3**, **d-4** (Fig.3-13 B, C, Fig.3-14).

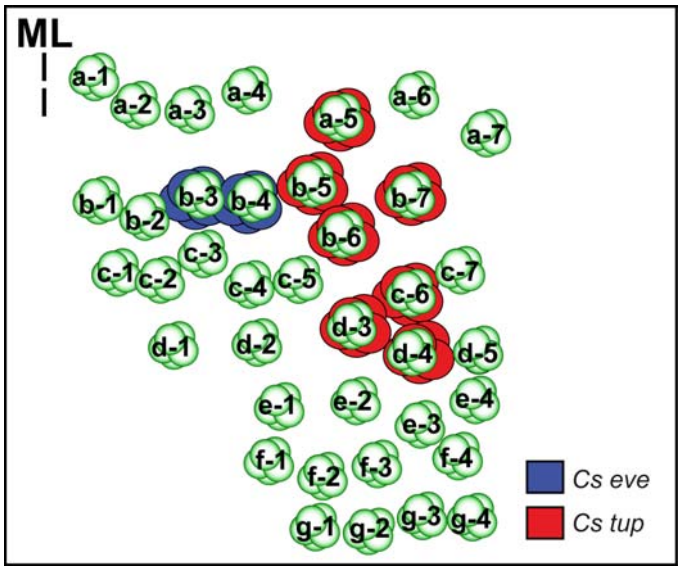

Figure 3-14: Schematic drawing of *even-skipped* and *islet* expression in cells of identified NPGs in *Cupiennius salei*.

Schematic drawing of *isl* and *eve* expression in identified NPGs in one leg hemineuromere after 200 hours of development. The differentiation marker *eve* (blue) is expressed in neural precursor cells which correspond to the NPGs **b-3** and/or **b-4**. Seven identified NPGs (**a-5**, **b-5**, **b-6**, **b-7**, **c-6**, **d-3**, **d-4**) contain differentiating neural precursor cells which express *isl* (red). The expression pattern of the two differentiation markers (*isl* and *eve)* do not overlap. Anterior is to the top.

As mentioned above, the two differentiation markers *even-skipped* and *islet* are never coexpressed in *Drosophila*. They are expressed in distinct subsets of moto- and interneurons which are generated by identified NB. In the spider, the two genes are not expressed in the neural cells of the same NPGs at about 200 hours of development (Fig.3-14).Therefore, their expression patterns seem to exclude one another like in *Drosophila*.

3.1.12 The DV patterning gene *msh* is involved in conferring specific identities to the NPGs in *Cupiennius salei*

The DV patterning gene *msh* plays a decisive role in the mechanism, which conveys identity to dorsal NBs in *Drosophila.* In *Drosophila msh* mutants, the dorsal NBs are formed, but their progeny are affected. In some cases, the NBs do not generate their

wildtypic lineage, which might be due to a change of dorsal to more medial NB fate. Furthermore in some of the progeny differentiation is affected what is reflected for example by incorrect migration.

The possible function of *msh* in neural precursor identity and differentiation in *Cupiennius* was investigated by analysing different aspects of neurogenesis in embryos with down- regulated *msh* expression. The reduction of *msh* expression was accomplished by using the RNA interference technique (see paragraphs 2.3.11 and 2.6.1). To study if all NPGs are generated in the lateral region and if they are arranged normally, Phalloidin stainings were done since this allows analysing in detail if there are changes in the number or the arrangement of the NPGs. To investigate a possible change in the identities of NPGs or differentiation defects, *in situ* hybridisations for *even-skipped* and *islet* were performed, because the expression of the differentiation genes should shed light on a possible involvement of *msh* in these steps of neurogenesis.

3.1.12.1 *msh* expression is reduced by the injection of double-stranded *msh* RNA interference

The reduction of *msh* expression, which is caused by the double-stranded RNAi is not consistent in all the injected embryos. The amount of injected dsRNA and also its effect differs from embryo to embryo, which leads to different levels of *msh*-transcript reduction (Fig.3-15). The development of uninjected embryos in one cocoon is not completely synchronised and the stages vary from a few hours up to one day. These differences are strongly enhanced up to almost two days in embryos treated with ds *msh* RNA and in embryos injected with ds GFP RNA (Fig.3-15). Also in both, control and *msh* RNAi embryos, about 30% of the embryos did not develop properly and were not used for further experiments. The same proportion of completely misdeveloped embryos in control and *msh* RNAi embryos shows that this is not due to the reduction of *msh* expression but rather to the injection procedure itself.

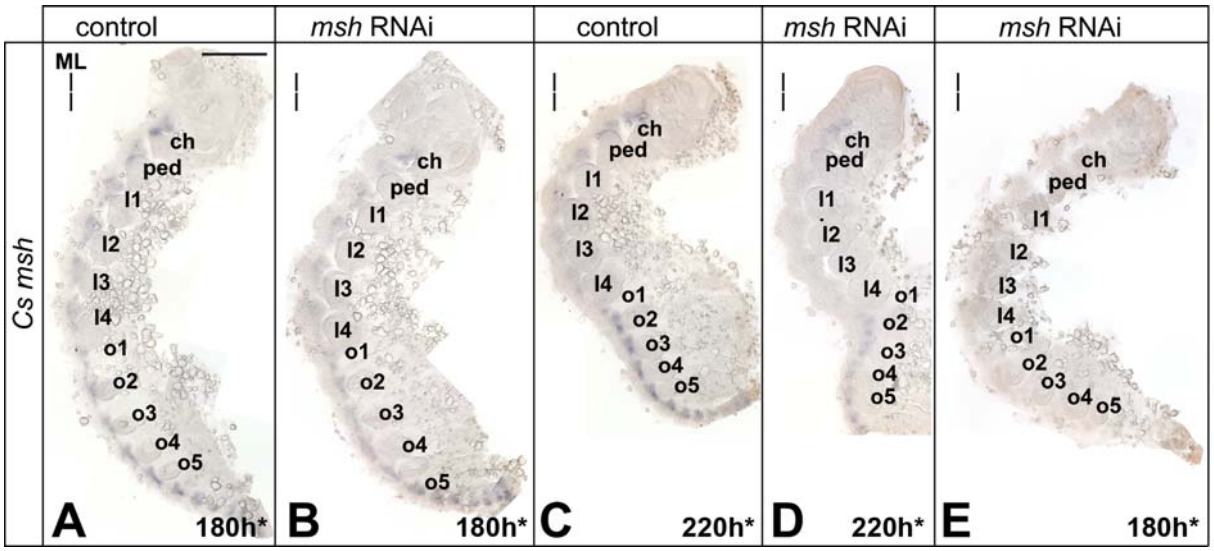

Figure 3-15: *msh* expression is reduced by double stranded RNA of *msh* in *Cupiennius salei*.

Flat preparations of one half of the germ band of control (**A**, **C**) and *msh* RNAi *Cupiennius* embryos (**B**, **D**, **E**); ventral views. The short enzymatic staining reaction after an *in situ* hybridisation with a DIG-labelled *Cupiennius msh* RNA antisense probe leads to a weaker staining, but allows a better comparison of different expression levels. Different developmental stages in control (ds RNA for *GFP* was injected) and *msh* RNAi (ds RNA for *msh* was injected) embryos are compared. (**A**) In the control embryo, at about 180 hours of development, *msh* is expressed normally in the lateral part of the hemineuromeres in all segments. (**B**) *msh* RNAi embryo, at a comparable stage as in A, shows slightly reduced *msh* expression. (**C**) The control embryo, at about 220 hours, shows *msh* expression in the lateral column of the hemineuromeres. The slightly weaker expression in leg hemisegments 2 to 4 is due to the legs which can cover this area during staining reaction and thus prevent strong staining there. (**D**) The *msh* RNAi embryo, at a comparable developmental stage, shows a clear reduction of *msh* expression. There is almost no expression detectable in the pedipalpal and the leg hemisegments and strongly reduced expression in the opisthosomal hemisegments. (**E**) 40% of the *msh* RNAi embryos do not express *msh* at all, or at least not in a detectable level. ***ch***, cheliceral segment; ***Cs***, *Cupiennius salei*; ***l1-l4***, leg segments 1 to 4; ***ML***, midline; ***msh***, *muscle segment homeobox*; ***o1-o5***, opisthosomal segments 1 to 5; ***ped***, pedipalpal segment. The hours of development are marked with asterisks as they do not reflect the actual time of development but rather describe the morphological developmental stage. Anterior is to the top. Scale bar: (A) 320μm in panel A-E.

The developmental stages of the injected embryos range from about 180 hours (Fig.3-15 A, B E) to 220 hours of development (Fig.3-15 C, D) in both, *msh* RNAi and control injected embryos, although they are all from the same cocoon. The staining reaction was stopped early to ensure that the embryos are not overstained and small differences in the level of expression can be detected. This results in a rather faint staining but reveals differences in staining intensities. All control embryos (n=6) express *msh* in the lateral part of the NE as described above (Fig.3-15 A, C). In the morphological older stage, the staining in the prosomal segments is weaker compared to the opisthosomal segments, because the legs cover the NE during the staining procedure (Fig.3-15 C). The morphological younger embryo shows a

continuous staining in all the segments, because the legs do not impair the staining in the NE of the prosomal segments in this stage (Fig.3-15 A). *msh* expression was downregulated in all analysed *msh* RNAi embryos (n=10). 60% of the embryos show a downregulation of *msh* expression and 40% of the embryos do not express *msh* on a detectable level. Among the embryos with reduced but detectable levels of *msh* expression, the two with the strongest staining are presented (Fig.3-15 B, D). Even in these embryos the intensity of the staining is slightly reduced compared to the control embryos, which indicates that *msh* is downregulated in all *msh* RNAi embryos. Furthermore 40% of the injected embryos do not express *msh* on a detectable level (Fig.3-15 E).

msh expression is reduced in all *msh* RNAi embryos, but the level of reduction varies from almost normal to no detectable expression.

3.1.12.2 *msh* does not influence the number and arrangement of NPGs

In *Drosophila, msh* is expressed in the lateral column of the NE before and during NB delamination, but is not required for the formation of NBs, as they are all present in *msh* mutant embryos (Isshiki *et al.*, 1997). To analyse this issue in *Cupiennius* Phalloidin staining was used for a detailed examination of the number and arrangement of the NPGs. Staining was performed in control and in *msh* RNAi embryos to analyse if *msh* has an influence on the formation of NPGs.

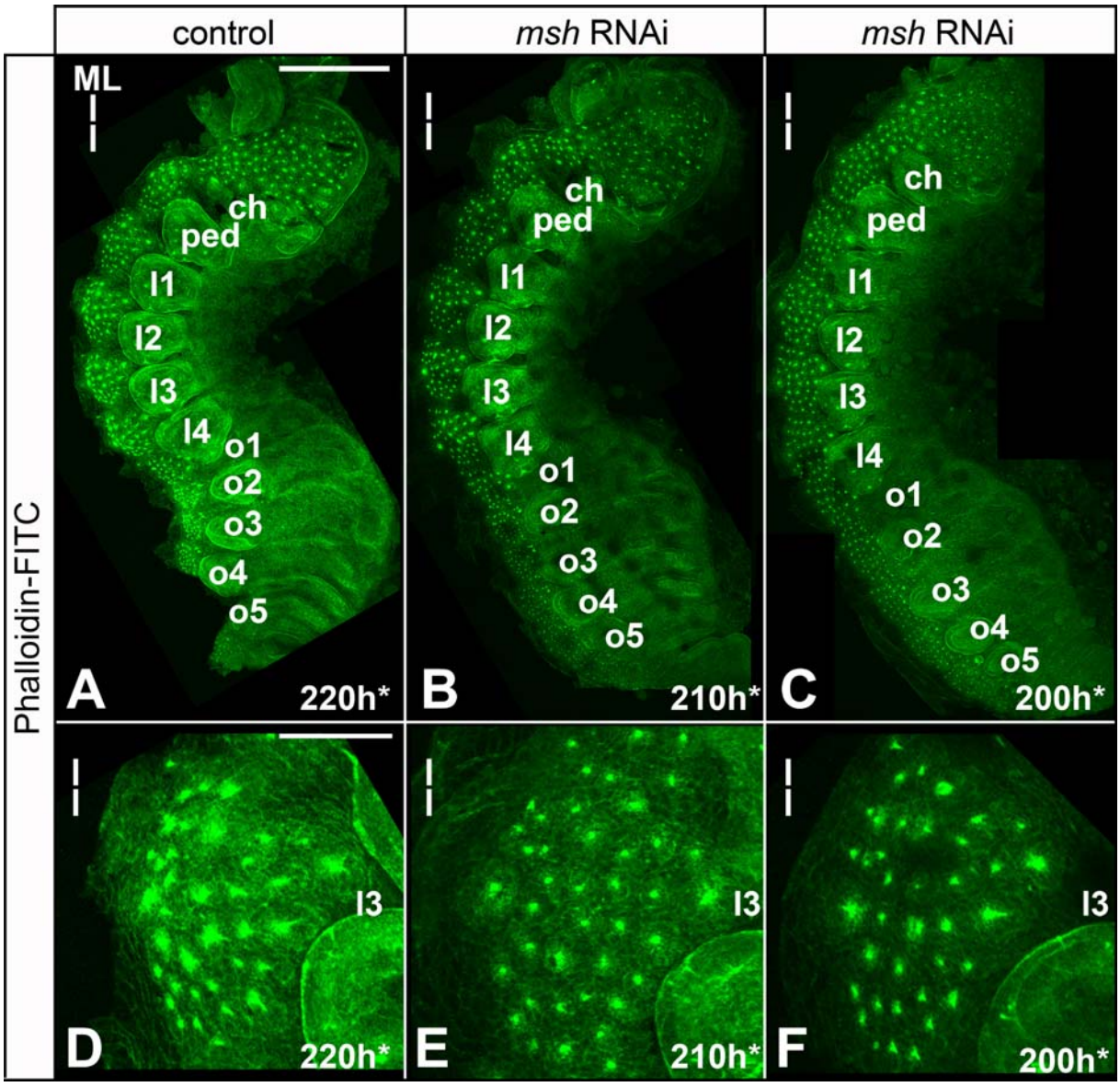

Figure 3-16: Reduced *msh* expression does not influence the formation of NPGs in *Cupiennius salei*.

Maximum projections of Phalloidin stainings in one half of the germ band of *Cupiennius* embryos (**A**-**C**) and hemineuromeres of the third leg segment (**D**-**F**) visualise the NPGs in a control (**A**, **D**) and two msh RNAi *Cupiennius* embryos (**B**, **C**, **E**, **F**); ventral views. (**A**) In the control embryo, at about 220 hours, all neuromeres contain the normal number of NPGs. (**D**) The 38 NPGs are arranged in a similar way as in uninjected embryos. (**B**, **C**, **E**, **F**) The *msh* RNAi embryos, at 210 (**B**, **E**) and 200 (**C**, **F**) hours of development, show normal amounts of NPGs (38) in the leg hemineuromeres. (**D**, **E**, **F**) Compared to the control, the arrangement of NPGs in the *msh* RNAi embryos seems to be slightly shifted. These small differences are not due to the reduced level of *msh*, but to the difference in developmental stage. ***ch***, cheliceral segment; ***Cs***, *Cupiennius salei*; ***l1-l4***, leg segments 1 to 4; ***ML***, midline; ***msh***, *muscle segment homeobox*; ***o1-o5***, opisthosomal segments 1 to 5; ***ped***, pedipalpal segment. The hours of development are marked with asterisks as they do not reflect the actual time of development but rather describe the morphological developmental stage. Anterior is to the top. Scale bars: (A) 320μm in panel A-C; (D) 80μm in panel D-F.

The number and arrangement of NPGs was analysed in 64 leg hemisegments of *msh* RNAi embryos, because all previous studies concerning the formation of NPGs have been done in these segments. In *Cupiennius msh* is not involved in the formation of the NPGs, because 100% of the studied hemisegments contain all the 38 expected NPGs (Fig.3-16 B, C, E, F). The slight differences of the arrangement of NPGs between the presented control embryo and the two presented *msh* RNAi embryos are due to the different morphological stages, since the NE undergoes morphogenetic movements during development (Fig.16). Apart from these insignificant differences, the number and positions of the NPGs are very similar in control and RNAi embryos (Fig.3-16 D, E, F).

As in *Drosophila*, the DV patterning gene *msh* is not necessary for the formation of the neural precursors in its expression domain in *Cupiennius*.

3.1.12.3 *msh* plays a role in neural precursor identity

By analysing the development of well known NB lineages in *msh* mutant *Drosophila* embryos, Isshiki *et al.* could show that *msh* is required for the proper development of dorsal NBs (Isshiki *et al.*, 1997). The lack of *msh* leads to abnormalities in the division and migration of the progeny of some dorsal NBs and furthermore it could be shown for at least one NB (4-2) that it might adopt the fate of its more medial neighbour.

Based on this data, the probable involvement of *msh* in neural precursor identity was analysed in the spider *Cupiennius* by *in situ* hybridisations for *islet* and *even-skipped* in *msh* RNAi embryos. Phalloidin staining already revealed that the number and arrangement of NPGs is not impaired by loss of *msh*. In further experiments, Prospero staining in embryos with a known level of *msh* should reveal if the arrangement and number of differentiating neurons might be affected by *msh* downregulation. Fig.3-17 shows the leg hemisegments of the same embryos as in Fig.15, which facilitates a direct comparison of the effect of varying levels of *msh* on the differentiation to neural cells in different stages.

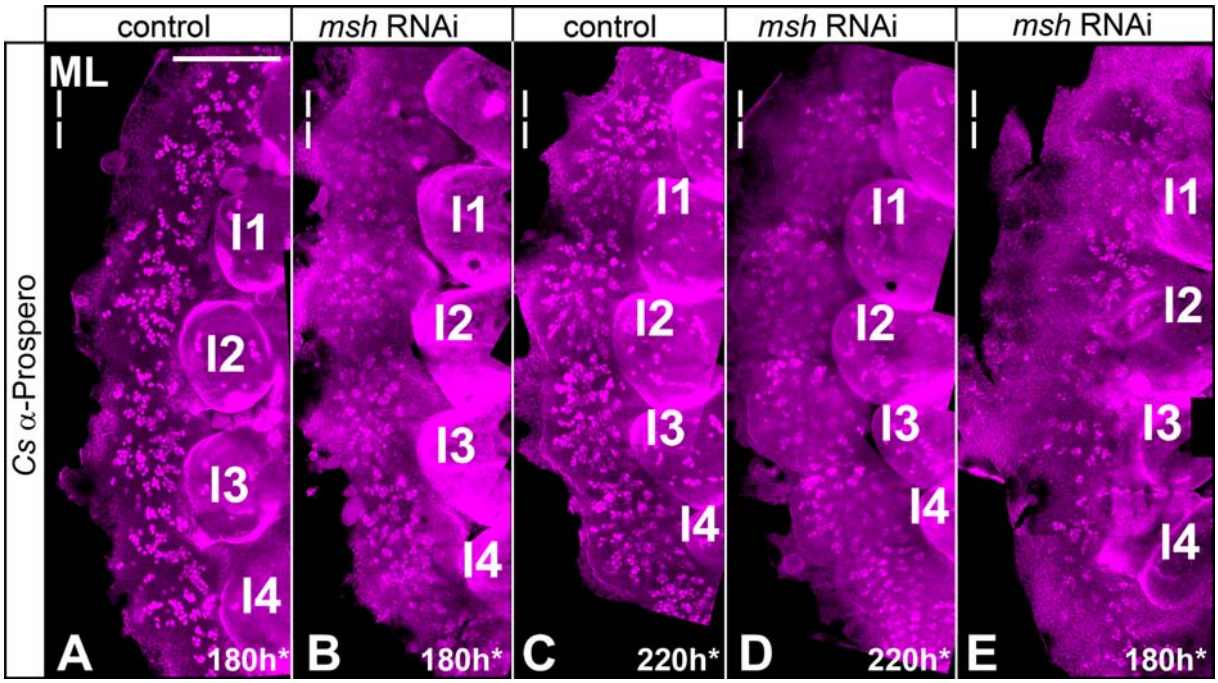

Figure 3-17: Reduced *msh* expression shows no striking phenotype in the Prospero expression pattern in *Cupiennius salei*.

(A-E) Staining against the *Cupiennius* Prospero antibody in flat preparations of leg hemineuromeres 1 to 4 in the same embryos which were presented in Fig.3-15 A-E; ventral views. The double-stainings of *msh* (Fig.3-15) and Prospero allow correlating the level of *msh* expression to the possible effects on neurogenesis, which are reflected by the Prospero staining of the differentiating neural precursors. (**A**) The control embryo with a morphological stage of about 180 hours and a normal *msh* expression (Fig.3-15 A) shows normal Prospero expression in the differentiating neural precursors of the invaginating NPGs. (**B**) Prospero staining in the *msh* RNAi embryo with a slightly reduced *msh* expression (Fig.3-15 B) at a similar morphological developmental stage shows no significant changes in Prospero expression compared to the control. (**C**) The control embryo which shows a morphologically older developmental stage (220 hours) and a normal level of *msh* expression (Fig.3-15 C) presents the normal amount and arrangement of differentiating neural precursor cells. (**D**) At a comparable stage, the Prospero staining in the *msh* RNAi embryo with strongly reduced *msh* expression (Fig.3-15 D) does not show significant differences. (**A**-**D**) Comparison of Prospero staining in different morphological stages in control embryos and embryos with reduced *msh* expression does not show a clear phenotype, but the staining in the *msh* RNAi embryos seems to be slightly weaker or shows a stronger background. (**E**) Even the embryo with no *msh* expression (Fig.3-15 E) shows Prospero-positive neural precursor cells in the lateral column. (**E**). ***Cs***, *Cupiennius salei*; ***I1-I4***, leg hemisegments 1 to 4; ***ML***, midline; ***msh***, *muscle segment homeobox*. The hours of development are marked with asterisks as they do not reflect the actual time of development but rather describe the morphological developmental stage. Anterior is to the top. Scale bar: (A) 160μm in panel A-E.

Although there is an obvious reduction of *msh* in both analysed morphological stages, Prospero staining, which shows differentiating neural cells, does not show strong differences compared to the staining in control embryos (Fig.3-15, A, B, C, D, Fig.3-17 A, B, C, D). Prospero staining is slightly weaker, or rather shows more background, in embryos with downregulated *msh*, but there is no significant difference in the amount of the stained cells in both, the morphologically older (Fig.3-15 A, B, Fig.3-17 A, B) and younger (Fig.3-15 C, D, Fig.3-17 C, D) embryos. Even in

the lateral part of the NE, where *msh* reduction should cause the strongest effects, a relatively normal number of Prospero-positive cell nuclei are present. But as the expression pattern of Prospero is very dynamic and most likely all differentiating neural precursor cells express the gene transiently, small changes in the number of Prospero expressing cells are difficult to detect. Not even a complete reduction of *msh* leads to a loss of Prospero expression in the lateral NE (Fig.3-15 E, Fig.3-17 E).

Possible phenotypes of incorrect migration or reduction in the number of cells in the NPGs, as has been described for the NB lineages in *Drosophila*, could not be uncovered in the spider. However, this does not rule out the possibility that the differentiation or the identity of the lateral NPGs is affected. The cells could still express Prospero even if their neural identity or differentiation program has been changed. The investigation of the expression of the differentiation markers *islet* and *even-skipped* offers the possibility to further examine these questions.

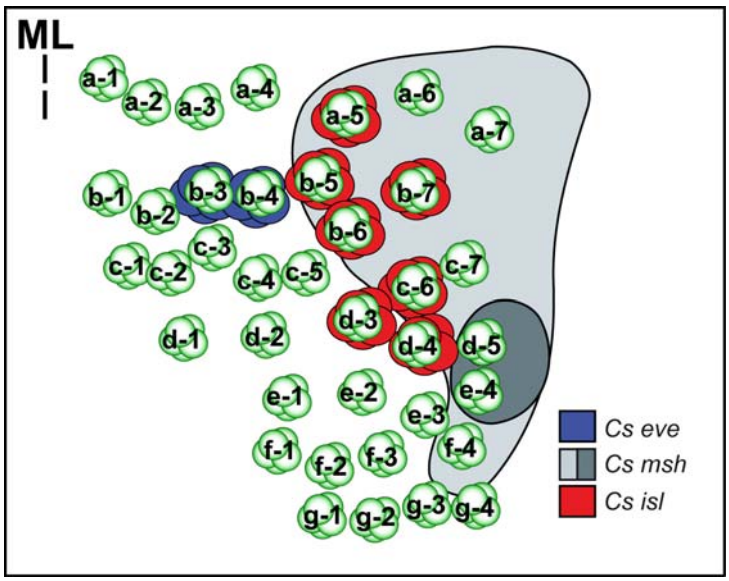

Figure 3-18: Schematic drawing of *msh*, *eve* and *isl* in correlation to the NPGs in *Cupiennius salei*.

The NPGs which contain *isl*-positive cells (red) delaminate from the region with *msh* expression (grey) or at the border of msh expression. The *eve* expressing cells (blue) correlate to the NPGs b-3 and b-4, which invaginate medially to the *msh*-positive lateral column. ***Cs***, *Cupiennius salei*; ***eve***, *even-skipped*; ***ML***, midline; ***msh***, *muscle segment homeobox*; ***isl***, *islet*. Anterior is to the top.

In *Drosophila,* the loss of *msh* possibly leads to a change of fate of the lateral NB (NB 4-3) to the fate of its medial neighbour (NB 4-2), which results in additional cells expressing the differentiation marker *even-skipped*. These cells were identified as

ectopic RP2 and aCC/pCC neurons which normally belong to the NB4-2 lineage and express *even-skipped* (Isshiki *et al.*, 1997). Another explanation for the phenotype could be that *msh* might influence the differentiation of the progeny cells. To investigate if there is a comparable effect of the reduction of *msh* expression in the spider, *in situ* hybridisations with *even-skipped* were done in *msh* RNAi and control embryos. As *even-skipped* expression is very dynamic in the spider, a strong phenotype with considerably more *even-skipped* expressing cells would be required to detect a change in identity similar to *Drosophila*. At about 200 hours of development *even-skipped* is expressed in a small cell cluster in the fourth leg segment and in the opisthosomal segments (Fig.3-18). At this stage no change in *even-skipped* expression was detected in *msh* RNAi embryos (data not shown). This does exclude the possibility of an influence of *msh* on neural precursor identity or neural cell differentiation. In *Cupiennius* the NPGs are generated in four waves. It is not possible to correlate the generation of identified NPGs with one of these waves because NPGs arise in-between the existing ones which leads to rearrangements of the pattern. NPGs can therefore only be unambiguously classified when the final pattern of all NPGs is present in the hemineuromeres. The NPGs adjacent to the *even-skipped* expressing NPGs could be generated later than their neighbours and therefore start expressing *even-skipped* later during neurogenesis. Due to the large number of *even-skipped* expressing cells during late embryogenesis and the morphogenetic movements, it would not be possible to trace the *even-skipped*-positive cells back to individual NPGs at later stages.

The expression of *islet* has not been analysed in *msh* mutant embryos in *Drosophila*. However, since all the NPGs which contain *islet*-positive cells are located within or very close to the *msh* expression domain, an influence of *msh* on the identity and differentiation of these neural cells can be expected (Fig.3-18).

islet expression was analysed in 30 control and 140 *msh* RNAi embryos. The enzymatic staining reaction was stopped after a short time to avoid overstaining, and to facilitate a detailed comparison of the *islet* expression levels in control and RNAi embryos (Fig.3-19).

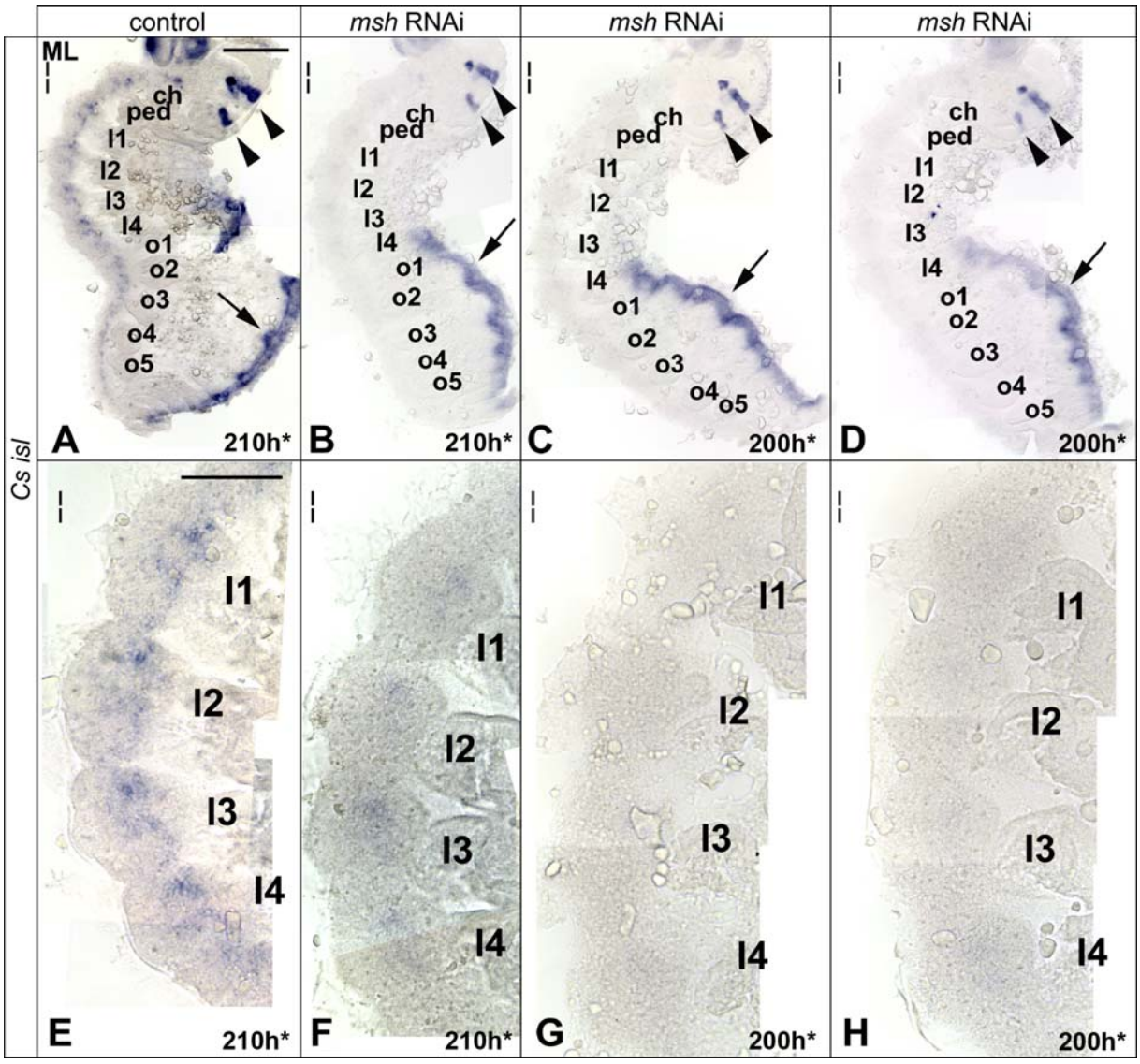

Figure 3-19: *islet* expression in the neural precursor cells is strongly reduced in *msh* RNAi *Cupiennius salei* embryos.

In situ hybridisation with a DIG-labelled *Cupiennius isl* RNA antisense probe in germ band halves in control (**A**) and in *msh* RNAi embryos (**B**-**D**) show different levels of *isl* reduction. (**E**-**H**) Magnifications of leg hemineuromeres in the same embryos. (**A**) In the control embryo, at about 210 hours of development, *isl* is expressed in two domains in the procephalic NE (arrowheads), in the developing heart (arrow) and in several clusters in the VNE. (**E**) The leg hemineuromeres of the control embryo show *isl* expression in the lateral part of the NE in the neural precursors of seven NPGs. (**B**, **C**, **D**, **F**, **G**, **H**) In *msh* RNAi embryos, at stages of 210 hours and 200 hours of development, *isl* expression is reduced to different levels in the VNE but not in the developing heart (arrows) and the procephalic NE (arrowheads). (**F**) There are some *msh* RNAi embryos which still express *isl*, but show a clear reduction of expression in the VNE. (**G**, **H**) In other *msh* RNAi embryos *isl* expression is reduced completely or to a level which makes detection impossible. ***ch***, cheliceral segment; ***Cs***, *Cupiennius salei*; ***l1-l4***, leg hemisegments 1 to 4; ***ML***, midline; ***msh***, *muscle segment homeobox*; ***o1-o5***, opisthosomal segments 1 to 5; ***ped***, pedipalpal segment; ***isl***, *islet*. The hours of development are marked with asterisks as they do not reflect the actual time of development but rather describe the morphological developmental stage. Ventral views; anterior is to the top. Scale bars: (A) 320μm in panel A-D; (E) 160μm in panel E-H.

All controls show a normal staining of *islet* with a strong expression in two domains of the precheliceral NE, the developing heart and in several clusters in the lateral part of the NE which is the focus of these studies (Fig.3-19 A, E). The *msh* RNAi embryos display a variety of phenotypes, which is probably due to different levels of downregulation of *msh.* All embryos show a reduction of *islet* expression in the VNE and cover a range from no detectable expression (53%) to strongly reduced expression (47%) (Fig.3-19 B, C, D, F, G, H). Apart from the comparison with the staining in the NE of control and *msh* RNAi embryos the staining in the developing heart and in the head presents the possibility of an internal control within the *msh* RNAi embryos. To point out the reduction of *islet* expression as a real phenotype and not only as a side effect of the injection procedure or the condition of the embryos, three examples are presented (Fig.3-19 B, C, D). There are embryos which express *islet* at a quite normal level in the heart and head and also show, even if strongly reduced, expression in the lateral part of the NE (Fig.3-18 B, F). Others which show at least the same intensity of staining in the head and heart do not express *islet* at a detectable level in the VNE (Fig.3-19 C, G). Compared to these embryos, the embryo in Fig.19 D and H shows a weaker staining in the head and heart but *islet* is expressed very weakly in the VNE. Thus, the strength of staining in the heart and head is not proportional to the staining in the VNE and the phenotype can not be caused by some background influences.

Furthermore this result was verified by the same experiment with a prolonged staining reaction. This leads to more background staining which prevents the detailed analysis of the expression, but clearly shows that *islet* is downregulated in the NE in *msh* RNAi embryos (Fig.3-20).

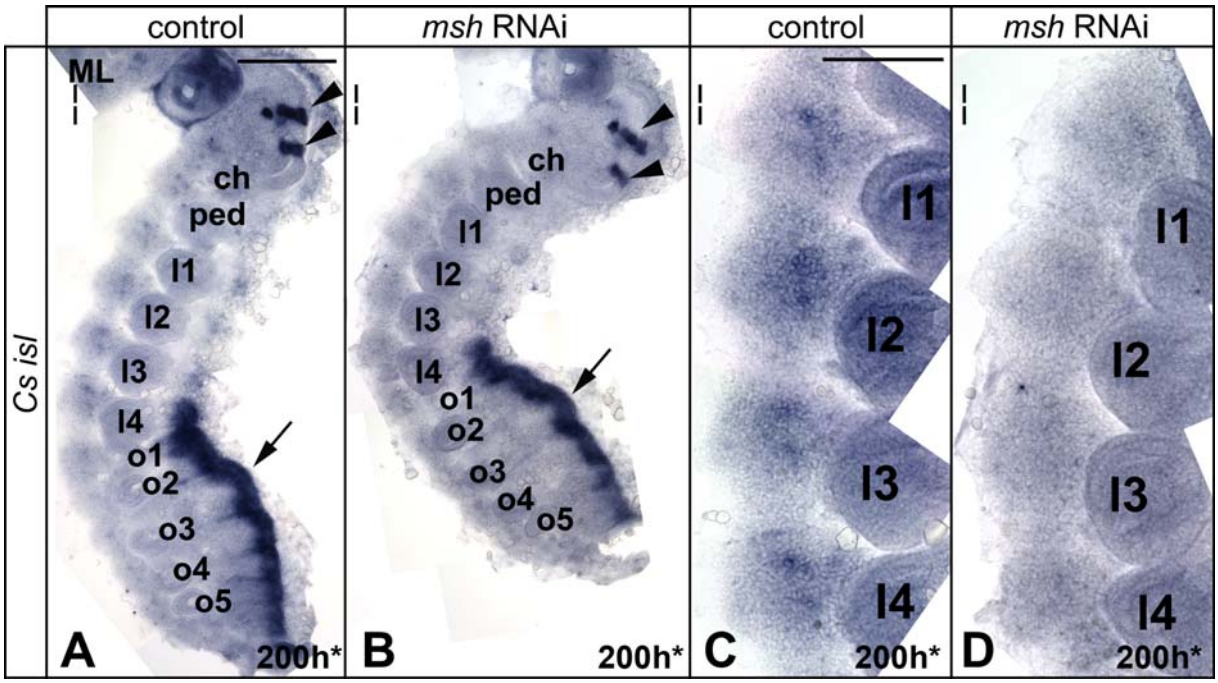

Figure 3-20: Verification of the reduced *islet* expression caused by *msh* RNAi in *Cupiennius salei*.

In situ hybridisation with a DIG-labelled *Cupiennius isl* RNA antisense probe in a control (**A**, **C**) and *msh* RNAi (**B**, **D**) embryo at a morphological stage of about 200 hours of development; ventral views. (**A**-**D**) To verify the results of Fig.3-19, the embryos which are presented in 3-20 were stained in a longer enzymatic staining reaction to confirm that *isl* expression is reduced in the VNE of *msh* RNAi embryos. (**A**) In the control embryo *isl* is expressed in the developing heart (arrow), in two domains in the procephalic NE (arrowheads) and in cell clusters in the VNE. (**C**) The expression in the VNE is restricted to the lateral column in the hemineuromeres of the leg segments. (**A**, **B**) The *msh* RNAi embryo shows *isl* expression in the developing heart (arrow) and in the procephalic NE (arrowheads) at the same level as the control embryo, but the expression in the VNE is strongly reduced. (**D**) The NE of the leg hemineuromeres does not express *isl* in *msh* RNAi embryos. ***ch***, cheliceral segment; ***Cs***, *Cupiennius salei*; ***I1-I4***, leg hemisegments 1 to 4; ***ML***, midline; ***msh***, *muscle segment homeobox*; ***o1-o5***, opisthosomal segments 1 to 5; ***ped***, pedipalpal segment; ***isl***, *islet*. The hours of development are marked with asterisks as they do not reflect the actual time of development but rather describe the morphological developmental stage. Anterior is to the top. Scale bar: (A) i320μm in panel A and B; (C) 160μm in panel C and D.

The phenotype showing reduced *islet* expression is confirmed by this experiment because even after the long staining reaction, no specific staining is observed in the VNE (Fig.3-20 B, D). Furthermore the intensity of the staining in the heart and head is the same in control and *msh* RNAi embryos, but in the *msh* RNAi embryos there is nearly no specific staining in the VNE (Fig.3-20).

Together, the results of the *islet* staining strongly suggest an essential role of *msh* in conveying the identities of the lateral neural precursors and regulating their differentiation.

3.2 Brain development in chelicerates

As the CNS is separated into the VNC and the brain, the examination of the development of both structures is useful to analyse and compare different aspects of neurogenesis in arthropods. Neurogenesis in the VNC has been analysed in detail compared to neurogenesis in the procephalic region. Therefore this work is not only focused on early neurogenesis in the VNE, but also concentrates on mechanisms which are involved in brain development. Moreover, the examination of brain development in spiders offers an advantage compared to insects, because there is no metamorphosis in spiders which allows for a consistently observation of how the neural precursors contribute to the adult brain structures. In *Drosophila* the brain NBs generate large lineages which remain distinguishable during larval stages and form compartments. These compartments are incorporated into the main brain centres with specific functions, like the mushroom bodies or the central body. In the adult spider brain similar centres have been identified and the focus of this project was to analyse how these centres are formed and to compare the mode of brain development in different arthropods. How the single NPGs build compartments, which are then incorporated in brain centres was analysed by observing the morphogenetic movements in the procephalic region. The developing brain centres were furthermore identified by analysing marker gene expression.

3.2.1 Application of body axes and segment assignment in the developing brain of *Cupiennius salei*

To present the data on brain development in the spider it is necessary to mention general facts about the nomenclature of the body axes and the assignment of segments and brain regions.

The body of insects, myriapods and crustaceans is separated into three tagmata, which are head, trunk and abdomen. In contrast to that, in chelicerates there are only two tagmata, the anterior prosoma and the posterior opisthosoma. Still, the CNS in spiders is subdivided similar as in the other arthropods. It is organised in the supraoesophageal ganglion, which is positioned above the oesophagus, and the suboesophageal ganglion, which is located beneath the oesophagus.

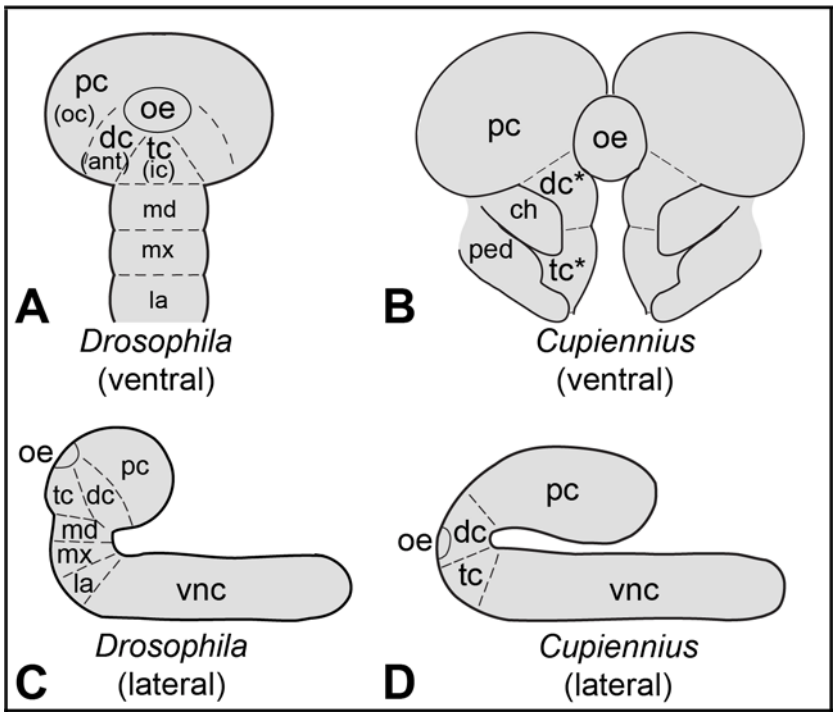

Figure 3-21: Comparison of the subdivision of the CNS in *Drosophila melanogaster* and *Cupiennius salei*

The schematic drawing presents a ventral view (**A**,**B**) and lateral view (**C**,**D**) of the CNS. (**A**-**C**) Both in *Drosophila* and *Cupiennius* the CNS consists of the supra- and the suboesophageal ganglion. The brain is subdivided into the protocerebrum (ocular segment), deutocerebrum and tritocerebrum. In *Drosophila* the deutocerebrum corresponds to the antennal segment and the tritocerebrum to the intercalary segment. In the spider the deutocerebrum might correspond to the cheliceral segment and the tritocerebrum to the pedipalpal segment. The asterisks in B indicate that the allocation of the neuromeres is still debated. (**D**) In the spider the supraoesophageal ganglion is folded on top of the suboesophageal ganglion. ***ant***, antennal segment; ***ch***, cheliceral segment; ***dc***, deutocerebrum; ***ic***, intercalary segment; ***la***, labial segment; ***md***, mandibular segment; ***mx***, maxillary segment; ***oc,*** ocular segment; ***oe***, oesophagus; ***pc***, protocerebrum; ***ped***, pedipalp; ***tc***, tritocerebrum; ***vnc***, ventral nerve cord.

In the spider the supraoesophageal ganglion contains the proto- and the deutocerebrum and builds the major part of the brain. The protocerebrum comprises of the precheliceral lobes and the deutocerebrum most likely corresponds to the cheliceral segment. In contrast to the proto- and deutocerebrum, the tritocerebrum, which belongs to the pedipalpal segment, is located under the oesophagus and thereby part of the suboesophageal ganglion (Fig.3-21 A, C, Fig.3-22).

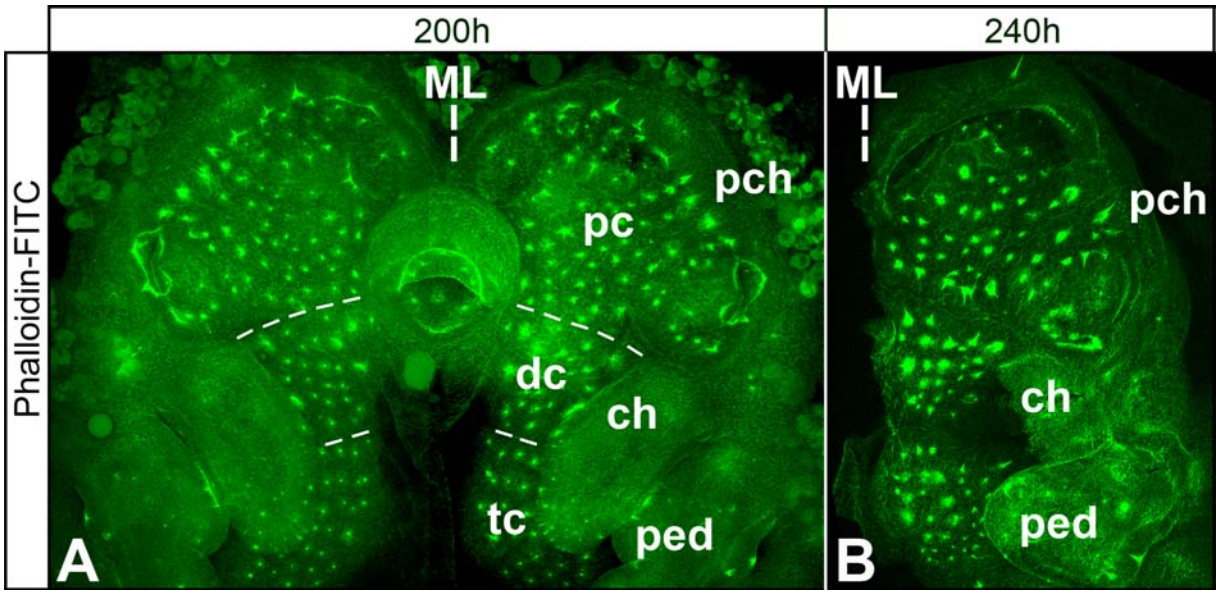

Figure 3-22: Segmental borders between the head segments in *Cupiennius salei*.

(**A**, **B**) Maximum projections of Phalloidin stainings in flat preparations of the precheliceral, the cheliceral and the pedipalpal neuromere in *Cupiennius* embryos; ventral views. (**B**) At 240 hours of development the neuromeres containing the NPGs are more condensed as compared to younger stages. This feature allows determining the segmental boundaries in a stage were all the NPGs are detectable. (**A**) Transferring the arrangement of the NPGs to the developmentally younger embryo (200 hours) allows determining the segmental boundaries between the precheliceral, the cheliceral and the pedipalpal segment. ***ch***, cheliceral segment; ***dc***, deutocerebrum; ***ML***, midline; ***pc***, protocerebrum; ***pch***; precheliceral segment; ***ped***, pedipalp; ***tc***, tritocerebrum. Anterior is to the top.

The segmental boundaries were determined with the help of Phalloidin stainings. In older embryos the neuromeres are more condensed and the NPGs of the individual neuromeres are clearly separated (Fig.3-22 B). The arrangement of NPGs in these stages was analysed and the neuromere boundaries in younger stages then determined by comparing the arrangement of NPGs (Fig.3-22). The correspondence of the tritocerebrum to the pedipalpal, the deutocerebrum to the cheliceral and the protocerebrum to the precheliceral segment is also supported by the expression pattern of the columnar gene *msh* in these segments (Fig.3-23). In *Drosophila* the DV patterning gene *msh* is expressed in the deuto- and the tritocerebrum from early neurogenesis onwards, whereas the expression in the protocerebrum starts clearly later (Urbach and Technau, 2003). *Cupiennius msh* shows a similar expression mode in the head segments. It is expressed in all the segments excluding the precheliceral segment from early neurogenesis onwards in the lateral column of the NE (Fig.3-9, Fig.3-23). In the precheliceral segment its expression starts later, at about 200 hours of development, in a small cell cluster (Fig.3-23 E, arrowheads). Expression in the precheliceral segment and in all the other segments is then maintained throughout

remaining neurogenesis (Fig.3-23 E-F). The temporal expression pattern of *msh* in *Drosophila* and *Cupiennius* supports that the protocerebrum is located in the precheliceral segment, which was also shown with other patterning genes such as *engrailed* and Hox genes (Damen *et al.*, 1998).

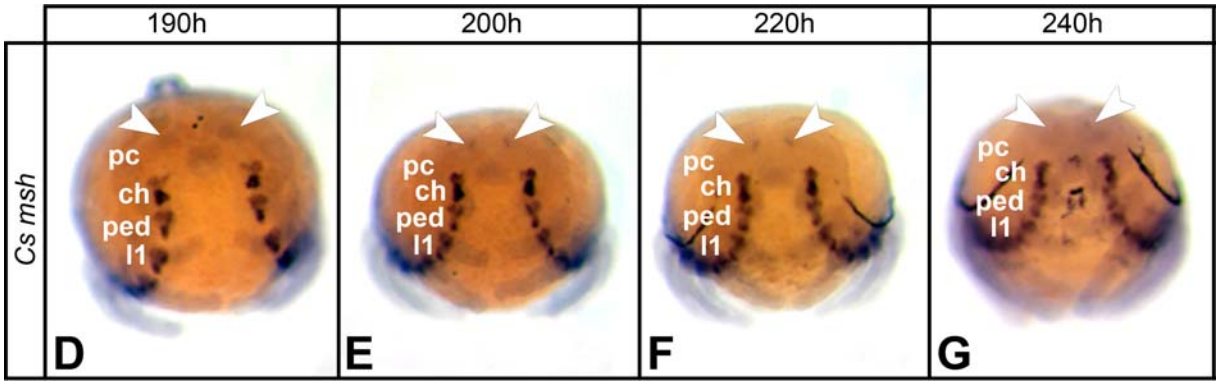

Figure 3-23: Temporal expression pattern of *msh* in the procephalic NE of *Cupiennius salei*.

(**D**, **E**, **F**, **G**) The same embryos as presented in Fig.3-9. Whole mount *Cupiennius* embryos stained with a DIG-labelled *msh*-RNA antisense probe; anterior view. (**D**) At 190 hours *msh* is expressed in the lateral column of the VNE in all segments except for the precheliceral segment. (**E**) Slightly later, after 200 hours of development, *msh* expression starts in a small cell cluster in the precheliceral NE (arrowheads). (**F**, **G**) *msh* expression is maintained in the cell cluster in the precheliceral NE during further development (arrowheads). ***ch***, cheliceral segment; ***Cs***, *Cupiennius salei*; ***l1***, leg segment 1; ***msh***, *muscle segment homeobox*; ***pch***; precheliceral segment; ***ped***, pedipalp. Anterior is to the top.

During embryogenesis, the supraoesophageal ganglion, which contains the proto- and the deutocerebrum, is folded on top of the anterior part of the suboesophageal ganglion in *Cupiennius* (Fig.3-21 D). This complicates the nomenclature of the body axes, since studies on the adult brain use anterior as term for the part where the oesophagus is positioned at the end of embryogenesis (Fig.3-21 D). But this work describes the development of the brain in the embryo and therefore the morphological terms are based on the embryonic body axes. This means that the most anterior part of the precheliceral lobes in the embryo corresponds to the posterior part of the adult (larval) brain.

3.2.2 Structure of the early larval brain of *Cupiennius salei*

In the following paragraph the brain structures of the early larva will be described, because this will help to trace back how the different three-dimensional centres

develop from the flat cell layer of the procephalic region in the early embryo. In the early larva the brain is separated into distinct brain centres (Fig.3-24). In adult spiders the main brain centres, which are the optic lobes, the mushroom bodies and the arcuate body, have already been characterised (Barth, 2002). Even if there are still a lot of undifferentiated neural precursors in the early larval brain, these brain centres can already be identified in the early larva (Fig.3-24).

The light micrographs of a medial (Fig.3-24 A) and a lateral (Fig.3-24 B) parasagittal section show the subdivision of the early larval brain into the supra- and the suboesophageal ganglion. At this stage most of the neural cells are still undifferentiated neural progenitors and arranged in two layers. One layer of epithelial vesicles covers the dorsal and the lateral surface of the larval brain. The other layer is located in a deeper position and consists of densely packed neural precursors, which have already segregated from the vesicles but are not differentiated yet. But there are also differentiated cells in the brain of the early larva. The cell bodies of these differentiated neurons form a cortex that surrounds the small central neuropile (Fig.3-24 A).

The distinguishable brain centres in the larva, which are the optic lobes, the dorsal protocerebrum (mushroom body and arcuate body), central protocerebrum, and the cheliceral neuropile, are positioned dorsal and lateral to the foregut (Fig.3-24). The optic lobes, which are located in the dorsal anterior part of the head, are separated from the posteriorly adjacent dorsal protocerebrum by muscle fibres. It is very probable that these muscles correspond to the muscles which move the principal eyes, because they insert at the dorsal and anterior head capsule (Fig.3-24 A-C) (Barth, 2002). Unlike all other arthropods, most spiders have eight eyes. The eyes are grouped into AP pairs and named according to their positions. Hence, the posterior-lateral (PL) and anterior-lateral (AL) eyes occupy the lateral positions of each half of the visual field, while the posterior-medial and anterior-medial pairs are located in the centre (Fig.3-24 G). Furthermore the eyes are subdivided into principal and secondary eyes according to structural differences. Although the anterior-lateral eyes might be poorly developed, they constitute the principal eyes (Barth, 2002). The secondary eyes have inverted photoreceptors and serve wide-field motion perception, whereas the forward directed principal eyes possess everted photoreceptors and can be moved by muscles (Barth, 2002). Similar to insects, three optic neuropiles can be distinguished in spiders which transmit and process the

visual information. Each secondary eye projects to its discrete first and second neuropile in spiders (ON1, ON2). The first optic neuropile corresponds to the lamina and the second optic neuropile to the medulla of the insect visual system (Barth, 2002). The secondary optic neuropiles of the three secondary eyes project to a common third neuropile. This third order neuropile presents the mushroom body (Hanstroem, 1928). In contrast to this projection pattern, the secondary optic neuropile of the principal eye projects to a separate part of the protocerebrum, which is called arcuate body (Fig. 3-24 D, F). In the larval visual system, the optic lobes contain mainly undifferentiated neural precursors, which build clusters interiorly to their respective eyes (Fig.3-24 C, D). At this stage only the optic lobes of the median eyes contain a detectably differentiated neuropile (Fig.3-24 A, B, D). The neuropile in this stage is subdivided in two distinguishable compartments – a small peripheral compartment and the neuropile of the dorsal protocerebrum. The former projects anteriorly towards a deeper neuropile, which in turn connects through a distinctive fibre bundle to the latter (Fig.3-24 D, arrows).

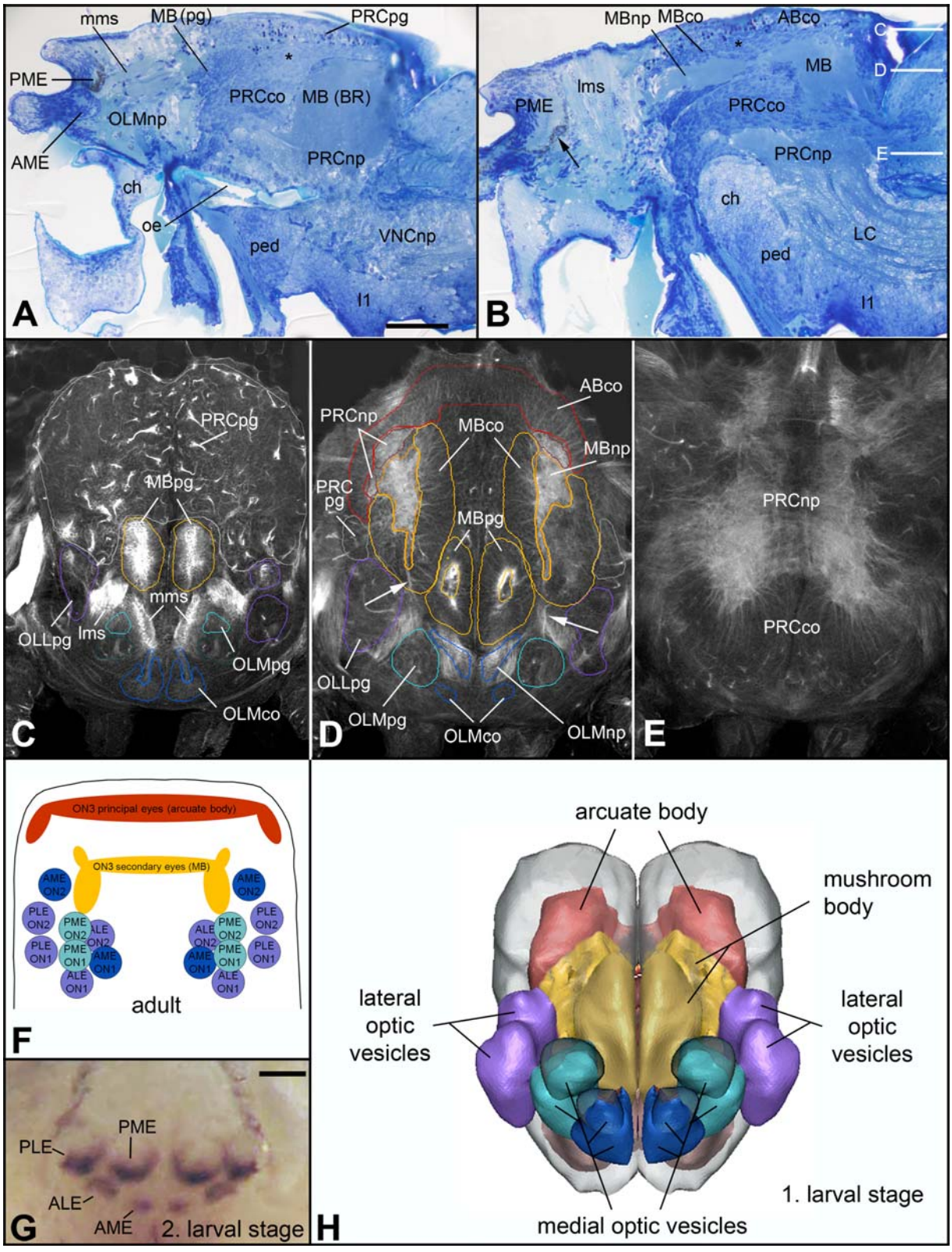

Figure 3-24: Structure of the early larval brain of *Cupiennius salei*.

Legend see next page (85).

Legend to Fig.3-24: Light micrographs of parasagittal sections through the brain of a larva (1st larval stage) (**A**, **B**), horizontal confocal optical sections of a larva stained with Phalloidin-FITC (**C**-**E**) and light micrograph of the visual field of a 2nd larva (**G**). (**A**, **B**) The medial (**A**) and lateral (**B**) sections show the subdivision of the early larval brain into the supra- and suboesophageal ganglion as well as the distinct neuropiles. Asterisks in A and B indicate undifferentiated neural precursors. The arrow in B points to pigment cells in the developing eye. The white lines and letters in B indicate the position of the optical sections shown in C-E. See text for details. (**C**, **D** ,**E**) The dorsal-anterior part of the larval brain is covered with epithelial vesicles that consist of protocerebral neural precursors (light-grey outline in C), the precursors of the mushroom bodies are located medially (yellow outline). The precursors of the lateral and medial optic lobes are outlined in purple and turquoise, respectively. The developing cortex and neuropile of the median eyes is outlined in blue. The cortex of the arcuate body is outlined in red and the protocerebral neuropile in dark red. Note that in publications that describe the adult brain structures of spiders, the optic neuropile behind the eyes is considered as the most anterior structure of the brain while the arcuate body is considered to be the most posterior brain centre. Here we are describing the development of the brain centres in the larva and embryo and therefore our morphological terms correspond to the embryonic body axes; for example the arcuate body forms in the most anterior part of the precheliceral lobe but it will eventually occupy the most posterior part of the adult brain since the protocerebrum folds on top of the suboesophageal ganglion (Fig.3-21 D). (**F**) Schematic drawing of the brain compartments in the adult *Cupiennius salei*. Note that this is a coarse presentation of the relative positions of the brain compartments which does not indicate the different DV positions of the neuropile. The colour code corresponds to the line colour shown in C-E. (**G**) Frontal view of the larval eyes (2nd larval stage). The red pigmentation becomes visible. Note the differences in the size of the posterior and anterior eyes. (**H**) The 3D-model shows the position of the brain compartments that we have identified in embryonic and larval stages. The colour code corresponds to the line colour shown in C-E. ***ALE***, anterior lateral eye vesicle; ***AME***, anterior medial eye vesicle; ***ABco***, arcuate body cortex; ***BR***, bridge of mushroom bodies; ***ch***, chelicerae, cheliceral neuromere; ***l1***, neuromere associated with the segment of walking leg 1; ***LC***, longitudinal connective of ventral nerve cord; ***lms***, lateral muscles; ***MB***, mushroom body; ***MBco***, mushroom body cortex; ***MBnp***, mushroom body neuropile; ***MBpg***, mushroom body precursor groups; ***mms***, median muscles; ***oe***, oesophagus; ***OLLpg***, lateral optic lobe precursor groups; ***OLMco***, medial optic lobe cortex: ***OLMpg***, medial optic lobe precursor groups; ***OLMnp***, medial optic lobe neuropile; ***ON1-ON3***, 1.-3. optic neuropile; ***ped***, pedipalpal neuromere; ***PLE***, posterior lateral eye vesicle; ***PME***, posterior medial eye vesicle; ***PRCco***, protocerebral cortex; ***PRCnp***, protocerebral neuropile; ***PRCpg***, protocerebral precursor groups; ***VNCnp***, ventral nerve cord neuropile. Scale bars: (A) 150 µm in panel A-E; (G) 150 µm in panel G. Anterior is to the left in A, B and at the top in C-E.

The protocerebrum can be subdivided into a dorsal and a ventral tier, which is most obvious on parasagittal sections of the larval brain (Fig.3-24 A, B). The dorsal tier is represented by an elongated layer of fibres, which is surrounded by neuronal somata in each brain hemisphere (Fig.3-24 B). In the anterior part, the fibres converge and build a commissural tract, which is part of the mushroom bodies (Fig.3-24 A, B).

The larval arcuate body is located further posterior and is represented by a thin, crescent-shaped fibrous layer that arches over the mushroom body and the central protocerebrum (Fig.3-24 B, D, E). The larval central protocerebrum is composed of a system of commissural tracts (supraoesophageal commissure). Layers of neuronal somata separate the commissural tracts of the central protocerebrum from the overlying dorsal protocerebrum (mushroom body) (Fig.3-24 B, E).

The fibres of the supraoesophageal commissure merge with the longitudinal connectives on both sides of the foregut. These connectives extend along the complete length of the neuraxis from the protocerebrum to the posterior tip of the opisthosomal ganglion (Fig.3-24 B).

Investigating how the above described brain centres develop from the embryonic NE was focus of this part of my work. The formation of the compartments can be followed backwards based on the characteristic topology of each brain centre and the mode of segregation from the ectoderm. The optic lobes, mushroom body and arcuate body are formed by large vesicles, which fold in the periphery of the protocerebral NE. These structures remain visible as vesicles, furrows or clusters during development. These features facilitated the correlation of the expression patterns of genes, which are known to be involved in brain development in insects, to the main compartments of the developing spider brain.

3.2.3 Morphogenetic movements of the developing brain in *Cupiennius salei*

To examine how the three-dimensional structures in the brain occur and build compartments, the morphogenetic movements in the embryo were observed. A centre of pronounced movements is found in the NE of the developing brain, where a two-dimensional single layer of neuroepithelial cells has to develop into a complex three-dimensional structure, which is subdivided into different compartments and centres. The morphogenetic movements were analysed by staining embryos against Tubulin and Phalloidin (Fig.3-25). This paragraph describes the gross morphogenetic movements which will then be interpreted and further described in following paragraphs where data about marker gene expressions contribute information.

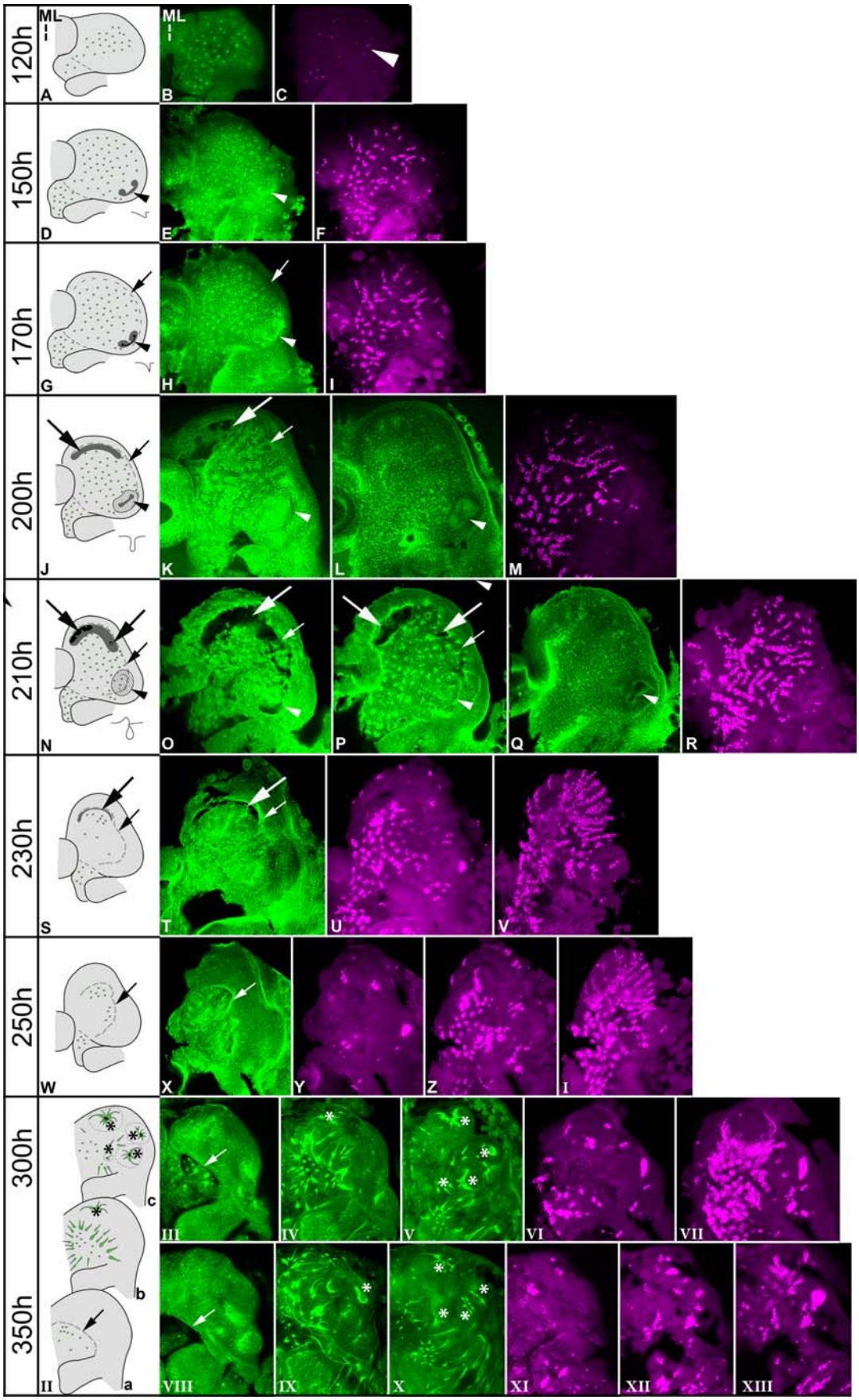

Figure 3-25: Morphogenetic movements and compartmentalisation in the precheliceral lobe.

Legend see next page (88).

Legend to Fig.3-25: (**A**, **D**, **G**, **J**, **N**, **S**, **W**, **II**) Schematic drawing of morphogenetic movements in the precheliceral lobe during development. (**B**, **E**, **H**, **K**, **L**, **O**, **P**, **Q**, **T**, **X**) Maximum projections and different horizontal sections of *Cupiennius* embryos at different stages stained for α- and β-Tubulin (green). (**III**, **IV**, **V**, **VIII**, **IX**, **X**) Horizontal sections of *Cupiennius* embryos at 300 and 350 hours of development stained with Phalloidin-FITC (green). (**C**, **F**, **I**, **M**, **R**, **U**, **V**, **Y**, **Z**, **I**, **VI**, **VII**, **XI**, **XII**, **XIII**) Maximum projections and different horizontal sections of *Cupiennius* embryos at the same age as presented with Phalloidin stainings and Tubulin stainings stained for Prospero (magenta). (**A**, **B**) Neurogenesis starts at about 120 hours of development with the formation of the first NPGs. In the flat epithelial sheet of the procephalic NE the NPGs are formed in a broad stripe which extends from medial to lateral. (**C**) At this early stage of neurogenesis only very few cells express the differentiation marker Prospero (arrowhead). (**D**, **E**) After 150 hours of development the precheliceral NE expands, more NPGs are recruited and a small furrow is formed at the posterior lateral edge (arrowheads). (**D**, **G**, **J**, **N**) The small scheme under the drawing of the embryo presents a cross section through this furrow. (**E**) The number of Prospero-positive differentiating neural precursors is increased after 150 hours of development. (**G**, **H**) Until 170 hours of development the precheliceral NE expands further (mainly towards anterior) and more NPGs are detectable. In this stage the epidermis starts to overgrow the precheliceral NE from the outer borders (arrows, dotted line). (**H**) The furrow at the lateral posterior edge deepens which was analysed by checking the Phalloidin staining in different layers form apical to basal with the confocal microscope. (**G**) This feature is pointed out by the drawing of a cross section of the deepening (arrowheads). (**I**) In the same embryo as presented in H the Prospero staining reveals a further increase of differentiating neural precursors. (**J**, **K**, **L**) After 200 hours of development the shape of the precheliceral NE undergoes slight changes from a rather round to a more elongated profile and the epidermis keeps overgrowing the NE (small arrows, dotted line). At this stage a second furrow (big arrows) is formed in the precheliceral NE in addition to the furrow at the posterior lateral edge (arrowheads). (**J**, **K**) The newly formed furrow emerges in the anterior half of the precheliceral NE which is shown by the Phalloidin staining in the apical part (big arrows). (**L**) As the surface of the NE deepens in the furrow, the NPGs which are positioned in the furrow are detected more basally and due to that feature they come to lie closer to each other. (**J**, **K**, **L**) The earlier formed lateral posterior furrow deepens further (arrowheads). In the apical layer (**K**) the borders of the furrow come closer to each other and in more basal positions (**L**) the furrow now builds a lumen. (**L**) This newly formed lumen is surrounded by cells which can be distinguished from the other cells in this region. (**M**) Until this stage the number of Prospero expressing cells increases slightly. (**N**, **O**, **P**, **Q**) Ten hours later the formation of the two furrows continues and the procephalic NE becomes more and more three-dimensional while it is further overgrown by the epidermis (small arrow, dotted line). The anterior furrow gets broader in the apical layer (**O**) and as it deepens toward basal it splits into two deepenings (**P**) (big arrows). (**Q**) As the NPGs are positioned in the precheliceral NE the NPGs which are located in the NE of the anterior furrow follow this deepening and can be detected further basal. This three-dimensional formation brings the NPGs closer to each other and might lead to a merge of NPGs. (**N**, **O**) At 210 hours the borders of the lateral posterior furrow meet in the apical layer after they have come closer together during earlier stages (arrowheads). (**N**, **P**, **Q**) Further basal the lateral anterior furrow builds a lumen which is surrounded by cells which can be distinguished from the neighbouring cells of the NE (arrowheads). (**N**, **O**, **P**, **Q**) Due to these morphogenetic movements the lateral posterior furrow builds a vesicle which is constricted at the apical surface. (**R**) The number of differentiating neural precursors clearly increased until 210 hours of development and these Prospero-positive cells seem to be arranged in compartments. (**S**, **T**) The epidermis keeps overgrowing the precheliceral NE (small arrow) and at 230 hours of development the lateral posterior vesicle is covered completely while the posterior border of the anterior furrow is still visible (big arrow). (**T**) The Tubulin antibodies can not reach their epitopes in more basal positions and thus only allow a view on the rather apical part where some single NPGs are still detectable. In contrast to Tubulin the Prospero expression can be presented in different layers from apical (**U**) to basal (**V**) after 230 hours of development. (**R**, **U**, **V**) Between 210 and 230 hours of development the amount of differentiating neural precursors increases significantly. (**U**) In the apical region the younger cells which newly express Prospero are positioned in a similar pattern as the NPGs in the central region of the precheliceral NE. (**V**) More apical the differentiating neural precursors are arranged in rows in which the older cells are pushed further by the newly delaminating neural precursors. (**U**, **V**) After 230 hours of development the first clear Prospero expression comes up close to the lateral posterior vesicle. (**W**, **X**) With ongoing development the epidermis covers the precheliceral NE further and at 250 hours of development the anterior furrow as well as the posterior lateral vesicle completely (arrows). But even at this late stage some NPGs still persist in the central part of the precheliceral NE. (**X**, **Y**, **Z**, **I**) The Tubulin antibody only shows the apical part of the procephalic region whereas the Prospero antibody allows detecting differentiating neural precursors in more basal regions. (**Y**) The most apical part

contains few clusters of Prospero-positive cells in the apical region. (**Z**) Further medial the differentiating neural precursors are arranged in a pattern which reflects the arrangement of the persisting NPGs in the central part of the precheliceral NE. (**I**) The major part of Prospero-positive cells is found in the basal part r. (**II**) The procephalic region becomes more and more three-dimensional and the schematic drawings present three parts from apical (a) to basal (c) to show all the morphogenetic movements and the formation of different compartments after 300 and 350 hours of development. (**III**, **IV**, **V**, **VIII**, **IX**, **X**) As the Tubulin staining did not allow following the morphogenetic movements in more basal layers, Phalloidin stainings were used to visualise cell shapes and compartmentalisation in the precheliceral NE after 300 and 350 hours of development. (**IIa**, **III**) At 300 hours of development the epidermis covers the major part of the procephalic NE (arrow). The small central part which is still not covered shows persisting NPGs. (**IIa**, **VIII**) After 350 hours of development the precheliceral NE is completely covered by the epidermis (arrow). (**IIb**, **IV**, **IX**) In the medial layers one anterior and one lateral vesicle are detectable after 300 and 350 hours of development (asterisks). (**IIc**, **V**, **X**) The Phalloidin staining in the basal layer clearly shows the formation of four distinct vesicles in the procephalic region (asterisks). (**VI**, **VII**) The number of Prospero expressing cell increased further during development and after 300 hours of development they are arranged in clusters and vesicles. (**VII**) The major part of differentiating neural cells is positioned in the basal part. During the next 50 hours Prospero expression stays similar; it is expressed in clusters in the apical (**XI**) and medial (**XII**) part and the majority of differentiating neural precursors is detected in the basal part (**XIII**). (**XIII**) Prospero expression in the basal part seems weaker which is due to the thickness of the tissue at this stage as it prevents the antibody from reaching its epitopes. ***ML***, midline. Ventral views; anterior is to the top.

Neurogenesis starts at 120 hours of development with the recruitment of some NPGs in a flat sheet of neuroectodermal cells (Fig.3-25 A, B). In few NPGs the differentiation to neural cells has already started, which is reflected by Prospero staining (Fig.3-25 C, big arrowhead). The number of NPGs, and with that the number of differentiating neural cells, increases with ongoing development and at 150 hours the rather flat precheliceral lobes show the first three-dimensional structures (Fig.3-25 D, E, F). In this stage a small furrow appears at the posterior lateral edge of the precheliceral lobe (Fig.3-25 D, E, small arrowheads). During further development this furrow becomes deeper and pointed while more NPGs are generated and more neural precursors differentiate (Fig.3-25 F, G, H). At this time the epidermis starts to grow over the precheliceral lobe from the periphery (Fig.3-25 F, G, small arrows). Between 180 and 200 hours of development a second three-dimensional structure is generated in form of a semicircular furrow in the anterior edge of the precheliceral lobes and the posterior lateral furrow changes its shape. The cleft becomes thinner in the apical layer, while it extends to form a vesicle in the basal layer (Fig.3-25 J, K, L, big arrows). The main portion of the NPGs are present in the apical layer, but the invagination sites which are located in the anterior furrow sink together with the surface of the furrow into a more basal position (Fig.3-25 K, L). Because of that the distance between them narrows and they are located very close to each other and might fuse during ongoing development (Fig.3-25 J, L, N, Q). This impression is

supported as the furrow becomes broader and deeper and splits into two lowerings at about 210 hours of development (Fig.25 N, O, P, Q, big arrowheads). At this point in time the lateral trough closes at the apical layer and the vesicle in the basal layer widens and is surrounded by a clearly confined layer of cells (Fig.3-25 N, O, P, Q, small arrowheads). During the last stages more cells start to differentiate and the epidermis is still growing over the precheliceral lobes (Fig.3-25 J-R). From about 230 hours onwards, the epidermis prevents that the Tubulin antibody reaches basal epitopes and because of that the stainings only show the apical surface of the embryo. This does not uncover further morphogenetic movements in the basal positions, but it shows how the precheliceral lobes are more and more covered by the epidermis (Fig.3-25 S, T, W, X, small arrows). The number of differentiating neurons increases very fast in this developmental time window. There are many newly Prospero expressing cells in further apical regions, where more and more NPGs are recruited and the basal layers contain the older differentiating neural cells which are arranged in compartments and clusters (Fig.3-25 U, V, Y, Z, I). For analysing morphogenetic movements in older embryos, Phalloidin stainings were used and examined in combination with Prospero stainings in embryos at the same stages (Fig.3-25 II-XIII). A view of the apical surface shows that the epidermis has grown over the precheliceral lobe almost completely and that there are still newly build NPGs with their cell processes in the apical layer, where the NE is not covered (Fig.3-25 IIa, III, VIII). But the main part of the NPGs is located more basally and their arrangement already suggests an association of certain NPGs to developing brain centres (Fig.3-25 IIb, IV, IX). This assumption is strongly supported by the staining in the most basal position, where the oldest NPGs are located. They are clearly organised in four vesicles per precheliceral lobe and one of these vesicles is further subdivided (Fig.3-25 IIc, V, X, asterisks). The compartmentalisation is also reflected in the arrangement of differentiating neural cells in clusters (Fig.3-25 VI, VII, XI, XII, XIII).

At the end of embryogenesis, the brain contains three main centres, which are the optic ganglia, the mushroom bodies and the arcuate body. After this general look on the morphogenetic movements, a more detailed description of the developing brain centres together with marker stainings revealed how these structures are generated in *Cupiennius salei*; these results are the content of the following paragraphs.

3.2.4 Expression of marker genes in the developing brain

3.2.4.1 Expression of the proneural gene *Cupiennius ASH1* defines the primordia of the brain

Early steps of neurogenesis can be analysed with *in situ* hybridisations for the proneural gene *ASH1*, which is responsible for the formation of neural precursors. Previous studies revealed that in the precheliceral lobe and all prosomal segments neurogenesis starts at about 120 hours of development with the expression of *ASH1* in *Cupiennius* (Stollewerk *et al.*, 2001).

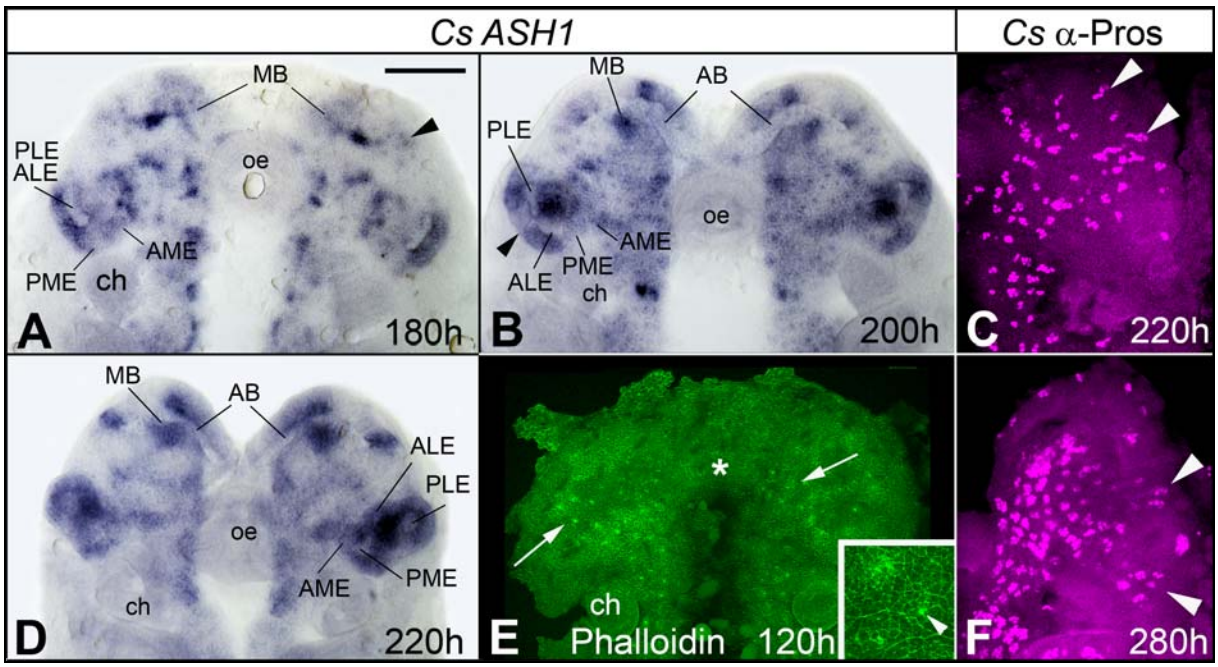

Figure 3-26: Expression pattern of the proneural gene *ASH1* in the cephalic lobe.

Flat preparations of embryos stained with a DIG-labelled *ASH1* RNA antisense probe (**A**, **B**, **D**), the anti-*Cupiennius salei* Prospero antibody (magenta; **C**, **F**) and Phalloidin-FITC (green; **E**); ventral views. (**A**) *ASH1* shows a dynamic expression in all areas of the cephalic lobe. At 180 hours, *ASH1* is expressed in the most anterior part of the procephalic NE in the area where the mushroom body (MB) vesicles will form during later development. *ASH1* transcripts are upregulated in the neural precursor groups (arrowhead). The optic ganglia of all eyes arise in the periphery of the precheliceral lobe. *ASH1* is expressed in the whole area and upregulated in patches of cells. Note that the grooves that give rise to the ALE and PLE ganglia are already visible. (**B**) Basal view. At 200 hours the bilateral grooves from which the arcuate body (AB) and the mushroom bodies develop have formed in the anterior part of the precheliceral NE. *ASH1* expression is visible in the grooves. In addition, *ASH1* is strongly expressed in two patches on either side of the midline which are most likely subsets of precursor groups that become incorporated into the MB vesicles. *ASH1* expression is maintained in the optic vesicles. The lateral groove has been subdivided into the PLE and ALE vesicles (arrowhead). Many additional neural precursor groups (dot-like staining) are visible in the developing central protocerebrum on either side of the oesophagus. (**C**) Prospero is expressed in postmitotic neurons that form regularly arranged small clusters of cells (arrowheads).
Legend continued on next page (92).

Legend to Fig.3-26: (**D**) The arcuate body grooves become incorporated into the developing brain and the two distinct strong expression domains on either side of the midline have increased in size and come closer together. *ASH1* continues to be expressed in the optic lobe anlagen. (**E**) At 120 hours of development the first neural precursor groups are generated in the precheliceral NE (arrows). The inset shows a higher magnification of three precursor groups (arrowhead). The asterisk indicates the area where the oesophagus will develop. (**F**) By 280 hours Prospero is expressed in additional precursor groups and can also be detected in the optic lobe anlagen (arrowheads). ***AB***, arcuate body; ***ALE***, anterior lateral eye vesicle; ***AME***, anterior medial eye vesicle; ***ch***, chelicerae; ***MB***, mushroom body; ***oe***, oesophagus; ***PLE***, posterior lateral eye vesicle; ***PME***, posterior medial eye vesicle. Anterior is to the top. Scale bar: (A) 150 µm in panels A-F.

The staining of *ASH1* in the spider shows that its expression is very dynamic during development in the precheliceral lobe (Fig.3-26). Like in the VNE, *ASH1* is expressed in a single layer of neuroepithelial cells in positions where the NPGs are recruited in the procephalic region (Fig.3-26 A, E, Fig.3-27). There, *ASH1* expression is transiently maintained in the NPGs which invaginate as vesicles after their formation (Fig.3-26 A, B Fig.3-27). The metameric pattern of the NPGs in the developing neuromeres of the VNE is also present in the cheliceral hemineuromeres, whose medial borders frame the central disc that develops into the foregut. The numerous small invagination sites in a more dorsal extended area will give rise to the central protocerebrum (Fig.3-26 E, G). Apart from this dotted expression, high levels of transcripts accumulate in some areas of the precheliceral lobe and persist throughout embryogenesis from about 180 hours onwards (Fig.3-26 B, D). Taking into account the analyses of the morphogenetic movements, this and other marker stainings, the areas with strong *ASH1* expression were identified as the developing optic lobes, the developing mushroom body (MB) and the developing arcuate body (AB) (Fig.3-26 A, B, D).

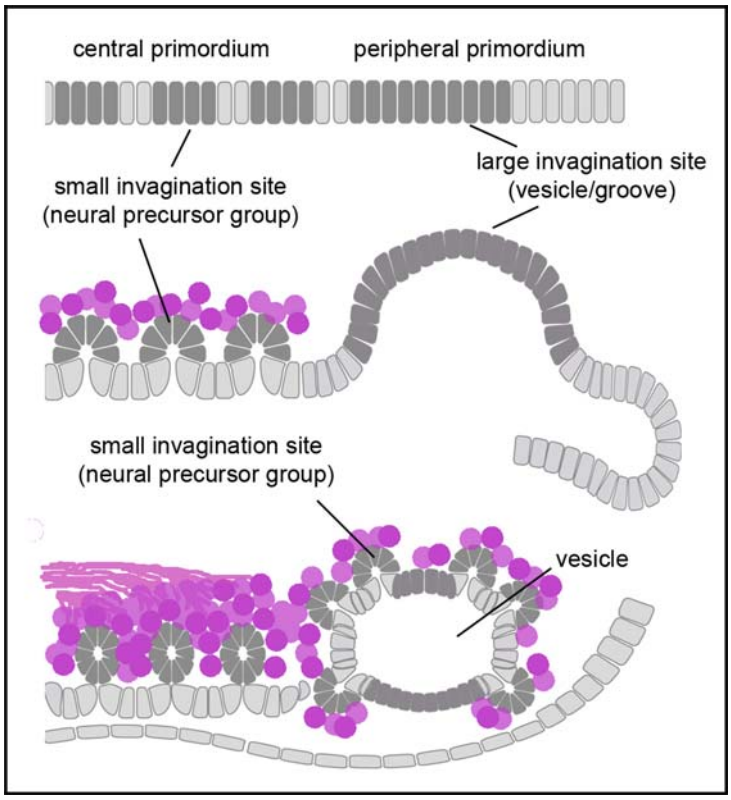

Figure 3-27: Formation of large and small invagination sites in the peripheral and central protocerebral primordia, respectively.

The central protocerebrum develops from numerous small invagination sites (neural precursor groups). In the peripheral precheliceral lobe large invagination sites (vesicles/grooves) segregate from the NE and develop into the mushroom bodies, arcuate body and optic anlagen. Small invagination site form in the walls of the vesicles/grooves. Light grey, neuroectodermal cells; dark grey, invaginating neuroectodermal cells; magenta, neural precursors/neurons.

At about 180 hours of development, Prospero can be detected in cells of the NPGs, which shows that these cells start to differentiate. The differentiating postmitotic neurons are detected in small clusters which correspond to the arrangement of the invaginations of the central protocerebrum (Fig.3-26 C). No significant Prospero expression can be detected in the peripheral domains, from where the optic lobes and the dorsal protocerebrum originate. But the *ASH1* expression in these areas of the developing visual system indicates that neural progenitors are continuously incorporated into the primordium (Fig.3-26 D). However, Prospero expression in these areas comes up during late embryogenesis (Fig.3-26 F).

3.2.4.2 Expression of *Cupiennius netrin*

In *Drosophila* two Netrins are known, which are Netrin A and Netrin B, whereas in the spider only one *netrin* (*net*) was identified until now (unpublished data). Its role in axonal pathfinding was already studied in some representatives of vertebrates and invertebrates. It is also involved in the development of the visual system in *Drosophila* and *Xenopus* and therefore a good marker to get insights in this issue in spiders. Netrin A and Netrin B are expressed in midline glia cells, in discrete subsets of muscles and in the developing lamina (Mitchell *et al.*, 1996). Due to its expression and function in the VNE *net* expression was analysed in the spider. These studies revealed that it is expressed in glia cells, which surround the NPGs and in cells of the midline (unpublished data).

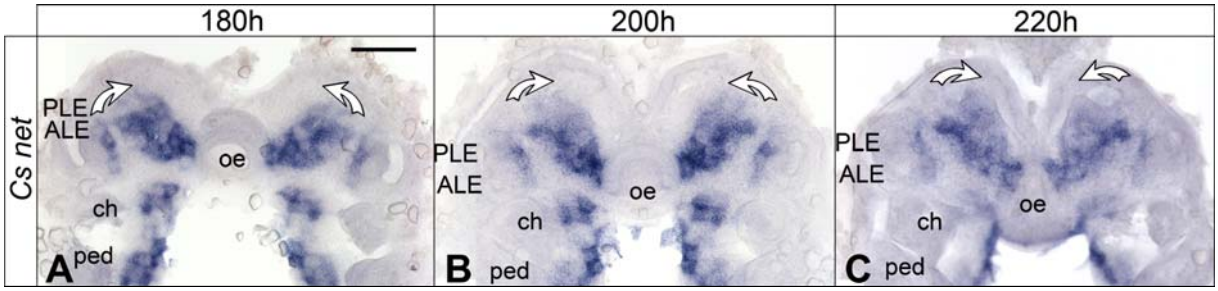

Figure 3-28: Expression pattern of *netrin* in the precheliceral lobe.

(**A**, **B**, **C**) Flat preparations of *Cupiennius* embryos stained with a DIG-labelled *net*-RNA probe; ventral views. (**A**) At 180 hours *net* expression covers a big area in the procephalic NE. In the precheliceral lobe are two *net*-positive domains; the bigger one extends from the oesophagus towards lateral in a broad domain and the smaller one is restricted to the medial border of the lateral furrow, which generates ALE/PLE. (**B**, **C**) The cells which expressed *net* at 180 hours maintain expression in later stages (200-220 hours). The differences in location of the expression domains are due to morphogenetic movements and not a change of expression areas. (**A**, **B**, **C**) *net* staining within the expression domains is not homogeneous as *net* is expressed in glial cells which surround the invaginating NPGs. That leads to weaker staining in the NPGs and stronger staining in the cells around the NPGs. ***ALE***, anterior lateral eye vesicle, ***ch***, chelicerae; ***Cs***, C*upiennius salei*; ***net***, *netrin*; ***oe***, oesophagus; ***ped***, pedipalp; ***PLE***, posterior lateral eye. Anterior is to the top. Scale bar: (A) 150 µm in panels A-C.

In the procephalic lobe in *Cupiennius netrin* expression covers a big domain which begins at the oesophagus and spreads towards lateral in all studied stages (Fig.3-28). As it is expressed in the glial cells which surround the NPGs the staining is not uniform within the expression domains but rather shows weaker staining where the NPGs are located and stronger staining in the surrounding glial cells. Observation of the expression in different stages shows that the *netrin*-positive domains move

towards anterior medial, which reflects the morphogenetic movements in the protocerebrum (Fig.3-28 arrows). Apart from this big expression area, there is another *netrin*-positive domain further lateral at the medial border of the lateral vesicle, which was described in paragraph 3.2.3 (Fig.3-25 D, G, J, N, Fig.3-28). This vesicle was identified as part of the developing visual system (ALE/PLE) and will be characterised later in more detail.

3.2.4.3 Expression of *Cupiennius dachshund*

During embryogenesis *Drosophila dachshund* is expressed in several populations of neural cells in the VNE and in the developing brain. In larval stages the expression level increases in the developing mushroom body and in the lamina, which represents the outer optic ganglion, and in the eye primordia. Its function in eye development, tissue specification, morphogenetic furrow initiation and photoreceptor selection in *Drosophila* is well studied (Kurusu *et al.*, 2000; Mardon *et al.*, 1994; Martini *et al.*, 2000; Noveen *et al.*, 2000). Based on this data the expression of *dachshund* was analysed in the precheliceral lobe in the spider to identify the areas of visual system development (Fig.3-29).

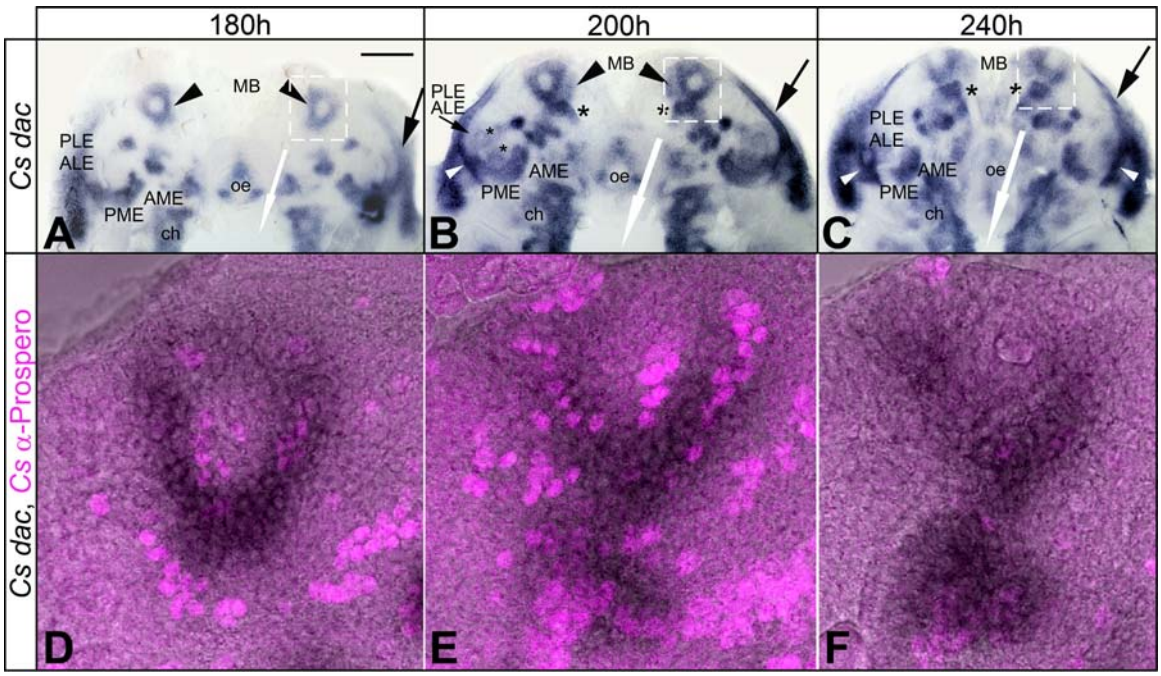

Figure 3-29: Expression pattern of *dachshund* in the precheliceral lobe.

Legend see next page (96).

Legend to Fig.3-29: (**A**, **B**, **C**) Flat preparations of embryos stained with a DIG-labelled *dachshund* (*dac*) RNA antisense probe; ventral view. (**D**, **E**, **F**) Double-staining of *dac* and Prospero in the developing mushroom body. (**A**) At 180 hours, *dac* is expressed in a ring-shaped structure that corresponds to the area where the large MB vesicles develop. The lateral expression domain covers the area of the optic anlagen. (**D**) The ring-shaped structure already contains some differentiating neural precursors at 180 hours of development. (**B**) At 200 hours the same expression domains are visible. *dac* is expressed in the separated PLE and ALE vesicles. Additional expression domains are visible posterior to the ring-shaped anterior structure in the MBs (asterisks). (**E**) At 200 hours the number of postmitotic differentiating neural cells increases in the ring-shaped structure and in the additional *dac* expressing cells posterior to the ring. (**C**) At 240 hours the same expression domains are visible as before. The asterisks indicate the same domains as in B. (**F**) Even if the presented Prospero staining is not increased in the ring shaped structure, the amount of Prospero expressing cells is actually higher. The stronger staining of *dac* and the thickness of the tissue at 240 hours prevents a strong fluorescent staining, but evaluation with the confocal microscope revealed an increase of Prospero expressing cells in the *dac*-positive domain. ***ALE***, anterior lateral eye vesicle; ***AME***, anterior medial eye vesicle; ***ch***, chelicerae; ***MB***, mushroom body; ***oe***, oesophagus; ***PLE***, posterior lateral eye vesicle; ***PME***, posterior medial eye vesicle. Scale bar: (A) 150 µm in panels A-C. Anterior is to the top.

In the spider, *dachshund* expression can be detected at 180 hours of development in distinct domains in the developing protocerebrum (Fig.3-29 A). Furthermore the gene is expressed in the developing sensory organs of the labrum, in the VNE including the cheliceral segment and in the developing appendages (data not shown). In the precheliceral lobe at about 180 hours of development, *dachshund* is expressed in the dorsal and lateral regions, where the vesicles of the optic lobe and the mushroom body are formed (Fig.3-29 A). While *dachshund* remains expressed in these areas and the expression increases throughout embryogenesis, transcripts accumulate in additional clusters between the ring-shaped anterior structure and the posterior expression domains (Fig.3-29 B, C). The lateral and dorsal *dachshund* expression covers the region of the developing mushroom body and the developing vesicles of the lateral eyes, whereas the more medial expression reflects the positions of the developing vesicles of the median eyes (Fig.3-29).

3.2.4.4 Expression of *Cupiennius islet*

In *Drosophila tailup* (*islet*) is expressed in different subsets of moto- and interneurons in the VNE and its expression in the NE of *Cupiennius salei* was already presented in paragraph 3.1.11. During the studies about *islet* (*isl*) expression in the NPGs of *Cupiennius* its strong expression in the developing brain was noticed (see Fig.3-19 A, Fig.3-20 A, Fig.3-30). Because of this very remarkable expression it was analysed in

different developmental stages in the procephalic region to analyse if the *islet*-positive domains can be related to the developing brain centres.

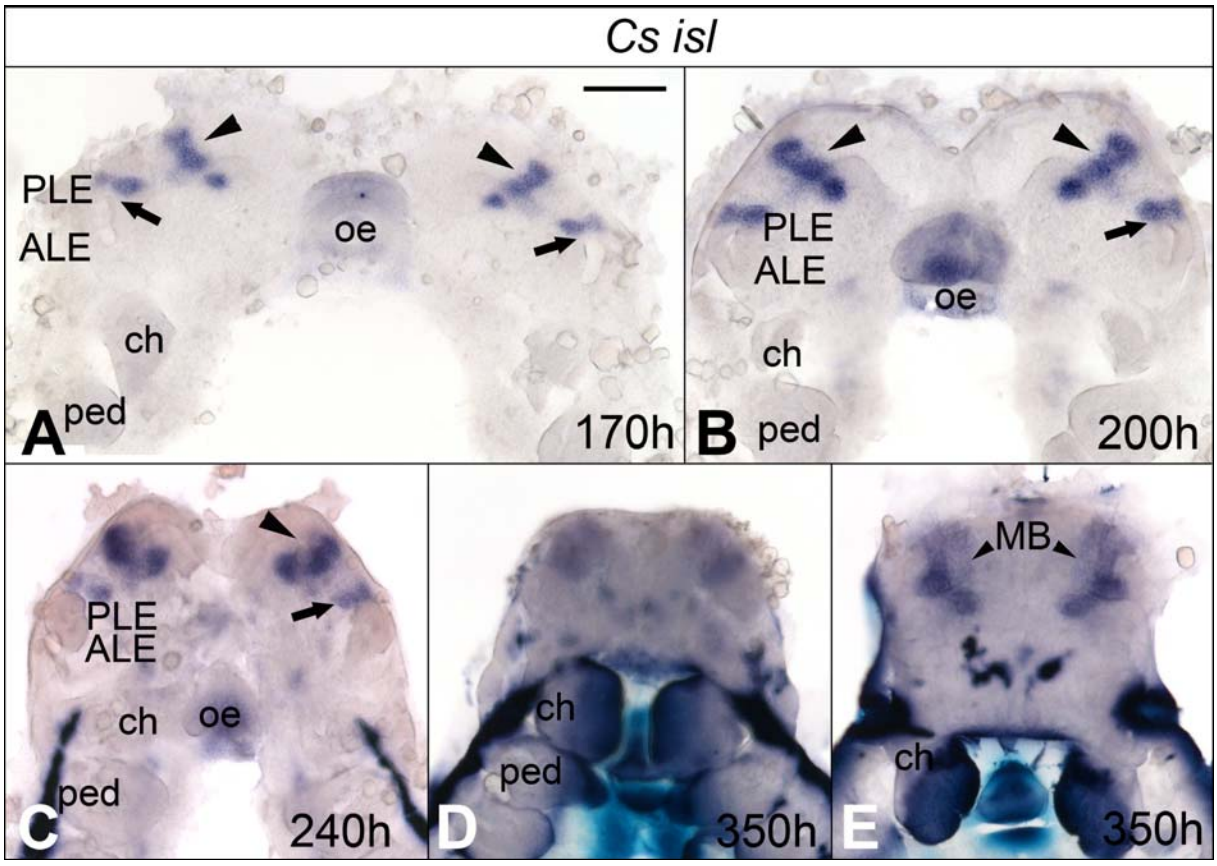

Figure 3-30: Expression pattern of *islet* in the precheliceral lobe.

(**A**-**E**) Flat preparations of *Cupiennius* embryos stained with a DIG-labelled *islet* RNA antisense probe; ventral view. (**A**) At 170 hours *isl* is expressed in two domains in the precheliceral NE. The anterior domain extends from the lateral border of the precheliceral lobe towards medial posterior (arrowheads) and the further posterior domain covers the anterior border of the developing ALE/PLE vesicles (arrows). (**B**) More *isl* transcripts accumulate in these two expression domains until 200 hours of development. The medial part of the bigger anterior expression domain crosses the lateral posterior end of the anterior furrow (arrowheads) and the smaller posterior domain occupies the anterior border of the PLE vesicle (arrow). (**C**) At 240 hours the shape of the anterior expression domain changes into a broader and rather round domain (arrowhead). The posterior domain is clearly located anterior to the PLE/ALE vesicles, but does not cover the cells of the vesicles (arrow). (**D**) The thick and upfolded supraoesophageal NE at late embryonic stages (350h) makes a clear evaluation of the *isl* staining difficult. (**E**) The upfolded supraoesophageal NE was downfolded to present *isl* expression at 350 hours of development. At this stage there is a continuous *isl* expression domain which quite possibly is the result of a fusion of the anterior and posterior expression domain. The shape and location of the *isl* expression suggest that the domain covers the developing mushroom body. ***ALE***, anterior lateral eye vesicle, ***ch***, chelicerae; ***Cs***, C*upiennius salei*; ***isl***, *islet;* ***MB***, mushroom body; ***oe***, oesophagus; ***ped***, pedipalp; ***PLE***, posterior lateral eye. Anterior is to the top. Scale bar: (A) 150 µm in panels A-C.

islet is detected at 170 hours in two domains, which extend from the lateral border of the precheliceral lobe towards medial (Fig.3-30 A). This expression is maintained and

increases, during further development (Fig.3-30 B). The larger anterior stripe crosses the big furrow in the anterior region (Fig.3-25, Fig.3-30 A, B, arrowheads) and the smaller posterior stripe is positioned at the anterior border of the lateral vesicle (Fig.3-25, Fig.3-30 A, B, arrows). At 240 hours expression of the anterior stripe (arrowhead) has changed slightly to a broader expression domain and the posterior stripe (arrow) is positioned directly anterior to -but not in- the lateral vesicles (Fig.3-30 C). In late embryonic stages (350h) the exact position of the staining is difficult to determine, because in this stage the tissue is very thick and the supraoesophageal NE starts to fold up on the suboesophageal NE (see paragraph 3.2.1) (Fig.3-30 D). But if the anterior part is folded back by preparation one continuous *islet*-positive domain can be detected in each procephalic lobe (Fig.3-30 E). The shape of that expression domain suggests that *islet* is expressed in the interneurons of the developing mushroom bodies, which will be described in more detail later.

3.2.5 Formation of the brain compartments by morphogenetic movements

In order to correlate areas of the analysed gene expressions (*ASH1*, *dachshund*, *netrin*, *islet*) to the developing brain centres the morphogenetic movements in these regions were investigated using Phalloidin staining. The earlier description of the general movements (see 3.2.2) was mainly done with Tubulin staining and did not describe the changes in more basal positions of the developing brain. For these more detailed analyses of the movements in the marker gene domains, Prospero and Phalloidin double-stainings were performed in different stages of development (Fig.3-31).

3.2.5.1 Embryonic development of the optic ganglia

The main morphogenetic movements, which lead to the generation of the three-dimensional structures of the two-dimensional sheet, start at about 180 hours of development (Fig.3-25, Fig.3-31). The rearrangements and movements of the NE are accompanied by the overgrowing of the ectoderm. The epidermal ectoderm which surrounds the NE dorsally and laterally slides forward/downward and thereby grows over the neural primordia (Fig.3-25, Fig.3-31, Fig.3-32). Another essential movement

is taking place in the centre of the head ectoderm, where the placode, which produces the foregut, invaginates. In addition to these rearrangements, the head NE itself undergoes morphogenetic movements. Thus, the primordia of the optic lobes and dorsal primordia, which give rise to the arcuate body and the mushroom body, fold inside and become attached to the surface of the brain primordia (Fig.3-31).

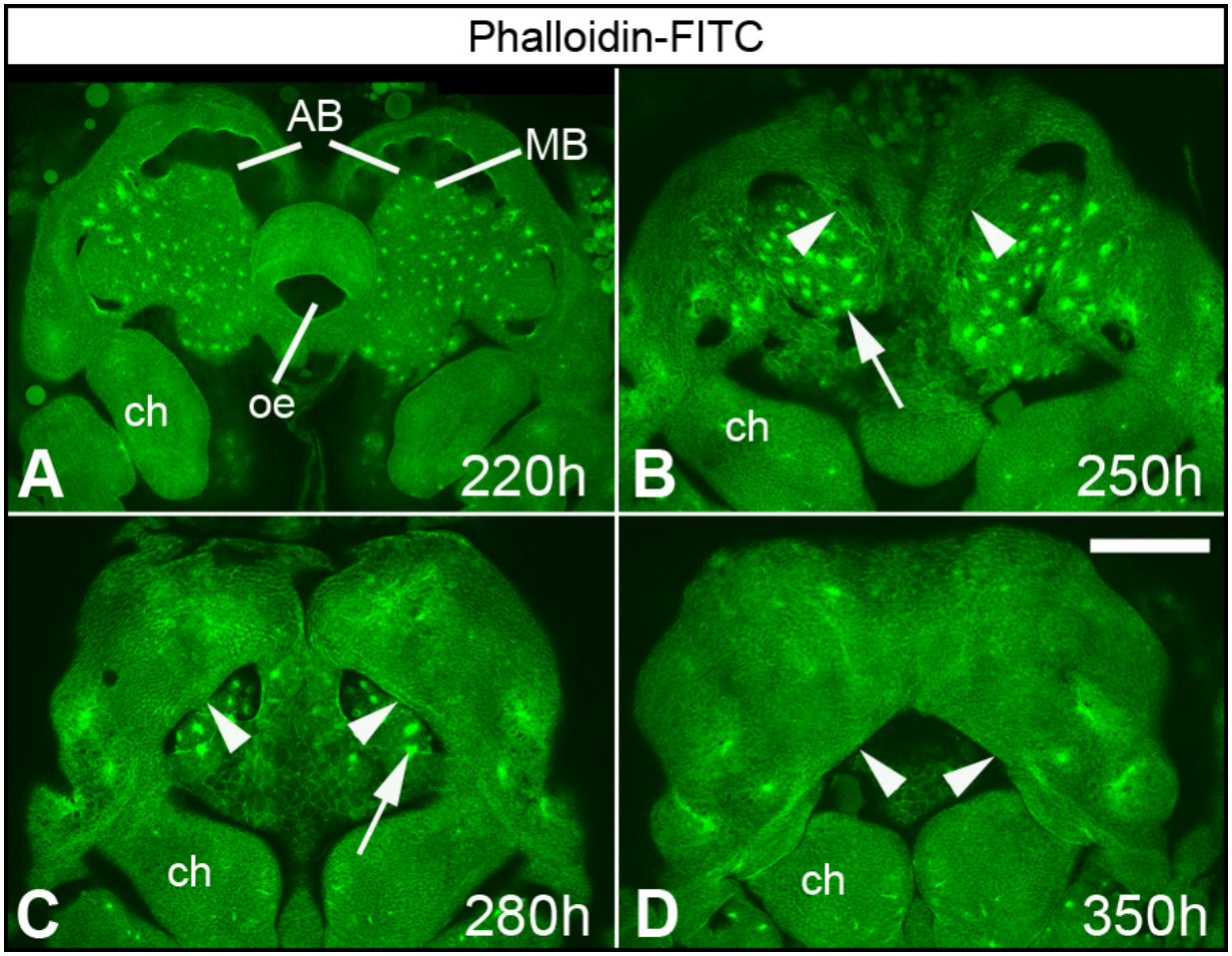

Figure 3-31: The epidermis overgrows the developing brain.

Confocal micrographs of flat preparations of embryos stained with Phalloidin-FITC in a ventral view. (**A**) At 220 hours the semi-lunar grooves and the oesophageal placode are visible. (**B**-**D**) The epidermis gradually overgrows the developing brain (arrowheads). The arrows point to neural precursor groups. ***AB***, arcuate body; ***ch***, chelicerae; ***MB***, mushroom body; ***oe***, oesophagus. Scale bar: Scale bar: (D) 150 µm in panels A-D. Anterior is towards the top.

Generation of the optic ganglia starts with the bilateral formation of grooves in the most lateral parts of the developing protocerebral hemisegments at about 180 hours (Fig.3-25, Fig.3-32 A). At this time the precheliceral lobe is a flat sheet of cells and other morphogenetic movements cannot be detected (Fig.25 G, H, Fig.3-32 A). The

grooves deepen and assume a wedge like shape at 220 hours. The borders of the groove are closer together at the centre (Fig.3-32 B). The lateral invaginations (grooves), called “lateral vesicles” in other arachnids, have been identified as the primordia of the optic ganglia of the lateral eyes (Legendre, 1959; Weygoldt, 1985). Around that stage no differentiating neural cells were detected in this area, but the Phalloidin staining shows that invaginating NPGs form in the apical surface of the grooves (Fig.3-32 B, magnification). At 250 hours the area of the grooves is occupied by two lateral vesicles which most likely arise from the partitioning of the grooves into a dorsal and a ventral half (Fig.3-32 C). The assumption that the relative positions of the optic ganglia to each other as well as their positions along the medio-lateral axis remain the same throughout ontogenesis suggests that these vesicles give rise to the optic ganglia of the posterior-lateral eyes (PLE) and the anterior lateral eyes (ALE) (Fig.3-25 F, G).

Interestingly, slightly later the most basal part of the posterior vesicle is further subdivided into two vesicles (Fig.3-32 F, arrowheads). At the same time two additional grooves form in each brain hemisphere which will probably develop into the posterior-median and the anterior-median eye neuropile, respectively (Fig.3-32 D, E). This assumption is based on the relative position as well as the size of the optic vesicles. According to Barth the size of the optic neuropiles reflects the size of the corresponding eyes (Barth, 2002). Since the PME is considerably larger than AME, the smaller median vesicle was identified as the developing AME ganglia and the larger vesicle between AME and PLE/ALE as the developing PME ganglia (Fig.3-32 F, H). The grooves arise in areas where NPGs have formed before. Basal optical sections reveal that NPGs have been incorporated into the groove and combined to form vesicles (Fig.3-32 E, arrowheads).

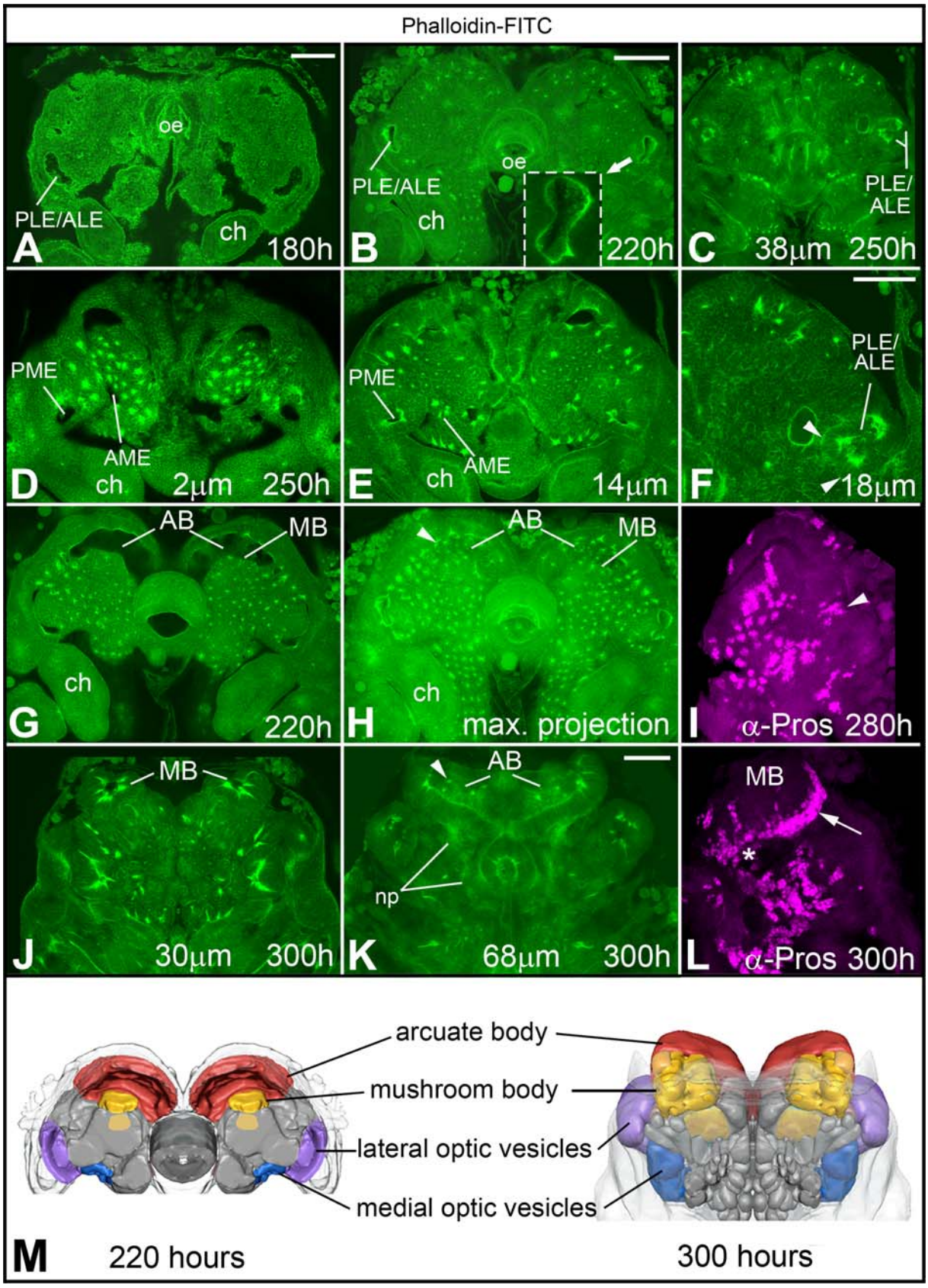

Figure 3-32: Development of the optic vesicles, mushroom bodies and arcuate body.

Legend see next page (102).

Legend to Fig.3-32: Confocal micrographs of flat preparations of embryos stained with Phalloidin-FITC (green) (**A**-**H**, **J**, **K**) and the *Cupiennius salei* anti-Prospero antibody (magenta) (**I**,**L)**; ventral view of precheliceral lobes. (**A**) At 180 hours of development the groove from which the ALE/PLE vesicles arise is visible in the lateral precheliceral NE. (**B**) The grooves have deepened and are constricted in the middle. The magnification of the groove shows the constriction in the middle and the presence of invaginating NPGs in the walls of the groove. (**C**) PLE and ALE vesicles have separated. (**D**, **E**, **F**) Apical and basal views of horizontal optical sections of the same embryo (2 µm, 14 µm, 18 µm). At 250 hours two medial invaginations form in each hemisphere which will give rise to the median optic lobes. (**F**) Basal view of the PLE/ALE vesicles showing further subdivision of the vesicles (arrowheads). (**G**) Apical optical section of the precheliceral lobe showing the half-moon shaped bilateral grooves that give rise to the arcuate body and the mushroom bodies. (**H**) Maximum projection of the same area showing the neural precursor groups that cover the surface of the groove (arrowhead). (**I**) At 280 hours, Prospero is expressed in the optic anlagen (arrowhead). (**J**) The large anterior MB vesicles are visible in the anterior part of the developing protocerebrum. (**K**) The flattened anterior vesicle develops into the arcuate body. The arrowhead points to a small invagination site (neural precursor group). (**L**) At 300 hours differentiating neural precursors (arrow) form a circular structure at the base of the MB vesicle. The asterisk indicates the area posterior to the MB vesicle which expresses *ASH1* and *dac* (compare to Fig.3-26 B and Fig.3-29 B). (**M**) 3D-models of the development of the arcuate body, mushroom bodies and optic lobes at 220 and 300 hours. ***AB,*** arcuate body*;* ***ALE***, anterior lateral eye vesicle; ***AME***, anterior medial eye vesicle; ***ch***, chelicerae; ***MB***, mushroom body; ***nap***, neuropile; ***oe***, oesophagus; ***PLE***, posterior lateral eye vesicle; ***PME***, posterior medial eye vesicle. Scale bars: (A) 150 µm in panel A; (B) 150 µm in panels B , D ,E; (F) 150 µm in panels F ,I , L; (K) 150 µm in panels C, J, K. Anterior is to the top.

3.2.5.2 Embryonic development of the mushroom body and arcuate body

At about 220 hours two large furrows in the anterior part of precheliceral NE occur, which deepen during development and build separated vesicles in the end (Fig.3-25, Fig.3-31, Fig.3-32 G, H, J, K). Through these bilateral furrows the entire dorsal part of the head NE is internalised (Fig.3-25, Fig.3-31, Fig.3-32 G, H, J, K). The NE of the large groove, which has been named semi-lunar groove according to its shape, gives rise to the mushroom body and the arcuate body (Brauer, 1895; Holm, 1952; Weygoldt, 1985; Yoshikura, 1955). At about 220 hours the semi-lunar groove contains two separated deeper parts (Fig.3-25 N-P, arrows, Fig.3-32 G, H). The smaller deepenings in the posterior part of the furrow form the vesicles that will become the mushroom bodies. The arcuate body is formed by the further anterior larger deepening within the semi-lunar furrow (Fig.3-25 N-P, arrows, Fig.3-32 G, H). During further development the two deepenings are more and more internalised until they form closed vesicles at about 300 hours. The vesicle of the mushroom body is characterised by building a dorsal “crown” on each brain hemisphere (Fig.3-32 J). As mentioned before, the arcuate body is formed by the further anteriorly located wider vesicle (Fig.3-32 K). With the morphogenetic movements in the procephalic region the two pairs of vesicles move medially during the next stages. Due to these movements toward each other the bilateral vesicles of the arcuate body meet at the

midline, where they fuse to give rise to the unpaired arcuate body of the larval brain (Fig.3-25 D, Fig.3-28).

The NPGs which are recruited in the NE of the furrows stay in the walls of the vesicles that are generated by the ongoing inversion of the furrows and furthermore new NPGs are build in the developing vesicles. Thus, the large invaginations which reflect the vesicles of the optic lobes, mushroom body and arcuate body contain numerous (additional) small invaginations (NPGs) after their separation from the surface (Fig.3-27, Fig.3-32 K, H). Counting of NPGs in the furrow which generates the arcuate body in four brain hemispheres revealed that approximately 25 NPGs are located in the NE of the developing arcuate body vesicle. As the small NPGs invaginate, some neural precursor cells in the basal part of the vesicles start to differentiate, which is reflected by the expression of the differentiation factor Prospero in these cells (Fig.3-32 I, L). The brain compartments (arcuate body, mushroom body, lateral and medial optic vesicles), which are generated by the described invaginating vesicles, are presented in a three-dimensional model at two distinct developmental stages (220 and 300 hours) in Fig.3-32 M. After 300 hours of development the postmitotic neurons start to produce rudimentary axons which are faintly labelled with Phalloidin. The axons then combine to form a small neuropile that encircles the foregut (Fig.3-32 K). Slightly later, between 350 and 400 hours the neuropile compartments of the (medial) optic lobes, mushroom body and arcuate body take shape. More specific markers for axon fascicles are required to follow neuropile differentiation in more detail.

3.2.5.3 Marker gene expressions in the developing optic lobe and mushroom body

In this paragraph conclusions about the identities of the vesicles will be supplemented by the data about the expressions of the analysed marker genes (*ASH1*, *dachshund*, *netrin*, *islet*) in these areas.

The presented data show that the optic ganglia of the spider arise from vesicles which are located in the area of *dachshund* expression. The grooves of the PLE and ALE seem to split into anterior and posterior vesicles. Further subdivisions might form the corresponding first and second optic neuropiles. In contrast, the PME and AME

ganglia arise from separate individual vesicles. At 180 hours, *ASH1* is expressed in the whole area of the PLE/ALE groove (Fig.3-26 A, asterisk), while *dachshund* expression is restricted to the posterior-lateral part of the groove (Fig.3-29 A). Both *ASH1* and *dachshund* are expressed in a domain medial to the PME vesicles which might correspond to the developing AME ganglia (Fig.3-26 C, arrowheads, Fig.3-29 A-C, small black arrowheads). The two other analysed marker genes, *netrin* and *islet*, are also expressed close to or in the developing PLE/ALE vesicle in distinct positions (Fig.3-28, Fig.3-30). *netrin* expression is located at the medial border (Fig.3-28), whereas *islet* covers the anterior border of the groove (Fig.3-30). The expression patterns of the marker genes strongly support the belonging of the vesicles to the visual system, particularly because the role of d*achshund* and *netrin* in visual system development was already described in other species.

The mushroom bodies are formed by two large anterior vesicles. *ASH1* is expressed in this area from the beginning of neurogenesis and shows a dynamic expression pattern in the developing mushroom bodies throughout embryogenesis (Fig.3-26 A-C). *dachshund* is expressed in a ring-shaped domain in the anterior region of the procephalic NE before the mushroom body vesicles protrude from the surrounding neuroepithelium (Fig.3-29 A). Double-staining with *dachshund* and the neural cell fate determinant Prospero revealed that this domain corresponds to the differentiating neural precursors of the mushroom bodies (Fig.3-26 D-F, Fig.3-32 M). The amount of cells that contribute to the ring-shaped structure increases during embryonic development and additional clusters of cells abutting the mushroom body vesicles on the posterior side are visible from 200 hours onwards (Fig.3-29 B, C, E, F, Fig.3-32 L, asterisk). At about 240 hours there seem to be less Prospero-positive cells, but this does not reflect the actual amount, since at this stage more cells coexpress Prospero and *dachshund*. But the presentation is difficult as the tissue is thicker in older embryos and this causes a weaker staining and a lower intensity when only one z-layer is shown (Fig.3-29 F). However, the generation of additional precursors is also reflected in the strong expression of the proneural gene *ASH1* in this area (Fig.3-26 B, C, asterisks). *islet* also shows strong expression in the area where the mushroom bodies develop (Fig.3-30 A, B, C). That this differentiation marker is expressed in cells of the mushroom bodies is supported by the expression pattern in later stages, were the expression shows the typical shape of the mushroom bodies (Fig.3-30 E).

Taken together, the expression pattern of *ASH1* indicates that it is required for the formation of neural precursors in the optic neuropiles. *dachshund* is partially expressed in parallel to *ASH1* and its expression is maintained in the areas of optic neuropile formation suggesting that it is required for the specification as well as the differentiation of the precursors in the visual system. The expression of *netrin* in parts of the developing visual system suggests that it is involved in visual system development which is also supported by data about its function in axon growth and guidance into and along the optic nerve (Livesey and Hunt, 1997; Wang *et al.*, 1996a). Furthermore the expression pattern of *islet* in its characteristic shape leads to the conclusion that this differentiation marker plays a role in the development of the mushroom bodies.

This study does not cover the question of how the eyes develop in the spider. In contrast to insects, spiders possess lens eyes that consist of a cornea, a lens, a vitreous body and a retina which contains monopolar photoreceptor cells. Based on the structural similarities to vertebrate eyes it is tempting to speculate that the optic vesicles which form the primary optic ganglia induce eye development during late embryogenesis. Similar to the VNC, the epidermis arises lateral and anterior to the cephalic lobe and gradually grows over the brain anlagen during late embryogenesis (Fig.3-25, Fig.3-31). At this time the optic vesicles could induce the formation of lens placodes in the overlaying epidermis which would then develop into the optic apparatus. This hypothesis is supported by the strong expression of *dachshund* in the presumptive head epidermis lateral to the optic vesicles of the PLE and ALE during late embryonic development (Fig.3-29, white arrowheads). Unfortunately it was not possible to trace *dachshund* expression during later development to confirm these findings for the remaining medially located optic vesicles (AME, PME) because the formation of the embryonic cuticle prevents the penetration of RNA probes during the last two days of embryonic development.

3.2.6 Expression pattern of the segment polarity gene *engrailed* in the developing brain

The evolution and segmentation of the arthropod head is subject to a long-standing debate. Expression domains of the segment polarity gene *engrailed* (*en*) have been

used to support the presence of six head segments – two preoral (antennal and ocular) and four postoral (intercalary/second antennal, mandibular, first and second maxillary) – in insects, crustaceans and myriapods (Scholtz and Edgecombe, 2006). The ocular (protocerebral) expression domain has been named head spot (Rogers and Kaufman, 1996). In insects and crustaceans additional domain(s) were identified in the developing protocerebrum, which have been associated with the visual system. Furthermore, a bilateral patch of *engrailed* expression has been observed in the precheliceral lobe of the spider *Cupiennius salei* which might correspond to the head spot in insects and crustaceans (Damen *et al.*, 1998). However, this domain could not be detected in another chelicerate, the mite *Archegozetes longisetosus* (Telford and Thomas, 1998). For a detailed study of *engrailed* expression in the precheliceral lobe and to evaluate if the protocerebral expression domain reflects the head spot, the expression of *engrailed* transcript was analysed in detail during different stages in the head segments.

In *Cupiennius* not all segments show the same developmental stage, because the opisthosomal segments are produced sequentially from a posterior growth zone. In all prosomal segments and the precheliceral lobe NPGs are generated at the same time, while neurogenesis proceeds from anterior to posterior in the opisthosoma (Stollewerk *et al.*, 2001). In the posterior region of the germ band *engrailed* is expressed in small dorsoventral stripes at the posterior border of the segments. In more anterior, developmentally advanced segments which are undergoing neurogenesis, *engrailed* expression covers a broader AP region in each developing neuromere that extends into the first row of NPGs of the next posterior segment as was described in paragraph 3.1.7 (Fig.3-7, Fig.3-33 A) (Stollewerk A. , 2006). This expression pattern can be detected in all segments including the cheliceral segment (Fig.3-33 A, white arrowheads).

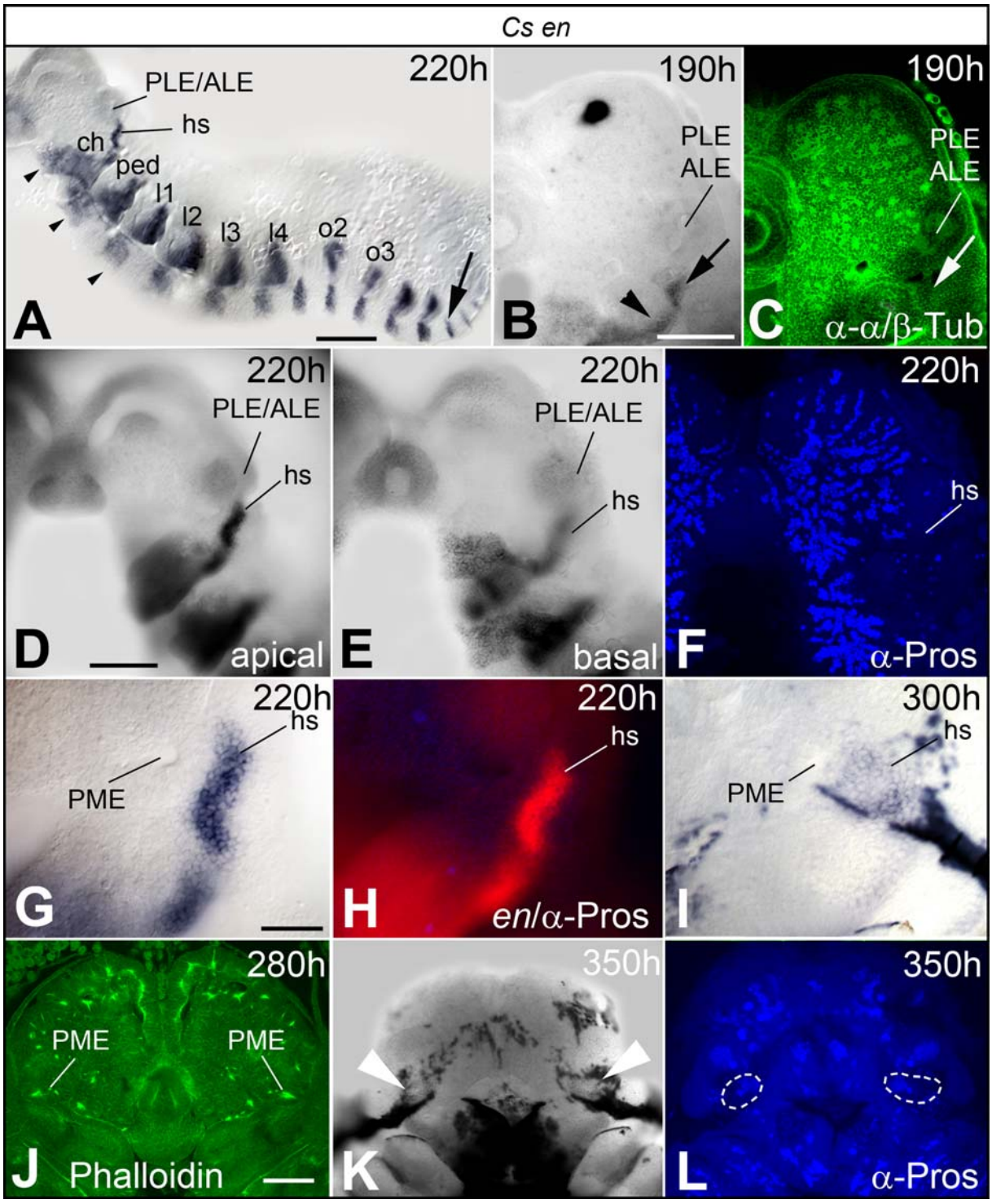

Figure 3-33: The expression pattern of *engrailed* in the precheliceral lobe.

Legend see next page (108).

Legend to Fig.3-33: Flat preparations of embryos stained with a DIG-labelled *engrailed* RNA-probe (**A**, **B** ,**D** ,**E** ,**G** ,**H** ,**I** ,**K**) and α-Spectrin (**C**), anti-Prospero (**F**, **H**, **L**) and Phalloidin-FITC (**J**); ventral views. (**A**) Light micrograph showing one half of the germ band. *en* is expressed in the posterior part of the limb buds and in square-shaped domains in the posterior part of the ventral neuromeres that extend into the anterior part of the next posterior neuromere (arrowheads). Before neurogenesis starts *en* is expressed in small stripes at the posterior border of the segments as seen in the developmentally younger segments in the posterior area of the germ band (arrow). (**B**) *en* is first expressed in a patch of cells at the posterior lateral border of the developing protocerebrum (arrow). The arrowhead points to *en* expression in the chelicerae. (**C**) Same optical section as shown in B. The α-Spectrin staining reveals that neural precursor groups are not present in the *en* expression domain at 190 hours (arrow). (**D**, **E**) Apical and ventral views of the head spot. At 220 hours *en* is expressed in a stripe at the posterior border of the precheliceral NE (hs). Note that *en* is expressed strongly in the apical area (**D**) whereas it is expressed further basal in the VNE (**E**). (**F**) Neural precursors are not present in the head spot domain at this time (arrowhead). (**G**) The procephalic *en* stripe abuts the PME vesicle. (**H**) Double-staining with *en* (red) and anti-Prospero (blue) reveals that neural precursors are absent in the *en* stripe at 220 hours. (**I**) At 300 hours, the *en* stripe has changed to a square-shaped domain which covers part of the PME vesicle. (**J**) The Phalloidin-FITC staining shows that neural precursor groups are present in the PME vesicles. (**K**, **L**) *en* remains expressed in the square-shaped domain (arrowheads in K) and at the end of embryogenesis neural precursor cells are present in this domain (encircled area in L, same optical section as in K) as revealed by *en*/anti-Prospero double-staining. ***ALE***, anterior lateral eye vesicle; ***ch***, chelicerae; ***hs***, head spot; ***l1-l4***, walking leg 1-4; ***o2-o3***, opisthosomal limb bud 2-3; **ped**, pedipalp; ***PLE***, posterior lateral eye vesicle; ***PME***, posterior medial eye vesicle. Scale bar: (A) 300 µm in panel A; (B) 150 µm in panel B,C. (D) 150 µm in panel D-F; (G) 50 µm in panels G-I; (J) 150 µm in panel J-L. Anterior is to the top.

Furthermore there is *engrailed* expression in the precheliceral lobe at the beginning of neurogenesis at the posterior-lateral border of the developing protocerebrum in the precheliceral segment, which presents the head spot (Fig.3-33 B, arrow). At this time no NPGs were detected in the head spot area (Fig.3-33 C, arrow). During the next day of development the spots elongate into stripes which extend anterior-laterally up to the PLE vesicles (Fig.3-33 D, E). NPGs as well as differentiating neural cells are still absent in this area (Fig.3-33 F, H). During 220 and 300 hours of development the *engrailed* expression domains change from stripes to squares (Fig.3-33 G, I). At this time invaginating NPGs are visible in the head spots (Fig.3-33 J) and slightly later differentiating neural cells can be detected (Fig.3-33 K, L). The NPGs correspond to the precursor groups that have been incorporated into the PME vesicle, which is reflected by the small trough (Fig.3-33 G, arrow). *engrailed* and Prospero expression does not completely overlap in the head spots as revealed by analysis of horizontal optical sections at different apico-basal levels. *engrailed* is mainly expressed in the apical epithelial cells, while Prospero transcripts accumulate in the basal differentiating neural cells in the head spot area. This apical-basal restriction of *engrailed* expression is different compared to the VNE, where *engrailed* expression extends from apical to further basal and shows intense overlaps with the differentiating neural cells in basal positions (Fig.3-7 E). However, in the head spot

there is an intermediate layer which expresses both *engrailed* and Prospero indicating that *engrailed* expression is switched off when differentiation is initiated in the neural precursors (Fig.3-33 K, L).

Unlike it is known from other arthropod groups (Urbach and Technau, 2003a) neither *engrailed* expression was detected anterior to the head spot nor a splitting of the head spot or the cheliceral *engrailed* domain into distinct groups could be observed in *Cupiennius*. These data show that the secondary head spot which has been described in insects and crustaceans is absent in the spider and that the 'primary' protocerebral head spot can be associated with the visual system in the spider.

3.2.7 Compartmentalisation is similar in *Achaearanea tepidariorum* and in *Cupiennius salei*

Phalloidin staining in the spider *Achaearanea tepidariorum* suggests that the major aspects of compartmentalisation are similar compared to *Cupiennius*. Due to the lack of marker staining in the head in *Achaearanea*, the compartments can not be clearly identified. But the analysis of Phalloidin staining in different stages of embryogenesis shows the general morphogenetic movements and the formation of compartments in the developing brain (Fig.3-34).

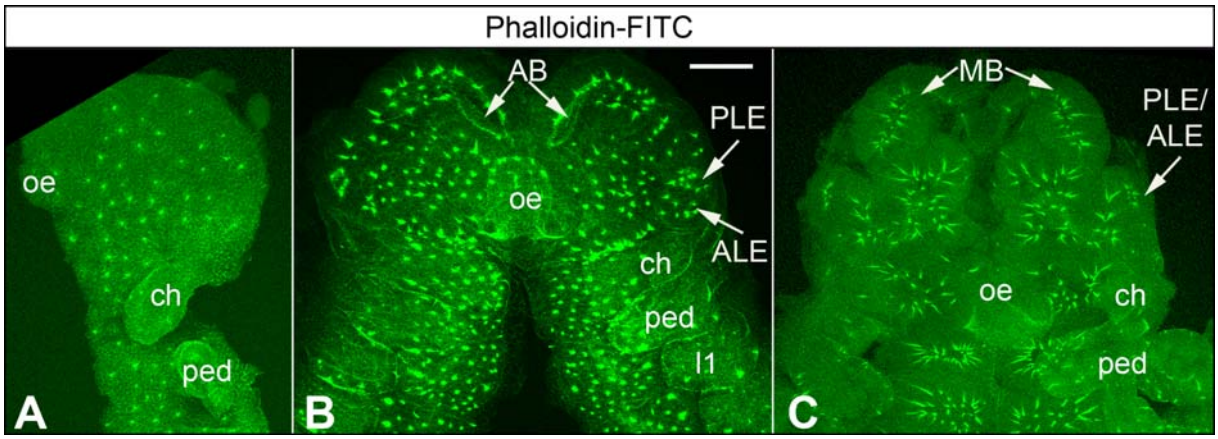

Figure 3-34: Compartmentalisation in the precheliceral NE of *Achaearanea tepidariorum.*

(**A**, **B**, **C**) Maximum projections of Phalloidin stainings in flat preparations of *Achaearanea* embryos; ventral views. (**A**) One half of the procephalic NE in *Achaearanea* at a stage which is comparable to 120-150 hours of development in *Cupiennius*. The precheliceral NE consists of a plane sheet in which the first NPGs are invaginating. (**B**) Both halves of the NE of the anterior segments in *Achaearanea* in a stage which is comparable to 200-220 hours of development in *Cupiennius*. The developmental features are very similar as in *Cupiennius*; there is also an anterior furrow with incorporated NPGs. Due to the similarities in *Cupiennius* it is assumed that these three-dimensional structures give rise to the AB and the MB. The posterior lateral vesicles which give rise to ALE and PLE in *Cupiennius* were also found in *Achaearanea*. (**C**) The complete procephalic region in *Achaearanea* in a stage which is comparable to 300-350 hours of development in *Cupiennius*. The three dimensional structures which are formed until that stage are very similar to the structures which build MB, ALE and PLE in *Cupiennius*. ***AB***, arcuate body; ***ALE***, anterior lateral eye vesicle; ***ch***, chelicerae; ***l1***, leg corresponding to the first leg segment; ***MB***, mushroom body; ***oe***, oesophagus; ***ped***, pedipalp; ***PLE***, posterior lateral eye vesicle. Anterior is to the top. Scale bar: (B) 80μm in panel A-C.

At a developmental stage, which is comparable to 120-150 hours of development in *Cupiennius*, the procephalic lobe in *Achaearanea* also consists of a single layer of epithelial cells in which the first NPGs invaginate (Fig.3-34A, Fig.3-25 A, D). Then the morphogenetic movements start and the increasing amount of NPGs partly assembles in the developing vesicles and compartments. During these stages, which are comparable to 200-220 hours of development in *Cupiennius*, similar structures as in *Cupiennius* arise in the protocerebrum of *Achaearanea* (Fig.3-34 B, Fig.3-22 A, Fig.3-25 J-N, Fig.3-34 B). As in *Cupiennius*, in *Achaearanea* a big semicircular furrow is formed in the anterior region of the precheliceral lobe. The significant similarity of this structure in the two spiders, suggests that the semicircular furrow in *Achaearanea* is comparable to the developing arcuate body in *Cupiennius*, which is also formed by a semi-lunar groove (Fig.3-34 B). In *Achaearanea* another vesicle is formed in the lateral posterior region, which seems to split in two vesicles. Comparing the position and morphology of this structure with the structure in *Cupiennius*, suggests that these vesicles are the developing PLE and ALE (Fig.3-34 B). This is

also supported by the Phalloidin staining in later stages, which are comparable to the developmental stage of about 300-350 hours in *Cupiennius*, where the further development of the lateral vesicles still shows strong similarities between the two spiders (Fig.3-34 C, Fig.3-32 K-P). In this later stage a structure which shows the characteristic shape of the developing mushroom bodies in *Cupiennius* is present in *Achaearanea*. There are also hints that the AME and PME vesicles are formed in similar positions as in *Cupiennius*, but the staining is not clear enough to evaluate that.

But, even if there are no marker stainings in the developing brain of *Achaearanea*, the morphogenetic movements and compartmentalisations indicate that ALE, PLE, the mushroom bodies and the arcuate bodies are formed similar as in *Cupiennius.*

4 Discussion

4.1 Early neurogenesis in arthropods

4.1.1 The NPGs of spiders seem to reflect the ancestral mode and arrangement of neural precursors

The most striking difference between neurogenesis in insects and crustaceans on one side and myriapods and chelicerates on the other side is probably the mode of neural precursor formation. Whereas in insects and crustaceans single NBs adopt neural cell fate in the VNE and later generate specific neural lineages, in chelicerates and myriapods whole groups of cells adopt the neural fate in the VNE before they invaginate as groups (Dove and Stollewerk, 2003; Kadner and Stollewerk, 2004; Mittmann, 2002; Stollewerk, 2002; Stollewerk et al., 2003; Stollewerk et al., 2001; Stollewerk A. , 2006). But, even if the mode of neural precursor formation is different in these arthropod groups, the neural precursors also share some features in the different arthropod groups. Similarities are for example reflected by the expression of proneural genes, the invagination of neural precursors in sequential waves and the number and arrangement of neural precursors (Dove and Stollewerk, 2003; Kadner and Stollewerk, 2004; Stollewerk, 2002; Stollewerk and Simpson, 2005; Stollewerk *et al.*, 2001). In *Drosophila* 30 NBs delaminate in a pattern of seven rows with three to six NBs per row in each hemineuromere (Goodman, 1993) (Fig.4-1 B). The number and arrangement of NPGs in representatives of chelicerates and myriapods is very similar to the NB pattern in the insect *Drosophila melanogaster* (Dove and Stollewerk, 2003; Kadner and Stollewerk, 2004; Stollewerk *et al.*, 2001). Earlier studies revealed that in *Glomeris* as well as in *Cupiennius* 30-32 NPGs are arranged similar to the pattern in *Drosophila* in seven transverse rows (Dove and Stollewerk, 2003; Stollewerk *et al.*, 2001). For analysing the mechanisms which are involved in neural precursor identity in *Cupiennius* and *Glomeris* in this work it was absolutely necessary to detect and distinguish all the NPGs. Therefore the number and arrangement were re-examined in detail in these two arthropods and furthermore in a second spider (*Achaearanea*). This led to the result of 37 NPGs in *Achaearanea*, 38 NPGs in *Cupiennius* and 33 NPGs in *Glomeris*, which are arranged in seven rows

with three to seven NPGs per row. The higher number of detected NPGs might be caused by longer – and thus more reliable - staining reactions with Phalloidin. Another reason is probably that the results were compared in a large number of embryos and stages. However, even if the number of NPGs is higher as compared to earlier data, the similarity of neural precursor arrangement between insects, myriapods and chelicerates is still striking (Fig.3-2).

An exact comparison of NPG number and arrangement in the two spiders *Achaearanea tepidariorum* and *Cupiennius salei* shows a remarkable conservation. The numbers of the NPGs are almost completely the same and the arrangement is, apart from slightly different shapes of the whole hemineuromeres, nearly identical (Fig.3-2 A, B). If the hemineuromeres are brought in similar shapes by using image editing software the single NPGs in the two spiders are almost exactly in the same positions. This indicates that this feature of neurogenesis is highly conserved in chelicerates. Thus, the NPGs in the two spider species are most probably homologous on a single NPG level. Based on the number and arrangement of neural precursors in *Cupiennius* the NPG which is missing in *Achaearanea* is the most lateral-posterior one which is identified as NPG **g-4**. Taking into account the different shapes of the hemineuromeres in *Glomeris*, *Cupiennius* and *Achaearanea*, even the comparison of neural precursor arrangement between spiders and diplopods shows such a similarity that the NPGs in these arthropod representatives can be considered as homologous. Regions where the related patterns of NPGs in these arthropod representatives are most obvious are for example those areas which are occupied by the outer NPGs (Fig.3-2). There, the NPGs of row **a** and even more obvious the NPGs of rows **f** and **g** and the slight dent at the posterior part of the lateral border reflect striking similarities in the NPG patterns. These pronounced similarities allow assignment of single NPGs in spiders and myriapods which lead to the assumption that the NPGs which are fewer in *Glomeris* compared to spiders are **a-1**, **a-7**, **b-6**, **c-3** and **e-4**. The detailed studies on the neural precursors in my work therefore show that the stereotyped arrangement of NPGs was a developmental feature of the last common ancestor of myriapods and chelicerates. If we assume that the number of NPGs has decreased rather than increased during evolution, the number and pattern of NPGs in *Cupiennius* could be considered as ancestral. Even if this work only contributed data about one representative of myriapods, the millipede *Glomeris marginata*, these results and assumptions are confirm with data about early

neurogenesis in two centipedes (*Lithobius forficatus* and *Strigamia maritima*) (Chipman and Stollewerk, 2006; Kadner and Stollewerk, 2004; Stollewerk and Chipman, 2006). There are differences in neurogenesis in the millipede *Glomeris* and the centipede *Lithobius* as opposed to the centipede *Strigamia* due to different modes of development. Both *Glomeris* and *Lithobius* show an anamorphic development (not all segments are generated during embryogenesis), while *Strigamia* exhibits an epimorphic development (all segments are build during embryogenesis). Since all the segments are generated successively during embryogenesis and are very narrow along the AP axis, the early arrangement of NPGs in *Strigamia* is different as compared to the common arrangement in seven rows. But as the neuromeres develop further, the early pattern of three rows of NPGs changes to the seven-row pattern. These data and the data of my work show a very similar number and arrangement of NPGs in chelicerates and myriapods and strongly suggest that the NPGs in chelicerates and myriapods are homologous on a single NPG level. The degree of similarity in the number and arrangement of the NPGs is striking considering that these two arthropod groups separated in the Cambrian, about 500 Mio years ago.

Given that insects were separated from the common ancestor of chelicerates and myriapods earlier, the similarity of neural precursor arrangement is still obvious. Even if the mode of neural precursor formation is different between these groups, the similar pattern suggests that the NBs in insects have emerged from NPGs. The single NBs in insects seem to be an adaptation to changed mechanisms in development like for example the organisation of the NE and the time span of development. While development takes between two and three weeks in *Cupiennius* and *Glomeris*, this process is fulfilled in about 24 hours in *Drosophila*. Compared to the mode of neurogenesis in chelicerates and myriapods where whole groups of cells adopt neural cell fate in sequential waves, the mode of single stem cells which proliferate after delamination allows neurogenesis in a much shorter period of time. Thus, the time saving in *Drosophila* is possible, because in insects the proliferation period which is necessary to replace the cells of the NPGs between the delamination waves in *Cupiennius* and *Glomeris* can be shortened by the use of single neural precursor cells which delaminate before proliferation. The shorter duration of neurogenesis in insects is accompanied by a smaller number of cells in the NE compared to the densely packed cells in the NE of spiders and myriapods. Both

features, the relatively fast neurogenesis and the reduced cell number in the NE are enabled by the formation of single NBs. All these data strongly suggest that the NBs reflect the diverged mode of neurogenesis and that the NPGs present the ancestral mode of neurogenesis of the last common ancestor of the arthropod groups. These assumptions are strongly supported by data about neurogenesis in onychophorans, which are considered to be basal in arthropodan clade (Nilsson and Osorio, 1998; Wheeler *et al.*, 1993, Aguinaldo *et al.*, 1887; Schmidt-Rhesa *et al.*, 1998; Giribet *et al.*, 2000; Manuel *et al.*, 2000). In the onychophoran *Euperipatoides kanangrensis* the whole central part of the hemisegments invaginate as single vesicles respectively to generate the CNS (Eriksson *et al.*, 2003). This mode of early neurogenesis is obviously closer related to the invaginating NPGs in chelicerates and myriapods as compared to the single NBs in insects and crustaceans.

My data confirm a rather conserved number and arrangement of neural precursors in arthropods and thus support the hypothesis that this is an ancestral feature in arthropods – at least in insects, myriapod and chelicerates. Our knowledge of the arrangement of NBs in crustaceans is fragmentary. NBs have been identified in higher crustaceans (malacostracans) but a complete map of NBs is not a available (Dohle, 1972; Dohle, 1976; Gerberding and Scholtz, 1999; Harzsch, 2001; Harzsch, 2003; Harzsch and Dawirs, 1996; Harzsch *et al.*, 1998; Harzsch S., 1994; Scholtz, 1984; Scholtz, 1990; Scholtz, 1992; Ungerer and Scholtz, 2008). However, based on different partial maps it has been suggested that the final number of NBs is similar to other arthropods which further supports a conservation of the amount and arrangement of NBs.

4.1.2 Patterning gene expression seems to be conserved in neural precursors

The similar number and arrangement of neural precursors in arthropods hints at a conserved mode of neurogenesis. But there are also differences in neurogenesis, as for example the mode of neural precursor formation. Because of that other steps in neurogenesis are compared in different arthropods to analyse which mechanism are conserved and how they were modified during evolution. One aspect, which is taking place in early neurogenesis, is the mechanism which gives neural precursors their

specific identity and thus allows them to generate specific progeny. Genes, which pattern the VNE in DV and AP direction are important factors in this mechanism and thus have been focus of many studies on neurogenesis in different species (Doe, 1992; Martin-Bermudo *et al.*, 1991; Skeath, 1999; Skeath and Carroll, 1992; Skeath *et al.*, 1992).

The proneural clusters, and thus the NBs which are recruited in these clusters, in the VNE of *Drosophila* are arranged in three longitudinal columns on both sides of the midline (Jimenez and Campos-Ortega, 1990; Skeath, 1999; Skeath and Carroll, 1994). The separation of the developing NBs in three columns was also observed in other insects such as the grasshopper *Schistocerca gregaria* and the flour beetle *Tribolium castaneum* (Doe and Goodman, 1985a; Wheeler *et al.*, 2005). Similar to arthropods the longitudinal subdivision of the developing nervous system is a feature in vertebrate neurogenesis, as the proneural clusters and primary neurons in *Xenopus laevis* and *Danio rerio* are arranged in three DV columns (Chitnis *et al.*, 1995; Haddon *et al.*, 1998). Hence, the partitioning of the developing nervous system in DV columns seems to be conserved. Furthermore, the genetic regulation which is involved in this separation shows similarities as well and thus supports an ancestral origin of this patterning mechanism. In vertebrates and insects the subdivision of neural precursors in three DV columns is regulated by the expression of homologous DV patterning genes (D'Alessio and Frasch, 1996). The lateral column of the vertebrate (for example frogs, birds and mice) neuroepithelium is specified by the expression of *Msx* genes (Davidson, 1995; Ramos and Robert, 2005; Su *et al.*, 1991). The genes which specify the intermediate neurogenic column of the vertebrate CNS are the *Gsh-1* genes (Deschet *et al.*, 1998; Valerius *et al.*, 1995). The medial column of the vertebrate neuroepithelium is marked by the expression of the DV patterning gene *NK-2.2* in mice, frogs and zebra fish (Barth and Wilson, 1995; Saha *et al.*, 1993; Shimamura *et al.*, 1995). In *Drosophila* the three columns are characterised by the expression of homologues of the DV patterning genes of vertebrates. *Drosophila vnd,* the homologous gene to *NK2.2*, is expressed in the medial, *Drosophila ind* (homologous to *Gsh-1*) in the intermediate and *Drosophila msh (*homologous to *Msx*), in the lateral column (D'Alessio and Frasch, 1996; Jimenez *et al.*, 1995; Mellerick and Nirenberg, 1995; Weiss *et al.*, 1998). The expressions of homologues of these DV patterning genes also specify the longitudinal columns in *Tribolium* (Wheeler *et al.*, 2005).

While there are data about DV patterning in several representatives of vertebrates and in insects (Barth and Wilson 1995; D'Alessio and Frasch, 1996; Davidson, 1995; Deschet *et al.*, 1998; Jiminez *et al.*, 1995; Mellerick and Nirenberg, 1995; Ramos and Robert, 2005; Saha *et al.*,1993; Shimamura *et al.*, 1995; Su *et al.*, 1991; Valerius *et al.*, 1995; Weiss *et al.*, 1998; Wheeler *et al.*, 2005), there were no data about this mechanism in other arthropods. Even if the data about this step of neurogenesis was described in detail in *Drosophila* and there are also data, which present a conserved mechanism in *Tribolium*, data about DV patterning in neurogenesis in non-insect arthropods were firstly described in this work.

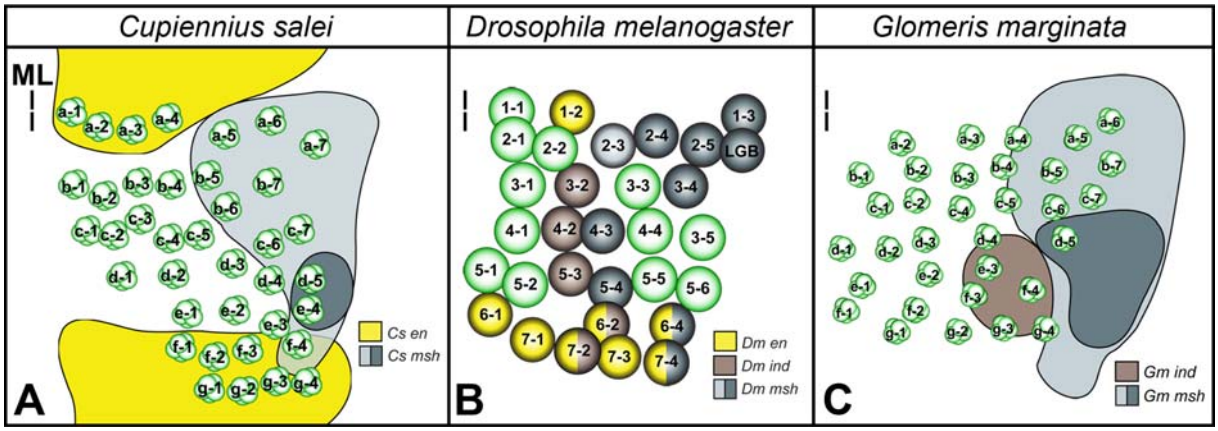

Figure 4-1: Schematic drawing of patterning gene expression in neural precursors in chelicerates, insects and myriapods.

(**A**) Schematic drawing of NPGs (green), *en* (yellow) and *msh* (grey) expression in one *Cupiennius* hemineuromere. (**B**) Schematic drawing of the NBs in one hemineuromere in *Drosophila*, modified after Doe, 1992. The NBs which express *en* are marked yellow, the NBs which express *msh* are marked grey, the NBs which express *ind* are marked brown. NB 2-3 is marked in light grey, as the expression in that NB is not that clear. (**C**) Schematic drawing of NPGs (green), *msh* (grey) and *ind* (brown) in one hemineuromere in *Glomeris*. (**A**, **C**) Light grey marks the expression domains of *msh*, dark grey marks the region with remarkable strong *msh* expression. The *msh* expression pattern is very similar in *Cupiennius* and *Glomeris*. (**A**, **B**) Similarly to *Drosophila*, some neural precursors of row a (=1 in *Drosophila*), f (=6) and g (=7) are recruited in the *en*-positive transversal stripe. (**A**, **B**, **C**) In *Drosophila*, *Cupiennius* and *Glomeris msh* is expressed in the lateral column in the NE. Even if the exact positions of the neural precursors which express *msh* are slightly different, the number of *msh* expressing neural precursors is similar in *Drosophila*, *Cupiennius* and *Glomeris* (ten to eleven NBs in *Drosophila*, eleven NPGs in *Cupiennius,* seven to eleven NPGs in *Glomeris*). (**B**, **C**) With regard to the neural precursors the *ind* expression in *Glomeris* and *Drosophila* shows similarities. Comparable to *Drosophila* where five NBs of the posterior five rows maintain *ind* expression after delamination, in *Glomeris* four to six NPGs are recruited in the intermediate posterior *ind* expression domain. ***Cs***, *Cupiennius sal*ei; ***Dm***, *Drosophila melanogaster*; ***en***, *engrailed*; ***Gm***, *Glomeris marginata*; ***ind***, *intermediate neuroblast defective*; ***ML***, midline; ***msh***, *muscle segment homeobox*. Anterior is to the top.

Like in the insects *Drosophila melanogaster* and *Tribolium castaneum*, the expression of *msh* covers the lateral column of the NE in the spider *Cupiennius salei* and the myriapod *Glomeris marginata* (Fig.4-1). Another indication of a conserved expression pattern of the columnar gene *msh* is provided by its expression in a similar number of neural precursors in arthropods (Fig.4-1). In *Drosophila msh* is expressed in the lateral column of the VNE and its expression is maintained in nine to ten NBs after they have delaminated from that region (Isshiki, 1997) (Fig.4-1 B). Similarly, in *Cupiennius msh* is expressed in the lateral column of the NE (Fig.4-1 A, Fig.3-8). The expression is not restricted to the neuroectodermal cell layer but the gene remains expressed in the eleven NPGs which invaginate from this area. (Fig.4-1 A, B). One difference is displayed by the positions of neural precursors which express *msh* after delamination. In *Cupiennius* all lateral NPGs (except **g-4**) express this columnar gene after delamination, whereas in *Drosophila* some of the lateral NBs do not express *msh* (Isshiki *et al.*, 1997) (Fig.4-1 A, B, Fig.3-8). In *Drosophila msh* is shortly downregulated in the NE after the NBs of the first two waves have segregated. Furthermore some of the NBs of the first wave loose *msh* expression after delamination. Despite this difference, the expression of *msh* seems to be rather conserved in insects and chelicerates regarding its general expression pattern in the VNE and the neural precursors which delaminate from that epithelial layer. Furthermore the expression pattern of *msh* in the myriapod *Glomeris marginata* is very similar as compared to *Cupiennius* and also shows similarities to *Drosophila*. *Glomeris msh* also covers the lateral part of the VNE and seven to eleven lateral NPGs invaginate from this expression domain (Fig.4-1 C, Fig.3-3). Even though it could not be shown if the cells of the NPGs, which have already invaginated, express *msh*, the general expression domain and the number of NPGs which are recruited in this domain in *Glomeris* are similar compared to insects and spiders (Fig.4-1). But a closer look on the expression pattern of *msh* also shows some differences in *Cupiennius* and *Glomeris*. These distinctions are most obvious in the posterior and lateral parts of the hemineuromeres. Whereas the *msh* expressing domain in *Cupiennius* ends more or less in the region where the most lateral NPGs invaginate, the expression domain extends far further laterally in *Glomeris*. This is even more striking since the broader *msh* expressing domain in *Glomeris* seems to contain fewer NPGs compared to the narrower domain in *Cupiennius*. The posterior *msh*-positive domain in *Cupiennius* contains the NPGs **d-5**, **e-4** and **f-4**, whereas in

Glomeris there is only **d-5** and one NPG (**g-4**) at the border of *msh*-expression. As in *Glomeris* the NPG **e-4** is missing anyway, the number of NPGs in the posterior region which are *msh*-positive in *Cupiennius* but not in *Glomeris* is reduced to one (**f-4**). Thus, the difference in the posterior part of the *msh*-expressing region considering the NPGs is rather small in *Cupiennius* and *Glomeris*. Therefore it is assumed that the NPG **g-4** at the border between *msh* and *ind* expression in *Glomeris* rather can be assigned to the *ind* domain, because the homologous NPG in *Cupiennius* is not within the *msh* domain but interestingly posterior-lateral to the *msh*-positive region. If we assume that the NPGs which are positioned at the border of *msh*-expression (**a-4**, **b-4**, **c-5**) in *Glomeris* do not reside in the *msh* expression domain based on the fact that in *Cupiennius*, where the staining is clearer, the homologous NPGs are recruited in the *msh*-negative region then the exact same number of homologous NPGs are recruited in the *msh*-positive region in *Cupiennius* and *Glomeris.* The only difference occurs due to the missing NPGs (**a-7**, **b-6**, **e-4**) in *Glomeris*. This interpretation shows that although the *msh* expression domain in the hemineuromeres slightly differs between *Glomeris* and *Cupiennius*, the mechanism which gives the lateral NPGs their identity is homologous on single NPG level in both animals. Thus, the differences in expression which are most obvious in the lateral extended expression in *Glomeris* and the missing expression in the most posterior part of the segments in *Cupiennius* do not influence the identity of the lateral NPGs. However, an explanation for the extended *msh* expression lateral to the NPGs could be found in the organisation of the NE and in the mode of detection of the invagination groups. With the Phalloidin staining only the apical cell processes of the NPGs are visualised as spots. The cell bodies of the neural precursors cover a larger area as compared to the apical processes. The NE of *Glomeris* contains more small cells as compared to the NE of *Cupiennius* (Dove and Stollewerk, 2003). Due to the limited space in the *Glomeris* NE and the fact that the medial part of the NE sinks into the embryo, it is very likely that the cells of the neural precursors in the lateral NPGs are pushed further laterally. Thus, the impression of a more laterally extended *msh* expression in *Glomeris* compared to *Cupiennius* regarding the positions of the NPGs relative to the expression domain could be the result of these features. Altogether, this data show an impressive conservation of the expression of the columnar gene *msh* in identified NPGs of *Glomeris* and *Cupiennius*.

The expression pattern of *msh's* medial neighbour, *ind*, also shows similarities between *Drosophila, Tribolium* and *Glomeris*. In these arthropod species, *ind* is expressed in the intermediate column of the NE (Dove, 2003; Weiss *et al.*, 1998; Wheeler *et al.*, 2005). Its expression regarding the neural precursors which are recruited in the expression domain hints at a conserved role in neural regionalisation, since *ind* expression is maintained in five intermediate NBs after delamination in *Drosophila* (Weiss *et al.*, 1998) and in *Glomeris* four to six intermediate NPGs are recruited in its expression domain (Fig.4-1 B, C, Fig.3-4). Even if this is more obvious in *Glomeris*, in *Drosophila* the neural precursors with *ind* expression are also not members of the most anterior rows. In *Drosophila ind* is expressed in the intermediate NBs of rows 3 to 7 and in *Glomeris* the intermediate NPGs of rows **d** to **g** are recruited in the *ind*-positive domain. Together with the fact that *ind* expression in *Tribolium* is also restricted to the posterior part of the intermediate column (Wheeler *et al.*, 2005) these data strongly suggest a conserved expression of *ind* in insects and myriapods.

4.1.3 The function of the columnar genes in neural precursor development seems to be conserved

Not only the expression of the analysed columnar genes but also their function seems to be conserved in arthropods and vertebrates. This was already shown for different insects but now the results of this work strongly suggest a similar role of DV patterning genes in chelicerates and myriapods.

In *Drosophila msh* is not required for NB formation in the lateral column but is essential for the proper development of NBs and their progeny (Isshiki *et al.*, 1997). Its role in NB specification is reflected by division and differentiation phenotypes in progeny of dorsal NBs in *msh*-mutants (Isshiki *et al.*, 1997). Similar functions were observed for *Tribolium msh*, which is also not involved in NB formation but in NB specification (Wheeler *et al.*, 2005). In contrast to *Drosophila*, this function of *msh* in *Tribolium* seems to be restricted to late forming NBs, which correlates with the late onset of *Tribolium msh* expression (Wheeler *et al.*, 2005).

The results of *msh* RNAi experiments in the spider *Cupiennius salei* now strongly suggest a conserved role of *msh* in spiders and insects. Like in insects, the formation

of neural precursors (NPGs) in *Cupiennius* is independent of *msh*, because a downregulation of *msh* expression does not lead to a reduction of lateral NPGs or a changed arrangement of these neural precursors (Fig.3-16). Although the dorsal NPGs are formed properly when *msh* expression is reduced, the further development of the neural precursor cells is affected. In *msh* RNAi embryos the expression of *islet*, a differentiation marker for moto- and interneurons in *Drosophila,* which is normally expressed in lateral neural precursors, is almost completely reduced (Fig.3-18, Fig.3-19). The fact that the formation of neural precursors is not affected but the differentiation marker is not expressed could be due to cell fate changes similar to the cell fate changes which were discussed in *Drosophila msh* mutants (Isshiki, 1997). Another explanation for the phenotype in *Cupiennius msh* RNAi embryos is reflected by the role of *msh* homologous in vertebrates (Liu *et al.*, 2003). In chicken embryos *Msx1* was shown to influence the expression of neural differentiation markers like for example *Isl1* indirectly. Regarding the phenotype in *Cupiennius msh* RNAi embryos where *islet* expression is strongly impaired in the complete dorsal part of the NE a role of *msh* in differentiation becomes more probable compared to a cell fate change. This is also supported by the fact, that in *Drosophila msh* mutant embryos *ind* expression does not extend laterally, which should be accompanied by a cell fate change from lateral to medial (Skeath, 1999). Since in *Drosophila msh* is required for differentiation it is discussed that the cell fate change is rather due to later functions of *msh* (Skeath, 1999; Isshiki, 1997).

Even though the RNAi experiments with *Glomeris msh* and *Glomeris ind* did not contribute data on a possible conserved role of the columnar genes in myriapods, detailed expressional studies allow some assumptions. A prerequisite for the proper functions of the columnar genes in neural regionalisation is a restriction of their expression to the three longitudinal columns. This restriction is regulated by themselves as the genes repress the expression of their lateral neighbours – *vnd* represses *ind* and *msh*, *ind* represses *msh* – and thus are not coexpressed (Cornell and Ohlen, 2000; McDonald *et al.*, 1998; Skeath, 1999; Weiss *et al.*, 1998).

The expression data from *Glomeris* suggest a similar regulation, as *msh* and *ind* expression seem to exclude one another. This is most obvious in the leg segments where the two expression domains fit together like pieces of a puzzle (Fig.3-5). This expression pattern suggests that *ind* functions in a similar way as in *Drosophila* in restricting the medial extension of *msh*. But, interestingly, the regulation in the head

segments from stages 3 - 4 onwards seems to be an exception of this known repressional cascade of the DV patterning genes (Fig.3-6). In the anterior neuromeres there is an area in the medial anterior region where *msh* and *ind* expression seem to overlap. This possible overlapping expression is most obvious in the mandibular, the maxillary and the postmaxillary segment. The coexpression of these two DV patterning genes implies that the function of *ind* in repressing *msh* is modified in *Glomeris* in the mentioned segments and stages. This modification of *ind* function in *Glomeris* is very interesting, as until now no other example of coexpression of these genes in arthropods was published. However, new data on DV patterning gene expression in *Drosophila* reveals that there is an exception of this known rule of repression of the lateral neighbour which is reflected by a transient coexpression of *msh* and *ind* (personal communication, Janina Seibert).

The functional and expressional data on *msh* in *Cupiennius* and the expression data on *msh* and *ind* in *Glomeris* strongly suggest a conserved role of these columnar genes in arthropods. Earlier data even proposed a conserved role in vertebrates and insects and now this new data suggest that the mode of DV neural regionalisation is an ancestral feature in vertebrates and arthropods (Arendt and Nubler-Jung, 1999; Cornell and Ohlen, 2000).

4.1.4 Small differences in the expression patterns of DV patterning genes reflect evolutionary modifications in arthropods

Even though the general expression pattern of the DV patterning genes in three longitudinal columns seems to be largely conserved in arthropods, small differences have been observed nevertheless. These differences in expression help to analyse how developmental processes can be modified to generate different nervous systems. Until now, detailed expression data on the columnar genes have only been available for the two insects *Drosophila melanogaster* and *Tribolium castaneum* (Isshiki *et al.*, 1997; Jimenez *et al.*, 1995; McDonald *et al.*, 1998; Weiss *et al.*, 1998; Wheeler *et al.*, 2005).

In *Drosophila msh* expression starts very early – at stage 5 – in some clusters in the NE. During further development the expression extends and forms a longitudinal stripe in the lateral (dorsal) part of the NE (D'Alessio and Frasch, 1996; Isshiki *et al.*,

1997; Lord *et al.*, 1995). While *msh* is downregulated in the NE after the generation of the first two waves of NBs (stage 8/9), the gene remains expressed in one of the delaminated S1 NBs. At stage 10 *msh* is re-expressed in lateral proneural clusters. The expression of the DV patterning gene is maintained in the S3-S5 NBs which are recruited from this area and in some of their progeny (Isshiki *et al.*, 1997). In late embryonic stages *msh* is expressed in neurons and glia cells which are mostly located in the lateral part of the developing central nervous system, but some of the cells migrate further lateral to contribute to the peripheral nervous system and some migrate towards medial (Isshiki *et al.*, 1997). Some of the cells which migrate medially were identified as longitudinal glia cells which enwrap the longitudinal axon tracts of the central nervous system (Beckervordersandforth *et al.*, 2008). Thus, *msh* expression extends over all stages of embryonic neurogenesis and the expression pattern is highly dynamic.

Similar to *Drosophila*, in *Tribolium* the columnar gene *msh* is also expressed in the lateral column of the NE, but in contrast to *Drosophila*, the expression in *Tribolium* starts clearly later (Wheeler *et al.*, 2005). The first expression in the NE comes up, when the formation of the NBs has already started. The expression is initiated in small clusters in the dorsal column and in the respective NBs. More and more clusters start to express *msh* in the lateral column which finally leads to a continuous longitudinal lateral stripe of *msh*-positive clusters in the NE of *Tribolium* (Wheeler *et al.*, 2005).

Compared to the dynamic expression of *msh* in these two insect species, *msh* is expressed rather continuous in the lateral column throughout neurogenesis in *Cupiennius* and *Glomeris* (Fig.3-6 A-D, Fig.3-9). Although changes occur in this expression pattern during development, the lateral portion of the VNE expresses *msh* in all observed stages. The expression in that region is already present before the first NPGs start to invaginate, which is more similar to *Drosophila* than to *Tribolium* (Dove and Stollewerk, 2003; Isshiki *et al.*, 1997; Stollewerk *et al.*, 2001; Wheeler *et al.*, 2005). In *Glomeris* and in *Cupiennius msh* expression starts in a continuous area which covers the lateral portion of the hemineuromeres and not in separated clusters like in insects. The earliest stages which were analysed do not show single clusters of *msh*-positive cells but rather a weak staining covering the whole lateral NE. It can be speculated that these differences in the spatio-temporal expression of *msh* have contributed to evolutionary changes of neural identities in the lateral area of the NE.

Future identification and comparative analysis of additional neural identity genes and differentiation markers will show if this assumption can be verified.

Apart form the continuous *msh* expression in the lateral column, *msh* is also expressed in more medial cells in later stages in *Drosophila*, *Glomeris* and *Cupiennius* (Fig.3-6 A-D, Fig.3-10) (Isshiki *et al.*, 1997). In *Drosophila, msh*-positive cells from the lateral region migrate towards medial and give rise to several cell types like longitudinal glial cells (Beckervordersandforth *et al.*, 2008; Isshiki *et al.*, 1997). From my analysis in *Glomeris* and *Cupiennius* it is unclear, if the medial cells that start expressing *msh* later than the lateral neuroectodermal cells have migrated away from the lateral column or if they express *msh de novo*. However, the detailed analysis of the *msh* expression pattern in different stages suggests – at least in *Cupiennius* - that the *msh*-positive cells in the medial position segregate from lateral NPGs and migrate towards a medial position. In any case, the medial expression of *msh* seems to be a conserved feature in insects, chelicerates and myriapods. Future analysis in *Cupiennius* and *Glomeris* will show if the medial cells are glial cells that associate with the longitudinal tracts similar to *Drosophila*. The identification of a glial marker or a marker which is specific for longitudinal glial cells would be the best tool.

In *Drosophila ind* is expressed in the intermediate column and shows a highly dynamic expression pattern (Weiss *et al.*, 1998). The first *ind* expression comes up at stage 5 in two broad parallel longitudinal columns (intermediate columns). The columns become narrower until they are only two cells wide as the embryo undergoes gastrulation. When the first NBs are recruited in stage 8 in the *ind*-positive domain they maintain expression. Shortly later *ind* is downregulated in the VNE but is still detectable in all NBs from the intermediate column. During further development *ind* expression becomes more and more restricted and at stage 9 mRNA expression can only be detected in the intermediate NBs of rows 6 and 7. The downregulation proceeds and *ind* mRNA disappears completely after stage 11 (Weiss *et al.*, 1998). In contrast to the temporally shifted *msh* expression in *Tribolium*, the expression of *ind* starts prior to NB formation (Wheeler *et al.*, 2005). *Tribolium ind* is expressed in the intermediate part of the NE and in the neural precursors of this region but not in a continuous longitudinal stripe. Whereas in *Drosophila ind* expression is restricted to the posterior part of the neuromeres, this restriction is present in *Tribolium* from the beginning. As in *Drosophila*, *ind* is downregulated with ongoing development in

Tribolium and by the end of germ band elongation is only maintained in two posterior intermediate NBs (Weiss *et al.*, 1998; Wheeler *et al.*, 2005).

In *Glomeris*, the temporal and spatial pattern of *ind* expression shows features of the expression in *Drosophila* and in *Tribolium* as well as differences. As in *Drosophila* and *Tribolium ind* expression in *Glomeris* starts early and is already expressed before the first NPGs invaginate. At the beginning of neurogenesis *ind* is expressed in a posterior intermediate and in a further anterior cluster. These two clusters seem to combine in most hemineuromeres during further development leading to an oval expression domain that does not cover the anterior intermediate part of the hemineuromeres (Fig.3-6 E). *ind* expression becomes more and more restricted to an intermediate posterior cell cluster during later stages (Fig.3-6 E-H). The restriction of *ind* expression to the posterior part of the NE throughout neurogenesis is seen both in *Tribolium* and *Glomeris* but not in *Drosophila* where the expression starts with a continuous longitudinal stripe. This suggests that the early function of *ind* in the anterior portion of the neuromeres could be modified in these arthropods. The absence of *ind* in the anterior intermediate column leads to the assumption that there might be another factor which regulates early neurogenesis (neural precursor formation) in this region in *Glomeris* and *Tribolium*. A similar observation of the expression domains of *ind* homologues in vertebrates confirms this possibility. The *Medaka* and *Mus ind* homologues *gsh1* and *gsh2* are not expressed during early neurogenesis (Cornell and Ohlen, 2000; Deschet *et al.*, 1998; Hsieh-Li *et al.*, 1995; Valerius *et al.*, 1995) suggesting that there is another factor in the intermediate column which is responsible for early steps in neural development. A candidate for this factor would be *pax6*, as it is expressed in the intermediate column in *Mus*. Furthermore *pax6* might have a similar role as *ind*/*gsh*, because *pax6* seems to repress the expression of *nkx2.2* (homologue of *vnd*) (Ericson *et al.*, 1997). However, these data and the expression data on *ind* in arthropods hint at the possibility that there is another factor which is involved in neurogenesis in the intermediate column. For further analyses the identification, expression pattern, and investigation of the function of *pax6* in representatives of arthropods would be necessary. The presence of another factor which has a similar role as *ind* in *Drosophila* could also be an explanation for the failure of *ind* identification in *Cupiennius*. Another reason could be that the *ind* sequence has considerable diverged in the lineage leading to

arachnids so that it is not possible to design primers and amplify ind fragments based on the alignments of known *ind* homologues.

4.1.5 NPGs versus NB lineages

The results of this work contribute new data to discuss the question if the NB lineages of insects and crustaceans are homologous to the cells of the NPGs in chelicerates and myriapods.

In chelicerates and myriapods not single cells, but whole groups of cells adopt a neural cell fate in the NE and invaginate in groups (Dove and Stollewerk, 2003; Kadner and Stollewerk, 2004; Mittmann, 2002; Stollewerk et al., 2001; Stollewerk A. , 2006). In myriapods the positions of the NPGs are prefigured by accumulation of mitotic divisions, whereas in spiders there is no correlation of cell proliferation and NPG position (Dove and Stollewerk, 2003; Stollewerk *et al.*, 2001). However, it is not clear if the cells of one precursor group are clonally related. But the striking similarity in the pattern of NPGs in chelicerates and myriapods indicates that the NPGs are homologous on single NPG level. Furthermore, as the number and the arrangement of the neural precursors are similar in all analysed arthropod groups, it would be exciting to examine if the cells of the NPGs can be considered as being homologous to the cell lineages of single NBs.

Even though some modifications in the number and arrangement of neural precursors or in the expression and possibly the function of patterning genes in the arthropod NE were found in the course of this work, the data suggest that these mechanism of early neurogenesis are conserved. Therefore it becomes quite possible that the NB lineages can be compared to the NPGs. Still, the obtained results can not prove or confirm this assumption, but adds some data for further suggestions. One option to get more insight was analysing the expression patterns of differentiation markers in *Cupiennius* and *Glomeris* and compare the data to *Drosophila* (Fig.4-2).

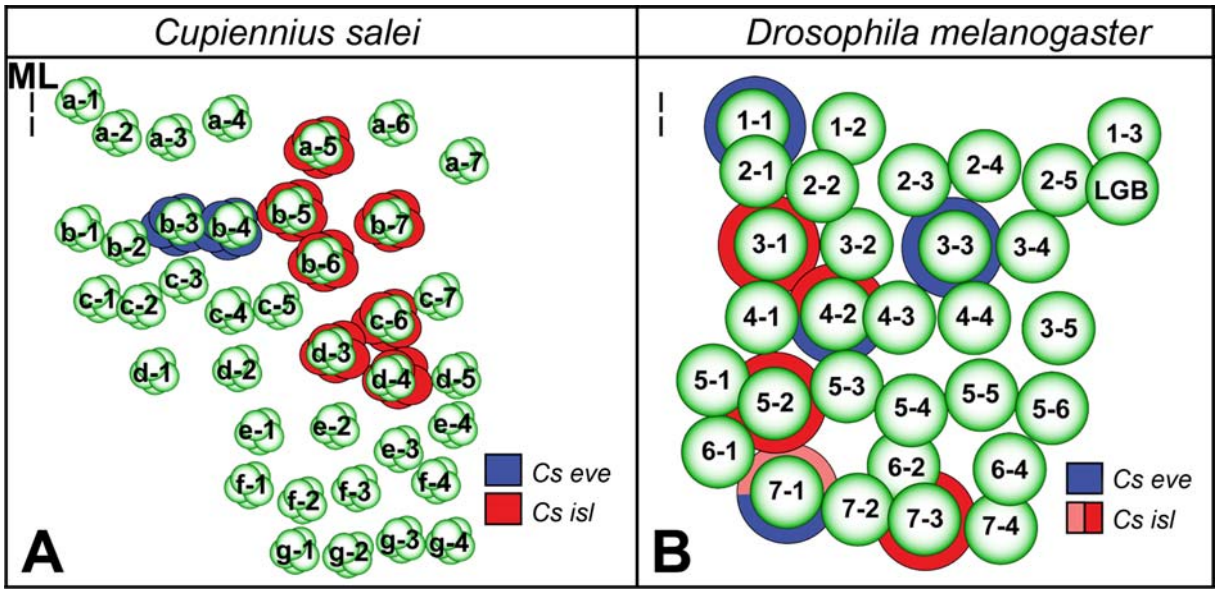

Figure 4-2: *even-skipped* and *islet* expression in NPGs of *Cupiennius salei* and progeny of *Drosophila melanogaster* NBs.

(**A**) Schematic drawing of the NPGs (green) and *eve* (blue) and *isl* (red) expression in cells of identified NPGs in one hemineuromere in *Cupiennius*. (**B**) Schematic drawing of NB in one hemineuromere in *Drosophila*; modified after Doe, 1992. The identified NBs whose lineages contain *eve* or *isl*-positive cells are highlighted in blue or red, respectively. (**A**) The NPGs b-3 and b-4 or rather unlikely just one of them, contain *eve*-positive cells. Seven identified NPGs (a-5, b-5, b-6, b-7, c-6, d-3, d-4) in a rather lateral anterior position contain *isl*-positive cells. (**A**, **B**) The positions of the neural precursors which give rise to *isl*-positive cells do not correlate in *Cupiennius* and *Drosophila*. In *Drosophila* these NBs are located in a posterior medial region within the neuromere and not in an anterior lateral region like the corresponding NPGs in *Cupiennius*. The four NBs which generate *eve*-positive cells in *Drosophila* are also not located in similar positions as the NPGs which contain *eve*-positive cells in *Cupiennius*. ***Cs****, Cupiennius salei;* ***Dm****, Drosophila melanogaster;* ***eve****, even-skipped;* ***isl****, islet*. Anterior is to the top.

In *Drosophila* each NB generates an individual cell lineage according to its identity. As the cells of the lineages differentiate they express distinct differentiation markers which allow identifying single cells and to assign these cells to individual NB lineages. Such differentiation markers are for example *islet* and *even-skipped*. These genes are expressed in identified neural cells which originate from specific NBs, which themselves do not express the differentiation markers (Certel and Thor, 2004; Landgraf *et al.*, 1997; Landgraf *et al.*, 1999; Thor *et al.*, 1999; Thor *et al.*, 1991; Thor and Thomas, 1997). *Drosophila islet* is expressed in serotonergic and dopaminergic interneurons and furthermore in a subset of motoneurons which innervate the ventral body wall muscles (Certel and Thor, 2004; Thor *et al.*, 1999; Thor and Thomas, 1997). All these cells descend from four to five known NBs which occupy a medial posterior position (Fig.4-2 B) (Landgraf *et al.*, 1997). *Drosophila even-skipped* is expressed in another subset of interneurons and dorsal muscle innervating

motoneurons (Landgraf *et al.*, 1999). These cells are generated by four NBs which are scattered within the hemineuromere (Fig.4-2 B) (Landgraf *et al.*, 1997).

The comparison of the expression domains of these differentiation markers in *Drosophila* and *Cupiennius* shows no similarities. In contrast to the medial posterior positions of the NBs which give rise to *islet* expressing progeny, the seven NPGs which contain *islet*-positive cells are located lateral anterior (Fig.4-2). Furthermore the positions of the NBs which generate lineages with *even-skipped*-positive cells and the *even-skipped* expressing NPGs do not share similar positions within the hemineuromeres (Fig.4-2). Hence, these data do not support the theory of a homology between the lineages of NBs in insects and crustaceans and the cells of the NPGs in chelicerates and myriapods.

However, these data also do not exclude a possible homology. All other results showed striking similarities between NB lineages and NPGs, like for example the number and position of the neural precursors and the AP (Stollewerk and Chipman, 2006) and DV compartmentalisation of the NE via patterning genes. Regarding the time span for how long ago these arthropod groups have been separated the level of similarities is very high which supports the theory that the NBs (and thus the NB lineages) derived from NPGs. The difference in the presented marker gene expression then would not disagree with the proposal that NPGs and NB lineages can be regarded as homologous but rather would reflect modifications in early neurogenesis and evolutionary divergence over time.

4.2 Differences and similarities in arthropod brain development

Although the organisation of the adult spider brain in different brain centres like the optic lobes, the mushroom body and the arcuate body, have been described earlier there existed no data on how these brain centres develop in the embryo. Here, I have analysed how the precheliceral NE of the spider *Cupiennius salei* is compartmentalised into the distinct areas which give rise to the main compartments (brain centres) of the protocerebrum. In the following, the development of these compartments in different arthropod groups will be compared.

4.2.1 Brain compartments and NPGs/NB lineages

The compartmentalisation of the developing brain mainly emanates from two areas of the initially flat precheliceral lobe. One area occupies the periphery of the precheliceral NE which gives rise to the optic lobe, the mushroom body and the arcuate body. The other area is located in the central part of the prechliceral lobe and thus builds the central protocerebrum. The data of this work furthermore revealed that the compartments which constitute the visual brain centres are generated by the formation of grooves and vesicles which arise in the peripheral two-dimensional precheliceral lobe. These furrows and vesicles contain smaller neural subunits, the NPGs. As the NPGs in the NE follow the movements of the furrows, they come closer to each other and some seem to merge and build larger subunits. However, the merging of single NPGs was not clearly shown and the majority of NPGs in the furrows is distinguishable throughout this process. As the single NPGs stay separated during these morphogenetic movements, the question arises if the single NPGs are functional subunits which are responsible for the formation of discrete parts of the brain centres. If each NPG can be considered as a functional subunit, this might imply that each NPG encodes spatial information according to the region from which it has been recruited. Thus, the spatial information could then determine in which compartments the NPGs are incorporated and how they integrate there. These assumption is in line with data from *Drosophila* where NBs and their lineages in the VNE carry such spatial information (Urbach and Technau, 2008). Furthermore, in late embryonic brain development in *Drosophila*, the progeny of individual brain NBs are grouped together and the outgrowing axons build fascicles which show characteristic projection patterns (Nassif *et al.*, 2003). These subunits, which are called primary axon bundles, reflect the pattern of larval lineages which build different compartments. As described before, the NBs in the embryonic VNE of *Drosophila*, get their spatial identity by the expression of DV and AP patterning genes (Skeath, 1999; Truman and Bate, 1988). Similarly, the larval lineages in the *Drosophila* brain express several patterning genes which are involved in conferring spatial information to NBs of the VNE (Sprecher *et al.*, 2007). Taken together, these data suggest that the NB lineages in the developing brain of *Drosophila* are comparable to the NPGs in *Cupiennius*. But further experiments have to be done to gain a better insight in this feature of neurogenesis. Therefore expressional and functional data about the

mentioned patterning genes in the developing spider brain would contribute necessary data.

4.2.2 The development of the first and second optic neuropiles

In the adult spider brain three distinguishable optic neuropiles are responsible for transmitting and processing visual information (Barth, 2002). In *Cupiennius*, the discrete first neuropiles (ON1) get their visual information directly from the photoreceptor cells, which project from the retina of each eye to the corresponding neuropiles. The neurons of ON1 send retinotopic projections to the discrete secondary neuropile (ON2) to transmit the visual information further. The further processing differs between the different types of eyes. If the information originates from secondary eyes, the ON2 neurons eventually send their projections to the third neuropile, which is common to all secondary eye projections (mushroom bodies). In contrast the projections of the principal eyes end in the arcuate body (Barth, 2002). Similar to chelicerates, also in insects and crustaceans three distinguishable optic neuropiles are involved in the visual information processing, but the processing itself slightly differs. In insects and crustaceans the photoreceptors of the compound eyes project either to the first and second optic neuropile, which are the lamina or the medulla, respectively. The information is then further relayed to the third optic neuropile, the lobula complex, and even further to higher order centres of the brain (Morante and Desplan, 2004). Comparing optic information processing between arthropods reveals that in contrast to the arcuate body of chelicerates, the central body in insects does not receive visual information (Barth, 2002; Strausfeld *et al.*, 2006). Another difference is reflected in the function of the mushroom bodies in insects and spiders. In insects the mushroom bodies are responsible for processing olfactoric information and are involved in learning and memory (Davis, 1993; de Belle and Heisenberg, 1994; Heisenberg, 1998; Strausfeld *et al.*, 2009). In contrast, the olfactory information in spiders is processed in the neuromere which corresponds to the appendage which receives the olfactory signals. Thus, the mushroom body in chelicerates is not involved in olfactoric processing but represents a visual neuropile (Barth, 2002). Hence, the first and second optic neuropiles share functional features in chelicerates, crustaceans and insects, whereas the third neuropiles are not comparable.

Within this work the development of the visual brain centres was analysed. The data suggest that the morphogenetic changes which are involved in the generation of the visual system are different as compared to the process which is involved in the formation of the other brain centres. The visual brain centres (optic lobes, mushroom bodies and arcuate body) are characterised by their origin from large furrows and vesicles which are formed in the precheliceral NE (Fig.3-27, large invaginations). In contrast, the other brain centres are generated by numerous, closely spaced small single NPGs (Fig.3-27, small invaginations). The morphogenetic movements which lead to the formation of the optic neuropiles start with the indentation of three medio-lateral areas in each protocerebral brain hemisphere. The developing grooves are then further subdivided into individual precursor groups. This is taking place prior to groove formation in the area of AME/PME or directly after the formation of the groove in the area of ALE/PLE. During further development the grooves form vesicles which integrate the individual single NPGs. Then the AME, PME, ALE and PLE vesicles are further subdivided into the corresponding ON1 and/or ON2 vesicles of the principal and secondary eyes. As the NPGs approach each other within the vesicles some of them might fuse, but most of the NPGs are maintained as distinguishable compartments.

Comparing these steps of optic lobe development to *Drosophila* shows that some are similar in insects and chelicerates (Meinertzhagen, 1993). One similarity is that the optic lobe of *Drosophila* develops from an invaginating placode that is located laterally in the head NE (Green *et al.*, 1993). Furthermore the neurons of the central brain are progeny of individual NBs. Thus, the lineages of these individual NBs in *Drosophila* might be comparable to the small invagination sites (NPGs) which form the central protocerebrum in *Cupiennius* (Fig.3-27).

In *Drosophila*, the optic lobe progenitors proliferate during the first half of the larval period. At the same time the progenitors maintain their epithelial morphology and development does not proceed until the first larval stage. Similar to the spider, they split into two partitions - an outer and an inner part - which generate the optic neuropiles. While the outer part gives rise to the lamina and the medulla (first and second optic neuropiles), the inner part forms the lobula complex (third optic neuropile) (Meinertzhagen, 1993). Thus, development of the lobula complex in insects and the comparable structures in spiders (mushroom body and arcuate body) are different. In the spider the mushroom bodies and the arcuate body are generated

by separate vesicles/grooves and thus develop independently from the outer optic anlagen.

4.2.3 The development of the mushroom bodies

As mentioned before, the data of this work revealed that the *Cupiennius* mushroom bodies (MBs) are generated by furrows in the peripheral part of the precheliceral NE. These furrows in the anterior-dorsal part of the developing protocerebrum are generated around mid-embryogenesis and build large invaginations throughout further development. The numerous NPGs which are located in the part of the NE where the furrow forms are incorporated in the developing vesicles and remain visible during development of the mushroom bodies.

Mushroom body development can also be traced by the expression of *dachshund*. This marker is expressed in neural precursor cells and in the differentiating neural cells in the mushroom body vesicles. Thus, the differentiating cells which correspond to the mushroom body can be identified by coexpression of *dachshund* and the cell fate determinant Prospero. With ongoing development *dachshund* expression extends towards posterior and these cells in the more posterior position also express Prospero. Due to this data it is assumed that differentiating neural precursors continuously delaminate from the mushroom body vesicles. Hence, these cells are either pushed into more posterior positions by later born neurons or these cells actively migrate into this more posterior position. These neurons might differentiate into the so-called globuli cells which form the ‘head’ of the mushroom bodies (calyx).

As the mushroom bodies in insects and chelicerates exhibit structural as well as functional differences, their possible homology has been challenged (Barth, 2002). One difference is the role of the mushroom bodies in olfactory information processing. In insects the mushroom bodies process olfactory information. In this process the calyx receives projections from the antennal lobes. This information is then transmitted to the peduncle and the lobes via Kenyon cells (insect globuli cells), whose dendrites form the calyx. The difference to spiders is that the mushroom bodies are not involved in processing of olfactory information as they do not receive input from olfactory sensory organs. Still, the mushroom bodies of insects and chelicerates seem to share some characteristics concerning development (Noveen et

al., 2000; Tettamanti, 1997). The development of the mushroom bodies was studied in detail in *Drosophila* and it was possible to trace the development from single identified NBs onwards (Noveen *et al.*, 2000). The four identified mushroom body NBs are recruited in the protocerebrum and at stage 9 they delaminate from the procephalic NE. After delamination the mushroom body NBs divide and generate lineages of 15 to 20 primary neurons which stay attached to the neural stem cells. The four mushroom body NBs and their progeny are marked by *dachshund* expression. These features of mushroom body development indicate that the mushroom body NBs of *Drosophila* can be compared to the NPGs which are incorporated in the mushroom body vesicles of *Cupiennius*. Another remarkable similarity in mushroom body development in insects and chelicerates is reflected by the characteristic arrangement of the neural cells from which they are built. The four mushroom body NBs in *Drosophila* divide further and generate the secondary Kenyon neurons. These neurons spread tangentially and thus form the calyx (Farris and Sinakevitch, 2003; Tettamanti *et al.*, 1997). In *Cupiennius* the circular expression of *dachshund* and the sequential extension of *dachshund* and Prospero coexpression towards posterior suggest a similar sequence of mushroom body development as compared to *Drosophila*. The described tangential extension of mushroom body neurons is unusual in both *Drosophila* and *Cupiennius*. Due to these morphological and molecular similarities it can be assumed that the mushroom bodies of insects and spiders are homologous structures which have diverged and adapted to distinct functions in the two arthropod groups. But, as there are differences in the structure and function of the adult mushroom bodies it is also possible that the mushroom bodies of insects and chelicerates can not be assumed to be homologous. Then, these brain subunits most probably have evolved by convergence in chelicerates and insects. For a better understanding of this issue more morphological and molecular data from all four arthropod groups are required.

4.2.4 The development of the arcuate body

Until now, it is questionable if the distinct midline neuropile is homologous in arthropods. The homology is commonly accepted for the central complex (central body) in insects, crustaceans and chilopods (myriapod), but has been challenged in chelicerates, because the arcuate body exhibits features which are not present in the

central bodies of other arthropod groups (Homberg, 2008; Loesel *et al.*, 2002; Strausfeld *et al.*, 2006). One distinct feature is that the midline neuropile in chelicerates is not embedded in the protocerebrum as it is in insects, crustaceans and chilopods. In chelicerates the arcuate body is located in a superficial position in the dorsal most posterior part of the adult brain (= most anterior part in the embryo). Another distinct feature is that the midline neuropile in chelicerates does not form complexes with satellite neuropiles as in the other arthropod groups (Barth, 2002). Furthermore, the stratiated appearance of the arcuate body in spiders is a specific characteristic of the spider midline neuropile. The stratiated structure reflects the parallel assembly of hundreds to thousands of neurons. Finally, the arcuate body of spiders is assumed to be a visual centre, which receives direct input from the second optic neuropiles (Barth, 2002). Thus the midline neuropile of chelicerates is involved in visual information processing and not in olfactory information processing as in the other arthropods.

The studies about brain development within this work revealed that the arcuate body in *Cupiennius* is generated in the anterior most part of the developing protocerebrum. The formation of the arcuate body starts in early neurogenesis by morphogenetic movements which form a bilateral pair of furrows. The furrows contain about 25 NPGs which are already present in the two-dimensional NE prior to groove formation. During further development the bilateral grooves converge until they fuse at the dorsal midline. In late embryogenesis the fused groove then segregates into the embryo.

The described grooves in the anterior most area of the developing brain were also presented in other publications about *Cupiennius* and other spider species. The authors of these publications named the corresponding grooves interior brain cavities or semi-lunar grooves ((Brauer, 1895; Holm, 1952; Weygoldt, 1985; Yoshikura, 1955). Furthermore, these data support the conclusion of this work that these grooves give rise to the arcuate body, as they also suggest that the semi-lunar grooves (or interior brain cavities) represent the anlagen of the arcuate and mushroom bodies. At the last larval stage the typical midline neuropile becomes visible in *Cupiennius*, but the elaborate adult structure is not found at this developmental stage. During this time span numerous new NPGs are formed in the anterior-most dorsal area of the protocerebrum. Thus, the NPGs which are newly

build in the first larval stage might later contribute to the adult arcuate body to form the elaborate adult midline neuropile.

Until now the embryonic development of the central complex has not been analysed in detail in arthropods other than orthopteran insects. The central complex of these arthropods is formed by progeny of NBs which are located in each half of the pars intercerebralis, which is part of the neuroendocrine system (Boyan, 1997; Malzacher, 1968; Williams and Boyan, 2008). This reflects a similarity of central complex/arcuate body development between orthopteran insects and chelicerates, because in both the unpaired midline neuropile is generated by bilateral groups of neural precursors. But drawing firm conclusions on a possible homology of the central complex and the arcuate body requires additional data concerning molecular and morphological features in all arthropod groups.

4.2.5 The protocerebral *engrailed* domain ("head spot") overlaps with the optic lobe

The segment polarity gene *engrailed* is expressed in transversal segmental stripes in the NE of arthropods. However, the expression pattern in the two pre-oral and two post-oral segments differs from the expression pattern in the trunk in insects, crustaceans and myriapods (Boyan *et al.*, 1995; Boyan and Williams, 2002; Rogers and Kaufman, 1996; Sintoni *et al.*, 2007; Urbach and Technau, 2003c). There *engrailed* is expressed in bilateral domains at the border of the developing protocerebrum which were named head spots (Rogers and Kaufman, 1996). As the *engrailed* expressing head spot occupies the posterior boundary of the acron/ocular segment it demarcates this border. In *Drosophila engrailed* expression starts very early, in a stage prior to NB recruitment in the VNE. Its expression is then maintained until it is downregulated concurrently to NB delamination. But in insects as well as in crustaceans there is a second phase of *engrailed* expression. This additional *engrailed* expression comes up in a neural cluster whose cells derive from the head spot. Thus, these clusters were named "secondary head spots" (Boyan and Williams, 2002).

It has been shown that the cells which originate from the head spot are involved in the development of the visual system. In *Drosophila* the head spot is adjacent to the

optic lobe placode and also the head spots in other arthropods contribute to the visual system. This was shown in grasshoppers and the marbled crayfish, where the neurons of the “secondary head spots” project to the optic nerve which connects the optic lobe with the central protocerebrum (Boyan and Williams, 2002; Sintoni *et al.*, 2007).

A similar *en* expression in bilateral patches in the precheliceral lobe was also observed in *Cupiennius*. This led to the assumption that these *engrailed* patches correspond to the head spots in insects and crustaceans (Damen *et al.*, 1998). Within this work *engrailed* expression in the prechliceral NE of *Cupiennius* was analysed in detail. *engrailed* expression demarcates the posterior lateral border of the developing protocerebrum. Its expression in this domain is maintained throughout embryogenesis and during all these stages no expression can be detected in further anterior positions. Interestingly, a similar expression pattern has been described in the representative of an ancestral group which is closely related to the arthropods, the onychophoran *Euperipatoides kanangrensis* (Eriksson *et al.*, 2009). In the spider the shape of the *engrailed*-positive domain changes during development. The stripe-like shape widens more and more and assumes a square-like shape in late embryogenesis. At these stages, the square-shaped expression domain covers the medial optic vesicle party. Thus, it seems that the head spot in *Cupiennius* is also associated with the visual system as was shown for insects and crustaceans. Even if the head spot expression represents a smaller *engrailed*-positive area as compared to the segmental expression domains in the VNE, the dynamic change in the *engrailed* expression is similar in all segments. *engrailed* expression starts with a sharply demarcated stripe at the posterior border of the segments. This transversal stripes widen during further development until the expression crosses the segmental boundaries and extends into the anterior part of the neighbouring posterior segment.

During these changes in the shape of the expression domain the NPGs are recruited in the NE and thus partly in the *engrailed*-positive region. Thus, *engrailed* initially determines the epithelial cell fate in the posterior compartment of the segments and is later involved in determining the fate of metamerically reiterated subsets of neural lineages. Transferring these assumptions to the protocerebrum, the neural precursors whose fates are determined by *engrailed*, form part of the optic lobe. The suggestion of an important and probably primary function of *engrailed* in the developing visual system is also supported by data on *engrailed* expression in

Archegozetes longisetosus. In the embryo of this blind mite no *engrailed* expression was detected anterior to the cheliceral neuromere (Telford and Thomas, 1998). Compared to the spider, these mites do not have a visual system which points out the correspondence between *engrailed* expression and visual system development in *Cupiennius*.

5 Summary

The central point of this work was the investigation of neurogenesis in chelicerates and myriapods. By comparing decisive mechanisms in neurogenesis in the four arthropod groups (Chelicerata, Crustacea, Insecta, Myriapoda) I was able to show which of these mechanisms are conserved and which developmental modules have diverged. Thereby two processes of embryonic development of the central nervous system were brought into focus. On the one hand I studied early neurogenesis in the VNC of the spiders *Cupiennius salei* and *Achaearanea tepidariorum* and the millipede *Glomeris marginata* and on the other hand the development of the brain in *Cupiennius salei*.

While the nervous system of insects and crustaceans is formed by the progeny of single neural stem cells (neuroblasts), in chelicerates and myriapods whole groups of cells adopt the neural cell fate and give rise to the VNC after their invagination. The detailed comparison of the positions and the number of the neural precursor groups within the neuromeres in chelicerates and myriapods showed that the pattern is almost identical which suggests that the neural precursors groups in these arthropod groups are homologous. This pattern is also very similar to the neuroblast pattern in insects. This raises the question if the mechanisms that confer regional identity to the neural precursors are conserved in arthropods although the mode of neural precursor formation is different. The analysis of the functions and expression patterns of genes which are known to be involved in this mechanism in *Drosophila melanogaster* showed that neural patterning is highly conserved in arthropods. But I also discovered differences in early neurogenesis which reflect modifications and adaptations in the development of the nervous systems in the different arthropod groups.

The embryonic development of the brain in chelicerates which was investigated for the first time in this work shows similarities but also some modifications to insects. In vertebrates and arthropods the adult brain is composed of distinct centres with different functions. Investigating how these centres, which are organised in smaller compartments, develop during embryogenesis was part of this work. By tracing the morphogenetic movements and analysing marker gene expressions I could show the

formation of the visual brain centres from the single-layered precheliceral NE. The optic ganglia, the mushroom bodies and the arcuate body (central body) are formed by large invaginations in the peripheral precheliceral NE. This epithelium itself contains neural precursor groups which are assigned to the respective centres and thereby build the three-dimensional optical centres. The single neural precursor groups are distinguishable during this process leading to the assumption that they carry positional information which might subdivide the individual brain centres into smaller functional compartments.

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7 Appendix

A Chemicals, reagents and solutions

Solutions:

Aqua dest.
Deionised autoclaved H_2O.

Alkalic phosphatase detection buffer
0.1M NaCl
50mM $MgCl_2$
50mM Tris-HCl
0.1% Tween20
pH 9.5

DEPC-H_2O
0.001% (V/V) DEPC autoclaved in deionised H_2O.

Fixative for Phalloidin staining in *Cupiennius* and *Achaearanea*
50% (v/v) n-heptane
50% (v/v) 5.5% MeOH-free formaldehyde in PEMS or PBS.

Fixative for *in situ* hybridisation in *Cupiennius* and *Achaearanea*
50% (v/v) n-heptane
50% (v/v) 5.5% formaldehyde in PEMS or PBS.

Fixative for Phalloidin staining in *Glomeris*
1ml n-heptane
100µl MeOH-free formaldehyde (16%)

Fixative for i*n situ* hybridisation in *Glomeris*
1ml n-heptane
50µl formaldehyde (37%)

hybA (5ml)
50% (v/v) formamide
5x SSC, pH 5.5
2% (v/v) 10mg/ml boiled sonicated salmon testis DNA (ssDNA)
0.5% (v/v) 20mg/ml t
0.05% (v/v) 100mg/ml heparin stock
DEPC-H_2O is added to a total volume of 5ml.
Solution should be prepared freshly before using.

hybB (50ml)
50% (v/v) formamide
5x SSC, pH 5.5
0.1% Tween 20
DEPC-H_2O is added to a total volume of 50 ml.
Solution can be stored at -20°C.

LB-(Luria Bertani) medium
10g Bacto-Trypton
5g Bacto-yeastextract
10g NaCl
Components are dissolved in 900ml aqua dest.
pH 7 (with 6N NaOH)
Aqua dest. is added to a total volume of 1 litre.
Medium is autoclaved and stored at 4°C.

Loading buffer for RNA gels
50 % (v/v) formamide
5% formaledhyde (37%)
2.5% (v/v) ethidium bromide (10mg/ml)
1% (v/v) DEPC-H_2O
1x MOPS
1% (v/v) RNA sample

10x MOPS Stock solution
0,2 M MOPS (pH 7)
50 mM sodium acetate (pH 7)
10 mM EDTA (pH 8)

NBT/BCIP-Staining solution
1ml alkalic phosphatase detection buffer
3.5µl BCIP (5-bromo-4-chloro-3-indolyl-phosphate), 50mg/ml
4.5µl NBT (nitroblue-tetrazoliumchloride), 50 mg/ml in 70% DMF (N,Ndimethylformamide)

20xPBS (phosphate buffered saline) Stock solution
1.3M NaCl
0.07M Na_2HPO_4
0.03M NaH_2PO_4
pH 7.4

1xPBS working solution (PBS)
20x PBS 1:19 diluted

1xPBT working solution (PBTx)
1x PBS with 0.3% Triton-X-100

1xPBTween working solution (PBTw)
1x PBS with 0.1% Tween 20 (for *in situ* hybridisation)
1x PBS with 0.02% Tween 20 (for combined Phalloidin and antibody staining)

PEMS
100mM PIPES
1mM EDTA
2mM $MgSO_4$
pH 6.9

20x SSC Stock solution
3M NaCl
0.3 M sodium citrate
pH 7 (with 14N HCl)

50x TAE-stock solution (Tris-Acetat-EDTA), pH 8.3
242g Tris are dissolved in 500ml aqua dest.
100ml 0.5M Na_2EDTA, pH 8
57.1ml glacial acetic acid
Aqua dest. is added to a total volume of 1 litre.

Chemicals and reagents:

Acetic anhydride (Fisher)

Agarose (Seakern)

Anti-digoxigenin-AP (alkalic phosphatase), fab fragment, 0.75 u/µl (Roche)

2 log base pair DNA ladder, 0.1-10kb (New England Biolabs)

BCIP (5-bromo-4chloro-3-indolyl phosphate) (Sigma)

$CaCl_2$

Chlorbleach (Vortex Thick Bleach)

Chloroform (VWR)

DIG RNA labelling mix (DIG NTPs, ATP, CTP, GTP each 10 mM, 6.5 mM UTP, 3.5 mM DIG-11-UTP) (Roche)

dNTPs (ATP,CTP, GTP, TTP each 2.5 mM) (Sigma)

DTT (0.1 M, Dithiothreitol) (Invitrogen)

EcoRV restriction Enzyme (20 u/µl) (New England Biolabs)

Ethanol (EtOH)

EDTA (Ethylenediaminetetraacetic acid) (VWR)

Ethidium bromide

Formamide (Sigma)

Formaldehyde (37%) (VWR)

Formaldehyde (MeOH-free) (Polyscience Inc)

Glycerol (VWR)

n - Heptane (VWR)

Isopropanol

Klenow polymerase (5 u/µl) (New England Biolabs)

Kanamycin sulfate solution (50 mg/ml) (VWR)

LiCl (Fisher)

$MgCl_2$ (VWR)

Methanol (MeOH)

Mouse serum (Sigma)

NBT (Nitro blue tetrazolium chloride) (Sigma)

Phenol Chloroform (Sigma)

Proteinase K (41 u/mg) (Sigma)

pZErO™-2 (supercoiled, 1 µl/µg) (Invitrogen)

RNAse H (2 /µl) (Invitrogen)

RNAse-Inhibitor (40 u/µl) (Roche)

RNA polymerase Sp6 (20 u/µl) (Roche)

RNA polymerase T7 (20 u/µl) (Roche)

RNAse - Out (40 u/µl) (Roche)

Sheep serum (Sigma)

Sodium acetate

Superscript III (200 u/µl) (Invitrogen)

T4 DNA ligase (4 u/µl) (Invitrogen)

Taq polymerase (5 u/µl) + **Thermo pol Buffer** (New England Biolabs)

Triethylamine (TEA) (Fisher)

Triton X-100 (VWR)

Trizol (Invitrogen)

Tween 20 (Sigma)

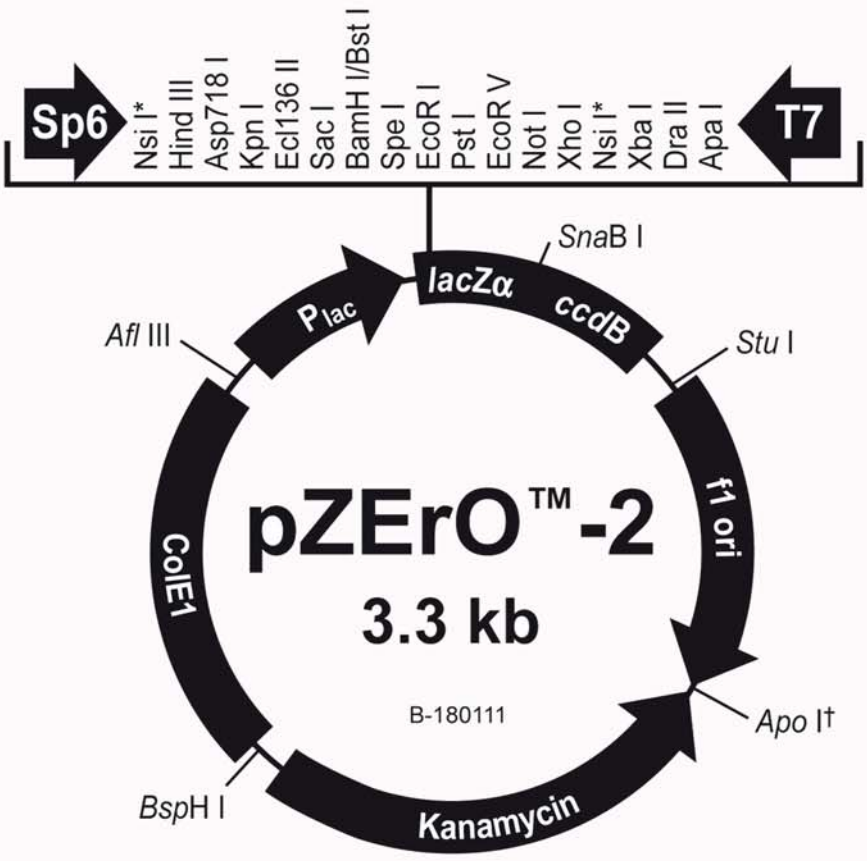
B Equipment

Label	Model	Producer
Balance	PM 4600 Delta Range PLJ360-3M	Mettler Kern
Binocular	Wild M3B	Wild
Cameras	Axiocam MRm 3CCD microscope camera	Zeiss Sony
Centrifuges	Centrifuge 5410 Centrifuge 5417R 3K10 IEC MICROCL 17/17R	Eppendorf Eppendorf Sigma Thermo electron corporation
Confocal Laserscanning Microscope	TCS SPII	Leica
Fluorescence binocular	MzFLIII	Leica
Fluorescence microscope	BX 50 WI	Olympus
Gelcamera	Quickstore	MS Laborsysteme
Gelelectrophoresis	HE 33 HE 99x EV243	Hoefer Jencons
Heatblock	TB1 Thermoblock Thermomixer compact QBD1	Biometra Eppendorf Grant
Incubators	KB-115 Friocell 707	WTB Binder MMM Medcenter Einrichtungen
Light microscope	Axioplan Axioskop 2	Zeiss Zeiss
Light source for binocular	Highlight 3100	Olympus
Magnetic stirrer	MR3001	Heidolph
PCR machine	TGradient TC-512	Biometra Techne

Label	Model	Producer
pH-meter	CG840 3510 pH Meter	Schott Jenway
Photometer	Biophotometer	Eppendorf
Pipettes	10, 100, 1000	Eppendorf
Power supply for electrophoresis	Gene Power Supply GPS 200/400 EV243	Pharmacia
Shaker Overhead shaker	Shaker Dos 20S REAX2 VX7 Mini gyro-rocker SSM3 Rotator SB3	neoLab Heidolph Janke und Kunkel Stuart Stuart
Table centrifuge	Mini Spin	Eppendorf
UV irradiation	Transilluminator N90 M	MS Laborgeräte
Vacuum centrifuge	Savant Speed Vac®	Thermo Life Siences
Vortexer	Vibrofix VF1 Vortex Genie 2TM	Janke&Kunkel Bender&Hobein AG
Waterbath	 SUB Aqua 12	Köttermann Grant

Table 7-1: Equipment

C Vector map



* The two *Nsi* I sites in the MCS are the only sites in the vector.

† There are two tandem *Apo* I sites at this location. *Apo* I also recognizes the *Eco*R I site.

Lac Promoter/Operator Region: bases 95-216
M13 Reverse Priming Site: bases 205-221
LacZα ORF: bases 217-558
Sp6 Priming Site: bases 239-256
Multiple Cloning Site: bases 269-381
T7 Promoter Priming Site: bases 388-407
M13 (-20) Forward Priming Site: bases 415-430
M13 (-40) Forward Priming Site: bases 434-450
Fusion Joint: bases 559-567
*ccd*B Lethal Gene ORF: bases 568-870
f1 origin: bases 895-1307
Kanamycin Resistance ORF: bases 2116-1322
ColE1 origin: bases 2502-3175

D Abbreviations

α	anti
AB	arcuate body
aCC	anterior corner cell
Achaearanea	*Achaearanea tepidariorum*
ant	antennal segment
AP	anteroposterior
ASH1	*achaete-scute homologue 1*
cDNA	complementary DNA
ch	chelicerae
CNS	central nervous system
Cupiennius	*Cupiennius salei*
Cy5	indodicarbocyanine
dac	*dachshund*
DEPC-H_2O	water treated with diethylcarbonate
DIG	digoxigenin
DNA	deoxyribonucleicacid
Drosophila	*Drosophila melanogaster*
ds	double stranded
DV	dorsoventral
EB	elution buffer
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
en	*engrailed*
et al.	and co-workers
EtBr	ethidium bromide
EtOH	ethanol
eve	*even-skipped*
FITC	fluorescin
g	gram
Glomeris	*Glomeris marginata*
GMC	ganglion mother cell
h	hour
ind	*intermediate neuroblast defective*
Hb	Hunchback
l	leg segment
LSM	confocal laserscanning microscope
M	molar

md	mandibular segment
mx	maxillary segment
MB	mushroom body
MeOH	methanol
mg	milligram
µg	microgram
min	minute
Mio	million
µl	microlitre
ML	midline
mM	millimolar
mRNA	messenger RNA
msh	*muscle segment homeobox*
NB	neuroblast
NE	neuroectoderm
NPG	neural precursor group
ng	nanogram
o	opisthosomal segment
ON	optic neuropile
pCC	posterior corner cell
PCR	polymerase chain reaction
ped	pedipalp
pH	potential hydrogenii
pmd	premandibular segment
pmx	postmaxillary segement
PNS	peripheral nervous system
RNA	ribonucleicacid
RNAi	RNA interference
RT	room temperature
sec	seconds
st	stage
Tab.	Table
Tribolium	*Tribolium castaneum*
isl	*islet*
V	volt
VNC	ventral nerve cord
VNE	ventral neuroectoderm
vol	volume
WT	wildtype

E Curriculum vitae

F Eidesstattliche Erklärung

Versicherung gemäß §11, Abs.3d der Promotionsordnung

1. Ich habe die als Dissertation vorgelegte Arbeit selbst angefertigt und alle benutzten Hilfsmittel (Literatur, Apparaturen, Material) in der Arbeit angegeben.

2. Ich habe und hatte die als vorgelegte Dissertation vorgelegte Arbeit nicht als Prüfungsarbeit für eine staatliche oder andere wissenschaftliche Prüfung eingereicht.

3. Ich hatte weder die als Dissertation vorgelegte Arbeit noch Teile einer Abhandlung davon bei einer anderen Fakultät bzw. einem anderen Fachbereich als Dissertation eingereicht.

Mainz, den

_____

Carola Döffinger

G Danksagung